Evaluation of host biomarkers for early diagnosis of tuberculosis disease in children

by

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Declaration

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Abstract

Background:

The diagnosis of tuberculosis (TB) remains a challenge in children. There is an urgent need for new tools for early diagnosis of TB disease in children

Objectives:

To evaluate the usefulness of a previously described 3-marker cerebrospinal fluid (CSF) biosignature (VEGF, IL-13 and cathelicidin LL-37) and other CSF biomarkers for diagnosis of tuberculous meningitis (TBM), and evaluate the utility of a previously identified adult 7-marker serum protein biosignature (CRP, IFN-γ, IP-10, CFH, Apo-AI, SAA and transthyretin) and other blood biomarkers for diagnosis of pulmonary TB (PTB) and TBM in children.

Methods:

CSF and serum samples were collected from children with suspected meningitis, whereas serum samples were collected from children with suspected PTB for investigation of biomarkers for the diagnosis of childhood TBM and PTB, respectively. Children in the TBM project were enrolled at the Tygerberg Academic Hospital, whereas those in the PTB study were enrolled at the Red Cross War Memorial Children's Hospital in Cape Town, South Africa. Children were classified as TBM or no-TBM and PTB or no-PTB, using combination of clinical, radiological and laboratory findings. Using a multiplex platform, the concentrations of 69 host biomarkers were evaluated in CSF and serum samples from children in the TBM study, whereas 40 host markers were evaluated in serum samples from children in the PTB study. The diagnostic accuracies of individual biomarkers were assessed by receiver operator characteristics (ROC) curve, whereas the General Discriminant Analysis (GDA) was used to assess the accuracies of combinations between different host biomarkers.

Results:

Of the 69 host biomarkers evaluated in CSF and serum samples from children in the TBM study, multiple individual host biomarkers showed potential as diagnostic candidates for TBM as ascertained by area under the ROC curve (AUC). The previously described 3-marker CSF biosignature was validated in the project. However, refinement of the biosignature by substitution of IL-13 and cathelicidin LL-37 with two new proteins (MPO and IFN-γ) resulted in a new biosignature with improved accuracy (AUC of 0.97). Furthermore, we identified a 4-marker CSF biosignature (sICAM-1, MPO, CXCL8 and IFN-γ), which also diagnosed TBM with AUC of 0.97. The adult 7-marker serum biosignature, modified by the replacement of transthyretin with NCAM1, diagnosed TBM with AUC of 0.80. However, a childhood TBM-

specific serum biosignature (adipsin, Aβ42 and IL-10) diagnosed TBM with AUC of 0.84. The adult signature performed with an AUC of 0.79 in children with PTB, showing no significant difference in the diagnosis of childhood PTB or TBM. However, novel childhood PTB-specific biosignatures performed better than the adult 7-marker signature.

Conclusion:

The adult 7-marker signature showed potential in the diagnosis of both PTB and TBM in children recruited from a high TB incidence area. We validated a previously established 3-marker CSF biosignature, but a refined signature showed much improved accuracy. The biosignatures identified in this thesis hold potential for development of new diagnostic tools for PTB and TBM in children for possible use at the point-of-care. Our findings require further validation in larger and multi-site studies.

Opsomming

Agtergrond:

Die diagnose van tuberkulose (TB) by kinders bly ‘n uitdaginig. Daar is ‘n dringende behoefte aan nuwe toestelle vir die vroeë diagnose van TB-siekte by kinders.

Doelwitte:

Om die doeltreffendheid van ‘n voorheen beskryfde 3-merker serebrospinale vloeistof (CSF) bioprofiel (VEGF, IL-13 and cathelicidin LL-37) en ander CSF biomerkers vir die diagnose van tuberkulose meningitis (TBM) te evalueer, asook die bruikbaarheid van ‘n voorheen geïndetifiseerde volwasse 7-merker serumproteïn bioprofiel (CRP, IFN-γ, IP-10, CFH, Apo-AI, SAA en transthyretin) en ander bloedbiomerkers te evalueer vir die diagnose van pulmonale TB (PTB) en TBM by kinders.

Metodes:

CSF- en serummonsters is verkry van kinders wat vermoedelik meningitis het, terwyl serummonsters van kinders met vermeende PTB verkry is om die biomerkers se vermoëns om kindertyd TBM and PTB, onderskeidelik, te diagnoseer te ondersoek. Die kinders in die TBM-projek is ingeskryf by die Tygerberg Akademiese Hospitaal, terwyl dié wat aan die PTB studie deelgeneem het by die Rooikruis Oorlogsgedenkhospitaal in Kaapstad ingeskryf is. Die kinders is geklassifiseer as TBM of nie-TBM, en PTB of nie-PTB deur gebruik te maak van ‘n kombinasie van kliniese-, radiologiese-, en laboratoriumbevindings. ‘n Multipleks-platform is benut om die konsentrasies van 69 gasheerbiomerkers in CSF- en serummonsters van kinders in die TBM-studie en 40 gasheerbiomerkers in serummonsters van die kinders in die PTB-studie, onderskeidelik, te evalueer. Die diagnostiese akkuraatheid van individuele biomerkers is met die ontvanger-operateur-eienskappe (ROC) kurwe geassesseer, terwyl die Algemene Diskriminant Analise (GDA) gebruik is om die akkuraatheid van kombinasies tussen verskillende gasheerbiomerkers te bepaal.

Resultate:

Van die 69 gasheerbiomerkers wat in die CSF- en serummonsters van kinders in die TBM-studie geëvalueer is, het verskeie individuele gasheerbiomerkers potensiaal getoon as diagnostiese kandidate vir TBM, soos vasgestel deur die area onder die ROC-kurwe (AUC). Validasie van die voorheen beskryfde 3-merker CSF bioprofiel is in die projek uitgevoer. Verfyning van die bioprofiel deur die vervanging van IL-13 en cathekicidin LL-37 met twee

nuwe proteïene (MPO en IFN-γ), het gelei tot ‘n nuwe bioprofiel met verbeterde akkuraatheid (AUC van 0.97). Daarbenewens, is daar ‘n 4-merker CSF-bioprofiel geïdentifiseer (sICAM-1, MPO, CXCL8 en IFN-γ) wat ook TBM met ‘n AUC van 0.97 gediagnoseer het. Die volwasse 7-merker serum bioprofiel, gewysig deur die vervanging van transthyretin met NCAM1, het TBM met ‘n AUC van 0.80 gediagnoseer. ‘n TBM-spesifieke serum bioprofiel (adipsin, Aβ42 en IL-10) het egter TBM met ‘n AUC van 0.84 gediagnoseer. Die volwasse teken het ‘n AUC van 0.79 in kinders met PTB opgelewer en dus geen beduidende verskil tussen die diagnose van kindertyd PTB of TBM getoon nie. Daarteenoor, het nuwe kindertyd PTB-spesifieke bioprofiele beter gevaar as die volwasse 7-merker profiel.

Afsluiting:

Die volwasse 7-merker profiel het potensiaal getoon om beide PTB en TBM by kinders afkomkstig van hoë TB-voorkomsgebiede te diagnoseer. ‘n Voorheen beskryfde 3-merker-CSF-bioprofiel is bevestig, maar ‘n verfynde profiel het heelwat verbeterde akkuraatheid getoon. Die bioprofiel wat in hierdie proefskrif geïdentifiseer is, het die potensiaal om gebruik te word om nuwe diagnostiese intrumente te ontwikkel vir punt-van-sorg gebruik by PTB en TBM in kinders. Die bevindings verg egter verdere validasie in groter en multi-setel studies.

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List of Abbreviations

°C	Degree Celsius
A1AT	alpha-2-antitrypsin
AFB	Acid fast bacilli
APCs	Antigen presenting cells
ATB	Active tuberculosis
AUC	Area under curve
Aβ40/42	Amyloid beta-40/42
BBB	Blood-Brain-Barrier
BC	Before Christ
BCG	Bacillus Calmette-Guérin
CC	Complement Component
CCL	Chemokine C-C Motif Ligand
CD	Cluster of Differentiation
CFD	Complement Factor D
CFH	Complement Factor H
CFP-10	Culture filtrate protein-10
CI	Confidence interval
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CT	Computed tomography
CXCL	Chemokine C-X-C ligand

DCs	Dendritic cells
dL	Decilitre
DTH	Delayed-typed hypersensitivity
EDCTP	European and Developing Countries Clinical Trials and Partnership
ELISA	Enzyme-Linked Immunosorbent Assay
EPTB	Extrapulmonary tuberculosis
ESAT	Early secretory antigenic target-6
GA	Gastric aspiration
GCS	Glasgow coma score
G-CSF	Granulocyte-colony stimulating factor
GDA	General Discriminant Analysis
GDF	Growth differentiation factor
GDNF	Glial cell-derived neurotrophic factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIV	Human immunodeficiency virus
IFN-γ	Interferon-gamma
IGRA	Interferon-Gamma Release assay
IL	Interleukin
IP-10	Interferon-gamma-Inducible protein-10
LAM	Lipoarabinomannan
LED	Light-emitting diode
LJ	Löwenstein-Jensen
LPA	Line probe assay
LTBI	Latent tuberculosis infection

M.tb	*Mycobacterium tuberculosis*
MBL	Mannose Binding Lectin
MCP	Monocyte chemoattractant protein
MDC	Minimum detectable concentration
MFI	Median Fluorescent Intensity
mg	Milligram
MGIT	Mycobacteria Growth Inhibitor Tube
MHC	Major Histocompatibility Complex
MIG	Monokine induced by gamma interferon
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
MTBC	*Mycobacterium tuberculosis* complex
NCAM	Neural Cell Adhesion Molecule
NPV	Negative predictive value
OD	Optical density
PAI-1	Plasminogen activator inhibitor-1 (total
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PCT	Procalcitonin
PDGF	Platelet derived growth factor
PEDF	Pigment Epithelium-Derived Factor
pg	Picogram

POC	Point-of-care
PPD	Purified Protein Derivate
PPV	Positive predictive value
PRRs	Pattern recognition receptors
PTB	Pulmonary tuberculosis
QC	Quality control
QFT	QuantiFERON
RIF	Rifampicin
RNA	Ribonucleic acid
RNIs	Reactive nitrogen intermediates
ROC	Receiver operator characteristics
ROS	Reactive oxygen species
SAA	Serum Amyloid A
SAP	Serum Amyloid P
SAPE	Streptavidin Phycoerythrin
SD	Standard deviation
SI	Sputum induction
sICAM	soluble Intracellular Adhesion Molecule
sRAGE	soluble Receptor for Advanced Glycation End Products
SU-IRG	Stellenbosch University-Immunology Research Group
TB	Tuberculosis
TBM	Tuberculous meningitis
TCR	T-cell receptor
TGF-α	Transforming growth factor-alpha

TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
TPA	Tissue Plasminogen Activator
TST	Tuberculin Skin Test
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation
ZN	Ziehl Neelsen
µl	Microliter

Chapter 1

Literature review

1.1 Introduction

Tuberculosis (TB) is one of the most important life-threatening diseases amongst the infectious diseases across the world (1). In 2016, TB was amongst the top 9 causes of death across the world, causing more deaths than HIV and malaria (2). TB is caused by members of the *Mycobacterium tuberculosis* complex (MTBC) species and this specie causes TB in both humans and animals (3) . *Mycobacterium tuberculosis (M.tb)* is the most frequent cause of TB in humans, while *M. bovis*, *M. caprae and M. pinnipedii* are known to be responsible for the disease in wild and domestic mammals (3). TB occurs following an inhalation of an aerosol droplet containing the causative agent of TB, *Mycobacterium tuberculosis*, also known as tubercule bacilli, from an individual who is sick of TB (4). Only 5 to 10% of people who are infected with *M.tb* progress to having active TB, with young children having a higher risk of progressing to active TB following an infection (13). The most common type of TB is pulmonary TB, which is TB that occurs in the lungs. The most common signs and symptoms seen in pulmonary TB include coughing, sputum expectoration, haemoptysis, breathlessness, weight loss, anorexia, fever, malaise and wasting (6).

TB also affects other organs of the body; and is known as extrapulmonary TB (EPTB). Other organs other than the lung, which TB frequently occurs in include the pleura, lymph nodes, abdomen, genitourinary tract, skin, joints, bones and meninges (7). The involvement of the central nervous system (CNS) may result in tuberculous meningitis (TBM). TBM is the most severe complication of TB and predominantly affects young children (8, 9). TBM is often referred as a medical emergency for which early treatment initiation is required (10). In the absence of treatment, TBM leads to severe consequences including high mortality and neurological sequelae in survivors (11, 12). The diagnosis of TB is mostly difficult in children and this often results in delayed initiation of treatment and consequently death (13). An estimated 210 000 children died from TB in 2016 (2).

1.2 History and epidemiology of tuberculosis

TB has been a threat to humankind throughout known history and human prehistory. It is estimated that the causative agent of TB disease, *Mycobacterium tuberculosis (M.tb)* may have killed more humans than any other microbial pathogen (14). It has been hypothesized that the genus mycobacterium originated more than 150 million years ago (15). Bone studies

provide evidence of the long existence of TB with a bone from the Neolithic period found in the region of Heidelberg in Germany showing evidence of TB features (Dated at 500 BC) (16). Similarly, skeletal deformities typical of TB were revealed in Egyptian mummies, dating back to 2400 BC (15). TB was endemic in the western world in the 18th century and it was then better known as consumption or phtisis (17). Hippocrates described phtisis as a fatal disease especially of young adults, with characteristic tubercular lung lesions (15). In 1882, Robert Koch became the first researcher to discover through staining experiments that TB is caused by tubercle bacillus (18). There is a trace evidence on the history of the involvement of central nervous system in tuberculosis. Smears taken from a psoas abscess in a well-preserved mummy of an Inca child dated at 700 BC showed acid-fast bacilli, indicating a case of TB of the lumbar spine (16). Fifty years after the discovery of the causative agent of TB, two pathologists, Rich and McCordock carried out a series of experiments in rabbits and post-mortem findings in children, which demonstrated that the release of *M.tb* bacilli into the meningeal space from focal sub-pial or sub-ependymal lesions was the cause of meningeal TB (reviewed in (19)).

To date, TB remains a global health challenge with about one third (30%) of the world population estimated to be infected with *M.tb* (20). According to the World Health Organisation (WHO), 10.4 million TB cases and 1.7 million deaths were reported in 2016 (21). 90% and 10% of these cases were in adults and children, respectively. The burden of TB varies amongst countries, as indicated in the report that the highest rates of incident cases in 2016 were observed in South-East Asia region (45%), African region (25%) and Western Pacific region (17%), while smaller numbers of incident cases were observed in the Eastern Mediterranean region (7%), European region (3%) and region of Americas (3%) (21). Currently, the incidence of TB is falling at about 2% per year, but in order to reach the first milestones of the WHO End TB strategy, this needs to improve to about 4-5% per year by 2020 (21).

EPTB represents an estimated 15% of all TB incident cases and approximately 20-30% of all cases in children (22). Central nervous system TB (CNS-TB) is estimated to represent up to 10% of all EPTB cases and 1% of all TB cases (8, 23). Amongst all CNS-TB cases, TBM is the most common form and most frequently occurs in children (8). As a result of difficulties in diagnosing TBM in children and under-reporting of TBM cases, the number of deaths caused by this disease across the world is unknown (24). However, it is estimated that up to 200 childhood deaths occur across the world every day due to TB (22). A meta-analysis study showed that the risk of death in children with TBM was 19.3% (95% CI, 14.0%-26,1%), indicating that TBM accounts to high number of deaths caused by TB in children (25). In the

Western Cape Province of South Africa, TBM is the most common form of bacterial meningitis, affecting predominantly children below the age of 13 years (8). The WHO aims to end preventable deaths of new-borns and children under 5 years of age by 2030, and have an overall vision of a world free of TB through the end TB strategy, of which the target is 95% reduction in TB deaths by 2035 (21).

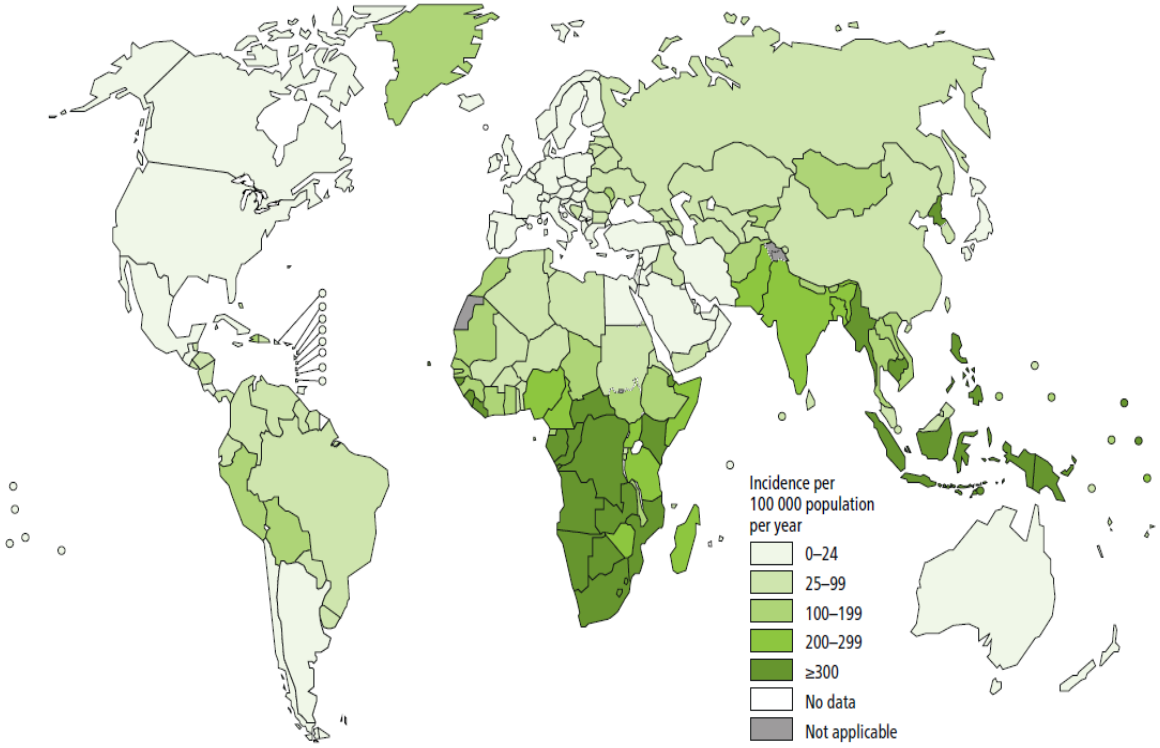

Figure 1.1: The map of the world showing the global estimated incidence of TB. Source: WHO GLOBAL TB report, 2017 (21).

1.3 General Immunology

Immunology is the study of the immune system, including its responses to microbial pathogens and damaged tissues, and its role in diseases (26). The immune system is the collection of cells, tissues, and molecules that mediate resistance to infections (27). The components of the immune system function together to protect the skin, respiratory passages, intestinal tract and other areas from invaders, such as bacteria, fungi, parasites, viruses, cancer cells and toxins (28). Most of the immune system's cells are produced after early childhood, from the primary lymphoid organs (bone marrow, foetal liver and thymus) and are further transported to the secondary lymphoid organs to optimally respond to invaders (29, 30). The immune

system comprises of two main classes: the innate immune system and the adaptive immune system (31).

The innate immune system is viewed as the first line of defence against intruding pathogens (28), and provides early and rapid host protection against infections. This system relies on the expression of germ line-encoded receptors for detection of pathogens. These receptors recognise the pathogen associated molecular patterns (PAMPs) shared by large group of microorganisms (28). Hence the innate immunity provides a non-specific response to foreign particles (32). The mechanisms by which innate immune system eliminate the invaders include prevention of entry via external barriers (anatomical and physiological), complement activation, phagocytosis, inflammation, cytokine secretion, and target cell lysis (33).

In contrast, the adaptive immune system provides a delayed, antigen-dependent and antigen-specific host immune response (22, 26). The specificity of this response is due to the antigen-specific receptors expressed on the surfaces of the adaptive immune cells (31). Both classes of the immune system are driven by several cells, proteins and other components.

The innate immune system consists of anatomical and physiological barriers (Such as the skin, mucous membrane, internal lining epithelium, cilia, and the gastric pH), antimicrobial peptides (defensins and cathelicidins) and proteins (lactoferrin and lysozyme) and humoral component (Such as complement components and acute phase proteins) (34, 35). This system also consists of the cellular components, which mainly include phagocytes (macrophages, neutrophils and dendritic cells [DCs]), mast cells, basophils, eosinophils and natural killer (NK) cells (22, 28). Both phagocytes share a similar function, which is to engulf microbes. In addition, neutrophils contain granules that when released, assist in the elimination of the pathogenic microbes (28), whereas DCs and macrophages function as antigen presenting cells (APCs), acting as messengers between innate and adaptive immunity (37). Macrophages are normally one of the first cells to engulf the invading pathogen and initiate inflammatory response that recruit neutrophils and natural killer cells to the site of infection. The effect of macrophages facilitate the maturation, differentiation and migration of DCs, which play a key role in stimulating the cells of the adaptive immune response (Particularly naïve T cells) (38).

The adaptive immune system mainly consists of the B and T lymphocytes, which are produced in the bone marrow. B lymphocytes mature in the bone marrow, whereas T lymphocytes mature in the thymus (39). B cells are mainly responsible for the humoral immune response via the production of antibodies, moreover, they also play a role in antigen presentation, and hence form part of the professional APCs (together with DCs and macrophages) (28, 32). T

cells express unique antigen-binding receptors on their membranes, known as T cell receptors (TCR), which recognise specific antigens presented by APCs in context of major histocompatibility complex (MHC) (22, 33). The two main types of T cells are cytotoxic T cells (CD8+ T cells) and T helper (Th) (CD4+ T cells) (28). Naïve CD4+ T cells differentiate into various effector T helper (Th) cells, such as T helper 1 (Th1) (which plays role in fighting intracellular pathogens), Th2 (which plays role in fighting extracellular pathogens), regulatory T cells (which regulates the immune response) and other subsets (Th9, Th17, Th22 and T follicular) (42). Activated CD4+ Th cells produce range of cytokines to serve their helper functions: helping B cells to produce antibodies, inducing macrophages to increase their microbicidal strength, recruiting other cells such as neutrophils, eosinophils, and basophils to the site of infection (39). Although CD4+ Th cells, which serve a helper function, are the most well-defined CD4+ T cells subsets, a subset of cytotoxic CD4+ T cells (CD4+ CTL) has been described, suggesting that CD4+ T cells also carry cytotoxic function (43). These CD4+ CTL serve their cytotoxic function by killing target cells in an antigen-specific manner upon direct contact through secretion of cytotoxic granules containing granzyme B and perforin (44). CD8+ T cells (cytotoxic T lymphocytes or CTLs) primarily play a role in immune response against intracellular pathogens, including viruses and bacteria and for tumour surveillance (45). CTLs kill infected and tumour cells via secretion of cytokines (TNF-α and IFN-γ), production and release of cytotoxic granules (containing granzymes and perforin) and Fas/FasL interaction (46).

While the above described T lymphocytes recognize antigenic peptides in the presence of MHC proteins, there are other T cells that recognize peptides without MHC and these cells are collectively called unconventional T cells (47). Unconventional T cells include CD1-restricted T cells, MR1-restricted mucosal associated invariant T cells (MAIT cells), MHC class 1b-reactive T cells and gamma-delta T cells (48). Gamma-delta T cells are the most well-described unconventional cells, which have been shown to mediate killing of target cells directly through their cytotoxic activity or to play their role indirectly by activating other immune cells (47). Immune system processes are orchestrated and regulated by cytokines and chemokines produced by leukocytes and non-leukocytes (49).These proteins are involved in cell proliferation, differentiation, activation, cell-cell interaction, and migration of immune cells (38, 39).

1.4 Immune response against *Mycobacterium tuberculosis*

Although the immune response underlying the pathogenesis of tuberculosis is complex and incompletely understood, it has been appreciated for a long time that the pathogenesis of TB

depends on the interaction between bacterial virulence and host resistance (40, 41). Upon inhalation through respiratory droplets and deposit into the alveoli, *M.tb* has been shown to first encounter the components of the innate immune system: the anatomical and the physiological barriers of the lung, which reduces the transport of microbes to the terminal alveolus (53). However, *M.tb* can manage to enter the terminal alveolus, in which, the *M.tb* is combated by antimicrobial peptides (defensins and cathelicidins) and proteins (lactoferrin and lysozyme) with both bactericidal and immunomodulatory effects (4, 43). These peptides and proteins are produced by multiple cell types including neutrophils, monocytes, macrophages and epithelial cells. Although the relevance of this peptides and proteins in childhood tuberculosis is yet to be established, but they are present in the airways from the early neonatal period (5).

Alveolar macrophages are the primary host cells to encounter mycobacterium tuberculosis (55). The mycobacterium products such as lipoarabinomannan (LAM) and lipoproteins activate these cells via the pattern recognition receptors (PRRs) (best studied PRRs are the two members of the toll-like receptor(TLR) pathway,TLR-2 and TLR-4) (53). Alveolar macrophages plays a vital role in the immune response against *M.tb* infection as they are involved in phagocytosis, killing of the mycobacterium, and the initiation of the adaptive T-cell immunity (56). Alveolar macrophages recognize the pathogen associated molecular patterns (PAMPs) such as LAM, through their PRRs. These pathogen recognition lead to intracellular signalling cascade in the alveolar macrophages, which result into the engulfing of the microbe and secretion of pro-inflammatory cytokines such as TNF-α (57). The alveolar macrophages also release various chemokines such as IL-8, MCP-1, MIP-2 and IP-10 which are thought to serve as attractants for other cells such as macrophages, neutrophils and T cells to site of infection (lungs) (58). Indeed other phagocytic cells including neutrophils, monocyte-derived macrophages and dendritic cells (DCs) are recruited to the infected lungs. These cells also ingest the bacteria and secrete more cytokines and chemokines (48, 49). In response, neutrophils release anti-mycobacterial effectors such as elastase, collagenase and myeloperoxidase (MPO) from their granules, which damages bacterial cells (61). DCs play an important role in initiation of the adaptive immune response. DCs are also one of the first types of cells to encounter the bacilli. When DCs recognises *M.tb* PAMPs, they phagocytose *M.tb*, mature and migrate to the local draining lymph node to present the antigen to the T cells (60). It appears that this migration is promoted by IL-12p40, and can be inhibited by IL-10 (Reviewed in (51)). This antigen-presenting process link the innate and adaptive immune system.

The primed antigen-specific T cells (CD4+T cells and CD8+T cells) then migrate to the site of infection (Lungs), guided by chemokines produced by the infected cells (62). In the infected lungs, effector T cells are thought to mediate protection in a two-step process, firstly, the activation of infected macrophages to produce anti-microbial substances and secondly, the formation of granulomas (63). The subsets of CD4+T cells, Th1 produce IFN-γ and TNF-α, which activate infected alveolar macrophages to mediate enhanced phagolysosome maturation and the induction of highly toxic antimicrobial substances such as reactive oxygen species (ROS) and reactive nitric oxide intermediates (RNIs) (63–65). The importance of IFN-γ in the control of *M.tb* infection have been well-investigated, for instance, studies in mice have shown that failure to produce IFN-γ lead to disseminated mycobacterial infection (Reviewed in 48). In another study carried out in CD4+ T cells deficient mice, it has been shown that IFN-γ produced by CD4+T cells and subsequent activation of macrophages determines the outcome of *M.tb* infection (67). In addition, infants vulnerability to TB have been associated with reduced levels and capacity of several pro-inflammatory cytokines (such as TNF-α, IL-1 and IL-12) and antimicrobial peptides (54).

Furthermore, the influx of T cells into the site of infection (lungs) also leads to the formation of granulomas. Granulomas are considered to be a hallmark of *M.tb* infection and are composed of macrophages, multinucleated giant cells, CD4+T cells, CD8+ T cells, B cells and neutrophils (55, 60, 68). IL-12, IFN-γ, and TNF-α are essential in the formation and maintenance of granulomas (69, 70). These is supported by studies done in mice, in which mice deficient of TNF-α and TNF-α receptor show increased susceptibility to *M.tb* and impaired granuloma formation following infection with *M.tb* (22, 60). The granuloma is vital in the containment of the bacteria, however, it also provide the bacteria with a niche in which they can persist in a latent form, and later get an opportunity to reactivate and spread (72). The necrotic breakdown of infected macrophages, which results into caseating granulomas, (with a cheesy appearance under macroscopic examination), is a major contributor to *M.tb* transmission. In contrast, apoptosis of infected macrophages is detrimental to the infected macrophages, thereby limiting the transmission (73). Generally, TB can result from either early progression of a primary granuloma during the infection process or reactivation of an established granuloma in a latently infected person (74).

1.5 Different stages of the MTB infection spectrum

Only about 5-10% individuals infected with *M.tb* will develop clinical manifestations of active TB disease within the first two years post-exposure and this is known as primary TB, whereas the majority of infected individuals present without clinical signs or symptoms and this is known

as latent TB infection (LTBI) (75). It is estimated that about one-third of the world's population is infected with *M.tb,* without clinical manifestation. The conventional and simplified classification of *M.tb* infection comprises the LTBI and active TB disease. However, LTBI has diverse presentations ranging from individuals who have completely cleared the infection, to those containing actively replicating bacteria in the absence of clinical manifestation (76). Individuals with LTBI represent a reservoir for potential future development of TB disease (77). Although latent infection is asymptomatic, there is a great danger of reactivation leading to active TB disease (75). Similar to LTBI, Active TB disease is characterized by diverse pathological presentations ranging from sterile tissue, to caseous hypoxic lesions containing variable numbers of bacteria, to liquified cavities with a massive load of replicating organism (76). Studies proposed that *M.tb* may therefore be viewed as a continuous spectrum including sterilized immunity, subclinical active TB disease and fulminant active TB disease (76).

1.5.1. Latent infection

Latent infection is described as a state in which an individual has evidence of *M.tb* infection as ascertained by positivity for immunologic tests (tuberculin skin test or interferon-gamma release assay [IGRA]), without clinical manifestation (75). This can be described as a state in which the host's immune response is able to control the infection but cannot completely eliminate the bacteria. This control is mediated by the formation of a granuloma. As highlighted in section 1.4, a granuloma is a hallmark of TB and it is composed of various inflammatory cells including macrophages, multinucleated giant cells, CD4+T cells, CD8+ T cells, B cells and neutrophils. The main function of the granuloma is to wall off the infection and prevent dissemination (78). Although latent infection is generally associated with the absence of clinical symptoms, recent researches propose new models for latent TB infection. A review by Barry et al (76) discussed that some of the findings observed in individuals with latent TB correspond to features seen in active TB disease. Following this, the review proposed that *M.tb* infection should therefore be viewed as a continuous spectrum (76).

1.5.2. Active TB disease

Active TB disease can either occur as primary TB disease following initial infection or can be due to reactivation of latent TB infection. Amongst the factors contributing to reactivation of latent TB are HIV, immune suppressive treatment, malnutrition, young age and tobacco smoke (75). Generally, those who are HIV infected and other immunocompromised individuals are at a much higher risk of reactivation (79). Majority of TB cases (90%) among adults results from

reactivation of latent TB infection (80). Young children are at high risk of infection following exposure and progresses more readily from infection into active TB disease (38). Correspondingly, the immune system of young children has been associated with vulnerability to tuberculosis (39, 40). The clinical manifestation of active TB disease can range from pulmonary TB to extrapulmonary TB. Pulmonary TB commonly present with symptoms such as cough, fever, weight loss, night sweat and fatigue. Children present with mild symptoms such as low-grade fever and unproductive cough (81).

Extrapulmonary TB is due to hematogenous and lymphatic spread of the *M.tb* bacilli to other organs including the lymph nodes, joints, pleura and brain meninges (7). The spread of the *M.tb* bacilli to the brain meninges results into tuberculous meningitis. TBM is more common in children and immunocompromised individuals (such as those living with HIV), and it is thought that the differences in immune responses between children and adults play a role (4). Bacillus Calmette-Guerin (BCG) vaccination provides some degree of protection against the severe forms of TB (milliary disease and TBM), although severe disease manifestations still occur in most TB-endemic areas (9).

1.5.2.1 Tuberculous meningitis

Fifty years after Robert Kock discovered the cause of tuberculosis, two pathologists, Rich and McCordock demonstrated using a series of experiments in rabbits and post-mortem findings in children, that the release of *M.tb* bacilli into the meningeal space from focal sub-pial or sub-ependymal lesions cause tuberculous meningitis (TBM) (82). Although TBM accounts for about only 1% of all cases of TB, it is the most dangerous form of TB, presenting with high morbidity and mortality, mostly in young children (83). TBM has been associated with neurological sequelae including, exudate obstruction of CSF flow resulting into hydrocephalus, formation of tuberculomas or abscesses resulting in focal neurological signs and obliterative vasculitis causing infarction and stroke syndromes (19). Similar to pulmonary TB, the pathogenesis of tuberculous meningitis is dependent on the interaction between the host immune response and the bacterial virulence. Absence of an adequate cell-mediated immunity has been associated with development of tuberculoma or tuberculous brain abscesses (84)

The development of tuberculous meningitis is a two-step process, firstly, *M.tb* bacilli enters the body through droplet inhalation, as described in pulmonary TB. Alveolar macrophages are the first cells to encounter the bacilli, and the subsequent mechanisms result into the formation of a granuloma. However, prior to granuloma formation, during the localised infection within

the lungs, bacilli are filtered into the draining lymph node and there is a short, but significant bacteraemia that can transmit bacilli to other organs in the body or the bacilli can gain its way to the central nervous system (CNS) by secondary reactivation from a “leaked granuloma” (70, 71, 73, 74). This extensive bacteraemia increases the probability that a sub-cortical focus will be established in the CNS (87). Although the CNS is protected by the Blood-Brain-Barrier (BBB), however, there are a number of bacterial and viral pathogens capable of crossing this barrier and causing subsequent meningitis or encephalitis (88). The mechanisms by which the bacilli evade the BBB are not fully elucidated. In theory, the bacilli can pass the BBB and access the meninges in one of the following ways: (i) As an extracellular organism or (ii) as an intracellular organism (Via infected monocytes or neutrophils) (88). Within the meninges or brain parenchyma, the bacilli form small sub-pial or sub-ependymal foci, called Rich Foci (82).

Secondly, the Rich Foci ruptures, releasing *M.tb* bacilli into the subarachnoid space, this triggers a robust inflammatory T cell responses along the meninges (76, 77). Although the immune responses within the brain during TBM remains poorly understood, the complications of TBM are largely dependent on the severity of inflammatory responses. In the CNS, microglia are considered the first line of defense against infectious agents such as *M.tb* and they carry their function through: migration to the site of infection, phagocytosis of self and non-self products, induction of Reactive Oxygen Species (ROS), production of cytokines and chemokines including MCP-1, RANTES, CXCL10, G-CSF, GM-CSF, IL-1, IL-1α, IL-1β, IL-10, IL-12p40,IL-6 and TNF-α (Reviewed in 71). The balance between pro-inflammatory and anti-inflammatory cytokines is thought to be crucial in the TBM disease progression, this is reflected by the up-regulation in CSF pro-inflammatory cytokines and anti-inflammatory cytokines in patients with TBM compared to patients with other forms of meningitis, such as other bacterial and viral meningitis (75, 79–81). Studies on CSF and serum cytokines levels in patients with TBM have found elevated levels of pro-inflammatory cytokines including TNF-α and IFN-γ, emphasising the inflammation occurring during meningeal TB (93).

Poor clinical outcomes of TBM have been associated with convulsions, headache, motor deficit, brainstem dysfunction and cerebral infarctions on neuroimaging (94). The Medical Research Council scale for prediction of TBM stage has criteria that classify TBM stages as follows ,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 and stage III –GCS < 11 (84, 85). This scale is used to classify the severity of TBM and relies on Glasgow coma scale (GCS) which is scaled between 3 and 15, where 3 is the worst and 15 is the best (reviewed in 61). The GCS depends on the assessment of three factors: best eye response,

best verbal response and best motor response (96). This classification is used to determine TBM disease severity.

1.6 TB vaccination and the influence of vaccines on diagnostics

Bacillus Calmette-Guérin (BCG) remains the only licensed vaccine for prevention of TB worldwide (97). BCG is a live attenuated bacterial vaccine derived from mycobacterium bovis that was originally isolated in 1902 (98). As of 1974, WHO Expanded Programme on Immunization recommends BCG should be given as soon as possible after birth in high TB prevalence countries, with coverage in infants exceeding 80% (99). Although the efficacy of BCG in preventing the development of adult pulmonary TB is controversial, BCG vaccination clearly protects infants and children from tuberculous meningitis and other severe forms of disseminated TB (100). A systematic review and meta-analysis of 14 case-control studies examining BCG vaccine efficacy against meningitis revealed that the incidence of TB meningitis was reduced by 73% (98). BCG vaccination of infants, at birth or as soon as possible after birth, is one of the key components of pillar 1 of the WHO End TB strategy (98). According to WHO, it is estimated that high global coverage of 90% and widespread use of BCG in routine infant vaccination programmes could prevent 115 000 TB deaths per birth cohort in the first 15 years of life (98).

BCG has been associated with false positive tuberculin skin test (TST) results. However, this is controversial, as other studies refutes this belief (101). It is thought that BCG vaccination causes some difficulties in the interpretation of TST results, as there is no reliable method to distinguish the reaction caused by vaccination, from that caused by infection with TB or other mycobacteria (102). A study has reported that BCG vaccination at the age of 0-2 months affects TST for a long period and this remains the case until 6 years of age (102). Other studies have demonstrated that BCG has no significant effect on TST positivity after 10 years or more of vaccination (103) .

1.7 Diagnosis of *M.tb* infection

The detection of latent TB infection is currently based on indirect methods which rely on the cell-mediated immunity (memory T cell response) to TB antigens (104). The current acceptable approaches include the tuberculin skin test (TST) and the interferon gamma (IFN-γ) release assays (IGRA). Although acceptable, these tests have well publicised limitations (104).

TST is the most commonly used diagnostic test for TB infection. This test employs Purified Protein Derivate (PPD) from various mycobacterial peptides (105). The test is based on the immune response to PPD as indicated by Delayed Type Hypersensitivity (DTH) reaction (88, 89). In individuals with cell-mediated immunity to this tuberculin antigens, a delayed type hypersensitivity is expected to occur after 48-72 hours, following intradermal administration of PPD (104). The DTH is measured as localized skin induration in millimetres after 48-72 hours at the site of injection (108). Diagnostic accuracy of TST is limited by inability of the test to distinguish active TB (ATB) disease from latent TB infection (LTBI) (107). TST has several limitations including false positive results due to prior or repeated BCG vaccination and false negative results due to low sensitivity in immunocompromised individuals (87, 91, 92).

Interferon-Gamma Release Assays (IGRAs) are commercially available and are increasingly used TB diagnostic tests. The only two commercially available IGRAs are QuantiFERON TB assay from Australia and the T.SPOT TB assay from Oxford (87, 91). Both assays are based on cell-mediated immune response to mycobacterial proteins (88, 91). These proteins stimulate patient's T cells to release IFN-γ *in vitro*. QuantiFERON TB assay is a whole blood assay which measures IFN-γ release by patients T cells in response to mycobacterial proteins, through ELISA, while T SPOT.TB assay measures the number of patient's T cells that releases IFN-γ, through ELISPOT (106). The QuantiFERON TB Gold in tube (QFT-GIT) assay measures IFN-γ released in response to *M.tb* antigens (ESAT-6, CFP-10 and TB-7.7). In contrast, the recent and enhanced QuantiFERON TB Gold plus (QFT-Plus) assay contains the TB1 and TB2 tubes. TB1 tube contains long peptides derived from ESAT-6 and CFP-10, which specifically stimulate the release of IFN-γ from CD4+ T cells, whereas in addition to the same long peptides, TB2 contains newly introduced shorter peptides to stimulate the release of IFN-γ from both CD4+ and CD8+ T cells (111).

QFT-Plus has a reported higher sensitivity of 95% (accurately identifies patients with TB infection) and a higher specificity of 98% (limit unnecessary follow-up and treatment) according to the manufacturer (112). In contrast to TST, the later developed immunological based TB diagnostic tests, IGRAs, have no cross-reactivity with BCG, because proteins used in the assay are specific to *M.tb* and are absent in BCG (113). False positive IGRA results have been reported in cases of other mycobacterial infections, such as *Mycobacterium mavium*, *Mycobacterium kansasii* and *Mycobacterium szulagas* (109). Like TST, false negative results have also been reported in IGRA, particularly in cases of immunodeficiency, or immunosuppression. IGRA has a sensitivity of 79-86% and specificity of 97-98% in immunocompetent children, but the diagnostic accuracy drops to sensitivity of 47% and specificity of 90% in immunocompromised children (109). IGRA have been reported to have

reduced sensitivity in younger and malnourished children (114). Thus, studies recommend IGRA to only be used in children aged 4 years or older and in combination with other clinical data. Unfortunately, IGRA cannot distinguish latent TB infection from active TB disease (113).

1.8 Diagnosis of TB disease

The diagnosis of childhood pulmonary tuberculosis is challenged by lack of a practical diagnostic gold standard (115). The South African National TB guidelines for children recommend that diagnosis of pulmonary TB in children must be based on a combination of clinical presentation, history of exposure, bacteriology (GeneXpert, smear microscopy, culture and drug sensitivity testing or line probe assay [LPA], chest X-ray and tuberculin skin test (Mantoux) (116). Clinical features associated with pulmonary TB include a fever and cough lasting longer than 2 weeks, loss of appetite, unusual fatigue, unexplained loss of weight and physical signs such as enlarged lymph glands and night sweats. Chest radiography are used to investigate lesions suggestive of TB disease (98, 99). However, these diagnostic parameters are not specific to TB disease and hence may provide a non-specific diagnosis (119). Clinical features and lung changes are not specific to pulmonary TB and may overlap with other lung diseases such as lung malignancies or pneumonia (120). Hence, in addition to clinical suspicion, further laboratory techniques (bacteriological) including GeneXpert, smear microscopy, culture and drug sensitivity testing or line probe assay (LPA) are done to confirm active TB disease (Presence of mycobacterium tuberculosis).

1.8.1 Laboratory confirmation of childhood TB disease

Although the microbiological confirmation of TB is essential for diagnosis of pulmonary tuberculosis (Adult-type), it is not done in many high burden settings for diagnosis of TB in young children due to difficulty in obtaining good quality specimen (121).

1.8.1.1 Specimen-type used

Microbiological diagnostic tests rely largely on the quality of the specimen. However, children with TB do not readily expectorate sputum, hence making it difficult to perform microbiological tests (102, 103). As a result, in the case of pulmonary TB, clinicians collect other specimen types including gastric aspirate (GA) to improve the microbiological diagnosis of TB in children and also in individuals with paucibacillary disease (124). It is recommended that a fasting and early morning GA should be collected to obtain sputum swallowed during sleep (125).

However, GA is invasive and not always possible in TB endemic areas (124). Sputum induction (SI) is relatively a non-invasive procedure that allows collection of sputum samples from individuals such as children, who are unable to produce or expectorate sputum (124). SI allows collection of sputum samples from children as young as one month of age and result in better performance than GA (125).

1.8.1.2 Smear microscopy

Ziehl Neelsen (ZN) sputum smear microscopy remains the primarily used diagnostic tool for bacteriological diagnosis of TB in both children and adult in most settings and it is recommended by WHO (16, 106). According to WHO, one smear-positive result is required for a diagnosis of pulmonary TB (21). To enable more rapid diagnosis, fluorescent microscopy was developed, and it has been found to be on an average of 10% more sensitive than conventional microscopy. However, the use of fluorescent microscopy is limited by its high cost, frequent burn-off of expensive mercury vapour lambs, continuous power supply necessity and need for dark room (102, 107). Presently, the light-omitting diode (LED)-based fluorescent microscopy technique has been developed as the best alternative to ZN-staining. It is cheap, robust, and consumes low energy (107, 108).

In adults the examination of acid fast bacilli (AFB) stained smears from clinical specimens is used to diagnose TB, however, children with pulmonary TB usually do not readily expectorate sputum, hence sputum sample is difficult to obtain (102, 103). As a result, gastric aspirate and induced sputum samples can be used when sputum samples cannot be obtained. Unfortunately, even with these methods only fewer than 20% of children are AFB smear positive (129). In a meta-analysis of 20 studies, the pooled percentage of children that were sputum smear positive was 6.8% (126). Although smear microscopy from gastric aspirate frequently yield positive results than the use of induced sputum, the rate of positivity of smear microscopy from both gastric aspirate and induced sputum specimen is <20% (126). Additionally, children present with paucibacillary TB disease and many may be smear-negative as smear microscopy has the detection range between 10^4 and 10^5 bacilli/ml (127).

1.8.1.3 Culture-based methods

Culture-based methods are often used to further confirm TB disease. Culture remains the reference standard for diagnosis of TB disease and it is recommended by WHO (21). Although culture-based methods are more sensitive than smear microscopy, *M.tb* is a slow growing bacteria, which requires about 4-8 weeks to grow, hence this delays appropriate treatment in

the absence of a confirmed diagnosis (130). Culture methods include the solid culture medium (Löwenstein-Jensen [LJ] medium) and the liquid culture (such as Middlebrook 7H9) (105, 111). In most high-burden countries, the use of LJ medium remains the common method for performing mycobacterial culture. LJ requires a long period to yield results (3-8 weeks). Automated liquid culture systems such as Bactec MGIT 960 OR 320 system (BD) are more rapid (10 days), providing faster detection of M.tb compared to solid medium system (125). However, automated liquid culture systems are costly and more susceptible to contamination (131). In paediatric TB, culturing mycobacteria was reported to confirm TB in only up to 30-40% (132). In a study on children with TB, culture revealed growth in only 16.2% of the specimen (133). Sputum and gastric aspirate cultures from children with pulmonary TB are sensitive in less than 50% of all cases (134).

1.8.1.4 Molecular diagnostic methods

Recently, a rapid molecular test called Xpert® MTB/RIF assay /GeneXpert (Cepheid, USA) has been developed. It can provide results within 2 hours. GeneXpert is currently recommended by WHO for diagnosis of Tuberculosis (21). GeneXpert was initially recommended (in 2010) for diagnosis of pulmonary TB in adults, and since 2013 the use of GeneXpert for diagnosis of childhood TB and other specific forms of extrapulmonary TB was recommended (21). GeneXpert MTB/RIF assay is a novel fully automated molecular diagnostic test, which is based on real-time PCR (115, 116). In addition to TB diagnosis, GeneXpert rapidly detects rifampicin resistance in clinical specimen (136). Studies on childhood TB shows high sensitivity and very high specificity of GeneXpert when using sputum specimen (117, 118). A pooled sensitivity for pulmonary TB diagnosis in children varied among studies and specimen type. Expectorated sputum offered 55%-90%, induced sputum offered 40%-100% and gastric lavage offered 40%-100% (139). The pooled specificity amongst these studies varied from 93% -100%, irrespective of specimen (139). Unfortunately, infrastructure, instrument, human resource requirements and cost of GeneXpert exceeds expectations of a point of care (POC) diagnostic test, hence a limitation to resource-limited areas (140).

GeneXpert Ultra is a recently developed improved diagnostic test for TB and it has been shown to overcome the shortcomings of GeneXpert MTB/RIF including significant improved TB detection especially in patients with paucibacillary disease (141). Xpert Ultra showed higher sensitivity in the diagnosis of pulmonary TB than GeneXpert MTB/RIF. In paediatric TB using respiratory samples, Ultra showed sensitivity of 71% compared to 47% for GeneXpert MTB/RIF (142). However, a multicentre comparative accuracy study reported lower sensitivity

of Xpert Ultra in the diagnosis of TB in children compared to adults and a reduced specificity in comparison with GeneXpert (143).

1.8.2 Diagnosis of EPTB disease

EPTB is typically more difficult to diagnose than pulmonary TB, because it presents with non-specific symptoms, and it requires invasive procedures for diagnosis. Most individuals with EPTB do not have lung involvement and are usually negative for sputum smear. Diagnoses of EPTB is even more difficult in poor settings due to lack of required facilities. The diagnosis of EPTB depends largely on the direct detection of *M.tb* bacilli in tissue or smear through the use of smear microscopy, culture methods, molecular tests (Xpert MTB/RIF) (144). Additionally, other indirect methods such as histopathology, cytology, TST, IGRA and adenosine deaminase assay are employed (144).

1.8.2.1 Diagnosis of TB meningitis

Although the guidelines for diagnosis of paediatric TBM are not clearly stated, several studies have reported that in the absence of bacteriological confirmation from cerebrospinal fluid (CSF), the diagnosis of TBM can be made on the combination of clinical presentation, CSF findings, brain imaging, evidence of TB elsewhere, and exclusion of alternative diagnosis (8, 125). Symptoms of TB include low-grade fever, malaise, headache, dizziness, and vomiting in the first few weeks and later the development of more severe headache, altered mental status, stroke, hydrocephalus and cranial neuropathies (8, 98). Brain imaging techniques such as Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) are also used to investigate brain changes associated with tuberculous meningitis, such changes include basal enhancement, pressure of exudates, hydrocephalus, periventricular infarcts, mild meningeal enhancement, basal exudates, small tuberculomas and lacunar infarcts (126, 127). In addition, routine CSF findings such as raised white cell count (100-500 cells/μL) with predominant pleiocytosis, and neutrophil predominance, elevated protein levels (100-500mg/dL) and hypoglycorrhachia (low CSF glucose level, less than 45mg/dL) may suggest tuberculous meningitis (147). Clinical features and brain changes are not specific to tuberculous meningitis and may also be seen in other bacterial meningitis or viral meningitis (6). Bacteriological confirmation of *M.tb* bacilli in CSF samples is required in order to make a definite diagnosis of TBM.

Although smear microscopy is the most widely used TB test, low sensitivity of CSF AFB microscopy has been reported. The reported sensitivity of AFB microscopy in CSF varies

amongst several studies, with many studies reporting poor sensitivity. A study reported sensitivity of 7.9% on CSF AFB microscopy and several other studies reported smear-positivity for AFB in CSF microscopy to be <10% (148). The sensitivity of CSF smear microscopy can be improved by collection of large volumes and centrifuging CSF at high speed, however the concept might not be practical in clinical settings, especially in young children. Culture remains the gold standard for detection of *M.tb* bacilli in human specimen. Although the use of CSF culture yields varying sensitivity (approximately 40-80%), it is more sensitive than smear microscopy and it is necessary in determining drug susceptibility (147). However, culture requires several weeks to yield results, it is prone to contamination and requires well-established microbiological laboratory. GeneXpert is recommended by WHO for use in the diagnosis of EPTB including TBM. However, poor sensitivities have been reported in the diagnosis of TBM. The use of large CSF volumes and centrifuging CSF at a high speed for use on GeneXpert provides better sensitivity (149). The use of large volumes of CSF was shown to improve GeneXpert sensitivity up to 72% (149). In another study, the use of large volumes of concentrated CSF and additional vortexing steps showed a sensitivity of 59% and a very high specificity of 99% for GeneXpert (150). A newly introduced molecular test, Xpert Ultra, is arguably more improved than GeneXpert. In a more recent study, Xpert Ultra showed higher sensitivity (95%) compared with either Xpert MTB/RIF or culture in the diagnosis of tuberculous meningitis in adults (151).

1.9 Diagnosis of childhood TB disease

According to childhood TB reports, everyday there are up to 200 children deaths caused by TB across the world (22). Childhood TB cases are often missed or overlooked due to non-specific symptoms and difficulties in diagnosis (22). The consequences of under-diagnosis are indicated by increased childhood morbidity and mortality related to TB. To a lesser extent, overdiagnoses of childhood TB also occurs, which results into unnecessary burdens of long-term treatment imposed on both the patient, family and the health care system (152). Difficulties in case finding make it hard to assess the actual magnitude of childhood TB epidemic, hence the childhood TB cases may be higher than currently estimated (22). Amongst the WHO strategies for ending TB-associated death, it is recommended that research should include children in clinical trials testing new diagnostics and that new diagnostics suitable for children should be developed (16, 17).

1.10 New approaches towards the development of diagnostic tools for TB disease

The shortcomings of the current diagnostic approaches results in delayed diagnosis and contribute to the poor outcomes associated with childhood TB (10). Studies shows that a better prognosis of this disease depends on early diagnosis and initiation of appropriate therapy (153). The world health organisation (WHO) recommend that new diagnostic tests suitable for use in childhood TB need to be developed (22).Preferably, a good diagnostic test should have high accuracy (High sensitivity and specificity), be rapid (at point of care), easy to use, cheap, and use non-invasive samples, such as blood, urine and saliva (154). Researches are currently on-going across the world, with aim of finding improved diagnostic methods for tuberculosis. Immunological biomarkers and gene expression biosignatures are amongst the widely investigated approaches for diagnosis of TB disease.

Following the development of IGRAs, studies have evaluated other immunological biomarkers other than the IFN-γ in whole blood stimulated with *M.tb* antigens (155–157). Indeed, these studies showed that immunological biomarkers other than IFN-γ were detectable in whole-blood stimulated with *M.tb* antigens and hold promise in the diagnosis of TB disease. Furthermore, a study by Chegou et al showed that host markers including IFN-α2, IL-1Ra, sCD40L, IP-10 and VEGF detectable following overnight stimulation of whole blood with *M.tb* antigens had potential in the diagnosis of TB in children (158). However, despite the potential shown by these immunological biomarkers, a good POC diagnostic should preferable be rapid, and hence, a test that requires overnight stimulation may not be convenient. As a result, recent studies focus more on the evaluation of biomarkers in unstimulated samples such as serum, plasma, urine and blood.

Another importantly shown aspect to the immunological host markers approach of diagnosis is that diagnostic accuracy of markers increased when markers were used in combinations (158). In a more recent large multi-centered study, a combination of seven serum host biomarkers (C reactive protein (CRP), transthyretin, IFN-γ, Complement Factor H (CFH), Apolipoprotein-A1 (Apo-A1), Inducible Protein 10 (IP-10) and Serum Amyloid A) detected from unstimulated serum samples diagnosed TB disease in adults with high accuracy (sensitivity of 93.8% and specificity of 73.3%). A five-marker salivary biosignature comprising of IL-1β, ECM-1, HCC1, IL-23 and fibrinogen diagnosed adult TB disease with sensitivity of 88.9% and specificity of 89.7%, regardless of HIV infection status (159). Another study identified 18 potential biomarkers including NCAM, CRP, SAP, IP-10, ferritin, TPA, I-309 and MIG in plasma samples, which diagnosed adult TB disease individually with area under the ROC curve (AUC) ≥0.80 and when the biomarkers were analysed on general discriminant analysis (GDA), a 6 marker plasma biosignature comprising of NCAM, SAP, IL-1β, sCD40L, IL-13 and

Apo-A1 was identified, which diagnosed TB disease with a sensitivity of 100% and specificity of 89.3%, irrespective of HIV infection status.

In a previous study, host immunological biomarkers were investigated in CSF and serum samples from children who were suspected of having TBM as potential diagnostic candidates. Although a limited panel of analytes (a single standard Luminex 27 panel only) was evaluated in these children, some of the host markers detectable in CSF showed potential in the diagnosis of TBM in the children. Importantly, a 3-marker CSF biosignature comprising of VEGF, cathelicidin LL-37 and interleukin (IL)-13 showed potential as a diagnostic biosignature for childhood TB, diagnosing the disease with a sensitivity of 52% and specificity of 95% (160). Another study on paediatric TBM suggested that elevated levels of IL-12p40, IP-10, monocyte chemoattractant protein 1 (MCP-1) were associated with tuberculomas, whereas elevated levels of tumour necrosis factor-α (TNF-α), macrophage inflammatory protein (MIP)-1α, IL-6 and IL-8 are associated with infarcts in the brain (12). 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 (161) and children (162). A study investigating RNA markers specifically in TBM identified four gene markers (glial fibrillary acidic protein (GFAP) , serpin peptidase inhibitor, clade A member 3 (SERPINA3), thymidine phosphorylase (TYMP/ECGF1) and heat shock protein 70 KDa protein 8 (HSPA8), which were associated with TBM in HIV positive individuals, using brain tissues (163).

Recent studies showed that host biomarkers can be translated into field-friendly diagnostic tools based on a simple lateral flow technology (144, 145). A simple test based on host biomarkers could overcome most of the shortcomings of the current available tests including high costs of operation, electricity usage and need for sophisticated laboratories. Such a test will allow screening for TB disease (pulmonary TB or extrapulmonary) at the point-of-care or bedside and can be a breakthrough in the diagnosis of childhood TB, including in resource limited settings.

However, most of the potential biosignatures identified so far were done in adult studies and majority of these host markers have not been evaluated in children. Therefore, the potential of these biosignatures is yet largely unknown in this population. There is a need to evaluate the potential usefulness of host biomarkers that have shown potential in adult studies and other new host biomarkers in children, especially as such biomarkers may contribute to the easier diagnosis of TB disease in this traditionally difficult to diagnose patient group. Furthermore, other biomarkers identified in childhood TB specific studies, for example, the 3-marker CSF biosignature that was identified by Visser et al (160) (IL-13, cathelicidin LL-37

and VEGF) require further validation in new cohorts of children with suspected meningitis. Furthermore, it is yet unknown whether such a biosignature (sensitivity of 52% and specificity of 95%) can be further optimised using new host biomarkers, including the biomarkers that have shown potential in adult studies, but which have not been evaluated in children.

In the present project, we therefore aimed to validate the previously identified CSF 3-marker biosignature in a new cohort of study participants, investigate whether the biosignature could be further optimised using new host markers, investigate whether better candidate biomarkers were available and also to assess blood-based biomarkers for the diagnosis of both paediatric pulmonary TB disease and TBM. Of particular interest was the performance of the previously identified adult 7-marker serum protein biosignature (166) in children with pulmonary TB and TBM.

1.11 Study specific aims and objectives

Specific aim 1: To evaluate the utility of a previously established 3-marker CSF protein biosignature in the diagnosis of TBM in a new cohort of children and explore other CSF-based biosignatures for the diagnosis of TBM.

Objective 1.1: To evaluate the usefulness of the previously identified 3-marker CSF biosignature (IL-13, VEGF, and cathelicidin LL37) in the diagnosis of TBM, in a new cohort of children with suspected meningitis.

Objective 1.2: To investigate whether the previously established CSF biosignature (objective 1) can be optimised by using new CSF biomarkers

Objective 1.3: To investigate whether any new CSF biosignatures show potential in the diagnosis of TBM in children

Objective 1.4: To collect and bank CSF samples from children with suspected meningitis for use in the discovery and validation of diagnostic biosignatures for TBM in future.

Specific aim 2: To evaluate the utility of a previously established adult 7-marker serum protein biosignature and other blood-based biomarkers in the diagnosis of pulmonary TB and TBM in children

Objective 2.1: To evaluate the usefulness of a previously established adult 7-marker serum protein biosignature (CRP, transthyretin, IFN-γ, CFH, Apo-A1, IP-10 and SAA) in the diagnosis of pulmonary TB disease in children.

Objective 2.2: To evaluate the usefulness of a previously identified adult 7-marker serum protein biosignature in the diagnosis of TBM in a cohort of children with suspected meningitis.

Objective 2.3: To investigate whether the previously established adult 7-marker serum protein biosignature could be optimised by using new serum biomarkers, for diagnosis of pulmonary TB or TBM.

Objective 2.4: To investigate whether any new childhood TB-specific serum biosignatures show potential in the diagnosis of pulmonary TB or TBM.

1.12 Study design

Different approaches were taken for different studies, based on the design used in the parent studies, in the case of the pulmonary TB diagnostic study. In the TBM project, a prospective design was used, in which participants with signs and symptoms suggestive of TBM were enrolled and classified into TBM and no TBM (included other causes of meningitis including bacterial meningitis, viral meningitis and other conditions) based on the routinely used diagnostic procedures employed at the tertiary hospital where patients were enrolled.

In pulmonary TB study, children with confirmed pulmonary TB (TB cases) and those diagnosed as unlikely TB (No-TB controls) were compared in a case-control design, using banked samples.

Chapter 2

Materials and Methods

2.1 Study setting

The studies described in the present thesis were carried out at the the Stellenbosch University Immunology Research Group (SU-IRG) laboratory, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences. The SU-IRG laboratory is accredited by the South African National Accreditation Agency under ISO 15189, as a specialist immunology research laboratory.

The institution is situated in the Tygerberg district, in the Western Cape Province of South Africa. The present studies enrolled children from the Tygerberg Children's Hospital and the Red Cross War Memorial Children's Hospital. These hospitals are situated in Cape Town, Western Cape Province of South Africa. According to the WHO report of 2017, the total TB incidence best estimate for South Africa was 781 per 100 000 population, and the uncertainty intervals were 543-1060 per 100 000. Previous surveys and studies showed that the Western Cape Province had the highest reported rate of all types of TB cases in South Africa (167). Furthermore, TBM was the most prevalent cause of paediatric bacterial meningitis in the Western Cape (168). The Tygerberg Children's Hospital is the tertiary academic hospital of the Faculty of Medicine and Health Sciences, University of Stellenbosch. Children with suspected TBM in primary day care hospitals, district and secondary level hospitals from the hospital's drainage areas are referred to the Children's Hospital to establish the diagnosis of TBM and to treat the complications associated with advanced disease (stage 2 and 3 TBM, e.g. Hydrocephalus). An average of 40 new TBM cases are admitted to the hospital's wards annually, even though this amount soured in 2017, probably owing to the shortage of BCG, hence the lack of BCG vaccination of children in recent years. The Red Cross War Memorial Hospital is a main referral institution for children under the age of 13 years in the Western Cape Province, South Africa. However, it also offers services to patients from all over South Africa and from other African countries.

2.2 Study Participants

2.2.1 TB meningitis diagnostic biomarkers study

The children included in this study were enrolled at the Tygerberg Children's Hospital, Parow Valley, Cape Town, South Africa, between December 2016 and November 2017. Children were enrolled if they presented with signs and symptoms suggestive of meningitis and requiring CSF examination for routine diagnostic purposes. Children were eligible for the

study if they were between the age of 3 months and 13 years, when written informed consent was provided by a legal guardian. Additionally, assent was obtained in children older than 7 years who had a normal level of consciousness, as assessed by the Glasgow Coma Score (GCS), whereby a GCS of 15/15 was considered as normal level of consciousness. Children were excluded from this study if they were older than 13 years and if written informed consent was not obtained. Some of the severely ill children admitted to the hospital during the study period (December 2016 and November 2017) were not recruited owing to these children being too ill and requiring emergency treatment. All the children enrolled into the study were TB treatment naïve.

2.2.2 Pulmonary tuberculosis diagnostic biomarkers study

Children who provided samples for this study were previously enrolled at the Red Cross War Memorial Children's Hospital, Cape Town, South Africa. Briefly, children presenting with signs and symptoms requiring investigation for pulmonary TB were recruited and samples collected and banked over multiple years. The children were later characterised as patients with TB disease or no-TB controls through extensive investigations using available clinical, laboratory and radiological investigations. Written informed consent was obtained from parents or legal guardians of all study participants prior to the enrolment of the children into the study.

2.3 Ethics statement

Where applicable (as discussed in 2.2.1 and 2.2.2), written informed consent was obtained from parents or legal guardians of the children enrolled in to the studies and where possible, assent was obtained from the child in addition to consent from the parents or guardians. The TBM study was approved by the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences of the University of Stellenbosch (Stellenbosch University ethics reference number: N16/11/142) (Principal Investigators; NN Chegou and R Solomons), and the pulmonary TB diagnostic study was approved by the University of Cape Town's Faculty of Health Sciences Human Research Ethics Committee (principal investigators, H Zar and Mark Nicol). Both studies were additionally approved by the respective hospitals (the Tygerberg Academic Hospital and the Red Cross War Memorial Hospital) respectively, and the City of Cape Town.

2.4 Sample collection and diagnostic work-up

2.4.1 TBM diagnostic biomarkers study

As none of the children enrolled into this study were recruited solely for purposes of the present

study, samples were collected during specimen collection for routine diagnostic procedures. That is, during the collection of samples (CSF and whole blood) for routine diagnostic purposes, all participants provided additional 1ml of whole blood and 1ml of CSF for the purpose of this research project. Following the collection of the routine diagnostic samples, whole blood was collected into BD Vacutainer® serum tubes and transported to the research laboratory (SU-IRG) at ambient conditions. In the laboratory, tubes were centrifuged at 1200 xg for 10 minutes, after which serum was harvested, aliquoted and stored at -80 °C until use. CSF was collected into a sterile (plain) tube and transported to the laboratory on ice. Upon arrival in the laboratory, CSF samples were centrifuged at 4000 xg for 15 minutes, after which supernatants were harvested, aliquoted and stored at -80 °C until use. All CSF samples were processed in a Biosafety level III (BSL-3) laboratory.

To be able to classify the study participants as having TBM, other causes of meningitis or no meningitis, a comprehensive clinical evaluation of all patients was done by a specialist paediatric neurologist (addendum 1). The routine investigations performed included clinical examination (signs and symptoms, history of TB contact, HIV test, GCS, tuberculin skin test, and chest X-ray), computed tomography (CT) and magnetic resonance (MR) imaging when clinically indicated and air-encephalography (in all children with hydrocephalus). Furthermore, lumbar puncture was performed and investigations including appearance and colour determination, differential cell counts, protein, glucose, chloride and other routinely investigated markers were assessed, followed by centrifugation of the CSF, Gram staining, India ink examination, culture of the centrifuged sediment on blood agar plates (for pyogenic bacteria), Auramine “O” staining and fluorescence microscopy (for *M.tb*), culture using the mycobacterium growth indicator tubes (MGIT)^{TM} method (Becton and Dickinson) and examination for *M.tb* using HAIN Genotype MTBDRplus kit (Hain Lifescience GmbH, Germany).

Using a uniform research case definition based on a scoring system consisting of clinical, CSF, and neuroimaging findings (145), diagnosis of probable TBM was made if two or more of the following criteria were present in combination with 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 TB (hilar lymphadenopathy), miliary TB 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 health cards, or positive identification of acid fast bacilli from gastric washings (microscopy or culture). A diagnosis of definite TBM was made if acid-fast bacilli were present in the CSF, *M.tb* was isolated from the CSF by culture, a nucleic acid

amplification test of CSF yielded positive results, or if there was histopathological evidence of *M.tb* from another CNS site. Children who could not be classified as either definite or probable TBM, and who had an alternative diagnosis such as other bacterial or viral meningitis, leukemia, milliary TB, viral pneumonia, autoimmune encephalitis, HIV encephalopathy and Guilain Barre syndrome were then classified as no TBM.

2.4.2 Pulmonary Tuberculosis diagnostic biomarkers study

Children suspected of having pulmonary TB at the Red Cross War Memorial Children's Hospital were recruited after which blood samples were collected into serum tubes (BD SST tubes) centrifuged, aliquoted and stored in a -80°C specimen biobank. Using routine diagnostic procedures including clinical, laboratory (microscopy and culture of gastric aspirates) and radiological (chest X ray) findings, children were classified as having definite, highly probable pulmonary TB disease or TB unlikely. Samples from only the culture confirmed pulmonary TB patients or children in the TB unlikely groups were selected from the biobank and used for the investigations described in the present dissertation.

2.5 Laboratory experiments

2.5.1 Luminex multiplex immunoassays

The bead-based Luminex multiplex assays were used to evaluate the concentrations of host biomarkers in the present study.

2.5.1.1 Principle of the Luminex technology

Luminex multiplex immunoassays are based on the Luminex® xMAP® technology. Luminex® utilizes color-coated microparticles that incorporate different proportions of two fluorescent dyes. This technology allows simultaneous multiplexing of up to 500 assays through precise concentrations of the two dyes, which can create 500 distinctively colored bead sets, each of which can be coated with a specific capture antibody. The different analyte specific beads are mixed and incubated with a test sample. If the test sample contains the analyte of interest, the analyte is captured by the bead via its specifically bound capture antibody at the surface of the bead. After addition of a cocktail of biotinylated detection antibodies, each detection antibody binds a different epitope of its specific analyte, thereby forming a sandwich on the surface of the bead. Addition of fluorescent dye (streptavidin-phycoerytherin; SAPE), which is the reporter molecule, completes the reaction on the surface of each microsphere. A dual-laser flow-based detection analyser (in the Luminex 200 or FlexMap 3D systems), or dual LED system (in the Luminex Magpix) is then used to read the assay results. One of the lasers or

LED lights (red) identifies and discriminates between the beads, whereas the second laser/LED (green) determines the magnitude of the PE-derived signal, which is directly proportional to the amount of analyte bound.

2.5.1.2 Luminex experiments for the TBM diagnostic study

The levels of 68 different analytes were investigated in the CSF and serum samples of all the children with suspected meningitis. These included analytes that were previously identified in previous studies either as potential biomarkers for TBM including VEGF A and interleukin (IL)-13 (160) or analytes that had shown potential as biomarkers for the diagnosis of adult pulmonary TB in previous studies (139, 146, 149, 150) and relatively new analytes, mainly complement proteins that have not frequently been evaluated as TB diagnostic biomarkers (Table 2.1). The experiments were performed using the reagent kits supplied by Merck Millipore, Billerica, MA, USA and R & D systems Inc. (Bio-Techne®), USA as indicated in table 2.1. All experiments were performed strictly according to the instructions of the respective kit manufactures, in a 96-well plate with the only modifications being the pre-dilution of the assay reagents 1:2. It should be noted that prior optimization experiments conducted at the SU-IRG laboratory as part of a previous MSc. project showed that a 1:2 pre-dilution of Luminex reagents prior to use did not result in any differences when compared with the standard procedure that is prescribed by the manufacturers, for the analytes assessed, with higher dilutions (≥1:3) resulting in significant differences or trends for some analytes when compared to the standard procedure (Jacobs R, MSc Thesis, 2016). As it is difficult to ascertain which analytes will perform well when a ≥1: 3 pre-dilution of reagents is used and the fact that a 1:2 pre-dilution worked well for all analytes evaluated, we chose to employ the 1:2 dilution in the current project.

Briefly, after bringing all reagents and samples to room temperature, the magnetic beads, and detection antibody cocktails were diluted 1:2 using the assay buffer supplied in the kits, whereas the assay controls (where available) and standards were prepared according to the manufacturer's instructions. Samples were then diluted or left undiluted as prescribed by the kit manufacturers or based on previous knowledge on the expected levels of these analytes as have been observed in previous studies conducted in the laboratory. After addition of the standards, controls and samples in to the appropriate wells of the 96-well plates, analyte-specific coupled beads were added to each well (in 25µL or 50µL amounts, as specified by the manufacturer). The plates were then incubated for 2 hours at room temperature (20-25°C) or overnight at 4°C on a plate shaker, following the recommendations of the kit manufacturer. After incubation, the well contents were gently removed, and the plates were washed. This was followed by the addition of the manufacturer recommended amount of detection

antibodies (25µL or 50µL), followed by a further 1 hour at room temperature on a plate shaker. Following the incubation, SAPE was added to each well containing the detection antibodies and the mixture was incubated for a further 30 minutes at room temperature on a plate shaker. After incubation, the well contents were removed, and the plates were washed, read on either the Bio Plex 200 Luminex system (Bio Rad laboratories) or Bio Plex Magpix system (Bio Rad laboratories) following the settings recommended by the manufacturer. All wash steps were performed using an automated magnetic bead washer (Bio Rad Laboratories, Hercules, USA) according to the instruction of the kit manufacturers. Beads were acquired and analysed using the Bio Plex manager software version 6.1 (Bio Rad Laboratories, Hercules, USA) for plates read on Bio Plex 200 system, or acquired using the Bio Plex Magpix software, followed by analysis using Bio Plex manager 6.1, for plates read on the Magpix instrument. Median Fluorescent Intensity (MFI) data were analysed using a 5 parameter logistic spline curve-fitting curve, for calculating analytes concentrations in the unknown samples. Quality control reagents supplied in reagent kits purchased from Merck Millipore and a laboratory internal quality control were evaluated on all plates. The levels of the analytes in the quality control reagent were always within the expected ranges.

Table 2.1: Reagent kits used for evaluation of host markers in TBM serum and CSF samples.

Analytes purchased from Merck Millipore, Billerica, MA, USA	
Panel names and catalogue numbers	**Analytes included in the kits**
Human Cardiovascular Disease (CVD) Magnetic Bead Panel 2 (HCVD2MAG-67K)	Von Willebrand factor-cleaving protease (ADAMTS13), D-dimer, growth differentiation factor (GDF)-15, Myoglobin, soluble intracellular adhesion molecule (sICAM)-1, myeloperoxidase (MPO), P-selectin, lipocalin2 (NGAL), soluble vascular adhesion molecule (sVCAM)-1, serum amyloid A (SAA)
Human Neurodegenerative Disease Magnetic Bead Panel 1 (HNDG1MAG-36K)	Apolipoprotein (Apo)-AI, Apo-CIII, complement factor H (CFH), complement C3 (CC3)

Human Neurodegenerative Disease Magnetic Bead Panel 2 (HNDG2MAG-36K)	C reactive protein (CRP), alpha-1-Antitrypsin (A1AT), pigment epithelium-derived factor (PEDF), serum amyloid P (SAP), CCL18 (MIP-4/ PARC), complement C4 (CC4)
Human Neurodegenerative Disease Magnetic Bead Panel 3 (HNDG3MAG-36K)	Brain-derived neurotrophic factor (BDNF), cathepsin D, ICAM-1, MPO, platelet derived growth factor (PDGF)-AA, CCL5 (RANTES), neural cell adhesion molecule (NCAM), PDGF-AA/BB, VCAM-1, plasminogen activator inhibitor (PAI)- 1 total
Human Neurodegenerative Disease Magnetic Bead Panel 4 (HNDG4MAG-36K)	S100 calcium-binding protein B (S100B), amyloid beta 1-40 (Aβ40), Aβ42, soluble receptor for advanced glycation end products (sRAGE) , glial cell-derived neurotrophic factor (GDNF)
Human Complement Magnetic Bead Panel 1 (HCMP1MAG-19K)	Complement C2 (CC2), CC5, CC4b, CC5a, CC9, complement factor D (CFD), mannose binding lectin (MBL), complement factor 1 (CF1)
Analytes purchased from R&D SYSTEMS Inc. (Biotechne®), USA	
Human Magnetic Luminex Screening Assay (LXSAHM- 24)	CCL1 (I-309), CCL4 (macrophage inflammatory protein (MIP)-1β), CXCL8 (IL-8), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-10, IL-17A, IL-6, matrix metalloproteinase (MMP)-8, CCL2 (monocyte chemoattractant protein (MCP)-1), CD40 ligand (CD40L), CXCL9 (MIG), interferon (IFN)-γ, IL-12/23p40, IL-21, IL-7, transforming growth factor (TGF)-α, CCL3 (MIP)-1α, CXCL10 (IP-10), granulocyte colony-stimulating factor (G-CSF), IL-1β, IL-13, IL-4, MMP-1, tumour necrosis factor (TNF)-α
Human Magnetic Luminex Screening Assay	MMP-7, CD56 (NCAM-1), vascular endothelial growth factor (VEGF)-A

(LXSAHM- 03)	
Human Magnetic Luminex Screening Assay (LXSAHM- 02)	Ferritin, MMP-9

-Serum samples for the HCVD2MAG-67K panel were pre-diluted 1:100 prior to analysis, whereas CSF samples were evaluated neat.

-Serum samples for HNDG1MAG-36K panel were pre- diluted 1:40 000, while CSF samples were pre-diluted 1:400 prior to analysis.

-Serum samples for HNDG2MAG-36K panel were pre- diluted 1:2000, while CSF samples were pre-diluted 1:200 prior to analysis.

-Serum samples for HNDG3MAG-36K panel were pre-diluted 1:200 prior to analysis, whereas CSF samples were evaluated neat.

-Serum samples for HNDG4MAG-36 panel were pre-diluted 1:200, while CSF samples were pre-diluted 1:3 prior to analysis.

-Serum samples for HCMP1MAG-19K panel were pre-diluted 1:200, while CSF samples were pre-diluted 1:2 prior to analysis.

-Serum samples for LXSAHM-24 panel were pre-diluted 1:2 prior to analysis, while CSF samples were evaluated neat,

-Serum samples for LXSAHM-03 panel were pre-diluted 1:2 prior to analysis, while CSF samples were evaluated neat,

-Serum samples for LXSAHM-02 panel were pre-diluted 1:50, while CSF samples were pre-diluted 1:2 prior to analysis.

All the dilution factors employed in the experiments were either as specified by the manufacturers of the respective kits or determined after prior optimisation experiments that were performed in the Stellenbosch University Immunology Research Group laboratory.

2.5.1.3 Luminex experiments for the pulmonary TB diagnostic study

We evaluated the concentrations of the seven proteins comprising a previously published seven-marker adult serum protein signature (166) namely; CRP, IFN-γ, CFH, SAA, Apo-A1,

IP-10 and transthyretin as well as proteins that showed potential in another adult study (6, 8) and other proteins selected from the literature as possible biomarkers for the diagnosis of paediatric pulmonary TB (Table 2.2). The procedures employed in evaluating the concentrations of these analytes in the serum samples, including bead acquisition and analysis of bead median fluorescent intensities were exactly as described under section 2.5.1.1.

Table 2.2: Reagent kits employed in the evaluation of biomarkers for the diagnosis of childhood pulmonary TB.

Analytes purchased from Merck Millipore, Billerica, MA, USA	
Panel names and catalogue numbers	**Analytes included in the kits**
Human Neurodegenerative Disease Magnetic Bead Panel 1 (HNDG1MAG-36K)	Apo-A1, CC3, Prealbumin (transthyretin), CFH
Human MMP Magnetic Bead Panel 2 (HMMP2MAG-55K)	MMP-1, MMP-2, MMP-9
Human Neurodegenerative Disease Magnetic Bead Panel 2 (HNDG2MAG-36K)	CRP, A1AT, SAP, CC4
Human Soluble Cytokine Receptor Panel (HSCRMAG-32K)	Soluble interleukin (sIL)-6R, sIL-4R, sIL-2Ra, soluble vascular endothelial growth factor (sVEGF)-R3
Human Cytokine/Chemokine Magnetic Bead Panel (HCYTOMAG-60K)	IL-13, macrophage-derived chemokine (MDC/CCL22), VEGF, IP-10, IL-6, IL-1β, MIP-1β, IL-10, IFN-γ, TGF-α, IL-12p40, G-CSF, MIP-1α, TNF-α, TNF-β, IFN-α2, IL-9
Analytes purchased from Bio- Rad Laboratories, Hercules, CA, USA	
Human Acute Phase Multiplex 4- Plex Panel	Alpha-2-macroglobulin (A2M), haptoglobin, CRP, SAP

Human Acute Phase Multiplex 5- Plex Panel	Procalcitonin (PCT), ferritin, tissue plasminogen activator (TPA), fibrinogen, SAA

-Samples for Human Acute Phase Multiplex 4-Plex panel were pre-diluted 1:10000 prior to analysis.

-Samples for Human Acute Phase Multiplex 5-Plex panel were pre-diluted 1:100 prior to analysis.

-Samples for HNDG1MAG-36K panel were pre-diluted 1:40000 prior to analysis

-Samples for HMMP2MAG-55K panel were pre-diluted 1:500 prior to analysis.

-Samples for HNDG2MAG-36K panel were pre-diluted 1:2000 prior to analysis.

-Samples for HSCRMAG-32K panel were pre-diluted 1:5 prior to analysis.

-Samples for HCYTOMAG-60K panel were evaluated neat.

As described for the experiments done on samples from children with suspected TBM, all the dilution factors employed in the experiments were either as specified by the manufacturers of the respective kits or determined after prior optimisation experiments that were performed in the Stellenbosch University Immunology Research Group laboratory.

2.6 Enzyme-linked Immunosorbent assay

The concentrations of cathelicidin LL-37, evaluated in the current project as a biomarker for TBM (160) in the serum and CSF samples from children with suspected meningitis were assessed by Enzyme-linked Immunosorbent assay (ELISA). This was done using a human LL-37 ELISA kit purchased from Elabscience Biotechnology Inc. (Catalog #E-EL-H2438).

2.6.1 Principle of ELISA

The ELISA kit test principle is based on the Sandwich-ELISA method. Briefly, the micro ELISA plate provided in the kit has been pre-coated with a capture antibody specific to Human LL-37. When the sample test is added to the micro ELISA plate wells and incubated, the antigen of interest (cathelicidin LL-37) binds to specific capture antibody. Then, any unbound antigen is washed away through recommended washing steps. Then a biotinylated detection antibody specific for human LL-37 and avidin-horseradish peroxidase (HRP) conjugate are added to each micro plate well and incubated. Then, any unbound conjugate is washed away. The

substrate solution is added to each well and only those wells that contain human LL-37, biotinylated detection antibody and avidin-HRP conjugate will appear blue in colour. The stop solution is then added to terminate the enzyme-substrate reaction and the colour turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nanometre (nm)± 2nm. The measured OD value is proportional to the concentration of human LL-37.

2.6.2 ELISA experiment for TBM diagnostic study

The experiment for this study was performed according to the manufacturer's instruction as follows: standards and samples were added into the plate wells and mixture was incubated for 90 minutes at 37°C. After incubation, the plate wells contents were removed, and biotinylated detection antibody was added, and the mixture was incubated for 1 hour at 37°C. After which the plates were washed 3 times according to the manufacturer's instruction, the HRP conjugate was added and the mixture was incubated for 30 minutes at 37°C. After incubation, the substrate reagent was added, and the mixture was incubated for 15 minutes at 37°C. Following incubation, the stop solution was added and the OD was determined at 450nm immediately, using an automated microplate plate reader (iMark™ Microplate Reader, Bio-Rad Laboratories).

2.7 Statistical analysis

Differences between any two groups being compared for example, TBM and No TBM or TB and No TB) were determined using the student's t-test or the Mann Whitney U-test, depending on whether the data were normally distributed. The diagnostic accuracy of individual host biomarkers was assessed by receiver operator characteristics (ROC) curve analysis. Cut-off values and sensitivity and specificity for individual analytes were determined using the Youden's index. General Discriminant Analysis (GDA), with leave-one out cross validation, was used to assess the predictive abilities of analytes in combination. P-values ≤0.05 were considered significant. All statistical analysis were performed using Statistica (TIBCO Software Inc., CA, USA) and Graph Pad Prism Version 6 (Graphpad Software Inc, CA, USA).

2.8 Role of the candidate in the research projects

Following the design of the studies and recruitment of the first study participant (for the TBM study) in December 2016, I subsequently joined the group as a Masters student to work on

these projects. From the commencement of my studies in January 2017, I sat in meetings regarding further planning of the studies and from then onwards, played the following roles: Following the collection of samples for the TBM project by the specialist neurologist, I went to the hospital wards, collected the samples and transported them to the laboratory (SU-IRG). Upon arrival in the laboratory, I processed and stored all the serum samples as per the standard operating procedures after which I went to the biosafety level 3 laboratory for processing and storage of the CSF samples. I therefore underwent all the necessary trainings and certifications while working on “my own” study samples. Towards the end of patient recruitment, I, together with my supervisor selected the analytes to be investigated in the project, after which I ordered all the reagent kits (for both Luminex and ELISA). I subsequently planned the experiments after undergoing the necessary training and certification as per the laboratory’s requirements, prepared all templates and performed all experiments, with assistance of a research assistant when needed.

For the pulmonary TB diagnostic study, I assisted in performing the experiments as the kits were already ordered by my supervisor prior to my arrival. For both studies, I cleaned all the Luminex and ELISA data in preparation for statistical analysis, merged the laboratory and clinical data, performed all the univariate data analysis including the preparation of the figures and tables and was assisted by a biostatistician from the Centre for Statistical Consultation of Stellenbosch University (Professor Martin Kidd) in performing the general discriminant analysis. I later assisted in interpreting of the data and wrote my thesis.

Chapter 3

Application of cerebrospinal fluid host protein biosignatures in the diagnosis of tuberculous meningitis in children from a high burden setting

Declaration:

The work presented in this chapter:

1) Was presented during oral presentation session at the Ninth EDCTP forum 2018, held from 17-21 September 2018 in Lisbon, Portugal.

***Abstract title**: Identification of new cerebrospinal fluid and blood-based biomarkers for the diagnosis of tuberculous meningitis in children. **Authors:** Charles Manyelo, Regan S Solomons, Gerhard Walzl, Novel N Chegou. Abstract no:* 8277

2) Was presented during poster discussion session at the 49th Union World conference on Lung Health, held from 24-27 October 2018 in The Hague, Netherlands.

***Abstract title:** Identification of new cerebrospinal fluid and blood-based biomarkers for the diagnosis of tuberculous meningitis in children. **Authors:** Charles Manyelo, Regan S Solomons, Gerhard Walzl, Novel N Chegou. Abstract reference number:* PS07-467-25

3) Formed part of a provisional patent application.

***Title:** Biomarkers for diagnosing tuberculous meningitis (Cerebrospinal fluid and blood-based biomarkers for the diagnosis of tuberculosis meningitis). **Inventors**: Chegou, NN, Walzl, G, Solomons, R, Manyelo, MC, **Applicant**: Stellenbosch University**, Application type**: Provisional Patent Application, **Country:** South Africa, **Application No**: 2018/03410; **Filling Date**: 23 May 2018, **Status**: Filed*

4) Has been written up as a manuscript and will be submitted for possible peer-review and publication as soon as my thesis is submitted:

***Title:** Application of cerebrospinal fluid host protein biosignatures in the diagnosis of tuberculous meningitis in children from a high burden setting. **Authors**: Charles M Manyelo, Regan Solomons, Candice I Snyders, Kim Stanley, Gerhard Walzl, Novel N Chegou; **Journal:** Paediatrics: American Academy of Paediatrics (with Clinical Infectious Diseases as backup if not accepted for publication in the Lancet Infectious Diseases).*

3.1 Background

Tuberculous meningitis (TBM) is the most severe form of extra pulmonary TB as it affects the central nervous system (CNS). It mostly occurs during early childhood and has high morbidity and mortality, mainly due to delayed diagnosis (171). It is well-established that diagnosing pulmonary TB disease in children is challenging, especially in resource-poor settings (172). It is even more challenging to diagnose extrapulmonary TB in this patient group including TBM. As a consequence, TBM frequently results in a poor outcome due to non-specific symptoms and signs (173). The limitations of both the most widely used diagnostic test for TB (smear microscopy) (174), and culture, the gold standard test, are well-publicised (172,174). These tests have been shown to not be very useful in the diagnosis of TBM (175,176). The GeneXpert MTB/RIF test®, the most important recent advance in the field of TB diagnostics, is limited by the large CSF volumes required and low negative predictive value (149). However, the use of the GeneXpert Ultra resulted in improved negative predictive value in a more recent study on HIV positive adults (151). The diagnosis of TBM is mostly based on a combination of clinical findings, multiple laboratory tests on the CSF, imaging findings and the exclusion of common differential diagnoses in routine clinical practice (10). Diagnosing the disease in most high burden, but resource –constrained settings is difficult due to the unavailability of most of these techniques, with children seen at primary and secondary healthcare facilities often having multiple missed opportunities; up to six visits before eventual diagnosis of TBM is made (177). New tests are urgently needed for the diagnosis of TBM.

Host biomarker-based tests may be valuable in the diagnosis of TBM as they have previously been shown to be potentially useful in other extrapulmonary forms of TB (178), and may be easily converted into point-of-care or bedside tests (164,165). In a previous study, we identified a 3-marker CSF host protein biosignature comprising of VEGF, IL-13 and cathelicidin LL-37, which showed potential in the diagnosis of TBM in children (160). As there have been multiple recent studies in which new, potentially useful TB diagnostic biomarkers were identified (17, 18), we hypothesized that at least some of these biomarkers which were identified in mostly adult pulmonary TB studies, will be useful in the diagnosis of TBM in children. We therefore aimed to evaluate the usefulness of our previously established 3-marker CSF biosignature in a new cohort of children with suspected meningitis, and to also evaluate the potential of other host biomarkers that have shown potential in adult pulmonary TB studies, as candidate markers for the diagnosis of TBM in children. We further hypothesised that the accuracy of the previously identified 3-marker CSF signature (160) may be improved if refined through the incorporation of some of these new biomarkers.

3.2 Methods

3.2.1 Study participants

As mentioned in chapter 2 (Section 2.2.1), children suspected of having meningitis and requiring CSF examination for routine diagnostic purposes were recruited from the Tygerberg Academic Hospital in Cape Town, South Africa. Children were eligible for participation in the study if they were between the ages 3 months and 13 years, provided that written informed consent was obtained from the parents or legal guardians. Assent was obtained from children older than 7 years if they had a normal level of consciousness i.e., a Glasgow Coma Score (GCS) of 15/15. The study was approved by the Health Research Ethics Committee of the University of Stellenbosch, Tygerberg Academic Hospital, and the Western Cape Provincial Government.

3.2.2 Sample collection

As mentioned in chapter 2 (section 2.4.1), after collection of CSF and blood samples for routine diagnostic purposes, an additional 1ml of CSF was collected into a sterile tube, followed by the collection of 1ml of whole blood into a serum blood tube (BD Biosciences). Samples were then taken to the immunology research laboratory for further processing for research purposes, within an average of 2 hours from collection. Briefly, blood samples were centrifuged at 1200 xg for 10 minutes, followed by collection of serum, aliquoting and storage at -80^{o}C until further processing. CSF samples were centrifuged in a biosafety level 3 laboratory at 4000 xg for 15 minutes, followed by aliquoting and storage at -80^{o}C until analysed.

3.2.3 Diagnostic work-up of study participants

All patients underwent a comprehensive clinical evaluation and classification according to a uniform clinical research case definition by a specialist paediatric neurologist as described in section 2.4.1

3.2.4 Immunoassays

As mentioned in chapter 2 (section 2.5.1.2), in addition to the three biomarkers that comprised our previous 3-marker model (160) (IL-13, VEGF and cathelicidin LL-37, we evaluated the concentrations of 66 other candidate biomarkers including markers that were previously investigated in adult TB studies (17, 18, 20, 21), and markers which have not been previously investigated in the TB field, as possible diagnostic biomarkers for TBM by ELISA (cathelicidin LL-37) or the Luminex platform (all other host biomarkers) (All markers are listed in Table 2.1).

As mentioned in chapter 2 (section 2.6.2), cathelicidin LL-37 levels in serum and CSF samples were evaluated using an ELISA kit purchased from Elabscience Biotechnology Inc. (Catalog #E-EL-H2438). Experiments were done according to the procedure recommended by the manufacturer after which optical densities (OD) were read at 450nm by an automated microplate reader (iMark™ Microplate Reader, Bio Rad Laboratories). The mean OD of the blank wells was subtracted from the OD of the sample wells and the background-corrected ODs used for statistical analysis.

3.2.5 Statistical analysis

As mention in chapter 2 (section 2.6), differences in the concentrations of host biomarkers between the TBM and the no-TBM group were assessed using the Mann Whitney U test. The receiver operator characteristics (ROC) curve analysis procedure was used to assess the diagnostic accuracy of individual host biomarkers for TBM. Optimal cut-off values and associated sensitivities and specificities were selected based on the Youden's index (180). The utility of combinations of biomarkers in the diagnosis of TBM was ascertained by general discriminant analysis (GDA), followed by leave-one-out cross validation. The data was analysed using Statistica (TIBCO Software Inc., CA, USA), and GraphPad Prism version 6 (Graphpad software, CA, USA).

3.3 Results

A total of 47 children in whom meningitis was strongly suspected, 30 (63.8%) of whom were males were included in the study. The mean age of all the children was 41.6 ± 41.5 months and six out of 37 with known HIV status (16.2%) were HIV infected. Using a composite reference standard based on a uniform research case definition of TBM (145), 23 of the children were diagnosed with TBM. The 24 children without TBM included children with bacterial meningitis (n=2), viral meningitis (n=2) and children with other diagnoses (Table 3.1).

Table 3.1: Clinical and demographic characteristics of children included in the study

	All	TBM	No-TBM[#]
Number of participants	47	23	24
Mean age, months± SD	41.6 ± 41.5	31.5±34.8	51.3±45.7
Males, n (%)	30(63.8)	13(56.5)	17(70.8)
HIV Positive, n/no tested	6 /37	0 /22	6 /15

[#]The no-TBM group included children with bacterial meningitis (n=2), viral meningitis (n=2), asphyxia (n=1), autoimmune encephalitis (n=1), febrile seizure (n=3), Guilain Barre (n=1), HIV encephalopathy (n=1), focal seizures (n=1), leukemia (n=1), Miliary TB (With lymyphocytic interstitial pneumonitis) (n=1), Developmental delay (n=1), Breakthrough seizure (n=1), Gastroenteritis (Caused by shock) (n=1), Idiopathic intracranial hypertention (IIH) (n=1), Viral Gastroenteritis (Adeno and Rota) and encephalopathy (n=1), Stroke (n=1), Mitochondrial diagnosis (n=1), viral pneumonia (This included also SAM and nosocomial sepsis) (n=1), Febrile Seizure and Acute gastroenteritis (n=1) and other (n=1).

3.3.1 Usefulness of the previously identified 3-marker CSF biosignature in the diagnosis of TBM

As we were interested in validating the diagnostic accuracy of the previously established 3-marker CSF biosignature (VEGF, IL-13 and cathelicidin LL-37), we first looked at the utility of individual analytes comprising this signature, followed by evaluation of combinations between different biomarkers comprising the signature.

As observed in our previous study (160), VEGF was the most useful individual biomarker in this signature as none of the other two markers showed significant differences between groups with the Mann Whitney U test. The median levels of all the three analytes were higher in children with TBM (Table 3.2). As reagent kits from different manufacturers were used in this study, in comparison with what was employed in the previous study, we performed receiver operator characteristics (ROC) curve analysis to ascertain the optimal threshold values for the analytes using these new reagent kits. Using these new cut-off values, we observed that combining all three biomarkers (that is, a patient yielding positive results with all three), or positivity with any two out of the three analytes, was inferior to the accuracy obtained with VEGF A alone. However, when considering values above the threshold for any one of the three markers was taken as a positive result, the accuracy of the 3-biomarker signature improved, with positive and negative predictive values of 59.5% (95% CI, 51.5-66.9%) and 90.0% (95% CI, 55.3-98.5%), respectively (Table 3.2).

Table 3.2: Utility of the previously established 3-marker CSF biosignature in the diagnosis of TBM in a new cohort of children with suspected meningitis. *Cut-off values shown for VEGF A and IL-13 are in pg/ml. Value shown for cathelicidin LL-37 is the optical density.

Biosignature	AUC (95% CI)	Cut-off Value	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV	NPV	Youden's Index (J)
VEGF A	0.81 (0.67-0.94)	>9.4	82.6 (61.2-95.1)	79.2 (57.9-92.9)	79.2 (63.0-89.5)	82.6 (65.6-92.2)	0.62 (0.39-0.84)
IL-13	0.58 (0.42-0.75)	>524.9	52.2 (30.6-73.2)	66.7 (44.7-84.4)	60.0 (43.0-74.9)	59.3 (46.6-70.8)	0.19 (-0.09-0.47)
*Cathelicidin-LL37	0.55 (0.38-0.71)	>0.045	69.6 (47.1-86.8)	50.0 (29.1-70.9)	57.1 (45.1-68.4)	63.2 (45.1-78.2)	0.20 (-0.08-0.47)
VEGF+ IL-13+ Cathelicidin-LL37	0.61 (95% CI, 0.50-0.72)	N/A	30.4 (5.6-50.9)	91.7 (74.2-97.7)	77.8 (44.8-93.8)	57.9 (50.6-64.9)	0.22 (0.00-0.44)
Any two out of the three biomarkers	0.73 (0.60-0.85)	N/A	78.3 (58.1-90.3)	66.7 (46.7-82.0)	69.2 (55.1-80.5)	76.2 (58.4-84.4)	0.45 (0.20-0.70)
Any one out of the three biomarkers	0.67 (0.56-0.77)	N/A	95.7 (79.0-99.2)	37.5 (21.2-57.3)	59.5 (51.5-66.9)	90.0 (55.3-98.5)	0.33 (0.12-0.54)

3.3.2 Utility of alternative host biomarkers in the diagnosis of TBM

When the concentrations of the 66 other host markers investigated in our study were compared between children with and those without TBM using the Mann Whitney U test, the levels of multiple host biomarkers were significantly different ($p \leq 0.05$) between the two groups (Table 3.3). When the data for individual host markers were assessed by ROC curve analysis, the area under the ROC curve (AUC) was above 0.70 for 45 of the 66 proteins. Of note, the AUCs for 28 of these proteins including IFN-γ, MIP-4, CXCL9, CCL1, RANTES, IL-6, TNF-α, MPO, MMP-9, MMP-8, CC2, IL-10, PAI-1, CXCL8, IL-1b, A1AT, CXCL10, G-CSF, CC4, CC4b, GM-CSF, PDGF AB/BB, Apo-AI, MBL, ferritin, CC5a, SAP and CC5 were ≥ 0.80 (Table 3.3, Figure 3.1).

As all the six known HIV infected children were in the no-TBM group, we excluded these children and re-analysed the data for individual host biomarkers, to assess the possible influence of HIV infection on the accuracy of the biomarkers. After excluding the HIV infected children, the median levels of PEDF, IL-12/23p40, MMP-1, CD40L and GDF-15 were no longer significantly different between the children with TBM Vs. no-TBM. CD40L and GDF-15 showed trends for significant differences ($0.05 \leq$ p-value ≤ 0.09), whereas the observations for all other host markers were unchanged (data not shown).

Table 3.3: Utility of host biomarkers detectable in CSF samples from children with suspected meningitis in the diagnosis of TB meningitis. Median levels (inter-quartile ranges in parenthesis) of all host markers and accuracies in the diagnosis of TBM as determined by ROC curve analysis are shown. Cut-off values and associated sensitivities and specificities were selected based on the Youden's index. #Values shown are in ng/ml, all other analytes are in pg/ml.*Values shown are absorbance and not concentration values.

Host marker	Median in TBM (IQR)	Median in No-TBM (IQR)	p-value	AUC (95% CI)	Cut-off Value	Sensitivity % (95% CI)	Specificity % (95% CI)
VEGF	45.4 (15.1-150.2)	3.1 (2.5-8.1)	0.000300	0.81 (0.67-0.94)	>9.4	82.6 (61.2-95.1)	79.2 (57.9-92.9)
IL-13	671.68 (246.08-1409.68)	378.2 (89.0-870.4)	0.322220	0.58 (0.42-0.75)	>524.9	52.2 (30.6-73.2)	66.7 (44.7-84.4)
***Cathelicidin-LL37**	0.1 (0.1-0.1)	0.0 (0.1-0.1)	0.619521	0.55 (0.38-0.71)	>0.045	69.6 (47.1-86.8)	50.0 (29.1-70.9)
IFN-γ	469.9 (194.0-818.1)	10.3 (3.9-45.7)	<0.0001	0.98 (0.95-1.00)	>99.5	91.3 (72.0-98.9)	91.7 (73.0-99.0)
#MIP-4	47.5 (31.0-105.4)	0.3 (0.2-0.8)	0<0.0001	0.97 (0.94-1.00)	>11.4	91.3 (72.0-98.9)	95.8 (78.9-99.9)
MIG/CXCL9	9846.2 (4983.6-29684.1)	1349.7 (929.7-2205.9)	<0.0001	0.95 (0.90-1.00)	>4855.0	82.6 (61.2-95.0)	95.8 (78.9-99.9)
I-309/CCL1	156.6 (127.2-318.9)	5.4 (3.8-11.4)	<0.0001	0.95 (0.87-1.00)	>74.6	91.3 (72.0-98.9)	95.8 (78.9-99.9)
RANTES	22.3 (14.6-52.0)	3.8 (0.1-5.7)	<0.0001	0.95 (0.87-1.00)	>9.9	91.3 (72.0-98.9)	91.7 (73.0-99.0)
IL-6	524.8 (196.3-2659.9)	2.8 (1.1-12.3)	<0.0001	0.95 (0.88-1.00)	>100.7	87.0 (66.4-97.2)	95.8 (78.9-99.9)

TNF-α	69.2 (50.9-137.6)	1.2 (0.0-8.5)	<0.0001	0.93 (0.85-1.00)	>19.8	95.7 (78.1-99.9)	87.5 (67.6-97.3)
MPO	62078.8 (49640.6-73505.9)	1430.5 (495.4-5436.1)	<0.0001	0.93 (0.83-1.02)	>25823.0	95.7 (78.1-99.9)	91.7 (73.0-99.0)
MMP-9	4074.6 (2081.8-7163.1)	8.6 (0.0-198.8)	<0.0001	0.91 (0.81-1.00)	>963.9	95.7 (78.1-99.9)	91.7 (73.0-99.0)
MMP-8	8640.1 (2811.4-23467.6)	257.3 (0.0-1075.2)	0.000002	0.91 (0.82-1.00)	>1695.0	91.3 (72.0-98.9)	83.3 (62.6-95.3)
#CC2	2188.4 (1229.9-180000.0)	87.5 (41.8-558.7)	0.000006	0.89 (0.78-0.99)	>712.0	87.0 (66.4-97.2)	83.3 (62.6-95.3)
PAI-1	6090.8 (2456.8-12786.0)	401.6 (194.4-1189.3)	0.000008	0.88 (0.77-0.99)	>2163.0	82.6 (61.2-95.1)	87.5 (67.6-97.3)
IL-1β	47.9 (24.2-64.3)	0.0 (0.0-9.7)	0.000009	0.87 (0.76-0.99)	>12.9	82.6 (61.2-95.1)	79.2 (57.9-92.9)
IL-8/CXCL8	970.6 (519.7-1550.8)	110.2 (50.2-331.2)	0.000010	0.88 (0.77-0.99)	>394.8	87.0 (66.4-97.2)	79.2 (57.9-92.9)
IP-10/CXCL10	44900.0 (2102.7-44900.0)	257.6 (85.5-837.4)	0.000016	0.86 (0.75-0.97)	>1200.0	95.7 (78.1-97.2)	79.2 (57.9-92.9)
#A1AT	2209.8 (916.0-6488.9)	338.4 (236.8-866.1)	0.000023	0.87 (0.75-0.98)	>715.3	91.3 (72.0-98.9)	75.0 (53.3-90.2)
IL-10	47.8 (22.2-82.4)	5.3 (0.0-12.0)	0.000037	0.88 (0.76-1.00)	>15.3	91.3 (72.0-98.9)	87.0 (67.6-97.3)
G-CSF	400.2 (178.1-561.0)	0.0 (0.0-152.9)	0.000043	0.85 (0.73-0.97)	>137.5	91.3 (72.0-98.9)	75.0 (53.3-90.2)

#CC4	1201.2 (667.0-2196.0)	336.2 (232.7-593.2)	0.000060	0.84 (0.73-0.96)	>653.3	78.3(56.3-92.5)	79.2 (57.9-92.9)
#CC4b	565.7 (377.5-668.8)	172.7 (94.3-331.7)	0.000103	0.83 (0.71-0.96)	>364.7	78.3 (56.3-92.5)	79.2 (57.9-92.9)
GM-CSF	88.9 (64.7-105.1)	27.9 (13.4-60.5)	0.000112	0.81 (0.71-0.95)	>63.8	78.3 (56.3-92.5)	79.2 (57.9-92.9)
#Apo AI	1708.0 (980.1-7429.6)	150.8 (0.0-980.1)	0.000167	0.82 (0.69-0.95)	>365.4	91.3 (72.0-98.9)	70.8 (48.9-87.4)
CC5a	66.7 (35.1-93.0)	6.4 (4.4-42.8)	0.000173	0.81 (0.68-0.95)	>26.0	82.6 (61.2-95.1)	70.8 (48.9-87.4)
PDGF-AB/BB	12.9 (5.8-24.5)	5.0 (0.9-7.0)	0.000175	0.82 (0.69-0.95)	>7.7	69.6 (47.1-86.8)	87.5 (67.6-97.3)
#MBL	12.3 (3.7-56.2)	1.0 (0.6-6.6)	0.000221	0.81 (0.69-0.94)	>2.9	87.0 (66.4-97.2)	66.7 (44.7-84.4)
Ferritin	4697.7 (3261.4-300000.0)	705.7 (325.5-3376.9)	0.000236	0.81 (0.68-0.94)	>2729.0	91.3 (72.0-98.9)	75.0 (53.3-90.2)
#CC5	344.7 (166.1-724.3)	36.2 (20.8-178.0)	0.000309	0.81 (0.67-0.94)	>155.4	82.6 (61.2-95.1)	75.0 (53.3-90.2)
#SAP	63.4 (34.6-184.6)	9.6 (5.7-33.5)	0.000364	0.81 (0.67-0.95)	>30.8	87.0 (66.4-97.2)	75.0 (53.3-90.2)
#CFH	1242.8 (669.1-5717.9)	238.9 (82.8-795.3)	0.000629	0.79 (0.66-0.93)	>850.9	73.9 (51.6-89.8)	78.3 (56.3-92.5)

ICAM-1	2128.5(1610.6-4313.7)	499.8 (319.5-1190.1)	0.000743	0.79 (0.65-0.93)	>1372.0	82.6 (61.2-95.1)	79.2 (57.9-92.9)
#P-Selectin	1.2 (0.0-1.8)	0.0 (0.0-0.0)	0.001036	0.76 (0.62-0.89)	>0.3	73.9 (51.6-89.8)	83.3 (62.6-95.3)
PDGF-AA	13.6 (7.3-19.9)	5.5 (3.5-7.8)	0.001047	0.78 (0.64-0.92)	>6.6	82.6 (61.2-95.1)	75.0 (53.3-90.2)
TGF-α	10.0 (5.8-25.7)	3.7 (0.0-7.3)	0.001048	0.78 (0.65-0.92)	>8.6	73.9 (51.6-89.8)	83.3 (62.6-95.3)
#NGAL	77.8 (16.8-512.8)	1.7 (0.7-7.3)	0.001401	0.78 (0.61-0.94)	>16.8	78.3 (56.3-92.5)	95.8 (78.9-99.9)
#CC3	886.7 (357.8-1722.5)	192.8 (56.5-749.1)	0.002344	0.76 (0.62-0.91)	>528.6	73.9 (51.6-89.8)	69.6 (47.1-86.8)
MIP-1β/CCL4	356.3 (240.6-624.8)	185.9 (122.5-261.6)	0.002480	0.76 (0.62-0.90)	>261.6	69.6 (47.1-86.8)	75.0 (53.3-90.2)
IL-17A	14.9 (4.9-32.5)	0.0 (0.0-9.2)	0.002642	0.75 (0.60-0.89)	>2.6	82.6 (61.2-95.1)	66.7 (44.7-84.4)
#CRP	230000.0 (522.0-230000.0)	361.6 (64.1-230000.0)	0.003122	0.74 (0.60-0.87)	>116193.43	69.6 (47.1-86.8)	70.8 (48.9-87.4)
NCAM	30138.4 (18759.6-35617.2)	41021.7 (31229.8-52874.4)	0.003673	0.75 (0.61-0.89)	<36722.0	78.3 (56.3-92.5)	66.7 (44.7-84.4)
#CC9	43.1 (35.5-59.0)	27.3 (20.7-35.8)	0.004645	0.74 (0.59-0.90)	>36.6	73.9(51.6-89.8)	83.3 (62.6-95.3)

CD40L	471.7 (350.8-823.8)	263.7 (160.1-426.2)	0.006422	0.73 (0.58-0.88)	>369.6	73.9 (51.6-89.8)	75.0 (53.3-90.2)
#CF1	480.3 (246.4-970.6)	111.3 (83.2-369.5)	0.006448	0.73 (0.58-0.88)	>263.4	73.9 (51.6-89.8)	70.8 (48.9-87.4)
MIP-1α/CCL3	277.3 (208.8-348.8)	179.0 (35.1-262.5)	0.007518	0.73 (0.58-0.87)	>223.9	69.6 (47.1-86.8)	70.8 (48.9-87.4)
#D-dimer	98000.0 (1425.0-98000.0)	95.7 (2.5-1581.2)	0.007729	0.72 (0.56-0.87)	>49857.4	73.9 (51.6-89.8)	79.2 (57.9-92.9)
#Apo CIII	69.8 (22.7-442.0)	14.2 (6.5-48.4)	0.015650	0.71 (0.56-0.87)	>26.3	73.9 (51.6-89.8)	69.6 (47.1-86.8)
VCAM-1	119507.9 (45091.2-149043.0)	41549.9 (17719.0-122798.7)	0.020942	0.70 (0.55-0.85)	>79387.1	69.6 (47.1-86.8)	66.7 (44.7-84.4)
IL-12/23p40	249.0 (0.00-695.8)	0.0 (0.0-181.8)	0.018510	0.69 (0.54-0.84)	>168.7	69.6 (47.1-86.8)	75.0(53.3-90.2)
#Adipsin/Complement factor D	50.1 (37.6-168.9)	26.1 (15.8-64.7)	0.046258	0.67 (0.51-0.83)	>35.5	82.6 (61.2-95.1)	62.5(40.6-81.2)
#GDF-15	0.4 (0.2-0.5)	0.0 (0.0-0.2)	0.049282	0.67 (0.50-0.84)	>0.2	73.9 (51.6-89.8)	79.2 (57.9-92.9)
#PEDF	746.8 (667.8-837.2)	658.0 (575.6-819.8)	0.056793	0.66 (0.50-0.83)	>689.6	73.9 (51.6-89.9)	62.5 (40.6-81.2)

MMP-1	448.8 (328.8-1058.9)	308.9 (243.0-581.1)	0.058111	0.66 (0.50-0.82)	>318.9	78.3 (56.3-92.5)	58.3 (36.6-77.9)
#SAA	450.4 (1.5-230000.0)	6.5 (0.1-254.2)	0.059259	0.66 (0.50-0.82)	>204.9	60.9 (38.5-80.3)	75.0 (53.3-90.2)
Aβ40	580.0 (305.1-918.5)	800.5 (323.6-2195.3)	0.190601	0.61 (0.44-0.78)	<759.5	65.2 (42.7-83.6)	58.3 (36.6-77.9)
#ADMTS13	8.1 (6.3-15.9)	6.1 (0.5-9.7)	0.212312	0.60 (0.44-0.77)	>6.2	78.3 (56.3-92.5)	54.2 (32.8-74.5)
Aβ42	172.8 (54.3-288.2)	219.0 (81.9-645.3)	0.259233	0.60 (0.43-0.76)	<292.1	78.3 (56.3-92.5)	41.7 (22.1-63.4)
#Myoglobin	0.5 (0.1-1.1)	0.1 (0.0-0.9)	0.266718	0.60 (0.43-0.76)	>0.2	73.9 (51.6-89.8)	58.3 (36.6-77.9)
MCP-1/CCL2	812.5 (457.9-1348.7)	1076.2 (513.2-1423.7)	0.292145	0.59 (0.42-0.76)	<881.0	60.9 (38.5-80.3)	66.7 (44.7-84.4)
S100B	41.2 (41.2-2800.0)	41.2 (28.0-64.6)	0.311691	0.59 (0.42-0.77)	>64.6	38.9 (17.3-64.3)	75.0 (50.9-91.3)
MMP-7	101.5 (81.6-181.6)	101.5 (81.6-121.5)	0.329877	0.58 (0.42-0.75)	>111.5	43.5 (23.2-65.5)	70.8 (48.9-87.4)
IL-4	162.6 (107.9-229.2)	191.6 (132.7-248.9)	0.394463	0.57 (0.40-0.74)	<181.1	65.2 (42.7-83.6)	58.3 (36.6-77.9)
Srage	14.1 (12.8-15.3)	14.4 (12.8-16.6)	0.473024	0.56 (0.39-0.73)	<14.4	56.5 (34.5-76.8)	50.0 (29.1-70.9)

Cathepsin D	75722.1 (61184.2-91429.2)	66539.1 (50433.7-96857.8)	0.537070	0.55 (0.38-0.72)	>68062.2	69.6 (47.1-86.8)	54.2 (32.8-74.5)
IL-7	4.3 (0.0-7.0)	5.3 (2.4-7.9)	0.645745	0.54 (0.37-0.71)	<4.3	52.2 (30.6-73.2)	58.3 (36.6-77.9)
BDNF	0.6 (0.0-1.1)	0.5 (0.0-1.0)	0.674752	0.54 (0.37-0.70)	>0.5	60.9 (38.5-80.3)	50.0 (29.1-70.9)
IL-21	43.2 (12.7-78.2)	46.8 (30.9-61.1)	0.781809	0.52 (0.35-0.69)	<37.36	47.8 (26.8-69.4)	66.7 (44.7-84.4)
GDNF	2.2 (1.8-2.3)	2.1 (1.9-2.5)	0.828794	0.48 (0.31-0.65)	<2.1	47.8 (26.8-69.4)	41.7 (22.1-63.4)

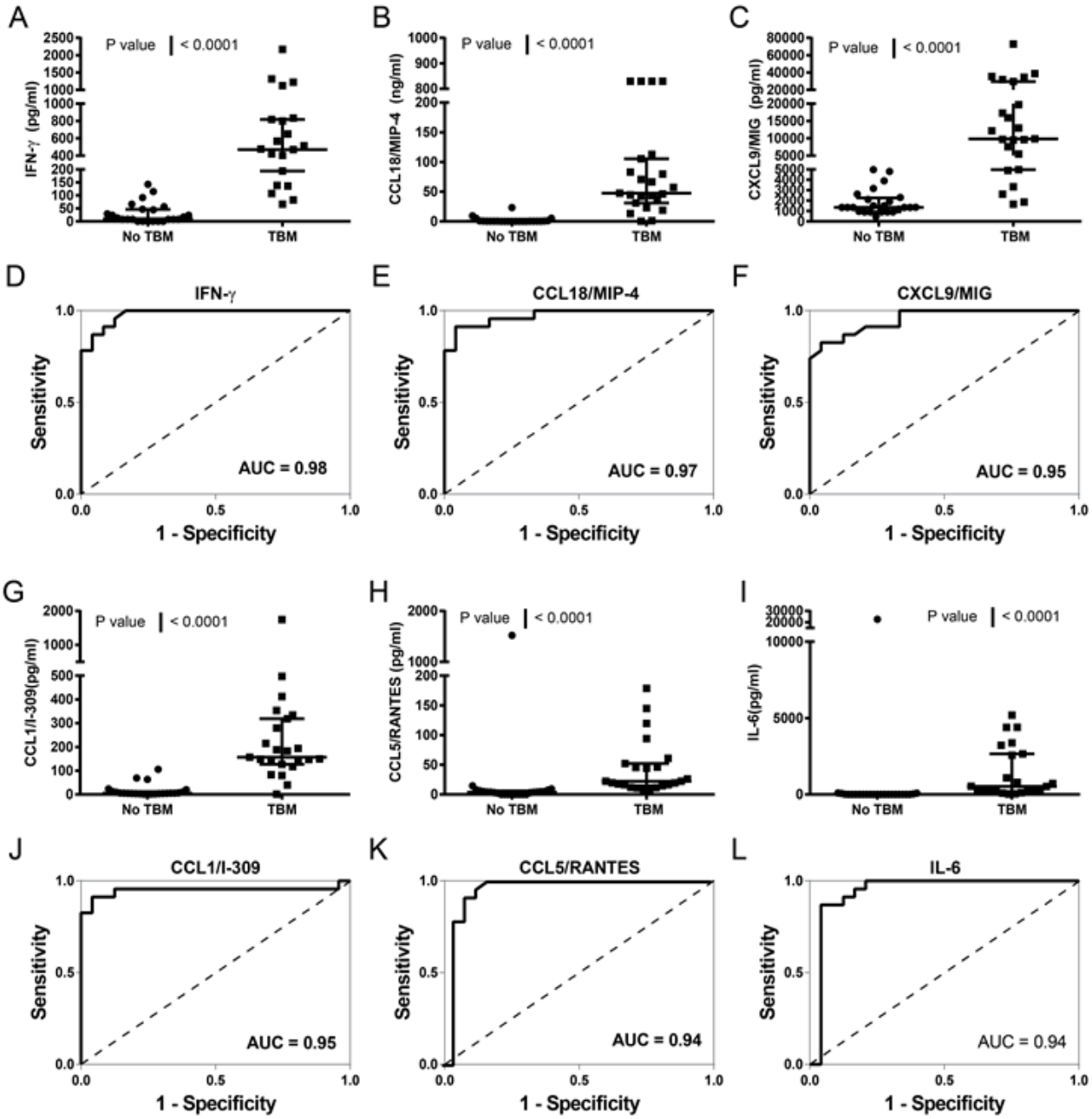

Figure 3.1: Representative plots showing the concentrations of biomarkers detected in CSF samples from children with and without TBM and ROC curves showing the accuracies of these biomarkers in the diagnosis of TBM. Error bars in the scatter-dot plots indicate the median and inter-quartile ranges. Representative plots for six analytes with AUC ≥0.80 are shown. The accuracies of all host biomarkers evaluated in the study are shown in Table 3.3.

3.3.3 Utility of combinations between other host biomarkers in the diagnosis of TBM

When the data obtained for all host markers (including VEGF A, IL-13 and LL-37) were fitted into General Discriminant Analysis (GDA) models irrespective of HIV status, optimal prediction of TBM was shown to be achieved with a combination of four markers. The most accurate four-marker biosignature comprising of sICAM-1, MPO, CXCL8 and IFN-γ diagnosed TBM with an AUC of 0.97 (95% CI, 0.92-1.00); corresponding to a sensitivity of 87.0% (20/23), (95% CI, 66.4-97.2%) and specificity of 95.8% (23/24), (95% CI, 78.9-99.9%). After leave-one-out cross validation, there was no change in the sensitivity (87.0%) and specificity (95.8%) of the four-marker biosignature. The positive and negative predictive values of the biosignature were 95.2% (95% CI, 74.5-99.3%) and 88.5% (95% CI, 72.7-95.7%), respectively. Further optimization of the four-marker biosignature by selection of optimal cut-off values based on Youden's Index resulted in both sensitivity and specificity 96% (Figure 3.2).

Given that VEGF has consistently shown promise as a biomarker for TBM (16, 23–25) and that we identified other candidate biomarkers with strong potential in the present study, we wondered whether the previous 3-marker VEGF-based biosignature could be further optimised using other analytes. A GDA model in which IL-13 and cathelicidin LL-37 were replaced with IFN-γ and MPO respectively, resulted in an improved AUC of 0.97 (95% CI, 0.92 - 1.00), corresponding to a sensitivity of 82.6% (19/23), (95% CI, 61.2-95.1%) and specificity of 95.8% (23/24), (95% CI, 78.9-99.9%). After leave-one-out cross validation, the sensitivity and specificity of the biosignature were 78.3% (18/23), (95% CI, 56.3-92.5%) and 91.7% (22/24), (95% CI, 73.0-99.0%) respectively. The positive and negative predictive values the refined VEGF-based biosignature after leave-one-out cross validation were 90.0% (95% CI, 70.1-97.2%) and 81.5% (95% CI, 66.8-90.6%), respectively. Further optimization of the biosignature through the selection of better cut-off values resulted in improved sensitivity and specificity of 92% and 100% respectively (Figure 3.3).

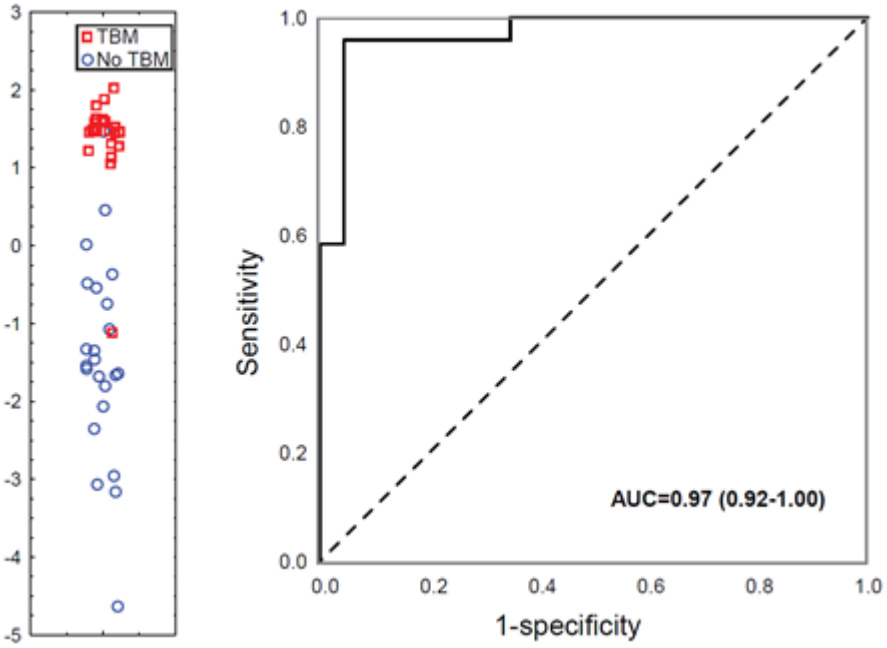

Figure 3.2: Accuracy of the 4-marker CSF biosignature (sICAM-1, MPO, CXCL8 and IFN-γ) in the diagnosis of TBM. Scatter plot showing the ability of the 4-marker signature to classify children as TBM or no TBM (left image). ROC curve showing the accuracy of the 4-marker biosignature (right image). Red squares; children with TBM; blue circles: children with no TBM.

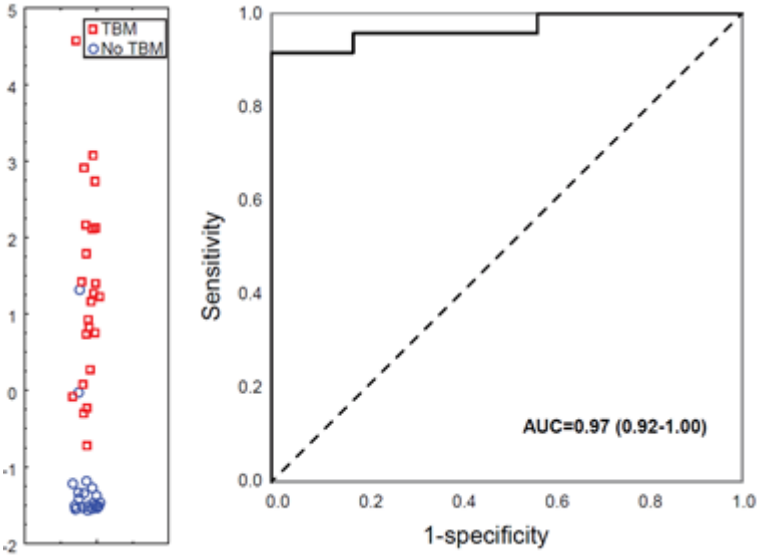

Figure 3.3: Accuracy of a new VEGF-based 3-marker CSF biosignature in the diagnosis of TBM. Scatter plot showing the ability of the 3-marker signature to classify children as TBM or no TBM (left image). ROC curve showing the accuracy of the 3-marker biosignature (VEGF, IFN-γ and MPO (right image). Red squares: children with TBM; blue circles: children with no TBM.

3.4 Discussion

We assessed the utility of a previously identified 3-marker CSF biosignature (IL-13, VEGF and cathelicidin LL-37) (160) as well as host biomarkers that have shown potential as pulmonary TB diagnostic candidates in recent adult studies, as tools for the diagnosis of TBM in children with suspected meningitis. Although we validated the diagnostic accuracy of the previously identified 3-marker biosignature, other major findings from our study included the identification of a novel four-marker CSF biosignature comprising of sICAM-1, MPO, CXCL8 and IFN-γ, and a modified 3-marker signature (VEGF, IFN-γ and MPO) which diagnosed TBM with promising accuracy. We also identified multiple host biomarkers that are detectable in CSF, and which showed strong potential as diagnostic candidates for TBM in children.

It is well-known that the diagnosis of TB disease in children remains a major challenge worldwide. This is mainly due to several well-publicised limitations in the currently available diagnostic tools (4, 6). It is even more challenging to diagnose extrapulmonary TB including TBM in this patient group, with unstandardized and cumbersome approaches without reliable diagnostic criteria, currently being used in routine clinical practice (184,185). In order to improve standardization of clinical diagnosis of TBM for research purposes, a uniform research case definition for both adults and children was proposed, categorizing patients as definite, probable, or possible TBM according to a composite score based on clinical, CSF, and neuroimaging findings (145). None of the tests that are used in the diagnosis of TBM in children performs with high accuracy individually (7, 8, 28, 29). The disease consequently results in high morbidity and mortality, due mainly to diagnostic delay (3, 30).

Host inflammatory biomarker based biosignatures have been shown to have potential in the diagnosis of TB disease in both adults and children in previous studies (17, 21, 31). Furthermore, these immulogical biomarker-based tests have the potential to be readily converted into user-friendly, point-of-care diagnostic tests (14, 15) with the development of such tools for the management of TBM especially in children promising to be a major breakthrough in future. In the present study, we validated the diagnostic accuracy of a previously identified CSF inflammatory biomarker-based biosignature (160). Although a diagnostic test with positive and negative predictive values of 59.5% and 90.0% respectively, will be imperfect, such a test may indeed contribute significantly to the management of children with suspected TBM if the test is a rapid, point-of-care or bedside test, considering that it currently takes up to six hospital visits before TBM is diagnosed in children, with the current diagnostic approaches, in a country such as South Africa, which is relatively well-resourced, compared to other lower and middle income countries (177). Our data indicates that replacement of two of the proteins in this previously identified signature (IL-13 and cathelicidin LL-37) with new biomarkers (MPO and IFN-γ respectively) has the potential to yield a test with

both sensitivity and specificity >95%. Furthermore, host markers comprising the alternative four-marker biosignature (sICAM-1, MPO, CXCL8 and IFN-γ) and other analytes that showed potential individually, may be back-up host markers that might be employed during the development of such a test. Our findings may therefore pave the way for the development of a prototype CSF biomarker-based test for the diagnosis of TBM in children.

During the development of such a test, the biosignature could be optimised further for use as a rule-in or rule-out test and the newly developed tests used as a screening test for TBM. If the test is based on a point-of-care diagnostic platform, such as the lateral flow technology, successful implementation of the test at the point-of-care or bedside would lead to a significant reduction in the costs and delays that are currently incurred in the diagnosis of TBM in children (177), with a consequent reduction in morbidity and mortality. Although a CSF-based point-of-care or bedside test will be useful in the management of TBM in children, the expertise required for lumbar punctures, opposed to that needed for the collection of other samples such as blood, saliva or urine may be a challenge in resource-limited settings, making the implementation of such a diagnostic tool difficult. That notwithstanding, such a test will also contribute to the management of the disease in children where sample collection is possible.

The fact that host-inflammatory biomarkers detectable in CSF show potential in the diagnosis of TBM is not surprising, given that previous studies identified VEGF and other candidate biomarkers (16, 23–25) as potential tools for the diagnosis of the disease. Such candidate biomarkers that are detectable in biological fluids including blood (17, 32–34), saliva (20, 21, 35), urine (191) and other specimens (13, 14, 37, 38) have been identified as TB diagnostic candidates in several previous studies. It is well-established that individual host biomarkers might not suffice as diagnostic tools for TB disease (17, 33, 34) owing to the fact that these inflammatory biomarkers will be raised in other diseases, including cancers. However, these specificity concerns may be addressed through the use of a panel of biomarkers as done in the present study. The main limitation of the current study was the relatively small sample size, especially the few children with alternative diagnoses including children with other forms of meningitis. However, as this was a TBM-suspect study, the design of the study was relatively strong and the number of participants enrolled into the study is consistent with the patient numbers were described in multiple previous studies. Validation of the previously established 3-marker CSF signature in the current study indicates that the novel biosignatures identified in the study have strong potential. Further studies should include larger numbers of study participants with suspected meningitis, including those who are HIV infected, and individuals with confirmed alternative meningitides. HIV infected children included in such studies should be appropriately staged with CD4 counts and viral loads, to

assess the possible influence of severe HIV infection on the accuracy of the diagnostic biosignatures. For the biosignatures described in the current study to be useful in the management of children with suspected TBM, the biosignatures would require incorporation into a point-of-care or bedside diagnostic test platform, for example, based on the lateral flow technology. Such prototype blood-based TB tests have been developed and successfully investigated in multiple African countries (14, 15), with multi-biomarker finger-prick based formats currently under development for the diagnosis of adult pulmonary TB disease (www.screen-tb.eu). Incorporation of host inflammatory biomarkers into such a platform may be relatively easier, and faster as lessons learned during the development of adult pulmonary TB tests will be beneficial.

In conclusion, we validated a previously established 3-marker CSF biosignature as a tool for adjunctive diagnosis of TBM in children and furthermore showed that modification of this signature through the substitution of two of the proteins with new protein biomarkers results in a strong biosignature for the diagnosis of TBM. These biosignatures will only be beneficial for people who would benefit from the urgently required new tools (children with suspected TBM, parents) if these signatures are further developed into point-of-care or bedside diagnostic tests. Our study therefore paves the way for the development of such a test.

Chapter 4

Application of blood-based host immunological biomarkers in the diagnosis of tuberculosis in children

Declaration:

1) The work presented in this chapter has been submitted for presentation as a late-breaker oral abstract at the 49th Union World conference on Lung Health, which will take place from 24-27 October 2018 in The Hague, Netherlands.

Abstract title: Application of a seven-marker adult serum protein biosignature in the diagnosis of tuberculosis disease in children from a high burden setting. ***Authors:*** *Novel N Chegou, Regan Solomons, Charles M Manyelo, Mark Nicole, Heather Zar, Gerhard Walzl*

2) The work presented in this chapter (section 4B) formed part of a provisional patent.

Title: *Biomarkers for diagnosing tuberculous meningitis (Cerebrospinal fluid and blood-based biomarkers for the diagnosis of tuberculosis meningitis).* ***Inventors:*** *Chegou, NN, Walzl, G, Solomons, R, Manyelo, MC,* ***Applicant:*** *Stellenbosch University, Application type: Provisional Patent Application,* ***Country:*** *South Africa,* ***Application No:*** *2018/03410****;*** ***Filling Date:*** *23 May 2018, Status: Filed*

3) The work presented in this chapter (section 4B) is being written up as a manuscript and will be submitted for possible peer-review and publication after submission of my thesis.

   Target Journal*: International Journal of Infectious Diseases*

4.1 Background

Optimal management of TB disease depends largely on early diagnosis, followed by early initiation of anti-tuberculosis therapy. Therefore, the improvement of case detection and early administration of curative treatment can reduce the morbidity and mortality associated with paediatric tuberculosis (194). However, the diagnosis of TB disease remains difficult especially in children, due to sub-optimal performance of the currently available diagnostic methods. Bacteriological confirmation of TB disease still relies mainly on smear microscopy worldwide (174). *Mycobacterium tuberculosis* (*M.tb*) culture remains the gold standard test, but has a long turn-around time and culture facilities are not widely available in resource constrained settings (4, 5). The GeneXpert MTB/RIF test® is a rapid molecular test for TB diagnosis and detection of rifampicin resistance in a clinical specimen within 2 hours (21). The GeneXpert is arguably the most significant advance in TB diagnosis and it is recommended by WHO for diagnosis of both pulmonary TB and extrapulmonary TB (EPTB) in adults and children (21). However, these tests require the availability of good quality sputum samples and hence not suitable in individuals with difficulty in providing good quality sputum samples and those with paucibacillary disease such as children (118). Young children with pulmonary TB disease usually do not readily expectorate sputum, hence sputum sample is difficult to obtain. In such cases, induced sputum or gastric aspirate is used to determine the presence of *M.tb* bacilli. Sputum induction is invasive and not easily done in resource poor settings. As discussed in Chapter 3, these tests are not useful in the diagnosis of EPTB, including TBM in children. Therefore, the diagnosis of paediatric TB remains difficult, often resulting in under- or over-diagnosis (195) in some cases. The world health organisation encourages development of new diagnostic tools suitable for paediatric tuberculosis (22). Immunodiagnostic methods may be valuable in the diagnosis of childhood TB especially if based on easily obtainable samples such as saliva, urine, and blood (including plasma and serum).

In a recent large multi-centred pan-African study, an adult seven-marker serum protein biosignature comprising of CRP, transthyretin, IFN-γ, CFH, Apo-A1, IP-10, and SAA diagnosed TB with high accuracy as ascertained by AUC of 0.91 (166). Other studies showed that host biomarkers detectable in saliva and plasma samples hold promise in the development of new diagnostic methods for tuberculosis in adults (139, 149, 150). Although CSF biomarkers were shown to diagnose TBM in previous studies and validated in the present thesis (Chapter 3), host biomarkers detected in blood may be more beneficial as blood can be easily collected and offers easier adaptation of biomarkers into finger-prick-based tests. Such a test was highly recommended by the WHO as described in the target product profiles for new diagnostics (196) and may be easier to implement in low resourced settings. The aim of

the present study was therefore to ascertain whether host biomarkers, most of which had previously been shown to have potential in the diagnosis of adult TB in serum and plasma samples (166,170) possessed diagnostic potential for childhood pulmonary TB and TBM. We were specifically interested in evaluating the performance of the previously established adult seven-marker serum protein biosignature (CRP, transthyretin, IFN-γ, CFH, Apo-A1, IP-10, and SAA) in the diagnosis of pulmonary TB and TBM in children.

The usefulness of the host biomarkers in the diagnosis of childhood pulmonary TB shall be discussed as section 4A whereas the accuracy of these biomarkers in the diagnosis of TBM shall be discussed in section 4B.

4.2 Materials and Methods

As discussed in chapter 2 (Section 2.2.2), children who provided samples for the pulmonary TB diagnostic study were previously enrolled at the Red Cross War Memorial Children's Hospital, Cape Town, South Africa. Stored serum samples that were previously collected from these children as described in section 2.4.2 were used for Luminex experiments as described below. Children enrolled into the TBM study were the same children described in table 3.1 in chapter 3 and in Chapter 2 (Section 2.2.1)

4.2.1 Luminex multiplex immunoassay

For the pulmonary TB diagnostic study, we evaluated the concentrations of the seven markers comprising the previously described adult 7-marker serum protein biosignature (CRP, transthyretin, IFN-γ, CFH, Apo-A1, IP-10, and SAA) and 33 other biomarkers (as listed in table 2.2) selected based on a review of the literature as discussed in chapter 2 (Section 2.5.1.3). For TBM diagnostic study, we evaluated the concentration of six markers (CRP, IFN-γ, CFH, Apo-A1, IP-10, and SAA) amongst the 7 markers comprising the previously described 7-marker serum biosignature (as transthyretin (196) was not available at the time that the study was conducted) and 63 other biomarkers (as listed in table 2.1), also selected based on a review of literature as discussed in chapter 2 (Section 2.5.1.2).

All the experiments were performed in a blinded manner on the Bio Plex 200 Luminex system (Bio Rad laboratories) or Bio Plex Magpix system (Bio Rad laboratories), according to the instructions of the respective kit manufacturers (Bio Rad and Merck Millipore, and R&D Systens). As described in Section 2.5.1.1, bead acquisition and analysis of median fluorescence intensity was done using the Bio Plex manager version 6.1 or Bio Plex MP, on either the Bio Plex 200 system (Bio-rad) or Bio Plex Magpix (Bio-rad). The concentrations of host markers in the quality control reagents supplied by the kit manufacturers as well as a laboratory internal QC were within the expected ranges.

4.2.2 Statistical analysis

As discussed in chapter 2 (Section 2.6), the differences in the concentrations of host markers detected in serum samples from children with TB disease and children without TB (Not TB) or those with TBM Vs those without TBM were evaluated using the Mann Whitney U-test for non-parametric data analysis. The diagnostic accuracies of individual host markers were assessed by receiver operator characteristic (ROC) curve analysis. Optimal cut-off values and associated sensitivity and specificity were selected based on the Youden's Index. General Discriminant Analysis (GDA), with leave-one out cross validation, was used to assess the predictive abilities of analytes in combination. P-values ≤0.05 were considered significant. All statistical analysis were performed using Statistica (TIBCO Software Inc., CA, USA) and Graph Pad Prism Version 6 (Graphpad Software Inc, CA, USA).

4.3 Results

4.3.1 Section 4A: Usefulness of blood-based biomarkers in the diagnosis of childhood pulmonary TB

4.3.1.1 Study participants

A total of 40 children, 20 of whom were culture positive TB patients and 20 matched controls, in whom TB was unlikely, were investigated in this part of the project. The mean age of all study participants was 25.6±27.5 months. Twenty-six (65%) of all study participates were males. However, there was no difference in the proportion of males between children with or without TB. All children included in the study were HIV uninfected. The clinical and demographic characteristics of study participants are shown in Table 4.1.

Table 4.1. Clinical and demographic characteristics of children with pulmonary TB or no TB

	All	**TB cases**	**No TB controls**
Number of participants	40	20	20
Mean age, months± SD	25.6 ± 27.5	25.6 ± 28.0	25.6 ± 27.7
Males, n (%)	26 (65)	13 (65)	13 (65)
Mantoux positive, n/ number done	14/38	14/18	0/20

Abbreviations: TB=Tuberculosis. SD=Standard Deviation

4.3.1.2 Utility of the previously established adult 7-marker serum protein biosignature in the diagnosis of active TB disease

When the concentrations of the individual host markers comprising the 7-marker adult protein biosignature (CRP, transthyretin, IFN-γ, IP-10, CFH, Apo-AI and SAA) were evaluated in serum samples from children with pulmonary TB vs those without TB, significant differences were obtained for IFN-γ only. The concentrations of IFN-γ were higher in children with pulmonary TB. Trends ($0.05 \leq$ p-value ≤ 0.09) towards higher levels of CFH were observed in children with pulmonary TB. After ROC curve analysis, the most informative single host marker from this signature, as determined by area under the ROC curve (AUC) was IFN-γ (Table 4.2).

When these host markers were evaluated in combination, the area under the ROC curve for the signature (AUC) was 0.79 (95% CI, 0.65-0.93) (Figure 4.1 A and B). The sensitivity of the biosignature was 65.0% (13/20) and specificity was 75.0% (15/20). After leave-one-out cross validation, the sensitivity of the biosignature was 50.0% (10/20) and specificity was 60.0% (12/20). The positive and negative predictive values of the biosignature after leave-one-out cross validation were 55.6% (95% CI, 38.5-71.4) and 54.6% (95% CI, 40.5-67.9), respectively. When alternative cut-off values were carefully selected in order to maximise both sensitivity and specificity, the sensitivity and specificity of the biosignature improved to 75.0% and 70.0%, respectively. To contextualise these findings, this biosignature diagnosed pulmonary TB in adults from five different African countries, 24% of whom were HIV infected with a sensitivity of 93.8% and specificity of 73.3% in the previous adult study (166). When a first assessment of the potential usefulness of all host biomarkers evaluated in the study was done, by fitting all host markers, including the seven into general discriminant analysis (GDA) models in an unbiased manner, it was realised that only IFN-γ, CFH, IP-10 and Apo-AI featured amongst the analytes that contributed to the top 20 most accurate biosignatures (Figure 4.1 C).

Table 4.2: Usefulness of analytes comprising the previously established adult 7-marker serum protein biosignature in the diagnosis of pulmonary TB disease in children. Median levels (inter-quartile ranges in parenthesis) of all host markers detected in serum samples from children with TB or not TB and accuracies in the diagnosis of TB disease as determined by ROC curve analysis. Cut-off values and associated sensitivities and specificities were selected based on the Youden's index. #values shown are in ng/ml, values for all other host markers are in pg/ml.

Host marker	Median in Not TB (IQR)	Median in TB (IQR)	p-value	AUC (95% CI)	Cut-off Value	Sensitivity % (95% CI)	Specificity % (95% CI)
IFN-γ	4.9 (1.7-10.3)	15.5 (9.6-22.8)	0.001863	0.79 (0.64-0.94)	>6.83	85.0 (62.1-96.8)	70.0 (45.7-88.1)
#CFH	467324.7 (395553.7-553443.1)	575842.9 (409906.2-639034.7)	0.072046	0.67 (0.50-0.84)	>499011.0	60.0 (36.1-80.9)	60.0 (36.1-80.9)
IP-10	344.4 (196.4-1020.8)	701.1 (402.8-1511.0)	0.171931	0.63 (0.45-0.81)	>468.5	75.0 (50.9-91.3)	60.0 (36.1-80.9)
SAA	2435.5 (329.0-4684.4)	4061.9 (1446.5-5587.7)	0.297655	0.60 (0.42-0.78)	>4616.0	50.0 (27.2-72.8)	75.0 (50.9-91.3)
#CRP	8831.4 (1527.8-24656.0)	10586.0 (4236.4-21927.8)	0.473481	0.57 (0.39-0.75)	>5018.0	70.0 (45.7-88.1)	50.0 (27.2-72.8)
#Apo-AI	553386.3 (361307.5-759960.8)	534096.4 (402057.4-616430.1)	0.579094	0.55 (0.37-0.74)	<529657.0	50.0 (27.2-72.8)	55.0 (31.5-76.9)
#Transthyretin	93085.2 (65840.6-162001.4)	102319.7 (73968.4-123449.0)	0.871063	0.52 (0.33-0.70)	>95663.0	60.0 (36.1-80.9)	55.0 (31.5-76.9)

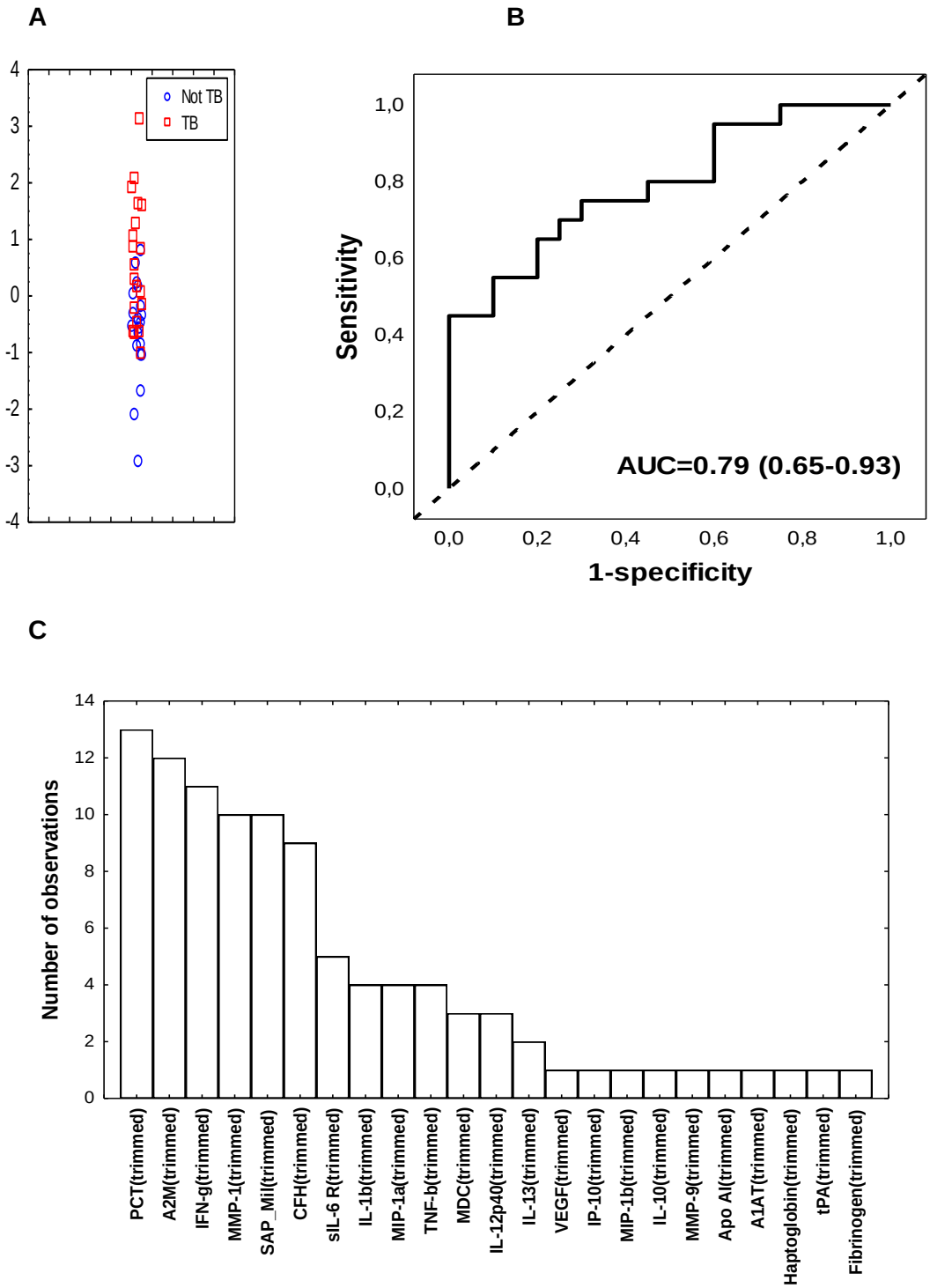

Figure 4.1: Accuracy of the previously identified adult 7-marker serum protein biosignature (CRP, transthyretin, IFN-γ, IP-10, CFH, Apo-A1, and SAA) in the diagnosis of pulmonary TB disease in children. Scatter plot showing the ability of the 7-marker signature to classify children as TB or not TB (A). ROC curve showing the accuracy of the 7-marker biosignature (B). Frequency of analytes in the top 20 most accurate models in the diagnosis of pulmonary TB, if host biomarkers are not pre-specified (C). Red squares in (A); children with TB; blue circles: children without TB (Not TB).

4.3.1.3 Usefulness of alternative host biomarkers detected in serum samples in the diagnosis of pulmonary TB in children

When the concentrations of the 33 other host markers evaluated in the study were compared between children with TB disease and children without TB disease using the Mann Whitney U-test, the median levels of MMP-1, MMP-9, PCT, sIL-6R, and IL-6 were significantly (p-value≤0.05) higher in children with TB disease compared to children without TB disease. Furthermore, trends (0.05≥p-value≤0.09) towards higher levels of sIL-2Ra, and sIL-4R were observed in children with TB disease, whereas trends (0.05≥p-value≤0.09) towards higher levels of MDC were observed in children without TB disease (Table 4.3). When the data for individual host markers were assessed by ROC curve analysis, the area under the ROC curve (AUC) was above 0.70 for 5 proteins including MMP-1, MMP-9, PCT, sIL-6R, and IL-6 (Table 4.3, Figure 4.2).

Table 4.3: Utility of alternative host biomarkers detectable in serum samples in the diagnosis of pulmonary TB in children. Median levels (inter-quartile ranges in parenthesis) of all host markers detected in serum samples from children with TB or not TB and accuracies in the diagnosis of TB disease as determined by ROC curve analysis. Cut-off values and associated sensitivities and specificities were selected based on the Youden's index. #values shown are in ng/ml, values for all other host markers are in pg/ml.

Marker	Median in Not TB (IQR)	Median in TB (IQR)	p-value	AUC (95% CI)	Cut-off Value	Sensitivity % (95% CI)	Specificity % (95% CI)
MMP-1	11462.4 (0.0-28694.4)	42440.1 (19336.1-737391.4)	0.002674	0.78 (0.63-0.92)	>26122.0	70.0 (46.0-88.0)	75.0 (0.51-0.91)
MMP-9	168289.4 (89630.6-274360.8)	564467.0 (353524.8-674156.1)	0.002924	0.78 (0.62-0.93)	>310782.0	80.0 (56.0-94.0)	80.0 (56.0-94.0)
PCT	1648.9 (827.5-3261.8)	3830.1 (2656.0-4634.3)	0.014408	0.73 (0.55-0.90)	>2656.0	75.0 (51.0-91.0)	70.0 (46.0-88.0)
sIL-6R	5739.1 (4117.6-6206.6)	7210.4 (5744.5-8276.4)	0.023904	0.71 (0.54-0.88)	>6119.0	75.0 (51.0-91.0)	70.0 (46.0-88.0)
IL-6	5.1 (1.2-9.0)	10.6 (4.8-26.9)	0.024738	0.71 (0.55-0.87)	>6.3	70.0 (46.0-88.0)	65.0 (41.0-85.0)
sIL-2 Ra	1258.4 (729.6-1755.5)	1819.3 (1416.6-2463.2)	0.061966	0.67 (0.50-0.85)	>1532.0	75.0 (50.9-91.3)	70.0 (45.7-88.1)
sIL-4R	0.0 (0.0-182.7)	200.2 (0.0-547.4)	0.081010	0.66 (0.48-0.83)	>164.9	55.0 (31.5-76.9)	75.0 (50.9-91.3)
MDC	1373.2 (1164.6-1651.9)	907.8 (544.0-1567.1)	0.085856	0.66 (0.48-0.84)	<1269.0	65.0 (40.8-84.6)	75.0 (50.9-91.3)

TGF-α	1.9 (0.8-13.5)	6.6 (4.0-12.2)	0.107298	0.65 (0.47-0.83)	>2.1	85.0 (62.1-96.8)	60.0 (36.1-80.9)
IL-1β	1.1 (0.9-2.1)	1.5 (1.1-2.0)	0.140179	0.64 (0.46-0.82)	>1.2	65.0 (40.8-84.6)	60.0 (36.1-80.9)
#Haptoglobin	259374.4 (195442.5-352633.0)	332038.9 (219377.2-417738.6)	0.147848	0.64 (0.46-0.81)	>305379.0	70.0 (45.7-88.1)	75.0 (50.9-91.3)
MMP-2	1361.7 (0.0-100430.9)	0.0 (0.0-20155.8)	0.150155	0.62 (0.44-0.80)	<1362.0	70.0 (45.7-88.1)	50.0 (27.2-72.8)
VEGF	271.9 (99.6-397.1)	309.4 (189.9-548.1)	0.167585	0.63 (0.45-0.81)	>266.8	55.0 (31.5-76.9)	50.0 (27.2-72.8)
#A1AT	6060.5 (5244.0-9269.6)	8536.1 (5181.4-10849.7)	0.255535	0.61 (0.43-0.79)	>7118.0	65.0 (40.8-84.6)	65.0 (40.8-84.6)
Fibrinogen	2977.3 (2470.9-4028.3)	3699.4 (3169.9-4418.5)	0.260451	0.61 (0.42-0.79)	>2977.0	85.0 (62.1-96.8)	50.0 (27.2-72.8)
MIP-1α	19.1 (12.1-34.8)	17.7 (10.6-25.1)	0.267363	0.60 (0.43-0.78)	<17.9	55.0 (31.5-76.9)	55.0 (31.5-76.9)
TPA	2204.4 (1549.0-4097.1)	2890.7 (2586.5-3872.0)	0.284944	0.60 (0.41-0.78)	>2586.0	75.0 (50.9-91.3)	60.0 (36.1-80.9)
Ferritin	8374.0 (5517.2-23885.4)	14162.7 (7067.7-29837.8)	0.297519	0.60 (0.42-0.78)	>10035.0	65.0 (40.8-84.6)	60.0 (36.1-80.9)
#SAP_Mil	65177.3 (44292.0-67930.3)	48612.6 (31511.3-70107.2)	0.297678	0.60 (0.41-0.78)	<44382.0	50.0 (27.2-72.8)	75.0 (50.9-91.3)
#Complement C3	10101.0 (7339.0-13321.0)	12327.3 (8083.5-16955.8)	0.424797	0.58 (0.39-0.76)	>9947.0	65.0 (40.8-84.6)	50.0 (27.2-72.8)

G-CSF	74.4 (42.1-179.6)	91.5 (70.9-145.7)	0.432710	0.57 (0.39-0.76)	>83.1	70.0 (45.7-88.1)	55.0 (31.5-76.9)
TNF-α	17.3 (8.7-24.7)	17.2 (12.8-25.1)	0.440750	0.57 (0.39-0.75)	>16.4	70.0 (45.7-88.1)	50.0 (27.2-72.8)
#α2M	1036000.0 (677326.6-1685750.0)	1233450.0 (758434.6-2484500.0)	0.440750	0.57 (0.39-0.75)	>1125850.0	55.0 (31.5-76.9)	55.0 (31.5-76.9)
IL-10	19.2 (6.7-58.0)	17.8 (6.5-26.)	0.456909	0.57 (0.39-0.75)	<22.5	65.0 (40.8-84.6)	50.0 (27.2-72.8)
sVEGF-R3	379.1(216.2-512.3)	412.1 (321.5-699.4)	0.515557	0.56 (0.38-0.74)	>362.5	70.0 (45.7-88.1)	50.0 (27.2-72.8)
IFN-α2	0.0 (0.0-33.3)	6.8 (0.0-21.4)	0.650211	0.54 (0.36-0.72)	>1.9	55.0 (31.5-76.9)	60.0 (36.1-80.9)
IL-9	1.3 (1.0-2.5)	1.6 (1.1-2.3)	0.674842	0.54 (0.36-0.72)	>1.3	65.0 (40.8-84.6)	60.0 (36.180.9)
IL-12p40	13.6 (8.4-34.0)	16.7 (5.9-22.4)	0.714647	0.54 (0.35-0.72)	<18.1	65.0 (40.8-84.6)	45.0 (23.1-68.5)
MIP-1β	36.7 (23.5-58.3)	34.2 (23.6-61.4)	0.892389	0.51 (0.33-0.70)	<36.3	55.0 (31.5-76.9)	55.0 (31.5-76.9)
#Complement C4	187321.8 (98162.7-239125.7)	168742.9 (119514.1-222998.7)	0.924573	0.51 (0.32-0.70)	<194156.0	60.0 (36.1-80.9)	50.0 (27.2-72.8)
IL-13	24.8 (0.0-150.5)	24.0 (0.0-127.0)	0.945141	0.51 (0.32-0.69)	<29.7	55.0 (31.5-76.9)	50.0 (27.2-72.8)
#SAP	28615.4 (16597.0-40008.2)	27356.1 (11787.1-43015.6)	0.989208	0.50 (0.32-0.69)	>27879.0	50.0(27.2-72.8)	50.0(27.2-72.8)
TNF-β	34.2 (0.0-250.1)	32.8 (0.0-205.7)	1.000000	0.50 (0.32-0.68)	<41.7	55.0 (31.5-76.9)	50.0 (27.2-72.8)

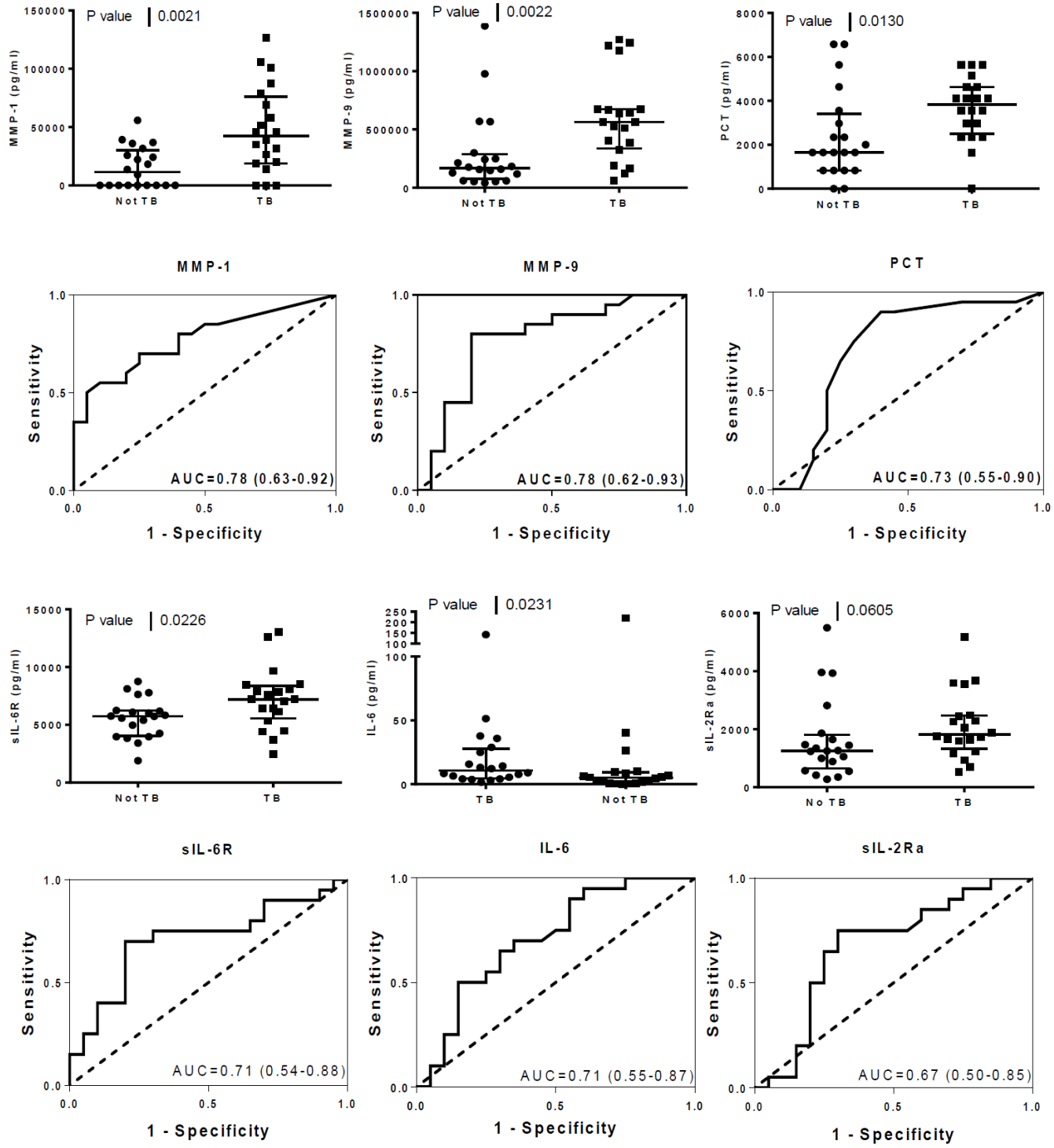

Figure 4.2: Representative plots showing the concentrations of biomarkers detected in serum samples from children with and without TB disease and ROC curves showing the accuracies of these biomarkers in the diagnosis of TB disease. Error bars in the scatter-dot plots indicate the median and inter-quartile ranges. Representative plots for six analytes with AUC ≥0.70 are shown.

4.3.1.4 Utility of combinations between alternative host markers detected in serum samples in the diagnosis of pulmonary TB in children

When the data obtained for all host markers (including CRP, transthyretin, IFN-γ, IP-10, CFH, Apo-AI and SAA) were fitted into General Discriminant analysis (GDA) models irrespective of HIV status, optimal prediction of TBM was shown to be achieved with a combination of up to seven markers. A new seven-marker serum protein biosignature comprising of PCT, MIP-1α, α2M, IFN-γ, IL-10, SAP, and CFH diagnosed pulmonary TB with AUC of 0.94 (95% CI, 0.87-1.00); corresponding to a sensitivity of 95.0% (19/20) and specificity of 90.0% (18/20) (Figure 4.3). After leave-one-out cross validation, there was no change in the performance of the seven-marker biosignature, with attempts in further optimization of the signature through the selection of better cut-off values not resulting in any improvement. The positive and negative predictive values of the seven-marker biosignature were 90.5% (95% CI, 71.8-97.3%) and 94.7% (95% CI, 72.6-99.2%), respectively.

In addition to the 7-marker signature described in the previous paragraph, two other novel host biosignatures showed potential in the diagnosis of pulmonary TB in children. A six-marker serum protein biosignature comprising of MMP-9, IFN-γ, α2M, fibrinogen, CFH and SAP diagnosed pulmonary TB with AUC of 0.94 (95% CI, 0.86-1.00); corresponding to sensitivity of 90.0% (18/20) and specificity of 95.0% (19/20) (Figure 4.4 A and B). After leave-one-out cross validation, the sensitivity of the six-marker biosignature was 90.0% (18/20) and specificity was 85.0% (17/20). The positive and negative predictive values of the six-marker biosignature were 85.7% (95% CI, 67.7-94.5) and 89.5% (95% CI, 69.3-97.0), respectively. Further optimization of the six-marker biosignature resulted in sensitivity of 90.0% and specificity of 95.0%. The other new biosignature comprised a combination between five analytes (IL-1β, IL-12p40, TNF-β, MMP-1 and α2M) which diagnosed pulmonary TB with AUC of 0.95 (95% CI, 0.89-1.00); corresponding to a sensitivity of 85.0% (17/20) and specificity of 95.0% (19/20) (Figure 4.4 C and D). After leave-one-out cross validation the sensitivity of the biosignature was 70.0% (14/20) and specificity was 95.0% (19/20). The positive and negative predictive values of the five-marker biosignature were 93.3% (95% CI, 67.0-99.0%) and 76.0% (95% CI, 61.7-86.2%) respectively. Further optimization of the five-marker biosignature by selection of optimal cut-off values resulted in sensitivity of 85.0% and specificity of 95.0%.

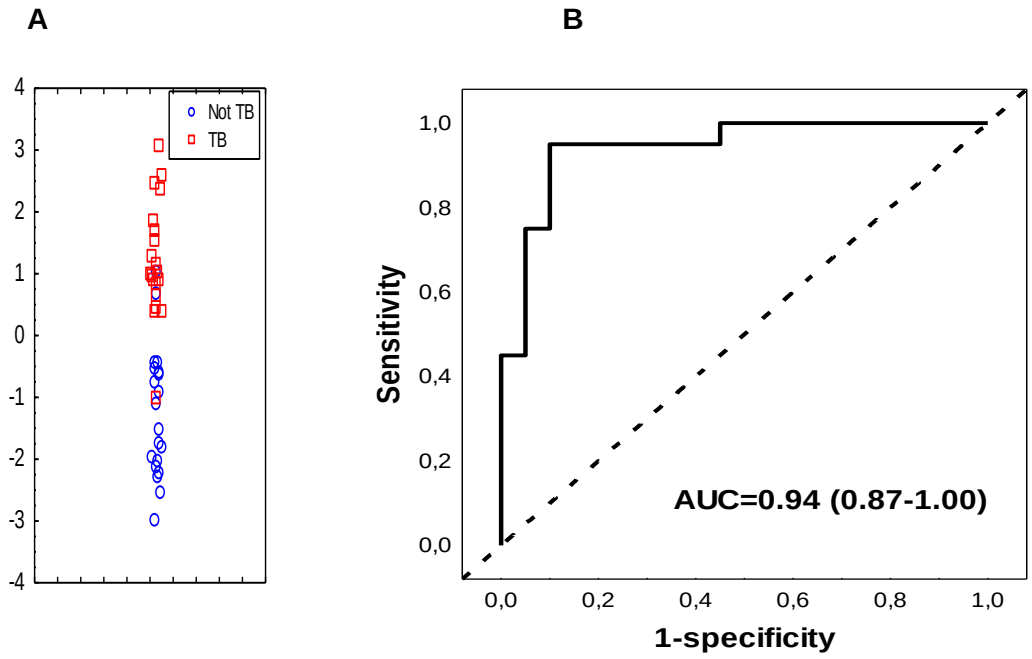

Figure 4.3: Accuracy of a new 7-marker serum protein biosignature (PCT, MIP-1α, α2M, IFN-γ, IL-10, SAP, and CFH) in the diagnosis of pulmonary TB disease in children. Scatter plot showing the ability of the 7-marker signature to classify children as TB or not TB (A). ROC curve showing the accuracy of the 7-marker biosignature (B). Red squares; children with TB; blue circles: children without TB (Not TB).

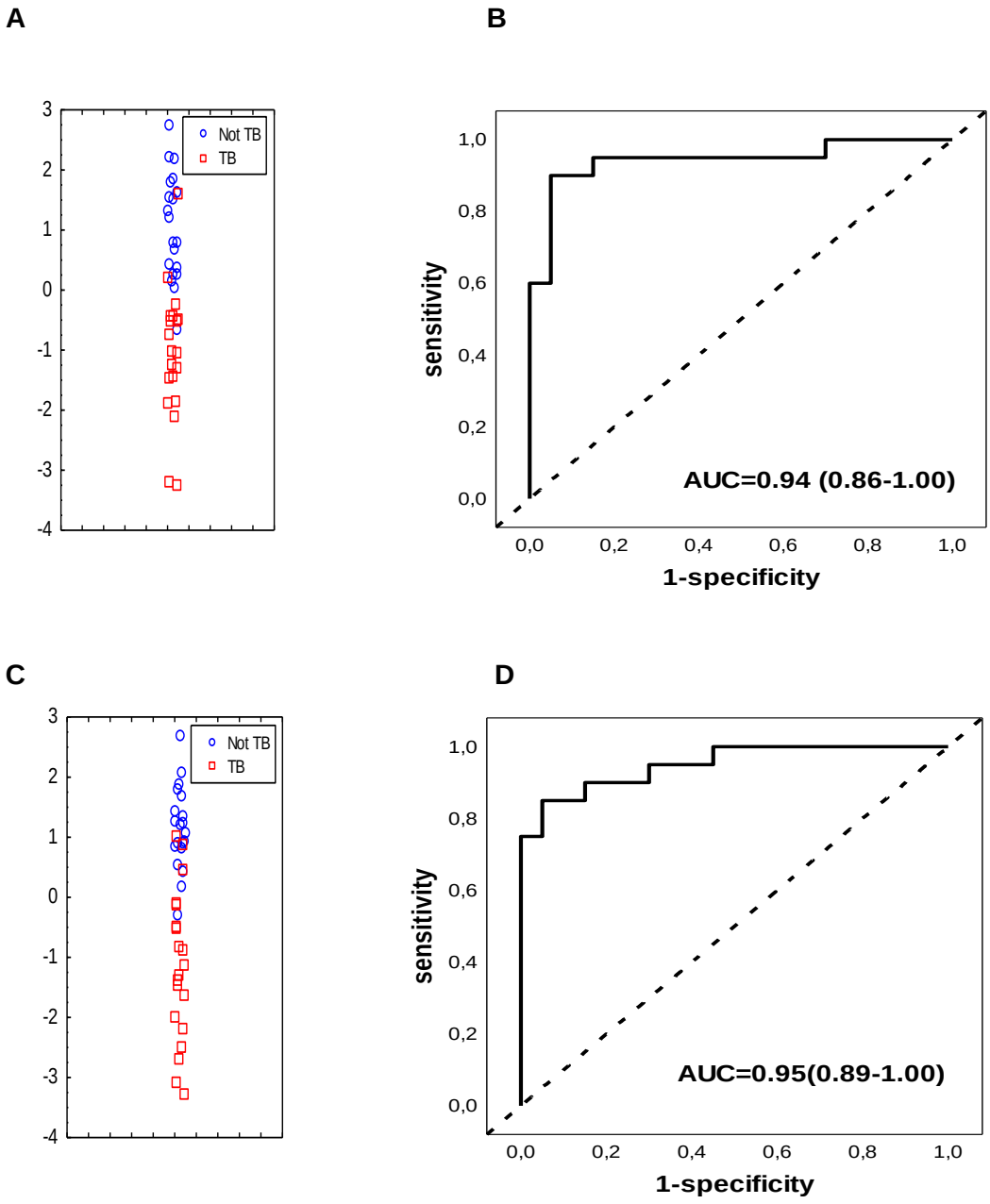

Figure 4.4: Accuracy of the new 6-marker (MMP-9, IFN-γ, α2M, Fibrinogen, CFH and SAP) and 5-marker (IL-1β, IL-12p40, TNF-β, MMP-1 and α2M) serum protein biosignatures in the diagnosis of TB disease. Scatter plot showing the ability of the 6-marker serum protein biosignature to classify children as TB or not TB (A). ROC curve showing the accuracy of the 6-marker serum protein biosignature (B). Scatter plot showing the ability of the 5-marker serum protein signature to classify children as TB or not TB (C). ROC curve showing the accuracy of the 5-marker serum protein biosignature (D). Red squares; children with TB; blue circles: children without TB (Not TB).

4.3.2 Section 4B: Usefulness of blood-based biomarkers in the diagnosis of childhood tuberculous meningitis

4.3.2.1 Application of the previously established adult 7-marker serum protein biosignature in the diagnosis of TBM in children

Of the seven markers comprising the 7-marker (CRP, IFN-γ, IP-10, CFH, Apo-A1, SAA and transthyretin) serum protein biosignature, transthyretin was not available on our analytes panel due to shortage of supply from the manufacturers. When the concentrations of the available six individual markers (CRP, IFN-γ, IP-10, CFH, Apo-A1 and SAA) of the 7 markers comprising the 7-marker adult serum protein biosignature (CRP, IFN-γ, IP-10, CFH, Apo-A1, SAA and transthyretin) were evaluated in serum samples from children with TBM Vs. those without TBM, significant differences were obtained for CFH only. After ROC curve analysis, the most informative single marker from this signature, as determined by AUC was CFH (Table 4.4)

As transthyretin was not available in our analytes panel, it was replaced NCAM1 as the levels of NCAM were previously shown to correlate with transthyretin levels in a previous study, even though that particular information was not included in the published manuscript (170).

When transthyretin was replaced by NCAM1, the modified seven-marker serum protein biosignature comprising of CRP, IFN-γ, IP-10, CFH, Apo-A1, SAA, and NCAM1 diagnosed TBM with AUC of 0.80 (95% CI, 0.67-0.92); corresponding to sensitivity of 73.9% (17/23) and specificity of 66.7% (16/24) (Figure 4.5 A and B). After leave-one-out cross validation, the 7-marker biosignature diagnosed TBM with sensitivity of 60.9% (14/23) and specificity of 58.3% (14/24). The positive and negative predictive values of the modified 7-marker biosignature were 58.3% (95% CI, 44.1-71.1%) and 60.9% (95% CI, 45.8-74.1%), respectively. Further optimization of the 7-marker biosignature by selection of optimal cut-off values resulted into sensitivity of 71% and specificity of 74%. When a first assessment of the potential usefulness of all host biomarkers evaluated in the study was done, by fitting all host markers, including the six available proteins from the 7-marker adult serum protein biosignature into general discriminant analysis (GDA) models in an unbiased manner, it was realised that only SAA featured amongst the analytes that contributed to the top 20 most accurate biosignatures, thus indicating that other biosignature (other than the adult 7-marker signature) might be more promising (Figure 4.5 C).

Table 4.4: Usefulness of analytes comprising the previously established adult 7-marker serum protein biosignature in the diagnosis of TBM in children. Median levels of host markers detected in serum samples from children with TBM or no TBM disease (Inter-quartile range in parenthesis) and accuracies in the diagnosis of TBM. Cut-off values and associated sensitivities and specificities were selected based on the Youden's index. #values shown are in ng/ml, values for all other host markers are in pg/ml.

Markers	Median in TBM (IQR)	Median in Non-TBM (IQR)	p-value	AUC (95% CI)	Cut-off Value	Sensitivity % (95% CI)	Specificity % (95% CI)
#CFH	415846.5 (363515.9-470137.5)	314294.0 (261691.8-412727.7)	0.009719	0.72 (0.57-0.87)	>350185.0	87.0 (66.4-97.2)	66.7 (44.7-84.4)
#Apo AI	302283.6 (267898.0-346446.2)	286350.3 (191698.6-320139.9)	0.160089	0.62 (0.46-0.78)	>287512.0	65.2 (42.7-83.6)	54.2 (32.8-74.5)
CXCL10/IP-10	55.9 (35.9-169.1)	75.8 (49.3-298.3)	0.213146	0.61 (0.44-0.77)	<57.2	52.2 (30.6-73.2)	66.7 (44.7-84.4)
#CRP	230000.0 (230000.0-230000.0)	230000.0 (63731.2-230000.0)	0.380342	0.56 (0.43-0.69)	>80721.0	87.0 (66.4-97.2)	33.3 (15.6-55.3)
#SAA	65700.0 (847.0-230000.0)	39439.7 (6551.9-226031.8)	0.656243	0.54 (0.37-0.71)	>59894.0	56.5 (34.5-76.8)	66.7 (44.7-84.4)
IFN-γ	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.928917	0.51 (0.39-0.63)	<61.5	87.0 (66.4-92.2)	20.8 (7.1-42.2)

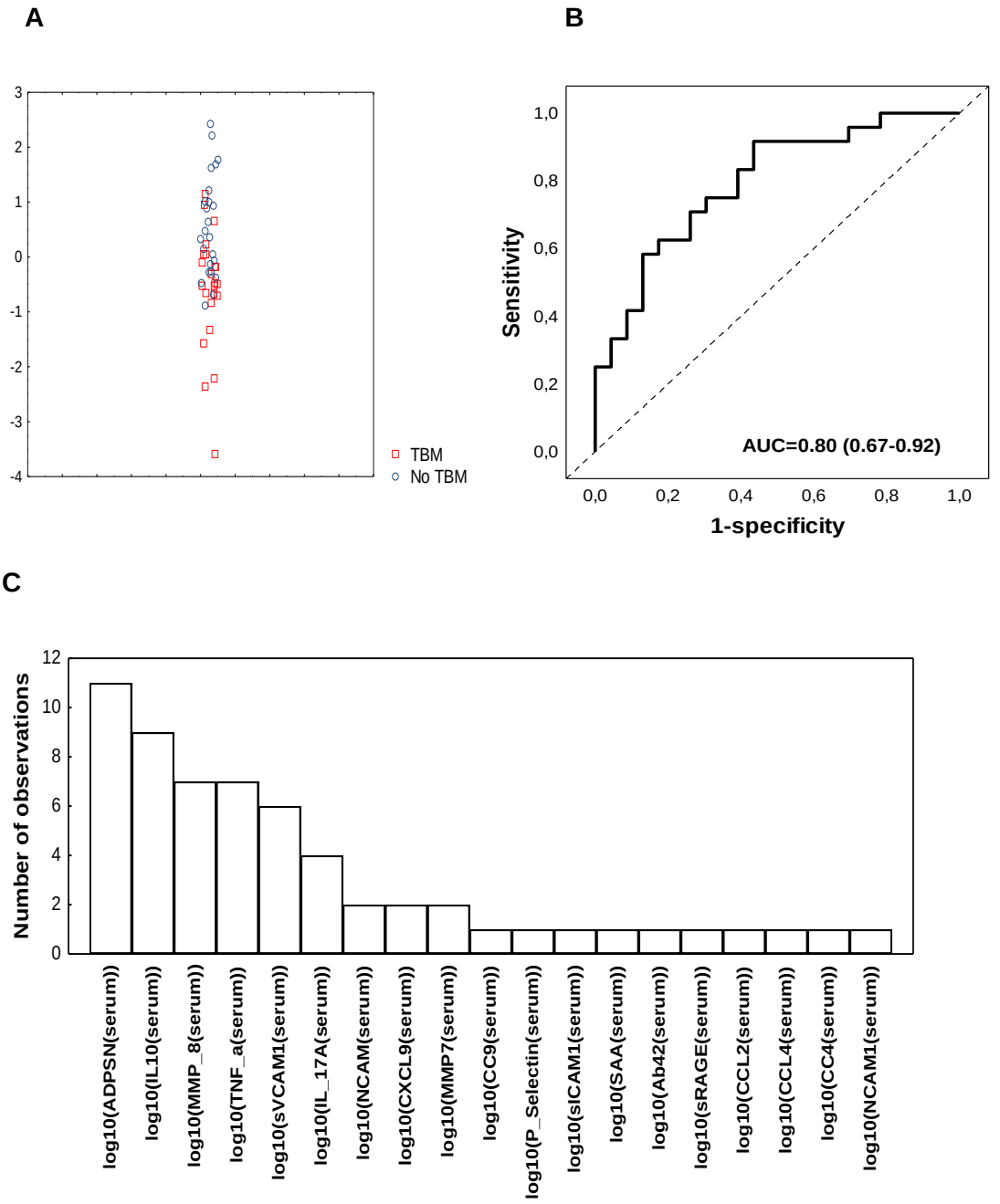

Figure 4.5: Accuracy of the modified 7-marker serum protein biosignature (CRP, IFN-γ, IP-10, CFH, Apo-A1, SAA and NCAM1) in the diagnosis of TBM. Scatter plot showing the ability of the 7-marker signature to classify children as TBM or no TBM (A). ROC curve showing the accuracy of the 7-marker biosignature (B). Frequency of analytes in the top 20 most accurate models in the diagnosis of TBM, if host biomarkers are not pre-specified (C). Red squares; children with TBM; blue circles: children with No TBM.

4.3.2.2 Potential of alternative blood (serum)-based host protein biomarkers in the diagnosis of TBM in children

When the concentrations of the 63 other host biomarkers detected in serum samples were compared between children with and those without TBM using the Mann Whitney U test, the median levels of 16 biomarkers including sVCAM1, CCL2, IL-4, TNF-α, CCL4, adipsin, SAP, CC5, G-CSF, IL-10, Apo-CIII, IL-17A, PAI-1, PDGF AB/BB, MBL and NCAM1 were significantly different ($p<0.05$) between the children with and those without TBM according to the Mann Whitney U test. The levels of five biomarkers (CC4b, MMP-1, CXCL8, CC4, sRAGE) showed trends ($0.05<p\leq0.09$) between the two groups. The concentrations of SAP, CC5, Apo-CIII, PAI-1, PDGF-AB/BB and MBL were significantly higher in serum samples of children with TBM whereas those of sVCAM-1, CCL2, IL-4, TNF-α, CCL4, ADIPSIN, G-CSF, IL-10, IL-17A and NCAM1 were higher in serum samples from children without TBM. When the diagnostic potentials of individual serum biomarkers were assessed by ROC curve analysis, 13 of the markers showed promise as ascertained by AUC ≥ 0.70 (Table 4.5, Figure 4.6). When only HIV uninfected children were considered, there were noticeable improvements in the performance of other host markers including MMP-1 and IL-7. The median levels of six markers including IL-10, MBL, sRAGE, CC4, CC4b and NCAM1 were no longer significantly different or showing trends between the two groups (Data not shown).

Table 4.5: Utility of alternative host biomarkers detectable in serum samples in the diagnosis of TBM in children. Median levels of host markers detected in serum samples from children with TBM or no TBM disease (Inter-quartile range in parenthesis) and accuracies in the diagnosis TBM**.** The data shown are raw, untransformed values. Cut-off values and associated sensitivities and specificities were selected based on the Youden's index. *values shown are the absorbance and not the concentration values. #values shown are in ng/ml, values for all other host markers are in pg/ml.

Markers	Median in TBM (IQR)	Median in Non-TBM (IQR)	p-value	AUC (95% CI)	Cut-off Value	Sensitivity % (95% CI)	Specificity % (95% CI)
VCAM-1	1197300.0 (952940.0-1543500.0)	1802000.0 (1456400.0-2521350.0)	0.000188	0.82 (0.70-0.94)	<1580000.0	78.3 (56.3-92.5)	66.7 (44.7-84.4)
MCP-1/CCL2	244.3 (165.5-390.9)	512.3 (319.7-994.8)	0.000262	0.81 (0.69-0.93)	<327.3	73.9 (51.6-89.7)	75.0 (53.3-90.2)
IL-4	82.7 (7.42-99.6)	136.8 (99.6-191.3)	0.001147	0.78 (0.65-0.91)	<116.7	78.3 (56.3-92.5)	62.5 (40.6-81.2)
TNF-α	4.8 (0.0-11.4)	23.3 (15.7-31.9)	0.001457	0.77 (0.62-0.91)	<12.9	78.3 (56.3-92.5)	79.2 (57.9-92.9)
MIP-1β/ CCL4	219.0 (158.1-296.8)	401.4 (275.7-667.2)	0.002148	0.76 (0.62-0.90)	<334.3	78.3 (56.3-92.5)	66.7 (44.7-84.4)
#Adipsin (CFD)	1950.4 (1611.1-2319.1)	2917.4 (2493.4-3938.5)	0.004065	0.75 (0.59-0.90)	<2393.0	78.3 (56.3-92.5)	79.2 (57.9-92.9)
#SAP	331539.9 (261542.1-655100.2)	167660.5 (88309.6-286067.7)	0.005664	0.74 (0.59-0.89)	>257478.0	78.3 (56.3-92.5)	70.8 (48.9-87.4)
#CC5	52307.0 (44989.9-59967.4)	38538.0 (28210.6-47089.8)	0.006660	0.73 (0.58-0.88)	>46742.0	69.6 (47.1-86.8)	75.0 (53.3-90.2)
#CFH	415846.5 (363515.9-470137.5)	314294.0 (261691.8-412727.7)	0.009719	0.72 (0.57-0.87)	>350185.0	87.0 (66.4-97.2)	66.7 (44.7-84.4)
G-CSF	14.0 (0.0-117.6)	147.6 (25.1-463.4)	0.010573	0.72 (0.57-0.86)	<76.0	65.2 (42.7-83.6)	70.8 (48.9-87.4)

IL-10	0.0 (0.0-4.1)	8.1 (0.0-21.2)	0.011193	0.70 (0.56-0.85)	<7.0	95.7 (78.1-99.9)	54.2 (32.8-74.5)
#Apo CIII	151289.3 (130100.8-181642.4)	95825.1 (63481.7-161543.8)	0.014822	0.71 (0.55-0.87)	>114926.0	87.0 (66.4-97.2)	62.5 (40.6-81.2)
IL-17A	0.0 (0.0-0.0)	0.0 (0.0-18.4)	0.018640	0.65 (0.53-0.76)	<11.3	95.7 (78.1-99.9)	37.5 (18.8-59.4)
PAI-1(total)	348736.6 (261199.3-456794.4)	246289.2 (175941.2-350988.5)	0.018694	0.70 (0.55-0.85)	>255621.0	78.3 (56.3-92.5)	58.3 (36.6-77.9)
PDGF-AB/BB	49576.6 (33649.3-83528.9)	33592.0 (14786.0-49751.6)	0.032444	0.68 (0.53-0.84)	>42307.0	65.2 (42.7-83.6)	66.7 (44.7-84.4)
#MBL	9533.4 (4686.1-30439.6)	3299.1 (901.1-14882.4)	0.033928	0.68 (0.52-0.84)	>4522.0	78.3 (56.3-92.5)	58.3 (36.6-77.9)
NCAM-1	246692.5 (164329.5-305706.5)	285446.4 (256271.6-342048.0)	0.036064	0.68 (0.52-0.84)	<264419.0	69.6 (47.1-86.8)	70.8 (48.9-87.4)
#CC4b	29843.2 (21128.5-42752.7)	25562.6 (17752.8-31264.4)	0.056822	0.66 (0.51-0.82)	>26285.0	69.6 (47.1-86.8)	54.2 (32.8-74.5)
MMP-1	5694.6 (3233.2-7609.0)	4084.8 (2174.5-6345.7)	0.068827	0.66 (0.50-0.81)	>4282.0	60.9 (38.5-80.3)	54.2 (32.8-74.5)
CXCL8/IL-8	37.1 (15.5-54.1)	55.4 (27.5-112.9)	0.072101	0.65 (0.49-0.81)	<42.1	60.9 (38.5-80.3)	66.7 (44.7-84.4)
#CC4	157528.9 (90929.3-209684.1)	85388.5 (48405.5-194821.2)	0.079129	0.65 (0.49-0.81)	>89484.0	78.3 (56.3-92.5)	54.2 (32.8-74.5)
sRAGE	855.2 (773.7-896.6)	875.8 (855.2-937.8)	0.094181	0.64 (0.48-0.80)	<875.8	73.9 (51.6-89.8)	50.0 (29.1-70.9)
TGF-α	60.3 (26.9-96.2)	28.5 (5.6-79.8)	0.110002	0.64 (0.48-0.80)	>29.9	69.6 (47.1-86.8)	54.2 (32.8-74.5)
IL-7	36.0 (22.9-55.8)	29.2 (12.4-37.7)	0.110363	0.64 (0.48-0.80)	>27.5	69.6 (47.1-86.8)	50.0 (29.1-70.9)
IL-6	6.8 (1.6-14.6)	8.9 (2.5-44.7)	0.135692	0.63 (0.47-0.79)	<8.0	56.5 (34.5-76.8)	58.3 (36.6-77.9)

GM-CSF	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.158099	0.57 (0.40-0.73)	<9.3	100.0 (85.2-100)	16.7 (4.7-37.4)
#Apo AI	302283.6 (267898.0-346446.2)	286350.3 (191698.6-320139.9)	0.160089	0.62 (0.46-0.78)	>287512.0	65.2 (42.7-83.6)	54.2 (32.8-74.5)
VEGF	152.4 (112.5-251.2)	106.7 (74.8-235.8)	0.169862	0.62 (0.45-0.78)	>111.2	78.3 (56.3-92.5)	54.2 (32.8-74.5)
Aβ40	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.171006	0.54 (0.37-0.71)	<72.1	100.0 (85.2-100.0)	8.3 (1.0-27.0)
#CF1	66236.8 (49972.0-99204.5)	54181.1 (45646.3-71882.7))	0.176578	0.62 (0.45-0.78)	>57835.0	65.2 (42.7-83.6)	62.5 (40.6-81.2)
MMP-7	808.0 (524.4-1584.1)	1175.0 (625.5-3399.8)	0.189921	0.61 (0.45-0.78)	<869.0	60.9 (38.5-80.3)	62.5 (40.6-81.2)
#Myoglobin	9.6 (4.4-20.3)	21.4 (4.9-51.0)	0.201135	0.61 (0.44-0.78)	<10.2	60.9 (38.5-80.3)	66.7 (44.7-84.4)
CXCL10/IP-10	55.9 (35.9-169.1)	75.8 (49.3-298.3)	0.213146	0.61 (0.44-0.77)	<57.2	52.2 (30.6-73.2)	66.7 (44.7-84.4)
PDGF-AA	8538.7 (5683.1-15788.5)	6995.0 (2635.5-12806.3)	0.221073	0.61 (0.44-0.77)	>6150.0	69.6 (47.1-86.8)	50.0 (29.1-70.9)
#MIP4	241.6 (172.5-366.9)	178.1 (119.2-342.3)	0.221073	0.61 (0.44-0.77)	>187.7	69.6 (47.1-86.8)	54.2(32.8-74.5)
Aβ42	0.0 (0.0-0.0)	0.0 (0.0-556.9)	0.240593	0.58 (0.45-0.72)	<278.4	73.9 (51.6-89.8)	41.7 (22.1-63.4)
#CC3	40885.9 (36448.0-74127.5)	46059.4 (25390.4-53871.9)	0.254876	0.40 (0.23-0.57)	>32056.0	91.3 (72.0-98.9)	41.7 (22.1-63.4)
#A1AT	18729.1 (14631.0-24621.2)	16819.0 (11711.3-27780.9)	0.287284	0.59 (0.42-0.76)	>17908.0	60.9 (38.5-80.3)	58.3 (36.6-77.9)
#P-Selectin	194.3 (102.1-352.1)	119.1 (54.4-274.0)	0.330420	0.58 (0.42-0.75)	>159.1	65.2 (42.7-83.6)	62.5 (40.6-81.2)
CC5a	2663.1 (1751.2-3946.9)	2423.2 (1559.1-3554.3)	0.349063	0.58 (0.41-0.75)	>2660.0	52.2 (30.6-73.2)	66.7 (44.7-84.4)

IL-12/23p40	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.349077	0.52 (0.35-0.69)	<620.1	100.0 (85.2-100.0)	4.2 (0.1-21.1)
IL-1β	0.0 (0.0-0.0)	0.0 (0.0-9.2)	0.358788	0.56 (0.43-0.68)	<8.3	91.3 (72.0-98.9)	29.2 (12.6-51.1)
MMP-8	24763.5 (12747.3-86623.8)	19342.7 (9257.2-35601.6)	0.360001	0.59 (0.41-0.75)	>22769.0	56.5 (34.5-76.8)	58.3 (36.6-77.9)
#CRP	230000.0 (230000.0-230000.0)	230000.0 (63731.2-230000.0)	0.380342	0.56 (0.43-0.69)	>80721.0	87.0 (66.4-97.2)	33.3 (15.6-55.3)
CCL3/MIP-1β	48.7 (0.0-65.1)	49.8 (0.0-209.1)	0.382647	0.57 (0.41-0.74)	<48.9	65.2 (42.7-83.6)	54.2 (32.8-74.5)
MMP-9	205449.19 (59802.48-556493.88)	174486.7 (73396.4-266465.9)	0.387093	0.57 (0.40-0.74)	>189764.0	56.5 (34.5-76.8)	58.3 (36.6-77.9)
IL-21	0.0 (0.0-0.0)	0.0 (0.0-15.9)	0.396732	0.55 (0.43-0.67)	<34.6	95.7 (78.1-99.9)	20.8 (7.1-42.2)
Cathepsin D	439708.5 (308272.3-728466.0)	493856.4 (331662.9-959098.3)	0.412583	0.57 (0.40-0.74)	<459422.0	60.9 (38.5-80.3)	54.2 (32.8-74.5)
ICAM-1	216547.6 (137559.5-286618.4)	215566.5 (171273.1-337326.2)	0.418679	0.57 (0.40-0.72)	<224039.0	56.5 (34.5-76.8)	50.0 (29.1-70.9)
#CC9	3295.9 (2497.1-4084.6)	3657.9 (2600.8-4489.9)	0.475866	0.56 (0.39-0.73)	<3502.0	65.2 (42.7-83.6)	58.3 (36.6-77.9)
MPO	4746700.0 (1779300.0-6026200.0)	3438600.0 (1669250.0-4934150.0)	0.475891	0.56 (0.39-0.73)	>4650000.0	52.2 (30.6-73.2)	70.8 (48.9-87.4)
CD40L	11633.0 (8228.9-16525.6)	10742.2 (6930.7-17042.3)	0.475891	0.56 (0.39-0.73)	>11151.0	65.2 (42.7-83.6)	54.2 (32.8-74.5)
#GDF-15	1.0 (0.6-1.6)	1.1 (0.6-3.1)	0.501871	0.56 (0.39-0.73)	<1.1	60.9 (38.5-80.3)	54.2 (32.8-74.5)

#D-dimer	9287.6 (1772.3-17900.1)	9102.9 (3021.2-41007.9)	0.550286	0.55 (0.38-0.72)	<9451.0	52.2 (30.6-73.2)	50.0 (29.1-70.9)
BDNF	15636.7 (10109.5-24406.5)	18107.0 (8952.6-28946.9)	0.572783	0.55 (0.38-0.72)	<17211.0	65.2 (42.7-83.6)	54.2 (32.8-74.5)
CXCL9/MIG	2309.5 (0.0-3311.4)	1800.7 (0.0-3557.3)	0.625319	0.54 (0.38-0.71)	>2114.0	52.2 (30.6-73.2)	62.5 (40.6-81.2)
#SAA	65700.0 (847.0-230000.0)	39439.7 (6551.9-226031.8)	0.656243	0.54 (0.37-0.71)	>59894.0	56.5 (34.5-76.8)	66.7 (44.7-84.4)
IL-13	0.0 (0.0-338.1)	0.0 (0.0-756.3)	0.681743	0.53 (0.38-0.69)	<74.6	56.5 (34.5-76.8)	45.8 (25.6-67.2)
#CC2	15903.9 (8706.0-31171.1)	15768.9 (6992.3-49343.7)	0.725481	0.53 (0.36-0.70)	<15990.0	52.2 (30.6-73.2)	50.0 (29.1-70.9)
Ferritin	52841.0 (14202.0-114067.7)	62740.4 (16776.2-169542.0)	0.740490	0.53 (0.36-0.70)	<56314.0	56.5 (34.5-76.8)	58.3 (36.6-77.9)
#PEDF	21756.5 (18654.6-25542.3)	21401.6 (18159.9-26348.1)	0.765743	0.53 (0.36-0.70)	>21725.0	52.2 (30.6-73.2)	54.2 (32.8-74.5)
RANTES	108231.8 (53485.5-169473.6)	92692.2 (39285.6-188178.1)	0.790226	0.52 (0.35-0.69)	>99016.0	56.5 (34.5-76.8)	54.2 (32.8-74.5)
#ADMTS13	901.2 (545.3-1092.7)	874.4 (600.0-1120.0)	0.823096	0.52 (0.35-0.68)	<962.3	60.9 (38.5-80.3)	45.8 (25.6-67.2)
#NGAL	394.1 (152.5-1046.1)	380.5 (189.3-560.3)	0.831299	0.52 (0.35-0.69)	>371.5	52.2 (30.6-73.2)	50.0 (29.1-70.9)
CCL1/I-309	15.0 (8.6-33.4)	15.2 (7.6-44.4)	0.848035	0.52 (0.35-0.69)	<15.2	52.2 (30.6-73.2)	50.0 (29.1-70.9)
GDNF	136.3 (120.1-152.7)	136.3 (136.3-152.7)	0.921886	0.51 (0.34-0.67)	<140.4	52.2 (30.6-73.2)	41.7 (22.1-63.4)
IFN-γ	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.928917	0.51 (0.39-0.63)	<61.5	87.0 (66.4-92.2)	20.8 (7.1-42.2)
***Cathelicidin-LL37**	0.5 (0.3-0.9)	0.5 (0.3-0.9)	0.974533	0.49 (0.31-0.66)	>0.4	60.9 (38.5-80.3)	34.8 (16.4-57.3)
S100B	2800.0 (2744.2-2800.0)	2800.0 (2744.2-2800.0)	0.986591	0.50 (0.34-0.66)	>2772.0	55.6 (30.8-78.5)	40.0 (19.1-64.0)

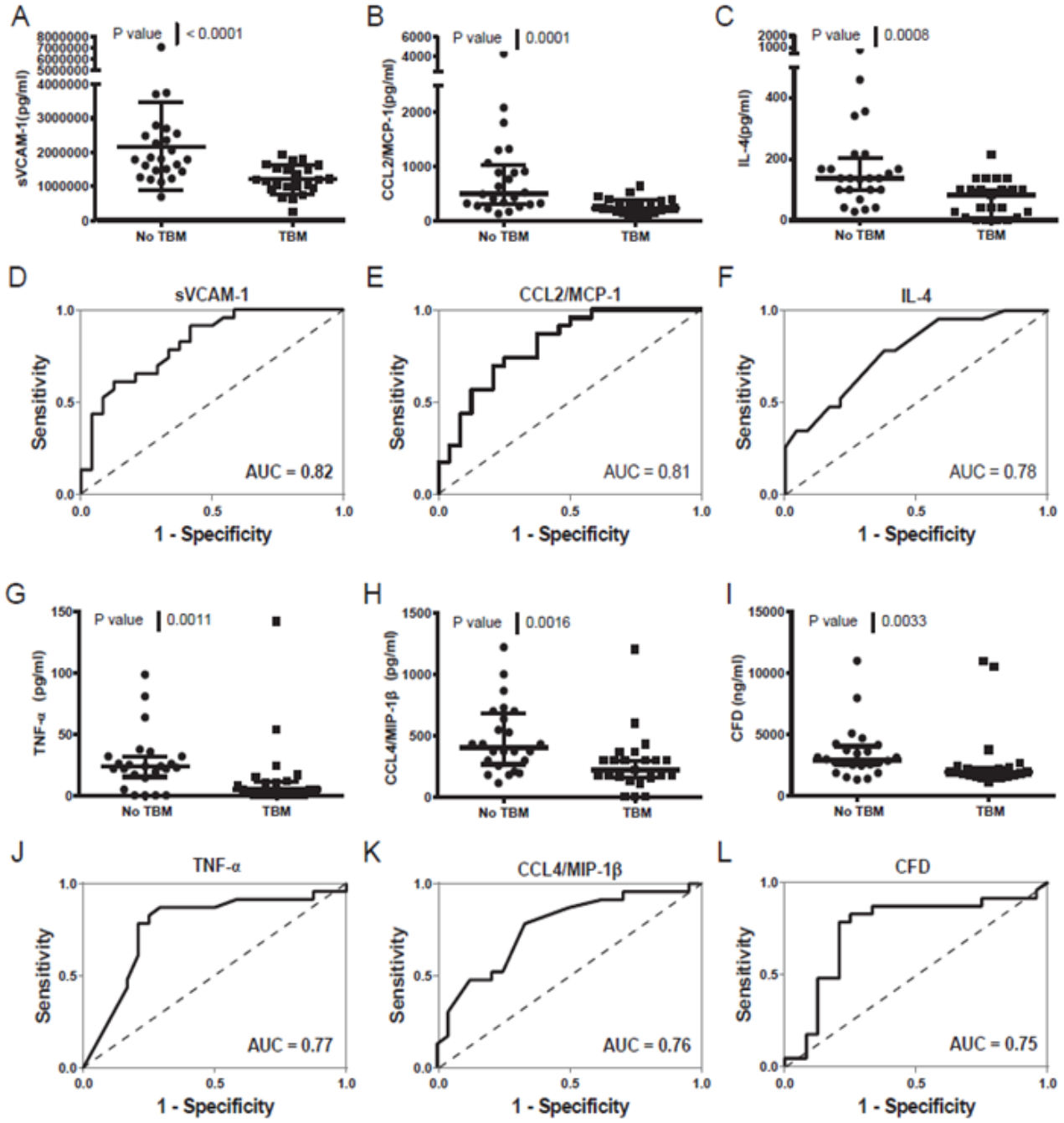

Figure 4.6: Representative plots showing the concentrations of biomarkers detected in serum samples from children with and without TBM and ROC curves showing the accuracies of these biomarkers as individual markers in the diagnosis of TBM. Representative plots for 6 analytes with AUC ≥0.75 are shown. Error bars in the scatter-dot plots indicate the median and inter-quartile ranges.

4.3.2.3 Potential of combinations between alternative blood (serum)-based host protein biomarkers in the diagnosis of TBM in children

When the data obtained from serum samples from all study participants were fitted into the General Discriminant Analysis (GDA) models regardless of HIV status, optimal prediction of TBM was shown to be achieved with a combination of three markers. The most accurate three-marker biosignature comprising of adipsin(CFD), Aβ42 and IL-10 diagnosed TBM with area under the curve (AUC) of 0.84 (95% CI, 0.73-0.96); corresponding to a sensitivity of 82.6% (95 CI, 61.2-95.0%) and specificity of 75.0% (95% CI, 53.3-90.2%). After leave-one-out cross validation, there was no change in the sensitivity of the 3-marker serum biosignature, whereas the specificity reduced to 70.8% (95 CI, 48.9-87.4%). The positive and negative predictive values of the biosignature were 73.1% (95% CI, 58.6-83.9%) and 81.0% (95% CI, 62.7-91.5%), respectively. Further optimization of the three-marker serum biosignature through selection of optimal cut-off values resulted in both sensitivity and specificity of 83% (Figure 4.7).

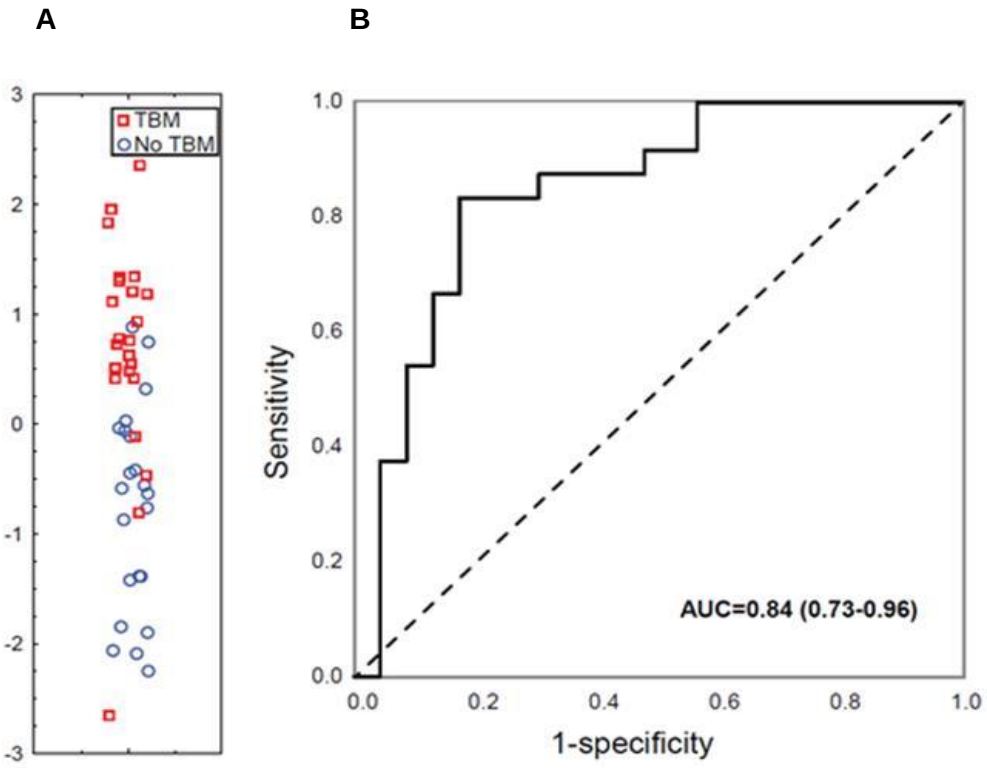

Figure 4.7: Accuracy of the 3-marker serum biosignature (Complement factor D/adipsin, Ab42 and IL-10) in the diagnosis of TBM. Scatter plot showing the ability of the 3-marker signature to classify children as TBM or no TBM (A). ROC curve showing the accuracy of the

3-marker biosignature (B). Red squares: children with TBM; blue circles: children with No TBM.

4.3.3 Differential expression of host biomarkers in cerebrospinal fluid Vs. serum samples

As the study participants whose samples were evaluated in this study (section 4B) were the same participants evaluated in chapter 3, with the same analyte panels being used in the two studies, data from both serum and CSF samples from each study participant was available. The levels of all 69 host biomarkers evaluated in serum samples in this study were therefore compared to the levels obtained in CSF samples from the same study participants in chapter 3, to assess the differential expression of the biomarkers in blood Vs. CSF. The data on the differential expression of host biomarkers between the two sample types could provide information on the best sample type for future experiments, for development of diagnostic tools and also for understanding of the immunology of TBM in children.

Using data obtained from the manufacturer's package inserts, we evaluated the proportions of participants in whom the detected levels of different host markers ≥ the published minimum detectable concentration (MDC). Out of all evaluated host markers, reliably published data on MDC was not available (NA) for 29 analytes (MIP-1α, IFN-γ, IL-10, IL-13, 1L-21, MIG, GM-CSF, G-CSF, IL-1β, IL12/23p40, CXCL8, IL-17A, IL-4, TNF-α, CD40L, TGF-α, IL-7, MMP-7, VEGF-A, NCAM-1, MMP-1, MMP-9, IP-10, IL-6, CCL2, ferritin, CCL1, MMP-8, and CCL4) purchased from R&D systems and for cathelicidin LL-37, which was analysed using ELISA kit purchased from Elabscience Biotechnology Inc. As a result, only analytes purchased from Merck Millipore were evaluated for proportions of participants in whom the detected levels were ≥ the published MDC. The concentrations of 19 markers (D-dimer, CC2, CC4b, CC5a, adipsin, CF1, ADAMTS13, Cathepsin D, ICAM-1, NCAM, sVCAM-1, PAI-1, Apo-CIII, CC3, CRP, A1AT, PEDF, SAP and MIP-4) were higher than the MDC in both serum and CSF in all study participants (100%). Furthermore, the levels of seven markers (CC5, CC9, MBL, PDGF-AA, sRAGE, CFH, and CC4) were higher than the MDC in both sample types in at least 90% of the study participants (Table 4.6).

When the levels of 69 host biomarkers were compared between CSF and serum samples using Mann-Whitney U-test analysis, the levels of 18 markers (Aβ40, Aβ42, MIP-1α, IL-8, IFN-γ, IL-10, IL-13, IL-21, IL-6, MCP-1, IP-10, MIG,GM-CSF, IL-1β, IL-12/23p40, IL-17A, IL-4, and TNF-α) were significantly higher in CSF samples in comparison to serum samples (Table 4.6, Figure 4.8). The levels of 19 markers (D-dimer, CC2, CC4b, CC5a, ADIPSIN, CF1, ADAMTS13, Cathepsin D, ICAM-1, NCAM, sVCAM-1, PAI-1, Apo-CIII, CC3, CRP, A1AT, PEDF, SAP and MIP-4) were significantly higher in serum samples of all study participants in

comparison to CSF samples (Table 4.6, Figure 4.9). The levels of 3 markers (CCL4, G-CSF and I-309) showed no difference between CSF and serum samples, while the other remaining markers were significantly higher in serum samples than in CSF samples (Table 4.6).

Table 4.6: Proportion of study participants with host markers above the minimum detectable concentration (MDC) in CSF and serum samples and the differences in median levels detected between the two sample types. Median levels of host biomarkers detected in CSF and serum samples from all study participants (n=47) and the proportion of study participants with host biomarkers above the minimum detectable concentration (MDC) in the two sample types are shown. MDC values were obtained from the package inserts provided by the kits manufacturers. *The Optical density (OD) is shown for these markers. #Marker levels are expressed in ng/ml, while the other markers are expressed in pg/ml. IQR: Inter-quartile range.

	Cerebrospinal Fluid (CSF)			**Serum**		
Marker	**MDC**	**% > MDC**	**Median (IQR)**	**% > MDC**	**Median (IQR)**	***P* value**
(A) Host markers more abundantly expressed in CSF						
Aβ40	1,4	97.9	647.6 (305.1-1407.0)	4.3	0.0 (0.0-0.0)	0.0001
Aβ42	9,7	93.6	200.6 (54.3-409.4)	34.0	0.0 (0.0-556.9)	0.0008
CCL3/MIP-1α	NA	NA	219.1 (118.9-327.5)	NA	48.7 (0.0-180.4)	<0.0001
CXCL8/IL-8	NA	NA	454.9 (107.8-1106.0)	NA	46.6 (20.4-78.0)	<0.0001
IFN-γ	NA	NA	91.7 (7.7-469.9)	NA	0.0 (0.0-0.0)	<0.0001
IL-10	NA	NA	16.0 (3.9-50.4)	NA	1.3 (0.0-8.8)	<0.0001
IL-13	NA	NA	378.2 (169.1-1171.0)	NA	0.0 (0.0-756.3)	0.0005
IL-21	NA	NA	44.7 (28.6-71.0)	NA	0.0 (0.0-0.0)	<0.0001
IL-6	NA	NA	86.8 (2.3-536.6)	NA	8.9 (1.8-25.9)	0.0017
CCL2/MCP-1	NA	NA	993.4 (502.2-1395.0)	NA	329.1 (228.9-640.0)	<0.0001

CXCL10/IP-10	NA	NA	1974.0 (189.8-44900.0)	NA	66.4 (39.3-195.4)	<0.0001
CXCL9/MIG	NA	NA	3163.0 (1350.0-9846.0)	NA	1801.0 (0.0-3311.0)	0.0021
GM-CSF	NA	NA	63.0 (20.7-93.0)	NA	0.0 (0.0-0.0)	<0.0001
IL-1β	NA	NA	13.9 (0.0-51.3)	NA	0.0 (0.0-0.0)	<0.0001
IL-12/23p40	NA	NA	0.0 (0.0-542.3)	NA	0.0 (0.0-0.0)	<0.0001
IL-17A	NA	NA	9.2 (0.0-20.5)	NA	0.0 (0.0-0.0)	0.001
IL-4	NA	NA	170.2 (113.3-246.1)	NA	99.6 (41.1-136.8)	<0.0001
TNF-α	NA	NA	22.1 (1.2-71.4)	NA	14.4 (0.0-24.9)	0.0736
(B) Host markers more abundantly expressed in serum						
#D-dimer	0.028	100.0	1448 (2.5-98000.0)	100.0	9283.0 (2437.0-21283.0)	0.7202
#CC2	0.25	100.0	774.4 (70.8-2297.0)	100.0	15904.0 (7911.0-37262.0)	<0.0001
#CC4b	0.28	100.0	351.9 (168.7-611.0)	100.0	28264.0 (19539.0-35483.0)	<0.0001
#CC5	0.68	93.6	166.1 (33.3-511.3)	100.0	46216.0 (34488.0-55936.0)	<0.0001
CC5a	0.0023	100.0	41.3 (6.4-88.0)	100.0	2530.0 (1645.0-3761.0)	<0.0001
#CC9	8.16	93.6	35.7 (24.6-50.0)	100.0	3423.0 (2584.0-4293.0)	<0.0001
#Adipsin/CFD	0.016	100.0	43.5 (21.2-119.4)	100.0	2468.0 (1791.0-3458.0)	<0.0001
#MBL	0.036	93.6	5.3 (0.9-25.6)	100.0	7544.0 (1636.0-18866.0)	<0.0001
#CF1	0.15	100.0	275.7 (97.6-699.5)	100.0	59466.0 (45700.0-77858.0)	<0.0001
#P-selectin	0.024	44.7	0.0-0.0-1.6)	78.7	166.5 (60.7-291.1)	<0.0001
sICAM1_67K	0.032	80.9	14.5 (0.5-58.5)	100.0	234.7 (146.8-372.8)	<0.0001

sVCAM1_67K	0.032	80,9	27.9 (6.8-129.4)	80.9	957.1 (631.5-1408.0)	<0.0001
#ADAMTS13	0.053	100.0	7.2 (0.6-13.3)	100.0	891.5 (545.3-1093.0)	<0.0001
#GDF-15	0.00011	80.9	0.2 (0.0-0.4)	95.7	1.0 (0.6-2.1)	<0.0001
#Myoglobin	0.007	80.9	0.2 (0.0-1.0)	100.0	11.1 (4.7-37.8)	<0.0001
MPO_67K	0.005	80.9	1.2 (0.0-46.8)	80.9	799.3 (313.5-1647.0)	<0.0001
#Lipocalin-2/NGAL	0.001	80.9	7.9 (0.8-78.4)	87.2	394.1 (170.6-691.6)	0.0004
#SAA	0.048	76.6	52.3 (0.2-2411.0)	80.9	46890.0 (4510.0-230000.0)	0.0018
BDNF	0.23	63.8	0.6 (0.0-1.0)	100.0	16046.0 (10109.0-25813.0)	<0.0001
Cathepsin D	8.08	100.0	73531.0 (55896.0-95805.0)	100.0	450337.0 (315790.0-746949.0)	<0.0001
ICAM1_36K	6.29	100.0	1503.0 (420.1-3384.0)	100.0	216548.0 (148077.0-303985.0)	<0.0001
MPO_36K	200.0	89.4	28780.0 (1383.0-63392.0)	100.0	3770000.0 (1690000.0-5750000.0)	<0.0001
PDGF-AA	0.22	95.7	7.3 (5.0-15.9)	100.0	8089.0 (3553.0-14143.0)	<0.0001
RANTES	1.20	83.0	9.3 (3.7-22.3)	100.0	102077.0 (49156.0-185835.0)	<0.0001
NCAM	4.81	100.0	33759.0 (26739.0-45387.0)	100.0	444783.0 (352121.0-542236.0)	<0.0001
PDGF-AB/BB	3.83	76.6	7.0 (4.1-12.9)	100.0	41696.0 (20984.0-69370.0)	<0.0001
sVCAM1_36K	6.44	100.0	89081.0 (24577.0-135239.0)	100.0	1510000.0 (1130000.0-1850000.0)	<0.0001

PAI-1	0.48	100.0	1706.0 (348.2-9135.0)	100.0	271962.0 (198549.0-387515.0)	<0.0001
S100B	3.1	80,9	41.2 (30.1-766.1)	80.9	2800.0 (2744.0-2800.0)	<0.0001
sRAGE	3,8	97.9	14.1 (12.8-16.0)	100.0	855.2 (773.7-937.8)	<0.0001
GDNF	0,9	80,9	2.1 (1.8-2.3)	83.0	136.3 (120.1-152.7)	<0.0001
Ferritin	NA	NA	3261.0 (621.2-8447.0)	NA	57058,0 (15940,0-137448,0)	0.0012
MMP-9	NA	NA	1480.0 (2.6-4385.0)	NA	188899.0 (59802.0-348674.0)	<0.0001
CD40L	NA	NA	363.4 (214.6-594.4)	NA	11509.0 (7489.0-16633.0)	<0.0001
MMP-1	NA	NA	398.5 (280.1-770.2)	NA	4363.0 (2518.0-7221.0)	<0.0001
TGF-α	NA	NA	7.3 (2.2-14.8)	NA	43.0 (22.6-91.9)	<0.0001
IL-7	NA	NA	5.0 (1.6-7.0)	NA	31.6 (14.8-46.3)	<0.0001
MMP-8	NA	NA	1985.0 (60.0-8791.0)	NA	21071.0 (11071.0-45371.0)	<0.0001
#Apo AI	0.300	85.1	980.1 (150.8-4219.0)	100.0	295552.0 (239391.0-324560.0)	<0.0001
#Apo CIII	0.001	100.0	29.4 (11.0-167.2)	100.0	134435.0 (86406.0-167953.0)	<0.0001
#CC3	0.012	100.0	657.6 (154.2-1449.0)	100.0	44827.0 (31909.0-67779.0)	<0.0001
#CFH	0.037	91.5	746.1 (200.7-2276.0)	100.0	374790.0 (293798.0-447723.0)	<0.0001
#CRP	0.0022	100.0	2387.0 (239.5-230000.0)	100.0	230000.0 (92176.0-230000.0)	0.0003
#A1AT (α1-Antitrypsin)	0.0362	100.0	916.0 (319.1-2721.0)	100.0	18344.0 (13298.0-25608.0)	<0.0001

#PEDF	0.008	100.0	735.7 (606.7-831.0)	100.0	21693.0 (18259.0-25919.0)	<0.0001
#SAP	0.009	100.0	34.6 (8.8-121.3)	100.0	269193.0 (140485.0-491860.0)	<0.0001
#MIP-4	0.0041	100.0	5.4 (0.3-47.5)	100.0	223.7 (146.9-366.9)	<0.0001
#CC4	0.0465	97.9	639.6 (297.1-1473.0)	95.7	146270.0 (70465.0-204543.0)	<0.0001
MMP-7	NA	NA	101.5 (81.6-121.5)	NA	930.0 (605.2-1748.0)	<0.0001
VEGF-A	NA	NA	10.0 (2.8-78.4)	NA	142.8 (77.1-241.8)	<0.0001
NCAM1	NA	NA	115167.0 (59829.0-172190.0)	NA	265229.0 (209949.0-339134.0)	<0.0001
***Cathelicidin-LL37**	NA	NA	0.0 (0.0-0.0)	NA	0.5 (0.3-0.9)	<0.0001
(C) Host markers showing no difference in expression levels between CSF and serum						
CCL4	NA	NA	240.6 (148.4-470.0)	NA	296.8 (179.8-431.0)	0.4363
G-CSF	NA	NA	173.4 (0.0-458.7)	NA	83.0 (0.0-162.9)	0.1172
CCL1/I-309	NA	NA	63.2 (5.13-156.6)	NA	15.0 (7.6-33.4)	0.13

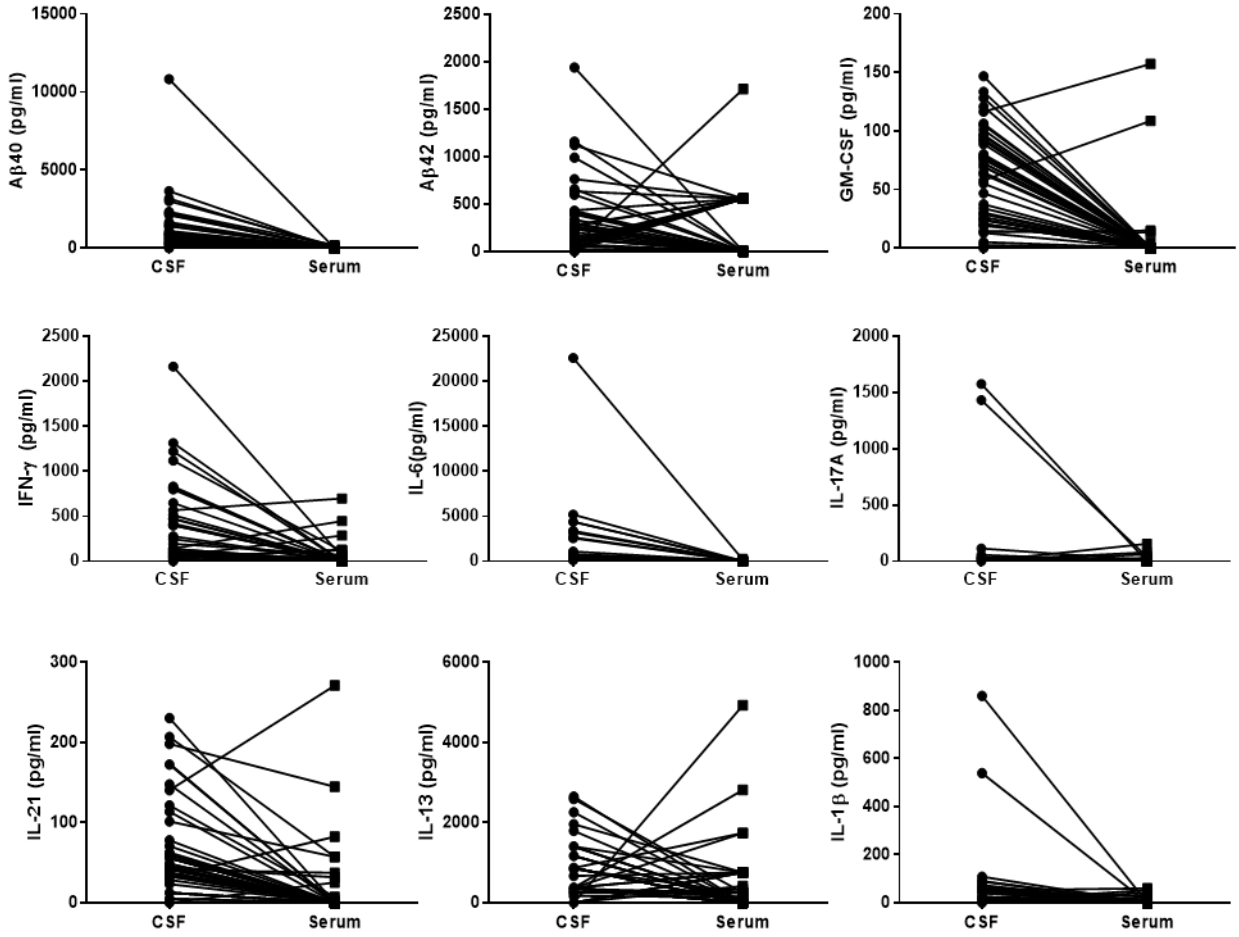

Figure 4.8: Representative plots showing the levels of host markers more abundantly expressed in cerebrospinal fluid. Samples of all study participants regardless of disease status or whether they were finally diagnosed with TBM or no TBM were included in the analysis **(n=47).** The levels of each host marker detected in cerebrospinal fluid sample was mapped to the levels detected in serum sample for each study participants. Only the top nine markers that were highly abundant in cerebrospinal fluid samples are shown (p-value <0.001).

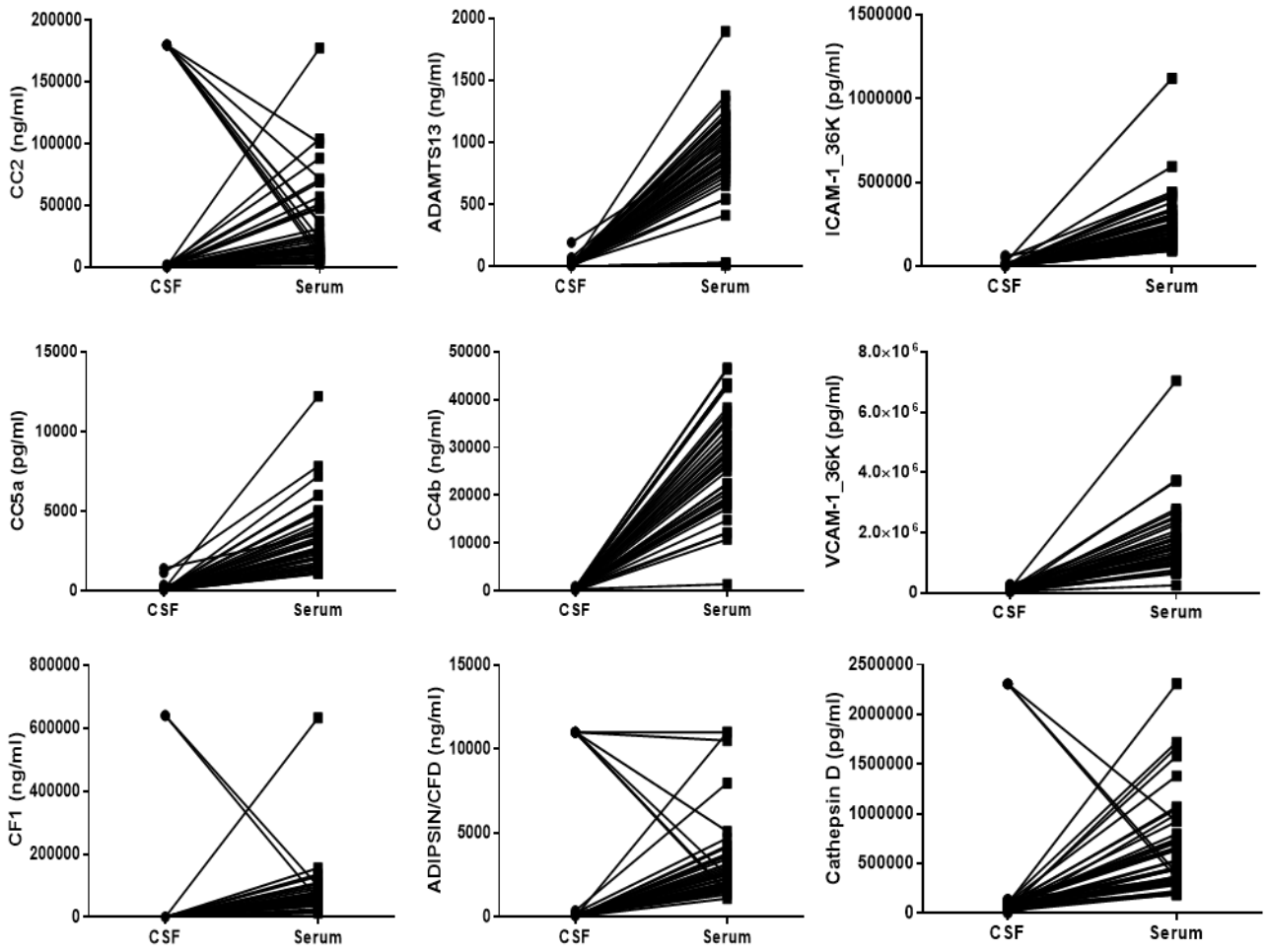

Figure 4.9: Representative plots showing the levels of host markers more abundantly expressed in serum. Samples of all study participants regardless of disease status or whether they were finally diagnosed with TBM or no TBM were included in the analysis **(n=47).** The levels of each host marker detected in serum sample was mapped to the levels detected in cerebrospinal fluid sample for each study participants. Only the top nine markers that are highly abundant in serum samples are shown (p-value <0.0001).

4.4 Discussion

In this study, the usefulness of a previously identified adult 7-marker serum protein biosignature (CRP, transthyretin, IFN-γ, IP-10, CFH, Apo-AI and SAA) as well as host biomarkers that have shown potential as pulmonary TB diagnostic candidates in recent adult studies were assessed as tools for the diagnosis of TB meningitis and pulmonary TB in children. In the first section of this study (4.3.1), it was observed that the adult 7-marker serum protein biosignature diagnosed pulmonary TB with promising accuracy, as ascertained by area under the ROC curve (AUC) of 0.79. Similarly, the same 7-marker adult protein signature

discriminated between children with or without TBM with an AUC of 0.80. Other major findings from this study included the identification of a novel 7-marker serum protein biosignature comprising of PCT, MIP-1α, α2M, IFN-γ, IL-10, SAP, and CFH and also five and six-marker signatures which diagnosed pulmonary TB in children with better accuracy than the adult 7-marker serum protein signature and also the identification of an alternative 3-marker serum protein biosignature which outperformed the adult 7-marker signature in children with TBM. Furthermore, there were several candidate biomarkers which showed potential as promising diagnostic candidates for both childhood pulmonary TB and TBM in the project.

The most promising individual host biomarkers with potential for diagnosis of pulmonary TB in children included IFN-γ, MMP-1, MMP-9, PCT, sIL-6R and IL-6. IFN-γ is a proinflammatory cytokine that has been shown to play an important role in the protection against mycobacterium tuberculosis (197). MMP-1 and MMP-9 are matrix metalloproteinases (MMPs), which play an important role in extracellular matrix remodelling, wound healing and angiogenesis (198) and are known to be associated with TB. A review by Salgame (199) demonstrated that *M.tb* induces tissue remodelling via induction of MMP-9 to favour its establishment in the host, whereas the excessive secretion of MMP-1 is responsible for matrix degradation and cavitation during reactivation of a latent infection. Another study reported that the levels of MMP-1 in plasma samples were significantly higher in children with pulmonary TB compared to healthy controls (200). Procalcitonin (PCT) is a 116 amino acid peptide that belongs to the calcitonin superfamily (201) and it is an inflammatory marker that is stimulated directly by bacterial peptides and indirectly by cytokines (IL-6 and TNF-α) (202). Furthermore, PCT is a biomarker that exhibits greater specificity for bacterial infections than other proinflammatory markers and its use in the diagnosis of bacterial infections has well been described, especially in sepsis (26, 27). It was previously demonstrated that serum PCT levels are lower in patients with pulmonary TB (PTB) in comparison to patients with community-acquired pneumonia (CAP), and that PCT was a useful biomarker for discriminating patients with pulmonary TB and patients with CAP (203). Similarly, another study showed that serum levels of PCT were higher in patients with CAP than in patients with pulmonary TB (204). A meta-analysis showed that PCT test had a pooled sensitivity of 42% and specificity of 87% for discriminating TB from pulmonary TB. In contrast, the current study demonstrated higher serum levels of PCT in children with pulmonary TB compared to children without PTB. IL-6 and sIL-6R are responsible for stimulation of cells that express only gp130 through a process referred to as trans-signalling (205). IL-6 has been reported as a potent biomarker for mycobacterium infection either as a stand alone or in combination with other markers (206). In the current study, both sIL-6R and IL-6 were significantly higher in serum samples of

children with pulmonary TB disease compared to children without pulmonary TB disease and showed potential in the diagnosis of pulmonary TB.

Given that the performance of the current diagnostic methods for pulmonary TB disease in children is limited by the difficulty in obtaining good quality sputum specimen and the paucibacillary nature of the disease in children, tests based on non-sputum samples such as blood could improve the diagnosis of pulmonary TB disease in children. In this study we showed the potential of host markers detectable in blood (serum) samples in the diagnosis of pulmonary TB disease. Blood is an easily obtainable sample requiring a non-invasive procedure. Previous studies have shown that host biomarkers can easily be incorporated into a field-friendly assays utilizing lateral flow format that allows quantitative detection of multiple biomarkers directly in several samples including serum samples (207). This assay could serve as a screening test for TB disease in children at a point-of-care in resource-limited areas. A similar test based on the 7-marker serum protein biosignature is currently being developed for diagnosis of TB disease in adults (www.screen-tb.eu).

In the current study we evaluated the usefulness of the adult 7-marker serum protein biosignature in children. Although the adult 7-marker serum protein biosignature performed poorly in children in comparison to adults, however, a test with sensitivity of 75% and specificity of 70% could be a breakthrough in the diagnosis of pulmonary TB in children, especially if it is a finger-prick blood-based formats. However, we identified alternative novel serum biosignatures with higher accuracies in the diagnosis of TB disease in children. These new biosignatures indicate a possibility for development of biomarker-based tests that are specific for childhood pulmonary TB diagnosis. The biomarker-based test could overcome most of the shortcomings of the current diagnostic methods, especially if it is rapid, cost-effective, user-friendly and accurate. The individual host markers that showed potential for diagnosis of TB disease in this study will serve as back-up markers for substitution of markers in the biosignatures in cases where problems are encountered during translation of Luminex-based data to lateral flow format. A successful biomarker-based test could improve case findings leading to early initiation of TB treatment and reduction of childhood death associated with tuberculosis. The main limitation of the current study was the relatively small sample size. However, this was a pilot study designed to generate preliminary data that could be used in further, larger validation studies. These future studies should include larger number of children with suspected pulmonary TB, including those who are HIV infected and children with confirmed other respiratory diseases.

The individual serum protein host biomarkers identified as potential biomarkers for TBM in the second part of this project included SAP, CC5, CFH, Apo-CIII, PAI-1, PDGF-

AB/BB, MBL, sVCAM-1, CCL2, IL-4, TNF-α, CCL4, ADIPSIN, G-CSF, IL-10, IL-17A, and NCAM1. The levels of SAP, CC5, CFH, Apo-CIII, PAI-1, PDGF-AB/BB and MBL were significantly higher in serum samples of children with TBM, whereas the median levels of sVCAM-1, CCL2, IL-4, TNF-α, CCL4, ADIPSIN, G-CSF, IL-10, IL-17A, and NCAM1 were significantly higher in serum samples of children with non-TBM. Although the diagnostic potentials of these markers were previously investigated in TB, most of them were not investigated in TBM, especially using serum samples. Serum amyloid A (SAP) is a member of positive acute phase proteins produced predominantly in the liver and secreted during an acute phase reaction (208). SAP attracts inflammatory cells, inhibits the respiratory burst of leukocytes and modulates the immune response (209). SAP has been reported as a potential diagnostic marker for TB in different body fluids (150, 170, 190). CC5, CFD, and CFH are the proteins of the complement system. CFH serves as a key regulator of the alternative pathway of the complement system (211). CFH has previously been investigated as a marker for TB diagnosis and higher levels of CFH were observed in patients with TB (32, 33). CFD is a protein of the alternative pathway and it is vital for cleavage of factor B. Patients deficient of CFD were reported to be unable to opsonize *Nessiaria meninigitidis* (212). In a complement cascade, the cleavage of CC5 generate a potent anaphylatoxin, C5a and leads to pathogen lysis, inflammation and cell damage (213). In the previous study, trends were observed towards TBM patients in the levels of IL-4 and G-CSF (160). However, in the current study we observed higher levels of IL-4 and G-CSF in TBM as compared to non-TBM patients. Neural cell adhesion molecule (NCAM) is widely expressed in the CNS and it plays a role in the intercellular adhesion among neurons, astrocytes, oligodendrocytes and myotubes (194, 195). NCAM was previously demonstrated as a biomarker for TB disease and higher levels were observed in plasma samples of individuals with other respiratory disease as compared to TB disease (170). Our findings that there are elevated levels of NCAM in children without TBM as compared to children with TBM are in line with the above-mentioned demonstration. In the previous study (33), NCAM was included in the plasma-based biosignature for diagnosis of adult TB disease, in our study, NCAM was included in the serum-based biosignature for diagnosis of TBM in children. This shows the consistency of NCAM detectable in blood samples in the diagnosis of *Mtb*-related disease/Infection. Mannose-binding lectin (MBL) is an acute phase reactant produced by the hepatocytes and it plays a major role in the recognition of pathogen-associated molecular patterns (PAMPS), leading to lysis of the pathogen via activation of the lectin complement pathways and opsonisation (216). Higher serum levels of MBL in patients with pulmonary TB have been reported (217). Our data is in line with this study, as we observed higher serum levels of MBL in children with TBM compared to children with non-TBM.

Although CSF biomarkers were shown to diagnose TBM in previous studies and validated in the present study (chapter 3), host biomarkers detected in blood may be more beneficial than those detected in CSF. Blood is an easily collectable sample as compared to CSF, which involves an invasive procedure called Lumbar puncture. Hence, the development of a blood-based screening test for TBM could reduce the number of lumbar punctures currently performed per case of meningitis in children. Another major advantage of using a blood-based test for the diagnosis of TBM is that such a test may be more easily applicable especially in resource-limited settings as blood based tests may be easily adaptable and used to develop a finger-prick blood as is currently being done in an ongoing study (www.screen-tb.eu), and would be a major breakthrough in the diagnosis of TBM disease. The blood biosignatures identified in this study hold a promise for development of a blood-based test for screening of TBM in children at a point-of-care or bedside.

Similar to the pulmonary TB part of the current chapter, the main limitation of this part of the project was also the relatively small sample size, especially the few children with alternative diagnoses including children with other forms of meningitis. However, as this study included individuals in whom TBM was suspected, the design of the study was relatively strong, and the number of participants enrolled into the study was consistent with the patient described in multiple previous studies. The same design was used in chapter 3, with the same study participants, in which the previously established 3-marker CSF signature was validated. This shows that the novel biosignatures identified in the current study have strong potential. As highlighted in chapter 3, further studies should include larger numbers of study participants with suspected meningitis, including those who are HIV infected, and individuals with confirmed alternative meningitides. HIV infected children included in such studies should be appropriately staged with CD4 counts and viral loads, to assess the possible influence of severe HIV infection on the accuracy of the diagnostic biosignatures. These future studies shall focus on the validation and refinement of the modified seven marker serum protein biosignature (CRP, IFN-γ, IP-10, CFH, Apo-A1, SAA, and NCAM1) and the 3-marker serum protein biosignature (adipsin/CFD, Aβ42 and IL-10).

Furthermore, in the last part of this study (4.3.3), we showed that host biomarkers are differentially expressed between CSF samples (in chapter 3) and serum samples (in chapter 4) from all participants in whom TBM is suspected, regardless of the disease. Majority of studies investigating host biomarkers for neurological disorders and diseases such as Alzheimer's disease, dementia, Parkinson's disease, multiple sclerosis, and meningitis, including TBM have focused mainly on the cerebrospinal fluid samples (80, 140, 198–202). There are only few studies that investigated the levels of host biomarkers in serum samples

for diagnosis of TBM. Furthermore, even in studies that investigated the levels of markers in serum and CSF samples, the differential expression of these markers between the two sample types were not compared. Currently, the routine TBM diagnosis requires the collection of CSF through a lumbar puncture. Studies report that the collection of CSF through lumbar puncture is well-tolerated and has fewer side effects (218). However, the collection of blood is easier and non-invasive, and therefore has a great advantage as compared to CSF. Studies showed the usefulness of host biomarkers detectable in serum samples in the diagnosis of TB. Our data shows that most of the markers investigated in this study are detectable in serum samples of all study participants. The abundant expression of these markers in serum may implies that these markers may be more reliably detected even with a lateral flow platform, which could be useful in the development of improved diagnostic tools for TBM. The data on the differential expression of host biomarkers between the sample types could provide helpful information in the sample type choice for future research. As the performance of the identified alternative childhood TB-specific signatures identified in this study have not been evaluated adults, it may be good to evaluate the performance of these biosignatures in adults in future studies, together with the adult 7-marker signature, so that a head-to-head comparison of the performance of the signatures could also be established in adults.

In conclusion, the current study showed that the adult seven-marker biosignature showed potential in the diagnosis of both pulmonary TB and TBM in children in a high burden setting. However, alternative childhood TB-specific biosignatures (pulmonary TB-specific and TBM-specific) were more promising. This may imply that different host biomarker-based tests might be required for adults and children. However, regarding the performance of the adult 7-maker serum biosignature in children, data from the current chapter demonstrates this specific biosignature may perform equally well in children with either pulmonary TB (AUC of 79%) or those with extra pulmonary TB, specifically TBM (AUC of 80%). The alternative biosignatures identified in the project, if further validated and refined, could be used to develop blood-based point-of-care or bedside diagnostic tests suitable for diagnosis of pulmonary TB and TBM in children. However, with an AUC of 79 or 80% in both pulmonary TB and TBM, diagnostic tests based on the adult 7-marker serum protein biosignature may still be useful in children, especially if they are based on simple, field friendly technologies such as the lateral flow technology as no other simple tests that perform with that level of accuracy currently exist. While waiting for further validation studies on the newly identified childhood TB-specific signatures, any test developed based on the adult signature, as is being done in the screenTB project (www.screen-tb.eu), could be implemented in the diagnosis of TB disease in children, especially as it will be a much simpler and field-friendly test, compared to any of the currently existing diagnostic tests for TB. This study therefore paves the way for the use of any tests

developed on the adult 7-marker signature, in the diagnosis of both pulmonary TB and TBM in children, pending the validation of the childhood TB specific signatures which may be more promising in that patient group.

Chapter 5

General discussion and conclusion

5.1 Introduction

As reviewed in previous chapters, TB remains a major health challenge worldwide. The bad outcomes resulting from TB are mainly due to difficulties in the diagnosis of the disease and/or delayed initiation of treatment. The current available diagnostic methods have several shortcomings as highlighted in chapter 1, especially in young children and in people presenting with extrapulmonary TB, including tuberculous meningitis (TBM). The bacteriological confirmation of *M.tb* heavily depends on the quality of specimen provided for diagnosis. However, young children do not readily expectorate sputum, which hinders the confirmation of TB in children. Even with new methods used for collection of clinical specimens (gastric aspiration and induced sputum), many cases are still missed. This emphasizes a need for development of new diagnostic methods suitable for use in young children, particularly sputum-independent methods.

It is even more difficult to diagnose TBM in children, especially in resource-limited areas. The diagnosis of TBM relies on the combination of clinical presentation, CSF findings, evidence of TB outside the CNS, brain imaging and where possible, bacteriological confirmation as discussed in chapter 1. However, access to these facilities requires admission into a tertiary hospital, in relatively well-resourced environments. Consequently, children who do not access these facilities, especially those in poor-settings, miss the opportunity for early diagnosis of TBM, with children living in relatively better-off provinces such as in the Western Cape Region of South Africa still presenting at the clinic an average of six times before proper diagnosis of TBM is made (223). An additional problem is that all the invasive tests currently used in the diagnosis of TBM perform poorly individually, hence the need for several admissions at which time, many of the children die. There is therefore an urgent need for rapid, cost-effective and accurate point of care tests for diagnosis of TBM in children.

Host biomarkers detectable in easily obtainable samples such as serum, plasma, saliva and urine have shown potential for development of point-of-care diagnostic tools, especially based on the lateral flow assay technology. A recent multi-centred pan-African study identified a 7-marker serum protein biosignature with potential for diagnosis of TB disease in adults (166). Considering that the 7-marker serum protein biosignature has never been investigated in children, this thesis evaluated the usefulness of this biosignature in the diagnosis of pulmonary TB and TBM in children. Another study identified a 3-marker CSF biosignature with potential for diagnosis of TBM in children (160). There was a need to further validate the performance of this biosignature in a new cohort of children. To address this, the diagnostic performance

of the 3-marker CSF biosignature was validated in a new cohort of children with signs and symptoms suggestive of meningitis. Furthermore, this thesis investigated other host markers that could be used in the diagnosis of pulmonary TB and TBM in children.

5.2 Summary of main findings

Application of cerebrospinal fluid host protein biosignatures in the diagnosis of tuberculous meningitis in children from a high burden setting

In chapter 3, the concentrations of 69 host markers were investigated in the CSF and serum samples from children with signs and symptoms suggestive of meningitis. The performance of the previously identified 3-marker CSF biosignature (VEGF, IL-13 and Cathelicidin LL-37) was validated in the current study, with positive and negative predictive values of 59.55 (95% CI, 51.5-66.9%) and 90.0% (95% CI, 55.3-98.5%), respectively. There were other important findings from this study. Although the levels of 46 individual host markers detected in CSF samples showed potential to diagnose TBM in children with high accuracy (AUC≥0.70), the most optimal performance was obtained with the combination of up to four markers. VEGF was the most consistent host marker, as it performed well individually in the previous studies and in the current study. As a result, VEGF was fitted into General Discriminant Analysis (GDA) models with other host markers and a modified 3-marker CSF biosignature comprising of VEGF, MPO and IFN-γ (that is MPO and IFN-γ substituted IL-13 and cathelicidin LL-37) was obtained. The modified VEGF-based CSF biosignature diagnosed TBM in children with high accuracy, as ascertained by AUC of 0.97 (95% CI, 0.92-1.00) corresponding to sensitivity of 92% and specificity of 100% after further optimization. When all the data obtained from CSF samples of all study participants was fitted into GDA models irrespective of HIV status, a four-marker CSF biosignature comprising of sICAM-1, MPO, CXCL8 and IFN-γ was identified. The four-marker CSF biosignature diagnosed TBM with an AUC of 0.97 (95% CI, 0.92-1.00); corresponding to both sensitivity and specificity of 96% after selection of optimal cut-off values.

Application of host immunological biomarkers detectable in blood in the diagnosis of childhood TB

As highlighted in chapter 4 (Part A), the concentrations of the seven markers comprising the previously described adult 7-marker serum protein biosignature (CRP, transthyretin, IFN-γ, CFH, Apo-A1, IP-10, and SAA) and 33 other biomarkers were investigated in serum samples obtained from children with confirmed pulmonary TB (PTB) and children with unlikely TB diagnosis. The study showed that the adult seven-marker serum protein biosignature has a reduced performance in children as shown by AUC of 0.79 (95% CI, 0.65-0.93) with

corresponding sensitivity of 75% and specificity of 70% after further optimization. Of note, multiple host markers including MMP-1, MMP-9, PCT, sIL-6R and IL-6 showed potential as diagnostic candidates for pulmonary TB in children. When fitting all the data obtained from serum samples of all study participants into the GDA models, a combination of up to seven host biomarkers showed an optimal prediction of pulmonary TB in children. A novel seven-marker serum protein biosignature comprising of PCT, MIP-1α, α2M, IFN-γ, IL-10, SAP and CFH diagnosed PTB with AUC of 0.94 (95% CI, 0.87-1.00); corresponding to sensitivity of 95% and specificity of 90%. Furthermore, a novel 6-marker serum protein biosignature comprising of MMP-9, IFN-γ, α2M, fibrinogen, CFH and SAP diagnosed PTB with AUC of 0.94 (95% CI, 0.86-1.00); corresponding to sensitivity of 90% and specificity of 95% after further optimization. The most accurate biosignature was the novel 5-marker serum protein biosignature comprising of IL-1β, IL-12p40, TNF-β, MMP-1 and α2M, which diagnosed PTB with AUC of 0.95 (95% CI, 0.89-1.00); corresponding to sensitivity of 85% and specificity of 95% after further optimization.

In part B (Chapter 4), we evaluated the levels of 69 host markers in serum samples from all study participants. The study participants and host biomarkers investigated in this study were the same as those included in chapter 3. The main aims of this study were to assess the usefulness of the adult seven-marker serum protein biosignature (CRP, IFN-γ, IP-10, CFH, Apo-AI, SAA and transthyretin) in the diagnosis of TBM in children and to identify other potential biomarkers. As highlighted in chapter 4 (Part B), transthyretin was not available in our analyte panel, therefore, six markers (CRP, IFN-γ, IP-10, CFH, Apo-AI, and SAA) out of the seven-marker serum protein biosignature were fitted into the GDA models with other markers and a modified seven-marker serum protein biosignature comprising of CRP, IFN-γ, IP-10, CFH, Apo-AI, SAA and NCAM1 (that is, NCAM1 substituted transthyretin) was evaluated. The modified seven-marker serum protein biosignature diagnosed TBM with AUC of 0.80 (95% CI, 0.67-0.92); corresponding to sensitivity of 71% and specificity of 74% after further optimization. When all the markers were evaluated individually, the median levels of SAP, CC5, CFH, Apo-CII, PAI, PDGF-AB/BB and MBL were significantly higher in children with TBM, whereas the median levels of sVCAM-1, CCL2, IL-4, TNF-α, CCL4, ADIPSIN, G-CSF, IL-10, IL-17A and NCAM1 were significantly higher in children without TBM (non-TBM). Furthermore, host markers including VCAM-1, MCP-1/CCL2, IL-4, TNF-α, MIP-1β/CCL4. ADIPSIN/CFD, SAP, CC5, CFH, G-CSF, IL-10, Apo-CIII and PAI-1 showed potential as diagnostic candidates for TBM in children as determined from the AUCs. When the data obtained from serum samples of all study participants was fitted into the GDA models regardless of HIV status, the optimal prediction of TBM was achieved with a combination of up to three host markers. A 3-marker serum protein biosignature comprising of complement

factor D (adipsin), Aβ42 and IL-10 diagnosed TBM with AUC of 0.84 (95% CI, 0.73-0.96); corresponding to both sensitivity and specificity of 83% after further optimization. It was also observed that there were vast differences in the expression of host biomarkers in serum Vs. CSF of children with suspected meningitis as discussed in Chapter 4B.

5.3 Significance of findings from the present thesis

As discussed in the previous paragraph, we validated a previously described 3-marker CSF biosignature (VEGF, IL-13 and cathelicidin LL-37) in chapter 3 of this thesis. Although this validated 3-marker signature will be useful in the diagnosis of TBM especially if developed into a simple, bedside or point-of-care test, we showed that modification of this signature by substituting IL-13 and cathelicidin LL-37 with two other proteins (MPO and IFN-γ) resulted in a much improved biosignature, with AUC of 97%. This paves the way for the development of a new CSF biomarker-based test for the diagnosis of TBM. During the development of the test, any of the analytes in the signature could be replaced by the analytes in the alternative new 4-marker signature which also performed with an AUC of 97%, or any of the other biomarkers that showed potential individually as shown in table 3.3. As such a test will be based on the evaluation of these proteins in CSF samples, collection of CSF by lumber puncture may be a problem as it will require expertise which might not be available, especially in resource limited settings. Considering the WHO Target Product Profiles (TPPs) (196), the key priority biomarker-based tests, especially tests that could possibly be used at the community health centre level as triage test should be preferably based on easily collected samples such as whole blood, urine, saliva amongst others. We therefore evaluated the utility of the adult 7-marker signature and other proteins in serum samples from all the children enrolled into the project as discussed in chapter 4. As collection of CSF may be problematic, the serum-based test, which may be easily adapted into a finger-prick tests as is currently done in our laboratory as well as in other countries may thus be useful in remote settings.

As also reported in chapter 4, we evaluated the adult seven-marker signature as well as other host biomarkers as tools for the diagnosis of pulmonary TB in children. Given the difficulties currently encountered in the diagnosis of TB disease, the adult 7-marker test may still be useful in the diagnosis of TB disease in children given the AUC of 79%, especially if based on a point-of-care lateral flow blood-based test as is currently done in the ScreenTB project. Such a test could be optimised, with better cut-off values to increase its sensitivity so that it is used as a rule-out test for TB disease in children. Individual diagnosed with positive test results would then be referred to higher levels of the healthcare system for confirmatory tests such as geneXpert and culture, thereby saving on costs on the currently extensive investigations that are done in all children that are suspected of having TB disease. Further optimisation of

the test with the new childhood TB specific biosignatures identified in the current study would lead to an even better performing test for TB disease in children.

The biosignatures identified in this study hold promise for the development of suitable POC diagnostic tools for TB disease (including both pulmonary TB and TBM) in children. The development of diagnostic tests suitable for use in children has been recommended by the WHO in order to reduce high morbidity and mortality in this population. The findings from this thesis suggest that the described biosignatures could be incorporated into a field-friendly platform, based on lateral flow technology. Similar blood-based TB tests have been developed and successfully investigated in multiple African countries (14, 15), with multi-biomarker finger-prick based formats currently under development for the diagnosis of adult pulmonary TB disease (www.screen-tb.eu). Such developed biomarker-based diagnostic tools will benefits children with difficulty in providing good quality sputum samples, those with paucibacillary disease, those with extrapulmonary TB (particularly TBM) and those living in resource limited areas with less opportunities to access the currently used diagnostic tools for TB disease.

5.4 Future investigations

Considering that the main common limitation amongst all the studies reported in the current thesis was the relatively small sample size, it will be important that future studies are done in larger numbers of children with suspected pulmonary TB or TBM. For the TBM study, further studies should include larger numbers of study participants with suspected meningitis, including those who are HIV infected, and individuals with confirmed alternative meningitides. HIV infected children included in such studies should be appropriately staged with CD4 counts and viral loads, to assess the possible influence of severe HIV infection on the accuracy of the diagnostic biosignatures. Given the wide distribution of age (3 months to 13 years) in children that were included in the TBM study, it might also be worthy in future studies to evaluate the performance of the biosignatures in young children or infants as compared to older children. The pulmonary TB diagnostic biomarkers study was a pilot study designed to select biomarkers that could be investigated further in larger validation studies and this will form part of my future studies. Such future studies should include larger number of study participants with suspected pulmonary TB, including those who are HIV infected and children with other respiratory diseases and should be conducted on children recruited from multiple countries and multiple continents, so as to assess the global application of the biosignatures. This will be the same for further work done on the biosignatures for the diagnosis of TBM. In the course of the above mentioned future studies the blood (serum) and CSF biosignatures identified in these thesis for diagnosis of pulmonary TB and TBM in children should be refined by evaluating various combinations between the different host biomarkers that showed potential

and also investigate the correlation structure between different promising analytes. Well-refined biosignatures could then be incorporated into field-friendly POC tests and in the course of development of such tests, biomarkers that correlate well with intended biomarkers for such tests may act as replacement markers, in case problems are encountered when attempting to convert the desired analytes into the POC tests.

When samples were being collected for the TBM study, PaxGene RNA whole blood was collected from all study participants. There are many recent studies that identified transcriptional biosignatures which showed potential as biosignatures for the diagnosis of active TB, biomarkers for progression from LTBI to active TB and biosignatures for monitoring of TB treatment response. As majority of these studies were done in adults, it will be interesting to see how transcriptomic biosignatures perform in the diagnosis of childhood TB. Future studies, depending on available funding may therefore make use of the collected PaxGene tubes to evaluate new candidate biomarkers for the diagnosis of TBM, through the RNA sequencing. Alternatively, targeted approaches could be employed to investigate the utility of the transcriptomic biomarkers that have been reported in the literature in children with either pulmonary TB or TBM. The most useful candidate biomarkers may then be selected for future development of tests for the diagnosis of childhood TB.

Metabolomics is an emerging science in the area of omics (224). Application of metabolomics offers ability to identify and quantify low-molecular weight metabolites and produce metabolite profiles which reflect the scale of a given biological system (224). Metabolomics has been widely applied in the search of biomarkers associated with TB disease. A study showed that specific metabolites detected in plasma samples including *M.tb*-derived glycolipids and resolvins, have potential as biomarkers of TB disease (225). A study by Mason et al. (226) Identified 16 CSF metabolites, which differentiated between TBM and non-TBM. Furthermore, a recent study identified 5 amino acids, namely alanine, asparagine, glycine, lycine and proline which were elevated in TBM cases (227). However, the exploration of metabolomics in TBM is still at very early stages, as compared to that of pulmonary TB. In my future studies, targeted metabolomics approaches will be used to evaluate *M.tb*-specific products including the antigen 85 (Ag85) protein complex, membrane lipids and fatty acid structures which are hypothesized to be present in CSF, the site of disease. Such *M.tb*-specific products may significantly enhance the specificity of host biomarker-based tests such as the ones based on the biosignatures identified in the current study.

The information gained about the differential expression of host markers between CSF and serum samples necessitates further investigation for immune cells subpopulations between children with TBM and those without TBM. CSF immune cells subpopulations have been

evaluated in several conditions, such as multiple sclerosis, Cryptococcal meningitis, and other neurological disorders (228–232). Furthermore, during the diagnosis of TBM, routine CSF investigations show raised CSF white blood cell count with pleiocytosis and predominance of neutrophils (18, 207). However, there is limited work on the immune cells subpopulations associated with paediatric TBM. Therefore, future studies shall investigate the differential immune cells subpopulations in CSF and peripheral blood (PB), as well as in comparison between children with TBM and those without TBM. Such investigations will provide information about the pathogenesis of TBM and information relating to understanding the mechanisms at play at the site of disease, especially when compared to the periphery.

5.5 Conclusion

In conclusion, the data presented in this thesis shows that host biomarkers detectable in CSF samples hold potential in the diagnosis of TBM. Furthermore, the adult seven-marker biosignature showed potential in the diagnosis of both PTB and TBM in children in a high burden setting. However, alternative childhood TB-specific biosignatures were more promising, indicating that different host biomarker-based tests might be required for adults and children. There is a need for further validations and refinements for these findings, especially the pulmonary TB diagnostic biomarkers pilot study, in new and larger cohorts of children before the development of biomarker-based diagnostic tools suitable for use in children. Pending these investigations, any point-of-care tests developed based on the adult 7-marker signature for use in adults may also be employed in children with pulmonary TB (AUC of 79%) or TBM (AUC of 80%). Cut-off values may then be adjusted so as to ensure that such a test when applied to children, is either a rule-out or rule-in test, pending the development of childhood TB-specific tests in future.

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Appendixes

Inclusion number | 0 | | | |

1

IDENTIFYING INFORMATION
KEEP SECRET

Faculty of Health Sciences
Stellenbosch University

Confident identifying patient information

Date study admission	…. /…. /…….											
Inclusion number	0											
Folder number												
Gender	M	☐	F	☐								
Date of Birth	…. /…. /…….											
First name												
Surname												
Address												
Zip-code						City						
Province						Country						
Mobile phone												
Home phone												
Other phone numbers												
E-mail address												

Identifying information of parents/carer

	Mother	Father
First Name		
Surname		
Date of Birth	…. /…. /…….	…. /…. /…….

Other contact person		Relation other contact person to patient	
First Name			
Surname		Father	☐
Address		Grand Mother	☐
Zip-code		Grand Father	☐
City		Aunt	☐
Province		Uncle	☐
Country		Neighbour	☐
Mobile phone		Other*	☐
Home phone		*If other specify:	
Email address			

Referring clinic

Name referring clinic	
Telephone number clinic	
Contact person clinic	

Contact information TB-contact

TB-contact	Yes	☐	No	☐								
First Name												
Surname												
Date of Birth	…. /…. /…….											
Hospital Name					Tel hospital							

Inclusion number | 0 | | | |

2

TBM-study group
History Sheet

General: Admission												
Faculty of Health Sciences Stellenbosch University	Date admission	…. /…. /…….										
	Ward	G-9	☐	G-10	☐	G-Ground	☐	Other	☐			
	Inclusion number	0										
	Date of Birth	…. /…. /…….										
	Initials	…	…									
	Gender	M	☐	F	☐							
	Name nearest clinic	……										

General: Discharge									
Date of discharge	…. /…. /…….								
Final Diagnosis	TBM	☐	Bact Men	☐	Viral Men	☐	Other*	☐	
*If Other please specify	……								
Referred to	Home tr.	☐	Brook. Chest	☐	Eben D.	☐	Brewelskloof	☐	
	Paarl	☐	St. Joseph	☐	Sonstraal	☐	Other*	☐	
*If Other please specify									

General: Consent Forms				
Genetic consent form Signed	Yes	☐	No	☐
General consent form Signed	Yes	☐	No	☐
DNA consent form Signed	Yes	☐	No	☐

History: Symptoms			
Presenting symptoms		**Duration (day's)**	**Extra information**
Decreased consciousness	☐	….	….
Fever	☐	….	….
Vomiting	☐	….	….
Diarrhoea	☐	….	….
Loss of milestones	☐	….	….
Weight loss	☐	….	….
Seizures	☐	….	….
Headache	☐	….	….
Change in behaviour	☐	….	….
Cough	☐	….	….
Poor feeding/ refusing feeds	☐	….	….
Other	☐	….	….

History: Health Professional (HP)											
First contact HP	…. /…. /…….										
Number of visits HP	1	☐	2	☐	3	☐	4	☐	>4	☐	
What kind of HP	Paediatrician	☐	GP	☐	Healthcare worker	☐	Nurse	☐			
What kind of clinic	Private	☐	district	☐	Academic	☐					

Inclusion number | 0 | | | |

3

History: TB-contact													
TB contact	Yes*	☐	No	☐	* please write down contact details at identifying information sheet .								
Sensitivity Mycobact.	N/A	☐			Normal	☐	SDR	☐	MDR	☐	XDR	☐	Unknown ☐
On treatment since	…. /…. /…….				N/A	☐							

History: HIV								
HIV-status	Unknown	☐	Negative	☐	Exposed	☐	Positive*	☐
*If HIV positive, please write down extra information at 'investigations'								

History: Previous diagnosis					
Is there any previous diagnosis?	Yes*	☐	No	☐	*If yes, please specify below

Diagnosis		When	Notes
Pneumonia	☐	…. /…. /…….	
Urinary tract infection	☐	…. /…. /…….	
Gastro-enteritis	☐	…. /…. /…….	
Bacterial meningitis	☐	…. /…. /…….	
Viral meningitis	☐	…. /…. /…….	
Asthmatic bronchitis	☐	…. /…. /…….	
Eczema	☐	…. /…. /…….	
Other	☐	…. /…. /…….	

History: Birth					
Gestational age	……	Weeks	Birth Weight	……..	Gram
Head Circumference	……	cm	Height	……..	cm

History: Feeding							
Breast milk	Yes	☐	No	☐	Duration Breast milk	……	Months
Formula milk	Yes	☐	No	☐	Type of Formula milk	……	
Multivitamin in last 6 months	Yes	☐	No	☐	Till age of	.....	Months
Ferro in last six months	Yes	☐	No	☐	Till age of	.....	Months
Vitamin A in last six months	Yes	☐	No	☐	When was last time	…/…/…..	

History: Mile stones								
Developmental mile stone age appropriate	Yes	☐	No*	☐				
*If No, specify	……							
School progress	Adequate	☐	Inadequate	☐	N/A	☐		
Hearing difficulties	Yes	☐	No	☐	Vision difficulties	Yes	☐	No ☐

History: Road to Health Chart								
Copy RtHC	Yes	☐	No	☐				
BCG done?	Yes	☐	No	☐	Unknown	☐		
Immunisation on schedule	Yes	☐	No*	☐	Unknown	☐		
*If No, specify	……							

Inclusion number | 0 | | | |

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History: Medication before inclusion							
Medication before inclusion ?	Yes* ☐	No ☐	*If yes, please write down all the drugs.				
TB-therapy started before inclusion	Yes ☐	No ☐	Days on treatment	.....	Days		
Steroid's started before inclusion	Yes ☐	No ☐	Hours on treatment	.....	Hours		

	Name medication	Date start medication
1		.... /.... /.....
2		.... /.... /.....
3		.... /.... /.....
4		.... /.... /.....
5		.... /.... /.....
6		.... /.... /.....
7		.... /.... /.....
8		.... /.... /.....

History: Admissions in the past				
Hospital admissions in the past ?	Yes* ☐	No ☐	*If yes, please specify below	

	Diagnose	Date
1		.... /.... /.....
2		.... /.... /.....
3		.... /.... /.....
4		.... /.... /.....
5		.... /.... /.....

History: Population group				
Population group	Black African	☐ →	If Black African:	
	Coloured	☐	Xhosa	☐
	White	☐	Sotho	☐
	Indian	☐	Tswana	☐
	Other	☐	Zulu	☐
			Other	☐

History: S.E.S.								
House	Formal ☐	Informal ☐	Monthly income	.....	Rand			
Running water	Yes ☐	No ☐	Child support grant	Yes ☐	No ☐			
Toilet in house	Yes ☐	No ☐	Mothers education level	.....	Grade			
Electricity	Yes ☐	No ☐	Number of adults in house	.....				

Caregiver	Mother ☐	Father ☐	Other* ☐	* Specify:			
Parents	Married ☐	Together ☐	Divorced ☐	No contact mother ☐			
	LAT ☐	No contact father ☐	Father died ☐	Mother died ☐			
Substance **use** in house	Alcohol ☐	Smoking ☐	Dagga ☐	Tik ☐	Other* ☐		
*If other specify							

Inclusion number | 0 | | | |

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Clinical Data

Clinical: Vital Signs									
Heart rate		/min	Skin turgor	Normal ☐	Delayed ☐				
Respiration rate		/min	Highest temperature in 24 hrs		°C				
Resp. distress	Yes ☐	No ☐		Capillary refill		seconds			
Saturation		%	Blood pressure		mmHg				

First impression / other symptoms:

Clinical: General									
Clubbing fingers	Yes ☐	No ☐							
Signs of rickets	Yes* ☐	No ☐	*If yes specify						
Lymph adenopathy	No ☐	Focal* ☐	Generalized ☐						
*If focal specify									
Skin-lesions	Yes* ☐	No ☐							
If Yes*	Maculopapular rash ☐	Purpura ☐							
	Vesicular lesions ☐	Candida Oral ☐							
	PEM-skin lesions ☐	Candida Genital ☐							
	Eczema ☐	Eschar ☐							
	Other ☐⇒								

Clinical: Upper respiratory tract					
Normal ☐	Otitis Media ☐	Pharyngitis ☐			
Tonsillitis ☐	Othorroea ☐	Parotitis ☐			
Rhinitis ☐	Mastoiditis ☐	Other			

Clinical: Abdominal investigation							
No abnormalities ☐	Hepatomegaly ☐	Splenomegaly ☐	Abdominal lymphnodes ☐				

Clinical: Anthropometry					
Weight		gram	Head Circ.		cm
Height		cm	MUAC		cm
BCG-scar	Yes ☐	No ☐			

Clinical: TBM-Stage					
TBM stage applicable	Yes* ☐	No ☐	*If yes, please specify below		
	Admission	**48 hours**	**Week 4**	**Discharge**	
Date	…. /…. /…….	…. /…. /…….	…. /…. /…….	…. /…. /…….	
Stage I	☐	☐	☐	☐	
Stage II a	☐	☐	☐	☐	
Stage II b	☐	☐	☐	☐	
Stage III	☐	☐	☐	☐	

Inclusion number | 0 | | | |

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Clinical: GCS						
			Admission	48 hours	Week 4	Discharge
	Date		…. /…. /…….	…. /…. /…….	…. /…. /…….	…. /…. /…….
Glascow Coma Scale	E	1-4				
	M	1-6				
	V	1-5				
	Total score					

Clinical: ICP								
Fontanel	N/A	☐	Normal	☐	Sunken	☐	Bombing	☐
Raised ICP	N/A	☐	Papiloedema	☐	Sun setting	☐	Sutures splayed	☐
Pupil Size	Right eye	….	cm	Left eye	…	cm		

Clinical: Brainstem					
No brainstem dysfunction	☐	Absent dolls eye reflex	☐	Neurogenic hyperventilation	☐
Unequal/ non-reactive pupils	☐	Decerebration	☐		

Clinical: Fundoscopy							
Normal	☐	Not done	☐	Abnormal*	☐		
*If abnormal	Papiloedema	☐	Optic atrophy	☐	Tubercles	☐	Other …….

Clinical: Cranial nerves								
Are there any cranial nerve palsies?	Yes*	☐	No	☐	*If yes, please specify below			
	Admission		48 hours		4 weeks		Discharge	
Date	…. /…. /…….		…. /…. /…….		…. /…. /…….		…. /…. /…….	
Side	R	L	R	L	R	L	R	L
N II (afferent pupil defect)	☐	☐	☐	☐	☐	☐	☐	☐
N III palsy	☐	☐	☐	☐	☐	☐	☐	☐
N IV palsy	☐	☐	☐	☐	☐	☐	☐	☐
N VI palsy	☐	☐	☐	☐	☐	☐	☐	☐
N VII palsy UMN	☐	☐	☐	☐	☐	☐	☐	☐
N VII palsy LMN	☐	☐	☐	☐	☐	☐	☐	☐

Clinical: Hemiplegia								
Are there any cranial nerve palsies?	Yes*	☐	No	☐	*If yes, please specify below			
	Admission		48 hours		Week 4		Discharge	
Date	…. /…. /…….		…. /…. /…….		…. /…. /…….		…. /…. /…….	
	R	L	R	L	R	L	R	L
Mild	☐	☐	☐	☐	☐	☐	☐	☐
Moderate	☐	☐	☐	☐	☐	☐	☐	☐
Severe	☐	☐	☐	☐	☐	☐	☐	☐

Clinical: Extra pyramidal signs								
Are ther any extra pyramidal signs?	Yes*	☐	No	☐	*If yes, please specify below			
	Admission		48 hours		Week 4		Discharge	
Date	…. /…. /…….		…. /…. /…….		…. /…. /…….		…. /…. /…….	
	R	L	R	L	R	L	R	L
Hemiballismus	☐	☐	☐	☐	☐	☐	☐	☐
Chorea	☐	☐	☐	☐	☐	☐	☐	☐
Tremor	☐	☐	☐	☐	☐	☐	☐	☐
Athetosis	☐	☐	☐	☐	☐	☐	☐	☐
Dystonia	☐	☐	☐	☐	☐	☐	☐	☐

Inclusion number | 0 | | | |

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Investigations

Inverstigations: HIV														
HIV rapid test	Positive* ☐	Negative ☐	Not done ☐	Date rapid test	…. / …. / …									
HIV PCR	Positive* ☐	Negative ☐	Not done ☐	Date PCR	…. / …. / …									
On HAART	Yes ☐	No ☐	Date start HAART	…. / …. / ……										
*CD4 count	…… x10*9/L	..... %	*Viral load	……. copies/ml										
Date investigations	…. / …. / ……		HIV-stage	1 ☐	2 ☐	3 ☐	4 ☐							

Investigations: TST														
TST done	Yes* ☐	No ☐	*If yes, please specify below											
TST (mm)	0-4 ☐	5-9 ☐	10-14 ☐	15-19 ☐	≥20 ☐									
Date TST	…. / …. / …….		Date result	…. / …. / ……										

Investigations: Continuous ICP														
Continuous ICP done	Yes ☐	No ☐	Date continuous ICP	…. / …. / ……										
ICP	Mean baseline	.....	mmHg	B-waves	Yes ☐	No ☐								
	Pulse pressure	.....	mmHg	Plateau-waves	Yes ☐	No ☐								

Investigations: CSF-culture														
CSF culture done	Yes ☐	No* ☐	* ⇨	No CSF obtained ☐	Not enough CSF ☐	CSF Lost ☐								
Culture	Positive ☐	Negative ☐	N/A ☐	Gram	……									
Date culture result	…. / …. / …….	Z.N.	Positive ☐	Negative ☐	Not done ☐									
Specimen	.....	Indian Ink	Positive ☐	Negative ☐	Not done ☐									
Sensitivity TBM	Unknown ☐	Normal ☐	SDR ☐	MDR ☐	XDR ☐	N/A ☐								
PCR virus	Not done ☐	Positive* ☐	Negative ☐											
*If positive specify	.....													

Investigations: CSF-cell count														
Cell-count done	Yes ☐	No ☐ ⇨	No CSF obtained ☐	Not enough CSF ☐	CSF Lost ☐									
Macroscopic aspect	Bloody ☐	Clear ☐	Turbid ☐	Xanth. ☐										
Erythrocytes		cells/µL	Protein		g/L									
Leucocytes (total)		cells/µL	Glucose		mmol/L									
PMN's		cells/µL	Serum glucose		mmol/L									
Lymphocytes		cells/µL	Date CSF-cellcount	…. / …. / …….										

Storage: CSF-Storage														
CSF storage for culture study	Yes ☐	No* ☐	Amount	.....	ml									
*If no, why not	N/A ☐	No CSF obtained ☐	Not enough CSF ☐	CSF Lost ☐										
Other notes	....													

Investigations: Blood culture														
Blood culture done	Yes* ☐	N o ☐	*If yes, please write down results below											
Blood culture	Positive ☐	Negative ☐	Date blood culture	…. / …. / …….										
Specimen	....	Possible contamination	Yes ☐	No ☐										
Sensitivity TBM	Unknown ☐	Normal ☐	SDR ☐	MDR ☐	XDR ☐	N/A ☐								

Inclusion number | 0 | | | |

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Chest X-ray									
Chest X-ray normal	Yes ☐	No* ☐	*If no, please specify						
Copy of Chest X-ray	Yes ☐	No ☐	No Chest X-ray done ☐						
Date	…. / …. / ……	Other specific findings:							
Miliary TB	Yes ☐	No ☐							
Hilar adenopathy	Yes ☐	No ☐							
Segmental lesion	Yes ☐	No ☐							
CT-brain									
Date CT-brain	…. / …. / ……	CT-brain normal?	Yes ☐	No* ☐	*If no, please specify				
Copy of CT-Brain	Yes ☐	No ☐	No CT-brain done ☐						
PV low densities	Absent ☐	Mild ☐	Moderate ☐	Severe ☐					
Subarachnoid space	Normal ☐	Decreased ☐	Increased ☐						
Hydrocephalus	Yes ☐	No ☐							
Periventr. lucency	Yes ☐	No ☐	L ☐	R ☐	*If other specify:				
Infarctions	Yes ☐	No ☐	L ☐	R ☐					
Position infarctions	Caudate/internal capsule	L ☐	R ☐						
	Thalamus	L ☐	R ☐						
	MCA	L ☐	R ☐						
	Other*	L ☐	R ☐						
Basal meningeal enh.	Yes ☐	No ☐							
Tuberculomas	Yes ☐	No ☐	Number ……						
Air encephalography									
Air-encephalography done?	Yes* ☐	No ☐	*If yes, please specify						
Date Air-encephalography	…. / …. / ……								
Copy of air-encephalography	Yes ☐	No ☐	No Air encephalography done ☐						
Hydrocephalus	Communicating ☐	Non-communicating ☐	N/A ☐						
MRI-brain									
MRI-cerebrum done?	Yes ☐	No ☐	Date MRI-cerebrum …. / …. / ……						
Copy of MRI	Yes ☐	No ☐	No MRI-brain done ☐						
Radiology report:									

Inclusion number | 0 | | | |

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Treatment

Medication

Drug name	Date started	Date stopped	Side effects
	…/…/……	…/…/……	
	…/…/……	…/…/……	
	…/…/……	…/…/……	
	…/…/……	…/…/……	
	…/…/……	…/…/……	
	…/…/……	…/…/……	
	…/…/……	…/…/……	

Neurosurgery

Neurosurgery done?	Yes ☐	No ☐	*If yes, please specify				
Date Neurosurgery	…. / …. / ……..						
What kind of surgey ?	VP-shunt(s) ☐	EVD ☐	ETV ☐				

Complications

Complications	Yes ☐	No ☐						
Admitted ICU	Yes* ☐	No ☐	Date adm.	…. / …. / ……..	Date disch.	…. / …. / …….		
*If yes, reason admission								
Died	Yes ☐	No ☐	Date	…. / …. / ……..	Cause of death			
Seizures	Yes* ☐	No ☐	Date	…. / …. / ……..				
*If yes, what type	Partial ☐	Generalized ☐	Status epilepticus ☐					
Infections	Yes* ☐	No ☐						
*If yes, what type	1.			Date	…. / …. / ……..			
	2.			Date	…. / …. / ……..			
	3.			Date	…. / …. / ……..			
IRIS (following INSHI criteria)	Yes ☐	No ☐						
Other complications :								

Other specific findings during admission/treatment

……

……