Immunological markers for active TB and early treatment response indicators

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Declaration

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Abstract

Background

The difficulty in diagnosing tuberculosis (TB) and evaluating TB treatment response are two major problems that are hampering the defeat of this infectious disease. The current TB diagnostic tools have several limitations and these call for the development of a simple, rapid and accurate diagnostic test that is suitable for use in poor-resource settings.

Aim and Objectives

This thesis aims to identify host markers for the development of a rapid and simple test for TB diagnosis and for monitoring early TB treatment response. The objectives are:

1. To investigate the diagnostic accuracy of host markers detected in *Mycobacterium tuberculosis* (*Mtb)* antigen-stimulated overnight whole blood culture supernatant.

2. To investigate the profiles of inflammatory markers of active TB patients undergoing treatment in a 14-day EBA trial for treatment monitoring potential.

3. To investigate the combined performance of the responses of IgG, IgM and IgA to selected mycobacterial antigens for their diagnostic potential.

Methodology

Participants were recruited as part of the recently concluded EDCTP-funded AE-TBC study and a 14-day phase II randomised clinical trial (early bactericidal activity (EBA) study of seven treatment arms). Sputum and blood samples were collected at different time points and multiplex cytokine array analysis performed on plasma or serum samples by Luminex and anti-mycobacterial antibodies detected by ELISA.

Results

After overnight stimulation of whole blood with ESAT-6/CFP-10, RV0081, Rv1284 and Rv2034, the most promising diagnostic markers were CRP, Ferritin, SAA and IP-10. Unstimulated host markers yielded the best discriminatory power. A six-marker biosignature comprised mostly of unstimulated cytokine levels shows promise for active TB diagnosis.

There were significant changes for CRP, IL-6, VEGF, sIL-2Rα, Ferritin, and sTNFRII from baseline to end of 14 day EBA evaluation in several treatment arms. However, none of these markers mirrored the decrease in the measured bacterial load in sputum. A four-marker combination only accounted for 20% of the variation in observed in both TTP and CFU.

The highest sensitivity and specificity was obtained with anti-16 kDa IgA (95%/95%) and anti-MPT64 IgA (95%/90%). A higher accuracy was obtained with a 3 or 4 antibody combination. Anti-16 kDa IgA and anti-16 kDa IgM, decreased significantly while anti-LAM IgG and anti-TB-LTBI IgG increased significantly at the end of month six anti-TB treatment.

Conclusion

Host biomarkers hold promise as a diagnostic tool in TB disease. In spite of the moderate accuracy of *Mtb* antigen-stimulated host markers, these could still have value in difficult to diagnose TB, like paediatric TB or extrapulmonary TB, and should be evaluated in future studies. Although host markers only explained a small degree of the variation in bacterial measures in early bactericidal activity studies, their potential role in overall treatment effect remains to be investigated. Serodiagnostic markers against novel *Mtb* antigens showed potential for future development into a simpler format for use at the point-of-care. These results should be validated in large scale studies in appropriate participant groups.

Opsomming

Agtergrond

Probleme met TB diagnosering en evaluering van die reaksie op TB behandeling is twee groot struikelblokke wat die effektiewe behandeling strem. Die huidige TB diagnostiese toetse het verskeie tekortkominge en dit is belangrik om ‘n eenvoudige, vinnige en akkurate diagnostiese toetse te ontwikkel wat effektief in hulpbron-beperkte gemeenskappe gebruik kan word.

Doelwitte

Die doel van die studie was om gasheer biomerkers te identifiseer vir die ontwikkeling van ‘n vinnige en eenvoudige TB diagnostiese toets en die waarneming van die reaksie op vroeë TB behandeling. Die doelwitte is:

1. Om die diagnostiese akkuraatheid van die gasheer biomerkers in ‘n *Mycobacterium tuberculosis* (MTB) antigeen-gestimuleerde bloed kultuur te bepaal.
2. Om die profiel van inflammatoriese biomerkers van pasiënte met aktiewe TB in ‘n EBA proef te analiseer.
3. Om die gebruik van IgG, IgM en IgA op geselekteerde mycobacterial antigene vir diagnostiese potensiaal te bepaal.

Metodes

Deelnemers was gewerf en deel van die onlangse gefinaliseerde EDCTP-befondsde, AE-TBC studie en die 14-dae fase-twee kliniese ewekansige proef (EBA-studie met sewe behandelings arms). Sputum en bloed monsters is op verskillende tye versamel, multiplex sitokinien, verskeidenheid ontleding is deur Luminex op plasma en serum monsters gedoen en anti-mycobacterial teenliggame is deur ELISA geïdentifiseer.

Resultate

Nadat die bloed oornag met ESAT-6/CFP-10, RV0081, Rv1284 en Rv2034 gestimuleer was, het CRP, Ferritin, SAA and IP-10 as die mees belowende diagnostiese merkers na vore gekom. Ongestimuleerde gasheer merkers het die beste onderskeidings vermoë getoon. Die ses-merker bio-kenmerk bestaan hoofsaaklik uit ongestimuleerde sitokiene wat se vlakke die belowendste resultate vir die diagnosering van aktiewe TB opgelewer het. Noemenswaardige veranderinge was vir CRP, IL-6, VEGF, sIL-2Rα, Ferritin en sTNFRII gesien teen oor die basislyn tot en met die einde van die 14 dag EBA evaluasie in verskeie van die behandelings afdelings. Alhoewel geeneen van die merkers die afname in die bakteriële lading in sputum weerspieël het nie. ’n Vier-merker kombinasie kon slegs vir 20% van die variasie in beide TTP en CFU verantwoord. Die beste sensitiwiteit en spesifisiteit was behaal deur die anti-16 kDa IgA (95%/95%) en anti-MPT64 IgA (95%/90%). ’n Hoër akkuraatheid is met ’n kombinasie van 3 of 4 teenliggame verkry. Anti-16 kDa IgA en anti-MPT64 IgA vlakke het beduidend afgeneem terwyl anti-LAM IgG en anti-LTBI IgG weer beduidend toe geneem het teen die einde van ’n ses maande anti-TB behandeling.

Gevolgtrekkings

Gasheer biomerkers as diagnostiese instrumente vir die toets van TB lyk belowend. Ten spite van die middelmatige akkuraatheid van *Mtb* antigeen-stimuleerde gasheermerkers mag hulle wel waarde hê in moeilik diagnoseerbare TB, soos kindertuberkulose of ekstapulmonale tuberkulose, en behoort verder evalueer te word in toekomstige studies. Alhoewel die gasheer merkers net vir ’n klein persentasie van die variasie in bakteriële lading kon verklaar in vroeë bakteriële dodingstudies, is daar ’n potensiële rol in die meet van algemene behandelings uitkomste wat verdere ondersoek benodig. Serologiese diagnostiese merkers teen nuwe *Mtb* antigene toon om belowend te wees vir die ontwikkeling van ’n eenvoudige punt van sorg toestel. Die resultate moet geëvalueer word in groter skaal studies in toepaslike deelnemergroepe.

Acknowledgements

I would like to express my profound gratitude to my supervisor Prof. Gerhard Walzl for his unrelentless contribution towards the completion of my PhD thesis. His teachings, advice, support and discussions even on issues that are not related to science and academics are remarkable. It is a great privilege working with such an inspiring leader. I also thank my co-supervisors Prof. Andreas H. Diacon and Dr. Novel N. Chegou for their guidance and suggestions.

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I am grateful to my families back in Nigeria especially my parents, brothers and sister and in South Africa my lovely wife Adetola and son Bimisiree for their patience, love, encouragement, support and prayer. My appreciation also goes to Dr. Atanda Raji and his wonderful family here in South Africa.

Lastly, I give glory unto God for given me the strength and courage to actualize my dream by successfully completing my PhD programme and witnessing his greatness through the world of science.

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Acronyms and Abbreviations

ADAM17:	ADAM metallopeptidase domain 17
AE-TBC:	African European Tuberculosis Consortium
AFB:	Acid fast bacilli
AlaDH:	Alanine dehydrogenase
ANOVA:	Analysis of variance
APC:	Antigen presenting cell
AUC:	Area under the operating characteristic curve
BCG:	Bacilli Calmette-Guérin
BSA:	Bovine Serum albumin
BSL:	Baseline
CFP:	Culture filtrate protein
CFU:	Colony forming unit
CI:	Confidence interval
CRP:	C-reactive protein
CT:	Computed tomography
CTL:	Cytotoxic lymphocyte
DC:	Dendritic cell
DNA:	Deoxyribose nucleic acid
DosR:	Dormancy survival regulator
DOTS:	Directly observed treatment short course
DTH:	Delayed type hypersensitivity
EBA:	Early bactericidal activity
EDCTP:	European and Developing Countries Clinical Trials Partnership
EGF:	Epidermal growth factor
ELISA:	Enzyme linked immunosorbent assay
ELISPOT:	Enzyme linked immunospot assay

ESAT:	Early secretory antigenic target
ESX:	Early secretory antigenic target -6 secretion system
FADD:	Fas-associated death domain
FcγR:	Fc gamma receptors
FGF:	Fibroblast growth factor
GDA:	General discriminant analysis
GM-CSF:	Granulocyte monocyte colony stimulating factor
HB:	Haemoglobin
HHC:	Household contacts
HIV:	Human immunodeficiency virus
HSP:	Heat shock protein
IFN:	Interferon
IFN-y:	Interferon gamma
Ig:	Immunoglobulin
IGRA:	Interferon gamma release assay
IL:	Interleukin
IP-10:	Interferon-inducible protein-10
IUALTD:	International Union Against Lung and Tuberculosis Disease
IU:	International units
LAM:	Lipoarabinomannan
LSD:	Least significant difference
LTBI:	Latent tuberculosis infection
LUMC:	Leiden Medical University College
MAPK:	Mitogen-activate protein kinase
MCC:	Medicines control council
MCP:	Monocyte cheoattractant protein
MDC:	Macrophage derived chemokine

MDR:	Multidrug resistant
MGIT:	Mycobacteria growth indicator tube
MHC:	Major histocompatibility complex
MIP-1β:	Macrophage inflammatory protein-1 beta
MMP:	Matrix metalloproteinase
NAAT:	Nucleic acid amplification test
NaOH:	Sodium hydroxide
NF-κB:	Nuclear factor kappa B
NK:	Natural killer cell
NO:	Nitric oxide
NPV:	Negative predictive value
OADC:	oleic acid-albumin-dextrose-catalase
OD:	Optical density
ORD:	Other respiratory diseases
PANTA:	polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin
PBMC:	Peripheral blood mononuclear cell
PBS:	Phosphate buffered solution
PDGF-BB:	Platelet derived growth factor BB
POC:	Point-of-care
PPD:	Purified protein derivative
PPV:	Positive predictive value
QFT-IT:	Quantiferon TB Gold In Tube
qPCR:	Quanitative polymerase chain reaction
RD:	Region of difference
RIF:	Rifampicin
ROC:	Receiver operator characteristics
ROS:	Reactive oxygen species
Rpfs:	Resuscitation promoting factors

SAA:	Serum amyloid A
SAP:	Serum amyloid P
sCD40L:	Soluble CD40 ligand
SD:	Standard deviation
sIL-2R α:	Soluble interleukin receptor alpha
sTNFRII:	Soluble tumour necrosis factor II
SSCC:	Serial sputum colony count
TACE:	Tumour necrosis factor alpha converting enzyme
TB:	Tuberculosis
TBK-1:	TANK binding kinase 1
TCR:	T cell receptor
Th:	Helper T cell
TLR:	Toll-like receptor
TMB:	Tetramethylbenzidine
TNF-α:	Tumour necrosis factor alpha
TNFR1:	Tumour necrosis factor receptor 1
TNFR2:	Tumour necrosis factor receptor 2
Tpx:	Thioperoxidase
TST:	Tuberculin skin test
TTP:	Time to positivity
TNF:	Tumour necrosis factor
UCP-LTA:	Upconverting phosphor lateral flow assay
VEGF:	Vascular endothelial growth factor
WBA:	Whole blood assay
WHO:	World Health Organisation
XDR:	Extreme drug resistant
ZN:	Ziehl Neelsen

Definition of key terminologies in the thesis

Sensitivity

This term refers to the correct identification of patients with disease. Thus, a test that is highly sensitive is used as a rule out test if a patient tests negative [1].

Specificity

This term refers to the correct identification of patients who do not have disease. Thus, a test that is highly specific is used a rule in test if a patient test positive. Both sensitivity and specificity are a measure of clinical test and do not depend on the population of interest being tested [1].

Positive predictive value (PPV)

This is defined as the number of patients that actually have a disease with a positive test result [2].

Negative predictive value (NPV)

This is defined as the number of patients that actually do not have a disease with a negative test result. Both PPV and NPV depend on the changes of disease prevalence in the interested population [2].

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Outline of thesis

This thesis contains three experimental chapters (2-4) and each chapter is written and structured for publication. Enzyme Linked Immunosorbent Assay (ELISA) and Luminex multiplex cytokine arrays were used for the experimental analysis. A detailed explanation of the ethical approval, recruitment of the study participants, sample collection, assay methodology and data analysis are found under each experimental chapter. These studies were carried out at the SUN Immunology Research Laboratory, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, University of Stellenbosch. This institution is located in Tygerberg (Cape Town) in the Western Cape Province of South Africa.

Chapter 1: Tuberculosis: Burden, Immunology, Diagnostics and Biomarkers

Chapter 2: Evaluation of cytokine responses against novel *Mtb* antigens as diagnostic markers for TB disease

Hypothesis

Supernatant levels of single or multiple cytokines produced in overnight whole blood culture after stimulation with *Mycobacterium tuberculosis* infection phase specific antigens will enable accurate diagnostic tests for active TB.

Objective

- To investigate the accuracy of host markers detected in *Mtb* antigen-stimulated whole blood culture supernatant in the diagnosis of TB.

Chapter 3: Evaluation of host markers for tracking early treatment response in newly diagnosed pulmonary TB patients

Hypothesis

Host biosignatures in body fluids, including serum/plasma, saliva or urine, will reflect changes in bacterial numbers during early TB treatment and will be suitable biomarkers for early TB treatment effect.

Objective

The main objective of this study is to discover biosignatures comprising host markers in the body fluids that correlate with a decline in bacterial burden and that are suitable adjunctive or even replacement tests for EBA.

Primary objectives

- To investigate the profiles of biomarker levels of patients infected with active TB undergoing treatment.
- To determine biomarkers that correlate with early treatment response as assessed by EBA.

Chapter 4: Combined specific IgG and IgA based diagnosis of tuberculosis in African primary healthcare clinic attendees with signs and symptoms suggestive of TB.

Hypothesis

The presence of or levels of specific antibodies to *Mycobacterium tuberculosis* will have diagnostic and treatment response tracking utility.

Objective

- To evaluate multiple antibody classes against selected *Mtb* antigen as diagnostic test for TB and for evaluating treatment effect.

Chapter 5: General discussion and conclusion

CHAPTER 1

Tuberculosis: Burden, Immunology, Diagnostics and Biomarkers

This chapter will be submitted to a journal as a review for publication

My contribution: Research of data

Literature searches

Planning of manuscript

Writing of manuscript

1.1 Tuberculosis: A threat to global health

Tuberculosis (TB) has been identified as a global health problem by the World Health Organization (WHO) since at least 1991 [1]. TB is an infectious disease, which is caused by *Mycobacterium tuberculosis* (*Mtb*) and spread by inhalation of aerosolized droplets. The disease affects about 10 million new cases and 1.7 million deaths are recorded yearly [2]. TB disease is a spectrum and based on the state of the disease, TB infection can be classified either as active or latent TB. An active TB state presents with active pathogens resulting in the spread of the disease to other uninfected individuals while people harbouring latent TB may not exhibit any clinical signs or visible symptoms but have a 5 to 10% chance of reactivation of the pathogen thereby becoming infectious at some stage of their lifetimes [1]. TB reactivation may be triggered by Human immunodeficiency virus (HIV) infection, immunosuppressive treatment – glucocorticoids, anti-tumour necrosis factor (TNF) therapy, anti-cancer therapies, malnutrition, tobacco smoking, alcoholism, malignancy, insulin dependent diabetes and renal failure. However, the exact cause of reactivation is unknown in most cases [1, 3-5]. A greater percentage of the infected individuals develop latent TB as reflected by the immunologic tests, tuberculin skin test (TST) or Interferon-gamma (IFN-γ) release assay [IGRA] [1]. The chances of developing active TB is about 20 to 30 times higher in people infected with HIV and TB is one of the leading causes of death amongst HIV infected people [6].

Despite the development of several new drugs in the past years, the TB epidemic is not yet under control and we are not going to reach the WHO targets of TB eradication [7, 8]. Some of the factors involved in the rise of TB incidence include poor programmes in TB management, poor treatment adherence, co-infection with HIV, poverty, imperfect diagnostic assays, limited vaccine efficacy and the emergence of drug-resistant *Mtb* strains [1, 9]. WHO has projected that there will be one billion new latent cases, thirty six million deaths and more than one hundred and fifty million people developing active disease by 2020 if TB treatment is not improved [9]. Clearly, this situation shows the shortcomings of the current

TB treatment strategies and the ineffectiveness of the public health systems due to their limitations, especially in resource poor countries that are faced with huge TB problems [10].

1.2 TB infection and immune response

Mycobacterium tuberculosis is an obligate aerobe with an ability to remain for long periods of time in humans as a result of a non replicating state associated with dormancy and/or non culturability [11]. Once infected with TB, an active TB patient will infect 10-15 people per year if left untreated [12]. After gaining entry into the human body, *Mtb* bacilli encounter several antimicrobial, intraphagosomal defense mechanisms as a result of phagocytosis by alveolar macrophages and dendritic cells (DC's) [13, 14]. They also face different forms of stresses due to low pH, reactive oxygen species (ROS) and nitric oxide (NO) and lysosomal hydrolytic enzyme action [13]. The DC's with bacilli and antigens move from the distal airways to the draining mediastinal lymph nodes where T cell responses are initiated. A granuloma is then formed when the lymphocytes and macrophages migrate to the primary site of infection [14]. Structurally, granuloma is an organisation of different immune cells that include B cells, T cells, macrophages, DC's, natural killer (NK) cells, neutrophils and fibroblasts. It is formed due to a pulmonary inflammatory response resulting from host cells stimulation by mycobacterial antigens [5, 15]. Multinucleated giant cells are formed when the granuloma macrophages differentiate into epithelioid cells. Additionally, macrophages filled with droplets of lipids, so-called "foamy macrophages", develop within the granuloma, acting as nutrient rich reservoir for persistent bacilli [16].

The TB bacilli, after several years of dormancy, can again change its metabolic state, reactivate and lead to necrotic cell death in the granuloma. Granuloma in *Mtb* infections may be vital to limiting mycobacterial growth, tissue damage and dissemination [17, 18]. NK cells also take part in mediating antimycobacterial activity. Once macrophages are infected with *Mtb*, vimentin will be upregulated and the infected mycobacteria are then lysed by NK cells via ligation of vimentin by the NKp46 molecule on NK cells [19-21]. In a study by Roy *et al*.,

[22] it was demonstrated that NK cells can also lyse the expanded T cells that are expressing a regulatory phenotype ($CD25^+$ $FoxP3^+$).

1.2.1 Cell mediated immune response

Cell mediated immune system is a key component in host defence against TB infection [23] and its development is within 2 to 6 weeks of infection [24]. The control of *Mtb* infection in resistant individuals is hinged on a robust Th 1 immune response development, which involves alveolar macrophages, DC's, T lymphocytes ($CD4^+$, $CD8^+$, Tγδ cells), release of pro-inflammatory cytokines, including IFN-γ, interleukin-2 (IL-2), IL-12, IL-18, tumour necrosis factor-α (TNF-α) and chemokines (IL-8, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 alpha (MIP-1α). All of these play a prominent role in recruiting additional cells to the site of infection, forming a granuloma that may contain and kill tuberculosis bacilli but may also act as a long time niche for latent tuberculosis infection (LTBI) [15, 25]. This internal accumulation of cells surrounded largely by lymphocytes especially $CD4^+$ T cells and $CD8^+$ T cells also include B cells and fibroblasts, which form a peripheral fibrotic capsule. Although there is a limited antigen-presenting cell (APC) function in the granuloma by T cells [26, 27] this is because the prime APC's in the initiation of T cell responses are the DCs [28]. Protective immune responses against *Mtb* infection rely largely on $CD4^+$ Th1 cells, which secret IFN-γ as is demonstrated by the inability of hosts to control *Mtb* when $CD4^+$ T cells are deficient (or major histocompatibilty complex class II is deficient) [29-31, 206]. Furthermore, $CD8^+$ T cell also contribute to immunity against *Mtb* by secreting IFN-γ, which activates macrophages to curb infection and/or by secreting products that can directly destroy the *Mtb* bacilli. However, the lack of $CD4^+$ T cells cannot be compensated for by $CD8^+$ T cells [30] [31].

1.2.2 Humoral mediated immune response

Humoral (B-cell-mediated) response in protecting against *Mtb* infection has been largely relegated towards irrelevance while much attention has been focused on the critical role of cell mediated immune response towards TB protection [5, 32]. However, the evolvement of adaptive immunity includes a collaboration of both cellular and humoral responses in mounting a defence towards an infectious pathogen [33]. There is promising evidence from studies that have shown the potential role B cells and antibodies may play in the containment of TB [34]. In one of such study, B cell-deficient mice showed a similar bacterial load to wild type mice but transfer of B cells to the B cell-deficient mice was able to decrease pathology and dissemination to levels seen in wild type animals [35]. The study suggests a more significant role for B cells in recruiting cells to granulomas through chemokine secretion [36]. In addition to antibody and cytokine production, antigen presentation to T cells has been described for B cells [5] (Figure 1.1). Although $CD3^+$, $CD4^+$ and $CD8^+$ T cells are present within the inflammatory lesion $B220^+$ cells dominate the lymphocyte population [37].

B cells through cytokine secretion polarize T cell responses and might enhance Th2 differentiation in mice through IL-10 [38, 39]. Furthermore, the immune system might be modulated within the granuloma through the interaction of the antibodies with the Fcγ receptors on cells such as macrophages [5]. This immunological pathway has been seen as a possible way to boost the immune system against intracellular pathogens [40]. Fcγ receptors are classified as either stimulatory or inhibitory and this depends on the presence of intracellular immunoreceptor tyrosine-based activation motifs (ITAM) or immunoreceptor tyrosine-based inhibitory motifs (ITIM) [41, 42]. Experimental evidence has reported the roles of stimulatory Fcγ receptors in contributing towards host defence against intracellular pathogens such as *Cryptococcus neoformans* [43] and *Mtb* [44]. IL-10 is an anti-inflammatory cytokine that is produced by various cells such as B cells, macrophages, DC's and T cells and it has been established that B cells play a major role in the secretion of IL-10

in the lungs [45]. Two different studies with *Mtb* infection in a B cell deficient murine model reported an increase in IL-10 production in the lungs [46, 47]. B cells have also been shown to influence the activation or inhibition of regulatory T cells which is a major source of IL-10 [48, 49]. Reappraisal studies on B cells and antibodies have been recommended [50-52] and future research on this arm of adaptive immunity should look into how to augment their potential in protecting against *Mtb* infection. In response to acute infection not many immune cells are likely to be recruited in containing infections leading to a reduced pathology as a result of decrease in mycobacteria burden while a sustained inflammatory response during response to chronic infection may eventually produce a better immunopathology (Figure 1.1A). However, more immune cells are likely required in response to acute infection in infection containment leading to increased pathology while there is an increased mycobacteria burden and decreased survival during chronic infection response (Figure 1.1B). In response to acute infection there is an increase in mycobacterial load in higher dose models and dysregulation of the granulomatous reaction. Recruitment of more immune cells is likely needed to contain infection leading to more pathology. A decrease in the continued activity of inflammatory response results in diminished immunopathology compared with wild type mice with B cells during response to chronic infection (Figure 1.1C).

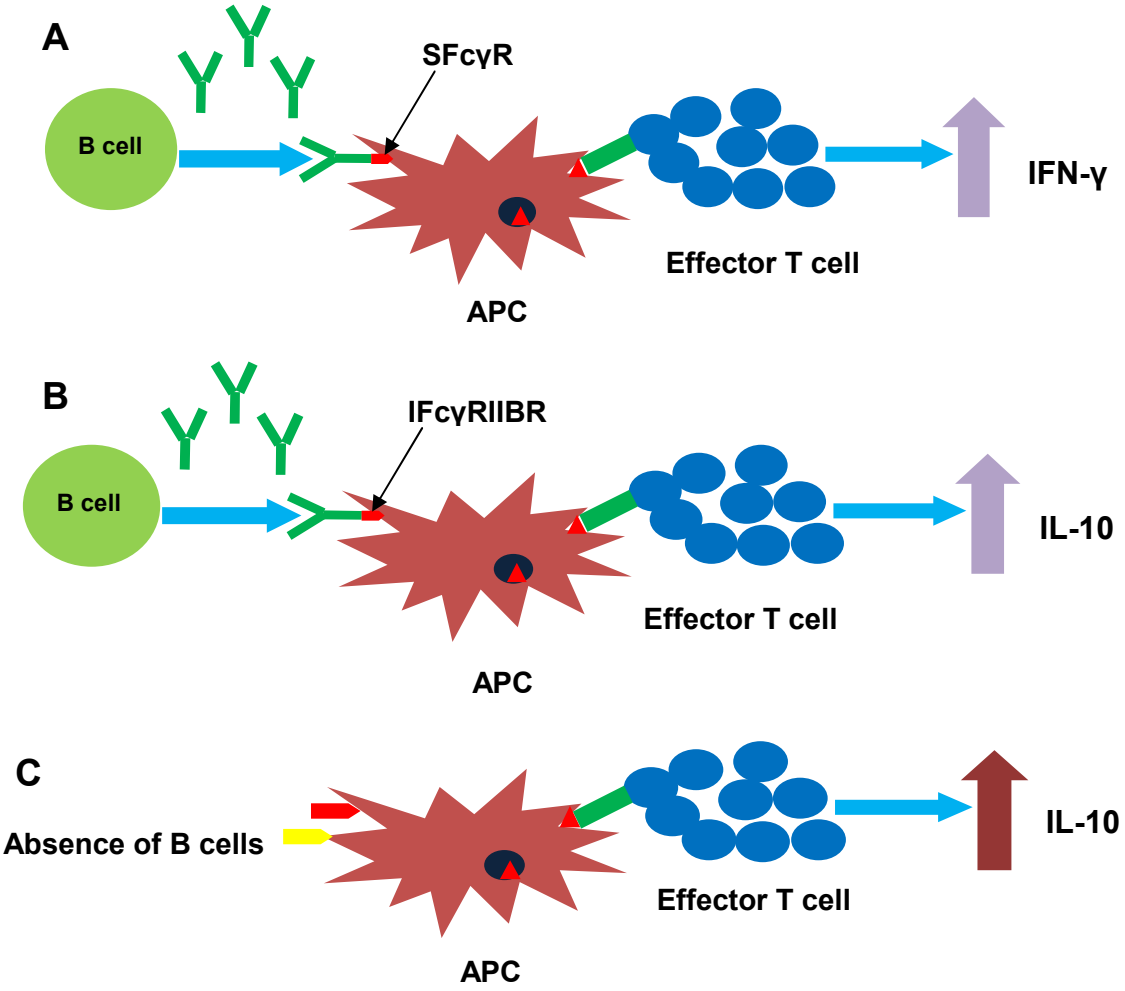


Figure 1.1 The role B cells play in enhancing immune response against Mtb. The murine host response against *Mtb* is modulated by B cells in numerous ways. During the acute infection stage, there is an increased Th 1 response which leads to the containment of mycobacteria with lesser inflammation due to the involvement of antibody-stimulatory Fcγ receptors complex (panel A). However, immunity against *Mtb* is compromised with a heightened IL-10 secretion by the engaged inhibitory FcγRIIB receptors (panel B). In the absence of B cells (panel C) an immunosuppressive phenotype temporarily suppresses the maximum containment of acute *Mtb* but inflammatory progression during chronic TB is delayed. APC=Antigen presenting cell, SFcγR=Stimulatory Fc-gamma receptors, IFcγRIIBR=Inhibitory Fc-gammaRIIB receptor. This figure was adapted from reference [34].

1.3 Diagnostics for tuberculosis

1.3.1 Immunodiagnostic biomarkers

The principle of TB immunological diagnostics is based on the hosts' immune response to *Mtb* antigens. Previous or current infection depending on the host's immune status could lead to the development of a positive result. The prominently used methods are TB antibody detection, Tuberculin skin test and IGRA. However, neither TST nor IGRA can distinguish between LTBI and active TB rather these two diagnostic tests measure immunity to response independently to infection from active disease [53].

1.3.1.1 Interferon-γ release assays (IGRAs)

The advent of IGRAs is regarded as a major development in the diagnosis of *Mtb* infection [54]. IFN-γ is a major cytokine that is secreted by the Th 1 cells and its response against primary *Mtb* infection is dependent on IL-12 [30]. IGRAs measure the amount and frequency of IFN-γ released from T lymphocytes in response to *Mtb* specific antigens. Early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) are two secreted proteins produced by *Mtb complex* but absent in BCG strains and in non tuberculous mycobacteria (NTM). These two antigens have been discovered to strongly induce the production of IFN-γ and their strong antigenicity has been the basis of IGRA development [23, 55-57]. Several studies have evidenced a higher specificity and sensitivity for IGRAs compared to TST for detection of latent *Mtb* infection [58-60].

Commercially, there are three T-cell dependent tests available. QuantiFERON-TB Gold (QFT-G), QuantiFERON-TB Gold In-Tube test (QFT-GIT) (Cellestis Ltd, Carnegie, Australia) and T.SPOT.TB assay (Oxford Immunotec, Abingdon, UK). Both QFT-G and QFT-GIT measure the IFN-γ response to mycobacteria antigens using Enzyme Linked Immunosorbent assay (ELISA). The QFT-G contains ESAT-6 and CFP-10 two antigens of the *Mtb* region of difference (RD1) while the QFT-GIT uses ESAT-6, CFP-10 and TB 7.7, a synthetic cocktail of three mycobacteria peptides, which are dried unto the walls of the collection tubes [61]. The T.SPOT.TB is an enzyme linked immunospot (ELISPOT) assay

that is based on measuring the frequency of IFN-γ producing T lymphocytes after stimulation with both ESAT-6 and CFP-10. The IFN-γ molecules released by the cells in response to the antigens specifically bind to immobilized anti-IFN-γ monoclonal antibodies on a plate and are seen as dark spots on the well membrane. The spots are counted with a magnifying lens or an automatic reader. In comparison to QFT-GIT test, the ELISPOT technique makes use of peripheral blood mononuclear cells (PBMC) separation [23]. Performing both TST and IGRAs alone using peripheral blood cannot differentiate individuals with LTBI, active or past tuberculosis [62, 63].

1.3.1.2 Tuberculin skin test (TST)

TST was named after Charles Mantoux and Clemens von Pirquet who experimented the test in 1907 after it was first described by Robert Koch in 1890 [64]. Tuberculin is a glycerol extract of mycobacteria, while purified protein derivative (PPD) is a precipitate of non specific antigens retrieved from mycobacterial culture filtrates. The test is based on the intradermal administration of PPD, which triggers a classical T cell mediated delayed type hypersensitivity (DTH) reaction. The induration of the skin is then measured after 2 to 3 days [207]. Usually, a dose of 2 international units (IU) of PPD RT23 (Statens Serum Institut, Copenhagen, Denmark) is used and a 6 to 10 mm reaction is considered positive. Criteria for positivity vary regionally and dependent on the dose and type of the PPD antigen that is used [65]. Even though TST has been prominently used in LTBI screening and to guide treatment decisions, the major impediment of the test is the low specificity [57]. The cross reactivity is due to over 200 PPD antigens that are not specific for *Mtb* but are shared with environmental mycobacterial strains and *Mycobacteria bovis* Bacilli Calmette-Guérin vaccine (BCG), commonly used for TB vaccination [5]. The sensitivity of the test has been described as low in people with HIV infection and in immunocompromised individuals with a high risk of progressing to active TB [66, 67]. A study by Santin *et al*. 2011 [66] showed that the TST reactivity in adults infected with HIV decreased distinctly as the $CD4^+$ cells dropped.

Efforts are been made in tackling some of the obstacles that are associated with TST as a way of improving its specificity. Such is the use of ESAT-6 in combination with CFP-10 instead of tuberculin. These two antigens that are specific to *Mtb* have been confirmed for its suitability and tolerability to increase its sensitivity in a recent phase I trial [54, 68].

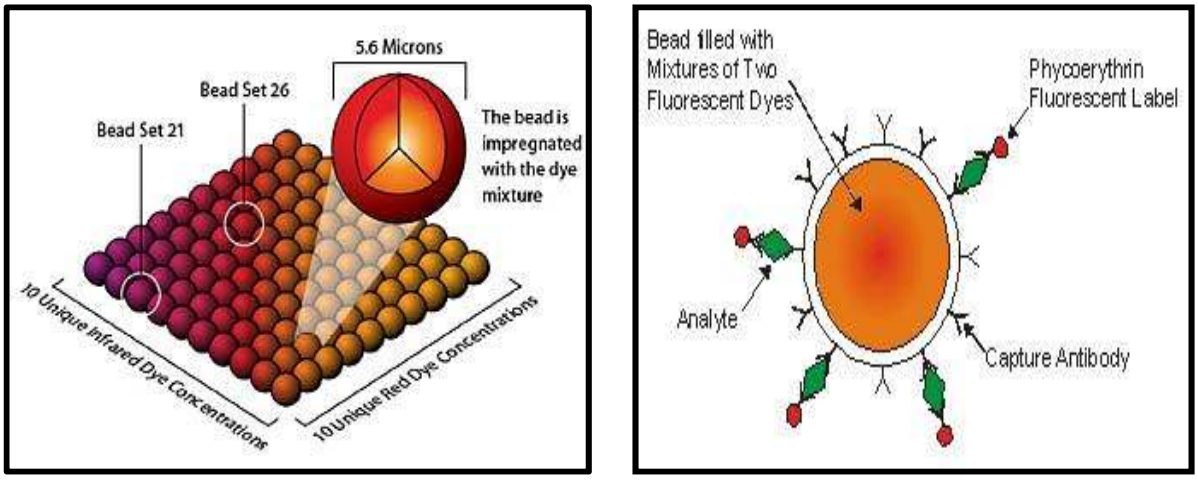
1.3.1.3 Antibody based serological test

Serological detection is being used for many infectious diseases and this may present a veritable potential for TB diagnosis [69]. During the early infection stage of TB, antibodies are produced by B lymphocytes against the TB antigens and these TB antibodies can be unmasked in the patient's blood using recombinant TB antigens as targets in ELISA assays, yielding results within 24 hours. Besides the RD1 antigens, ESAT-6 and CFP-10, which can be used in TB antibody detection due to their specificity for *Mtb* and not other mycobacteria species, other recombinant antigens such as TbF6, 38kDa, malate synthase, and MPT51 have been assessed for their sensitivity and specificity. These antigens were shown to have specificity of 93% to 97% and sensitivity 47% to 75%. This low sensitivity does not support clinical utility, which can even decrease further in people with suppressed immunity [54, 69]. However, a combination of several selected antigens has been shown to achieve a higher sensitivity than single antigens [69, 70]. Nevertheless, sensitivity of serological assays to date has not been high enough to replace the sputum microscopy [54].

Furthermore, this test cannot differentiate between active TB and LTBI but antigens cfp-21, Rv1978, nrdf1, mpt64, ppe57 and ppe59 encoded in RD-2 and RD-11 could offer a potential discrimination [71]. Despite some promising data concerning these candidate antigens, further efforts should be directed towards the evaluation of outcomes that look beyond specificity and sensitivity such as the efficiency of diagnostic algorithm in health care delivery and the effect of new tests in clinical judgement and effective treatment [69].

1.3.1.4 Luminex xMAP

Luminex x multi-analyte profiling (xMAP) technology is extensively employed in research for multiplexed detection and measurement of cytokines and signal transduction proteins. The technology of Luminex xMAP is a combination of many existing technologies such as flow cytometry, digital signal processing, biological chemistry and microsphere tools. Luminex xMAP is based on the use of polystyrene or paramagnetic 5.6-micron microspheres (beads) that are dyed internally with red and infrared flourophores of different intensities (Figure 1.2A). A bead region or unique number is assigned to each dyed bead and this allows for individual differentiation of the beads that are coated with antibodies, receptor, peptides and streptavidin specific for an analyte. With this process, multiple analyte specific beads can then combine with a small heterogenous sample volume during incubation allowing its capture and detection in a 96 well microplate (Figure 1.2B).



A **B**

Figure 1.2 Schematic of micro beads of the Luminex xMAP technology. Luminex xMAP technology uses paramagnetic 5.6-micron microspheres or beads that are dyed internally with red or green fluorophores thereby making each bead to either be red or infra red dominant. Each bead has a unique number or bead region that is associated with a capture antibody (panel A). Following this process, each well can be coated with up to 100 capture antibodies and the addition of a small sample volume containing the analyte of interest binds to the capture antibody on the bead. The formation of a new complex is then detected with the addition of detection antibodies and streptavidin-phycoerythrin (panel B). Figure adapted from www.luminexcorp.com

Within the Luminex analyzer, there are three major components: fluidics system, lasers and detectors. Inside the instrument, flow cytometry precision fluidics lines up the suspended beads in a single file when passing through the detection chamber allowing for individual bead analysis (Figure 1.3A). The beads are then individually interrogated by the green laser (532nm) which excites the streptavidin-PE for fluorescence intensity while the red laser (632nm) excites the bead's internal dye for the determination of bead colour or region. There are four detectors in the analyzer which measure the fluorescence and make the bead determination (Figure 1.3B). The reporter signal for each event based on the content of the dye and size is measured by a photomultiplier tube (PMT) linked to the green laser and all the captured information is then processed by the high speed digital signal processors. Although Luminex xMAP uses a robust immunoassay which allows for simultaneous detection of more than 100 analytes in a single well of a 96-well microplate this number is a small percentage of the total serum proteins. Non biased approaches such as proteomics using mass spectrometry can be used to quantittate low abundance proteins for biomarker research but in protein-rich samples matrixes like serum the masking effect of abundant proteins is problematic and removal of these proteins also affects the presence of proteins of interest. Multiplex cytokine arrays therefore do represent a very useful biomarker discovery tool.

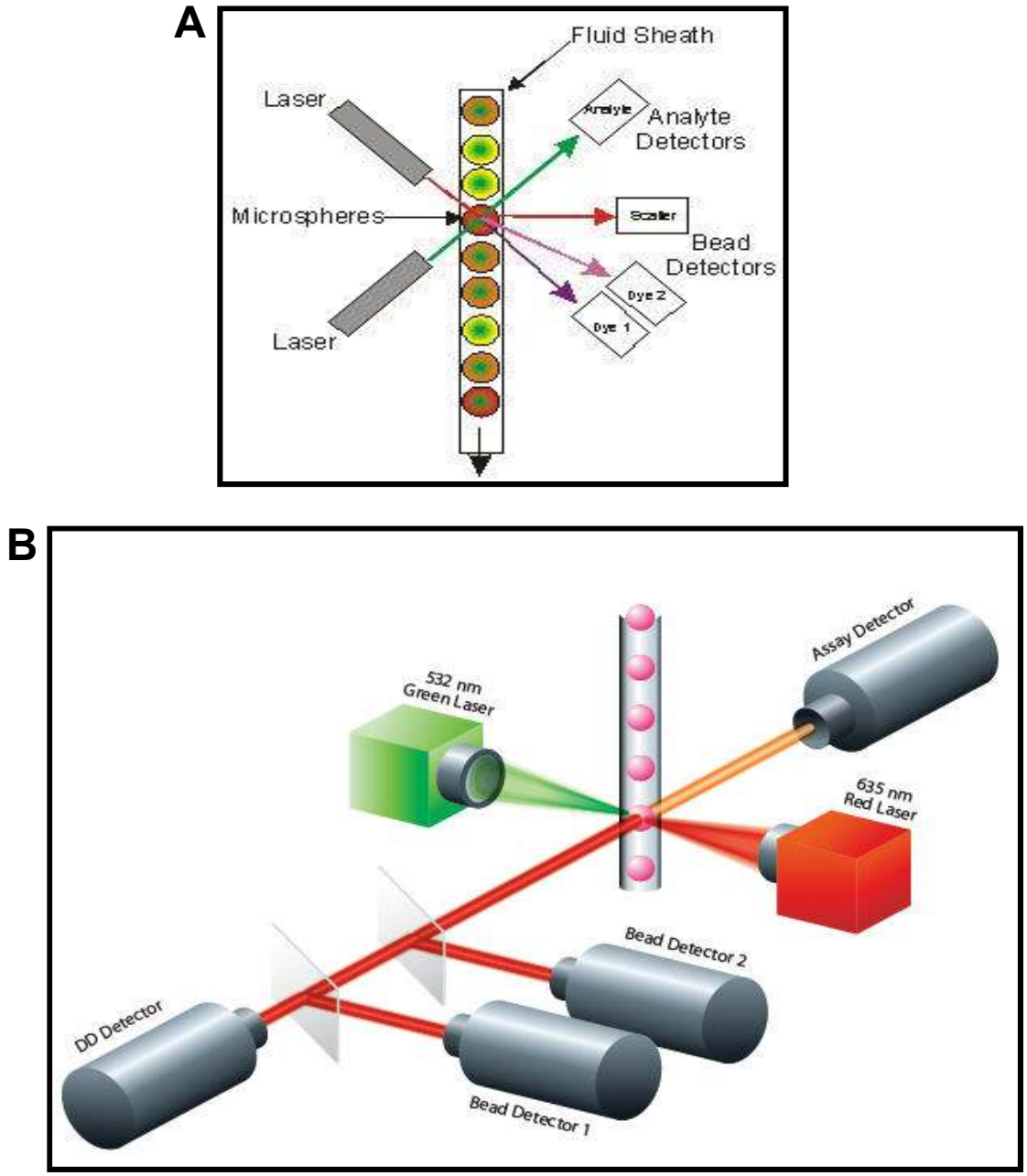


Figure 1.3 Interrogation and detection process of the beads by the Luminex reader. Inside the detection chamber, the fluidics system line up the suspended microspheres in a single file in a stream of sheath fluid and each bead is interrogated individually after the excitation of the two lasers (panel A). The fluorescence intensity of the bead is measured by the green laser (532 nm) which excites the streptavidin–PE while the excitation of the dye inside the bead by the red laser (635 nm) region determines the bead colour or region. There are four detectors within the Luminex analyzer which record all the captured information that are processed by the digital processor (panel B). Figure adapted from www.luminexcorp.com

More details can be found on:
http://www.luminexcorp.com/products
http://www.biotek.de/resources

1.4. Non-immunological biomarkers

The identification of *Mtb* involves imaging, microbiological and molecular methods. Imaging techniques for TB screening, diagnosis and treatment response follow up include chest X-ray and chest computed tomography (CT). Comparatively, high resolution CT has a greater sensitivity than X-ray to determine TB disease activity and identification of early parenchymal lesions or mediastinal lymph node enlargements [54]. Recent studies have shown promising research applications for [(18)F]-2-fluoro-deoxy-D-glucose positron emission tomography (PET) to assess responses to anti-TB chemotherapy [72, 73]. Although this technique is expensive, it could potentially contribute to the management of multidrug resistant (MDR) and extreme drug resistant (XDR) TB patients [54].

Sputum smear microscopy is a conventional method of TB diagnosis and involves identifying the presence of acid fast bacilli (AFB) in the sputum smear under the light microscope. This test is simple, cheap and rapid but has a low sensitivity which can be enhanced by repeating the examination three times [54]. However, fluorescent microscopy has been recognised as having the advantage of increased sensitivity over light microscope [74] but its affordability in the developing world is questionable. Culture of TB is seen as the gold standard in the detection of *Mtb* and the liquid cultures are more rapid and sensitive with a time to positivity (TTP) of about 14 days compared to Lowenstein Jensen solid medium culture with 27 days [54, 75, 76] .

A novel molecular technique for TB diagnosis and detection of resistance to rifampicin, GeneXpert MTB/RIF, was recently developed. GeneXpert MTB/RIF is an *ex vivo* *Mtb* gene amplification test and produces results within two hours. The advantage of this assay over the nucleic acid amplification test (NAAT) is its high sensitivity (98%), rapid test result and its ability to detect MDR strains. However, its use is limited as it is expensive and requires advanced equipment [77].

1.5 Biomarkers in treatment response

1.5.1 Colony forming unit (CFU)

CFU is the quantification of *Mtb* in sputum by counting viable CFU of the microorganism cultured on a solid media at different dilutions. For over three decades, this technique has been used extensively in the exploration of early bactericidal activity (EBA). Although the methodology has proven its usefulness, it is demanding and requires laboratories to be equipped with appropriate facilities [78] and a suitable replacement tool for evaluating treatment response would be needed.

1.5.1.1 Early Bactericidal Activity (EBA)

- EBA is defined as the mean daily decrease in CFU counted on agar plates and expressed as log CFU per ml of expectorated sputum [79, 208].

OR

- EBA can be referred to as the ability of an agent to kill *Mtb* in the pulmonary cavities during the first few weeks of treatment [80]

The history of modern day EBA study dates back to 1980 with the innovative study of Jindani *et al*., 1980 [79]. In the study, they gave a descriptive assessment of 27 anti-TB drugs and regimens in 124 sputum smear positive pulmonary TB patients by counting the sputum CFU before treatment and after every 2 days for 14 days on treatment. The efficacy of one or several anti-TB drugs in combination to kill *Mtb* in the first days of treatment is determined by enrolling TB patients into EBA studies [81]. Expectorated sputa are collected from participating patients at baseline and on each day until the end of treatment (day 7). The decline in bacterial load is determined during a limited time period of monotherapy with a new drug [82]. Drug combinations can also be assessed in this manner. Other treatment response biomarkers are assessed by various methodologies, which include AFB determination by Ziehl-Neelsen stain and microscopy, metabolic activity of viable bacteria by

liquid culture as surrogate of CFU count and detection and quantitation of mycobacterial DNA by NAAT or the novel GeneXpert test.

EBA has a discriminating power in identifying drugs like isoniazid or nitroimidazole PA-824 that actively act against replicating bacteria [83] but has power to assess sterilizing activity of drugs against dormant bacilli [84]. Regardless of the action of drug metabolism or regimen being tested, an ideal marker should be able to capture both rapid killing and sterilizing activity [85]. Nonetheless, EBA represents one of the major evaluation tools of new drugs and for dose finding but the method is time consuming and labour intensive. Thus, the discovery of novel biomarkers for TB treatment response is of significance to clinical practice and clinical trials of new anti-TB drugs [85].

1.5.1.2 Serial sputum colony counts (SSCC)

Serial sputum colony count (SSCC) is a valuable method in testing new TB drugs during clinical trials. This technique involves the quantification of *mycobacteria,* which is done by making several dilutions of bacterial suspension on a solid agar and then counting CFU after 21 days [86]. This procedure has been employed in Kenya, South Africa and Thailand [87] and combines the advantages of rapid EBA in the first 5 days with lower elimination rate in sterilization for 60 days and comparison of drug regimens based on the elimination rate in the sterilization phase [86, 88]. Several studies have demonstrated improved bacillary clearance in cases involving drug sensitive TB replacing ethambutol with 8-methoxyfluoroquinolones and multidrug resistant TB by adding TMC207 to standard therapy [89, 90].

SSCC is complex in nature due to its high contamination rate by bacteria and fungi [86]. However, selective destruction of nonmycobacterial organisms could be achieved by the decontamination of sputum with sodium hydroxide (NaOH) before culture inoculation although this practice could negatively affect *Mtb* recovery [91] and reduce bacillary load in sputum [92, 93]. Furthermore, it takes a long time for bacteria of interest to establish CFU's and the tendency of *mycobacteria* to aggregate results in multiple founders of a single

colony thus affecting the number of bacteria counted. Additionally, several dilutions are also required prior to plating in order to establish a definite count [94].

1.5.2 Time to positivity (TTP)

BACTEC (Bactenecin) mycobacteria growth indicator tube (MGIT) is an automated liquid culture system that uses the fluorescence of an oxygen sensor (fluoresces in the absence of oxygen as growing bacteria deplete oxygen) for the detection of *Mtb.* BACTEC MGIT is a useful tool in the assessment of treatment efficacy in the monitoring of the activity of the bacteria by sputum culture during anti-TB treatment. The TTP of *Mtb* in an automated liquid culture method demonstrates the metabolic activity of viable inoculated *Mtb* from sputum (or other body fluids, like pleural fluid of aspiration biopsies) and is being used mainly as a diagnostic and as measure of bacterial load. However, it is also being used as a substitute method to colony counting for exploring the bactericidal activities of novel anti-TB drugs [95]. Epstein *et al*., 1998 [96] demonstrated the potentiality of TTP as an early indicator of treatment effect. In this study, they showed an increase in TTP of *Mtb* samples of patients undergoing anti-TB treatment. TTP is less labour intensive using standardized calibrated equipment for its measurement and requires fewer steps in sputum preparation for analysis [83]. Several studies have established the use of liquid media for growing *Mtb* from sputum [97, 98] and also reported that more actively metabolizing bacteria can grow on both liquid and solid media whilst the more persistent bacilli grow only on liquid media [97, 99]. A study by Diacon *et al*., [80] in 2010, which showed a better performance of a novel drug TMC207 when measured with TTP but not on solid cultrure, further demonstrates the use of TTP as an early indicator of treatment monitoring. There is, however, a need to further examine the suitability of TTP as a biomarker of TB treatment efficacy especially in HIV positive patients [100]. The role of TTP as biomarker for month-six treatment outcome and for relapse-free cure for clinical management and clinical trials of new anti-TB drugs has to be assessed further.

1.6 TB biomarkers in serum: Hope for the future?

- **Biomarkers** are parameters that are measured and evaluated objectively as an indicator of normal biological process, disease monitoring or treatment response [101]
- **Surrogate marker** are intended as substitute for a clinical end point and should predict clinical benefit based on epidemiological, therapeutic and pathophysiological indices [101]

After several years of neglect, there is now a synergistic approach in developing new TB drugs and treatment regimens so as to cut short treatment duration and ultimately provide alternative drugs to resistant TB [102]. A major reason for the failure to control TB is simply because patients have to continue with the treatment for 6 months, which adversely affects treatment adherence [13]. Presently, the directly observed treatment short course (DOTS) recommended by WHO is only effective with drug sensitive *Mtb* with over 85% cure rate and 2 year subsequent disease free period [103]. The lack of an early surrogate marker for non relapsing cure has hindered the development of new TB treatment drugs. Although sputum culture conversion on solid medium at month 2 (M2) of treatment is still the most established surrogate biomarker of cure/indicator of treatment outcome [104] it has limited prediction [105, 106] particularly HIV co-infected patients are difficult to monitor. Sputum culture is also not applicable to extra pulmonary disease [107, 108]. This might constitute a huge problem especially in areas such as South Africa with high prevalence of HIV and TB co-infection. Thus, these limitations underline the need for surrogate markers that can determine treatment efficacy and clinical diagnosis [109]. These markers may include three different TB treatment-related biomarker categories: markers for relapse after initial cure, markers for early treatment effect and markers of baseline differences between patients [110]. The duration of clinical trials may be reduced by markers for relapse as conventional trials currently require at least 2 years of follow up to capture relapse.

Month two as earliest indicator of drug efficacy constitutes an unacceptably long delay. If TB patients could be stratified at diagnosis and/or early after onset of treatment into different treatment arms according to their risk for relapse and requirement for different treatment durations, equality across treatment arms could be ensured, resulting in smaller participant numbers in clinical trials [110]. This approach of patient's stratification requires suitable biomarkers that can be measured prior to or early during treatment.

The identification of patients with different treatment duration requirements might allow a shorter treatment regimen period in most patients even with current drug regimens, while reserving the longer treatment duration for the high risk group for recurrence. Additionally, such biomarkers could facilitate a faster way of evaluating new TB therapeutic interventions and be of significance in routine management of patients [109]. There is a need to engage in accelerated prospective studies on TB biomarkers, establishment of well characterized TB biobanks for biomarker evaluation and by encouraging many TB donors and/or sponsors to invest more in TB biomarker research so as to enhance discovery and validation efforts for novel surrogate biomarkers [111]. Early treatment response markers could also be indicative of drug resistance development, failure in treatment adherence and ineffective clinical drugs trials [103].

1.7 Candidate biomarkers of active TB and early treatment response: present study

In the past decades, several candidate biomarkers have been studied especially those that emerged from *Mtb.* Many of these biomarkers, when used singly, do not possess sufficient predictive power for clinical use. However, a combination of some of these biomarkers could demonstrate a potential ability in assessment of clinical cure and risk of relapse or reactivation. Several potential biomarkers of treatment, cure and relapse have been proposed from small patients' cohorts. These biomarkers should be validated in larger cohorts to enable their introduction into clinical trial or clinical use [111].

1.7.1 Interferon-gamma (IFN-γ)

IFN-γ is a pro-inflammatory cytokine and by far the most investigated in TB research [112]. Production of IFN-γ by the $CD4^+$ T cell is critical to the containment of the intracellular pathogen and evidence has shown that individuals with a deficient IFN-γ signalling pathway have an increase risk of tuberculosis [103, 113]. Seneviratne *et al*., [114] reported in 2007 that a HIV negative woman with disseminated TB as a result of an IFN-γ defect was completely healed after supplementing her TB treatment with IFN-γ. In protecting against TB infection, IFN-γ is produced in response to mycobacterial antigens by macrophages, which are then activated by IFN-γ in killing the intracellular pathogen through reactive nitrogen and oxygen production and phagolysosome formation [115-118]. The link between bacillary burden and the IFN-γ production in response to RD-1 specific antigens still remains uncertain [119]. However, the amount of IFN-γ produced can be determined by immunodiagnostic biomarker tests such as IGRA, which may serve as surrogate markers of bacillary load and treatment response [120].

1.7.2 Type I interferons

In 1957, Isaacs and Lindermann identified a secreted factor, which they termed "interferon" (IFN) while they were studying interference produced by heat-inactivated influenza virus [121]. Cytokines belonging to the interferon family are classified into three different types namely type I, type II and type III IFNs [206]. IFN-α/-β are the major effector cytokines of type I IFNs that are secreted by nearly all cell types including fibroblasts, endothelial cells and leukocytes. Much has been said about the viral interference of type I IFNs in host defence but further evidence has uncovered their roles in response to bacterial pathogens and immune modulation [209]. Type I IFNs in synergy with IL-18 and IL-21 regulated Th1 cell differentiation and effector functions *in vivo* [122-124] and may suppress the activity of Th2 and Th17 [122]. The induction of type I IFNs is regulated via Toll-like receptor (TLR) – dependent pathways [125, 126]. Berry *et al.,* in 2010 [127] reported that a

dominant IFN-α/-β signalling pathway is associated with TB pathogenesis and concluded that this type I IFN family of cytokines offer a potential diagnostic, vaccine and therapeutic development in curtailing the spread of TB disease. Similarly, the significance of IFN-α/-β as a candidate marker for monitoring TB was evident in the identification of a type I IFN molecular signature in a multi model TB gene expression study [128].

1.7.3 Tumour necrosis factor alpha (TNF-α)

TNF-α is an essential inflammatory cytokine that plays a prominent role in response to numerous conditions, including *Mtb* infection. Secreted primarily as a type II transmembrane protein and arranged in stable homotrimers, TNF-α can later be cleaved by the metalloprotease TNF alpha converting enzyme (TACE) to constitute a soluble form [129, 130]. In TB immunity, TNF-α acts against *Mtb* by inducing the production of chemokines [131], up-regulating adhesion molecules [132] and through induction of macrophage apoptosis [133]. Furthermore, TNF-α can exhibit pro and anti-inflammatory roles via activation of nuclear factor kappa B (NF-κB) or mitogen-activated protein kinase (MAPK) and recruitment of Fas-associated death domain (FADD) or caspase 8 by binding to the surface receptors TNFR1 and TNFR2 [130]. The significance of TNF-α as a candidate marker for TB treatment response was observed by Mattos *et al*., 2010 [134]. They found that TNF-α that increased in response to *Mtb* antigens ESAT-6, CFP-10 and 16kDa decreased after TB chemotherapy.

1.7.4 Matrix metalloproteinases-9 (MMP-9)

Gross and Lapiere [135] in 1962 discovered the first MMP by demonstrating a metamorphosis of collagenolytic activity in amphibian tissues and up to date 24 mammalian MMPs have been identified with overlapping specificities and functions [136]. MMPs belong to a class of zinc-dependent proteases consisting of two domains (predomain and catalytic domain) and are involved in the degradation of all the components of the extracellular matrix including collagens, laminin, fibronectin and proteoglycans [136]. Studies have shown that

MMPs are associated with pathologies including cardiovascular disease, Alzheimer's disease, atherosclerosis, arthritis, gastric ulcer, liver cirrhosis and cancer [137, 138]. Interestingly, MMPs are now gaining attention as major players in the progression of tuberculosis granuloma (creation of granuloma and lung tissue destruction) [139]. In one of such study, Marrero-Rivera and his team observed that the infection of mice with *Mtb* induced the expression of MMP-9 *in vitro* in macrophages [140] while Taylor *et al*., 2006 [141] demonstrated that a mice deficient in MMP-9 showed a reduction in macrophage recruitment to the lungs with formation of smaller granulomas. These studies offer the support for the rising paradigm that MMPs are responsible for lung tissue remodelling and initiation of granuloma formation [139]. Thus, investigating MMP-9 expression as candidate marker in TB patients would be interesting.

1.7.5 Soluble interleukin-2 receptor alpha (sIL-2Rα)

The IL-2 receptor is a complex of a trimer that consists of α, β and γ chains. The α subunit of the complex is also called Tac antigen or CD25. There is a growing consensus that serum levels of soluble IL-2Rα reflect the extent of activation and T cell expansion [142, 143]. sIL-2Rα is produced mainly by lymphocytic and monocytic cells and its levels might be useful for TB disease activity monitoring [144]. The level of sIL-2Rα in the serum was associated with pulmonary TB and decreased during anti-TB chemotherapy [145]. Thus, the measurement of sIL-2Rα in the sera might act as another TB biomarker.

1.7.6 Soluble interleukin-4 receptor (sIL-4R)

The role of Th 2 cytokines in TB remains controversial but this subject is crucial because IL-4 and IL-13 are known to be inhibitors in the effector mechanisms that control *Mtb* including cytotoxic lymphocytes (CTLs), apoptosis, autophagy and macrophage activation [146-149]. The production of sIL-4R is dependent either on a splice variant of the IL-4R mRNA transcript or on proteolytic shedding of the membrane bound receptor after encountering T cell receptor (TCR). sIL-4R is associated with the regulation of *in vitro* and *in*

vivo activity of IL-4 either as agonists or antagonists with a lesser affinity for IL-4 than the membrane bound receptor IL-4R [146] and therefore has potential as TB biomarker.

1.7.7 C-reactive protein (CRP)

CRP is an acute phase serum protein and a good biomarker for the detection of inflammation and bacterial infections [150]. CRP recognises nuclear antigens produced by apoptotic cells and binds to the surface receptors Fc gamma R (FcγR). It is an opsonin and induces macrophage mediated phagocytosis [151]. In patients with TB infection, levels of CRP have been shown to increase significantly in pulmonary TB and a marked decrease observed in its level after treatment with anti-TB therapy [150]. This finding was also corroborated by Kaminskaia *et al*., 2008 [210] in their study and this places CRP as a compelling candidate to further assess for early treatment response.

1.7.8 Interleukin-1β (IL-1β)

Autophagy is a cellular process that mediates cytoplasm degradation including entire organelles [152]. In the context of *mycobacteria*, IL-1β is said to be number one inducer of autophagy [153-155] and recently, this concept was confirmed by Pilli *et al.,* [156] in 2012. IL-1β induces autophagy and is needed in intracellular BCG killing in a TBK-1 (TANK binding kinase 1) dependent manner. IL-1β represents a classical example of control of cytokine secretion by autophagy [157] and its induction is hinged on stress factors such as intracellular pathogens which activate the inflammasome [158] and subsequently promotes pro- IL-1β processing into mature IL-1β by caspase-1 [159, 160]. As a result of a lack of a leader peptide required for conventional secretion, autophagy contributes to the secretion of IL-1β [157].

1.7.9 Interleukin-8 (IL-8)

IL-8/CXCL8 is a chemotactic cytokine for neutrophils and T cells and controls the recruitment of these cells to the pleural space in TB patients [161, 162]. IL-8 has been associated with a role in cancer pathogenesis, angiogenesis, tumour growth and metastasis

[163] and has also been linked to *Mtb* infection [164]. High levels of IL-8 have been found in the sera of TB patients and these correlated well with the restriction of *Mtb* infection [162, 164]. Few studies have been conducted on the role of IL-8 with TB treatment response and there is a possibility that IL-8 might produce more discerning predictive treatment response when used in combination with other biomarkers.

1.8 Soluble tumour necrosis factor receptor-2 (sTNFRII)

Primarily, TNF functions in augmenting cellular activation, tissue destruction and cytokine production [165] and its activity is regulated through the production of soluble TNF receptors (sTNFRI and sTNFRII) [166]. sTNFRII is a membrane bound protein that is secreted from the plasma membrane after TNFα converting enzyme (TACE) or ADAM metallopeptidase domain 17 (ADAM17) cleavage [167] and it is found as the active component of Enbrel® (a biological medicine that is used in treating inflammatory conditions such as rheumatoid arthritis) that has been associated with LTBI reactivation [168, 169]. Studies have demonstrated the blockade of host macrophage apoptosis by *Mtb* via the release of sTNFRII thus resulting in TNF inactivation [170] and this is consistent with a report by Richmond *et al.,* 2012 [166] who confirmed elevated levels of sTNFRII in mouse lung lavage fluid after *Mtb* infection. These results only indicate that sTNFRII is a potential indicator of early treatment response.

1.9 Potential of antigen-stimulated soluble markers in TB diagnosis

During the containment of *Mtb* infection in the granuloma, the bacilli have the ability to persist for long in the host in a non-replicative or slow replicative state, a stage that is described as being latent infection [171]. The host immune response mounts pressure continuously on the *mycobacterium* and it is believed that the response of the bacterium by self regulating the expression of its genes may interfere with the host immune response [172]. As *Mtb* undergoes physiological changes such as hypoxia, nutrient and oxygen deprivation, low pH and nitric oxide [173-175] during the transition from latency to active

disease, researchers have been able to identify several infection phase-dependent genes with diagnostic potential. The investigators were able to achieve this in *in vivo* and *ex vivo* models that mimic the stress conditions which the bacteria encounter in the granuloma [173, 176]. Based on their gene expression during the different infection states, these proteins have been classified accordingly and may be veritable diagnostic candidate genes.

1.9.1 Dormancy-related (DosR) antigens

DosR or latency antigens are a distinct set of genes that are expressed by *Mtb.* These genes are being regulated by the dormancy survival regulator (DosR) (Rv3133) [175]. It has been shown that these genes are upregulated especially in hostile conditions that mimic the stage of latent infection [177].

1.9.2 Resuscitation promoting factors (Rpfs)

The upregulation of rpfs is associated with active disease state of infection [178, 179]. Rpfs were identified in *Micrococcus leuteus* where it was shown that the dormant *M. leuteus* cells were resuscitated by *M. leuteus* rpfs. Furthermore, the growth of several microbacteria including *Mtb* was also stimulated. Similar genes to *M. leuteus* rpfs have been found in *Mtb* and encode mainly secretory proteins [180].

1.9.3 Reactivation antigens

Reactivation is defined as the re-emergence of *Mtb* from the state of latency after long spells to produce a clinical form of TB disease. The gene expression of *Mtb* changes from an inactive state to metabolically active during disease reactivation [181]. However, there is no much information on the genes or signals that are involved during *Mtb* reactivation from dormancy [182].

1.9.4 Classical TB antigens

The classical TB antigens are by far the most evaluated in TB research as candidates towards vaccine and diagnostics development [183]. These set of antigens are

expressed early during the infection stage [160] and include members of secreted antigen 85 complex (Ag85), members of the heat shock protein (HSP), ESAT-6/CFP-10 belonging to ESAT-6 secretion system (ESX), low molecular weight protein such as TB10.4 and lastly PPD [184]. Several *Mtb* antigens have been evaluated for use in immunodiagnostics by serology and T cell dependent tests. However, there is little information published on the possible utility of many of these antigens for diagnostics. The antigenicity of ESAT-6/CFP-10 forms the basis for the development of IGRA tests [55-57].

The diagnostic utility of these novel antigens have been investigated in several studies [173, 185]. Recently in our laboratory, we evaluated the immunogenicity of a total of 118 of these antigens in the diagnosis of TB in a high endemic setting. Out of all these antigens, 5 DosR antigens and rpfs were able to discriminate active TB disease from latent infection with an area under the operating characteristic curve (AUC) of more than 0.70 [186]. Response to IFN-γ in stimulated short or long term assays have long dominated the evaluation of the immunogenicity of novel *Mtb* antigens [186-188]. The sensitivity of short-term assays in the detection of recent *Mtb* infection increases with prolonged stimulation of whole blood [187].

2.0 Diagnostic utility of host markers in TB diagnosis

IFN-γ, a pro-inflammatory cytokine is secreted by the Th 1 cells in response to *Mtb* antigens and it is the most investigated in TB research [30, 112]. However, there have been lots of debates surrounding the use of IFN-γ as the best antigen-specific host marker for TB diagnostics. In a previous study, TNF-α was shown to perform better in discriminating active TB from latently infected cases [189] while other investigators have shown that multifunctional T cells (secreting TNF-α, IFN-γ , IL-2) possess a better diagnostic power compared to IFN-γ [190, 191]. Several other soluble antigen-stimulated and unstimulated cytokines have also shown diagnostic potential to measure immune sensitisation of *Mtb* antigen of which IFN-γ-inducible protein 10 (IP-10) is one.

IP-10 is a chemokine that is expressed by many innate cells such as monocytes, macrophages, neutrophils and it plays a major role in the course of infection acting as a chemoattractor to monocytes and lymphocytes at inflammatory foci [192]. Emerging experimental studies have provided evidence of the promising diagnostic ability of *Mtb* antigen-stimulated IP-10 [193-195]. It has been suggested as a replacement marker of latency in immunocompromised individuals and children [196] largely because of the advantages over IFN-γ including age independency and being less affected by immune suppression [197]. However, other studies have recorded conflicting results [194, 198, 199].

Besides IFN-γ and IP-10, other host markers that could be indicative of tissue damage in TB disease are being considered as biomarkers in the diagnosis of TB and these include vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and acute phase proteins. There is a high bacterial load during active TB in comparison to latent TB, which leads to tissue damage and resulting in a need for subsequent repair. Both EGF and VEGF are growth factors with major functions such as cell growth signalling and angiogenesis [200, 201], which could be important during TB disease. Higher levels of VEGF and EGF have been reported in acute TB compared to latent TB [202, 203]. The production of acute phase proteins, including serum amyloid A (SAA), C-reactive protein (CRP) and serum amyloid P (SAP) take place mostly in the liver as a result of inflammation and they may act in the protection of the immune system against TB. Previous studies have shown the potential usefulness of SAA and CRP in TB diagnosis [204, 205]. Enzyme-linked immunosorbent assay (ELISA) and multiplex assays such as Luminex are the two major methods for the detection of TB soluble biomarkers.

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CHAPTER 2

Evaluation of cytokine responses against novel *Mtb* antigens as diagnostic markers for TB disease

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My contribution: Planning of project

Generated data through laboratory experiments

Interpretation of results and data

Writing of manuscript

Summary

Objective: We investigated the accuracy of host markers detected in *Mtb* antigen-stimulated whole blood culture supernatant in the diagnosis of TB.

Methods: Prospectively, blood from 322 individuals with presumed TB disease from six African sites was stimulated with four different *Mtb* antigens (Rv0081, Rv1284, ESAT-6/CFP-10, and Rv2034) in a 24 hour whole blood stimulation assay (WBA). The concentrations of 42 host markers in the supernatants were measured using the Luminex multiplex platform. Diagnostic biosignatures were investigated through the use of multivariate analysis techniques.

Results: 17% of the participants were HIV infected, 106 had active TB disease and in 216 TB was excluded. Unstimulated concentrations of CRP, SAA, ferritin and IP-10 had better discriminating ability than markers from stimulated samples. Accuracy of marker combinations by general discriminant analysis (GDA) identified a six analyte model with 77% accuracy for TB cases and 84% for non TB cases, with a better performance in HIV uninfected patients.

Conclusions: A biosignature of 6 cytokines obtained after stimulation with four *Mtb* antigens has moderate potential as a diagnostic tool for pulmonary TB disease individuals and stimulated marker expression had no added value to unstimulated marker performance.

2.1 Introduction

The diagnosis of tuberculosis (TB) disease in resource-poor settings remains challenging. Several independent studies have reported on the limitations of current techniques in diagnosing TB [1-4]. There is a lack of simple field-friendly diagnostic tools and markers of immune activation and modulation of cytokine networks during intracellular infections might provide opportunities to develop appropriate tools [5-11].

The Interferon gamma (IFN-γ) release assays (IGRAs) with high specificity and accuracy in the diagnosis of *Mycobacterium tuberculosis* (*Mtb*) infection have been widely employed in the immune-based diagnosis of *Mtb* infection and have some advantages over the tuberculin skin test [12]. However, IGRAs are mainly useful in low incidence settings and for research advances in high burden areas as their major disadvantage is the inability to differentiate between active and latent TB [12, 13]. The discovery of secreted biomarkers similar to the gene expression signatures that were recently identified and that differentiate between these two infection states and which can be further developed into a rapid point of care test would be a major boost in TB diagnosis [14].

Recently, there has been an upsurge in the alternative use of novel *Mtb* antigens and host markers besides IFN-γ in *Mtb*-specific antigen stimulated whole blood culture assay for exploring the diagnosis of TB [15]. We have previously measured many of these host markers including tumour necrosis factor (TNF-α), interferon-inducible protein (IP-10), epidermal growth factor (EGF), macrophage inflammatory protein (MIP)-1β, vascular endothelial growth factor (VEGF) and soluble CD40 ligand (sCD40L) after stimulation with novel *Mtb* infection phase-dependent antigens (including TB vaccine candidate antigens, dormancy (DosR) regulon encoded antigens, TB reactivation antigens, TB resuscitation promoting factors (rpfs) and other stress response-associated antigens) in whole blood culture supernatants and some of these antigens look promising in TB disease diagnosis [16. 17]. However, in these studies, long term (7 day) whole blood assays were employed, which is not ideal for diagnostic purposes. In a follow up to these studies, we evaluated the

potential of some of these promising antigens to elicit a host response in a short term (overnight) whole blood assay compared to the long term (7 day) whole blood assay [18]. This study also evaluated the accuracy of some of these previously reported novel candidate antigens but in a larger study employing a short term (overnight), more field-friendly whole blood assay.

2.2 Materials and methods

2.2.1 Study participants

All the participants presumed of having pulmonary TB who participated in this study were recruited as part of the EDCTP funded African European Tuberculosis Consortium (AE-TBC) study that was conducted across six different African countries (www.ae-tbc.eu). Participants included in the present study were recruited from field sites serving Stellenbosch University, South Africa; Makerere University, Uganda; Medical Research Council Unit, The Gambia; and Karonga Prevention Study, Malawi. Participants presented with symptoms suggestive of pulmonary TB disease such as persistent cough for more than 2 weeks and one of the following: fever, recent loss of weight, night sweats, haemolysis, chest pain or loss of appetite. Participants were eligible for the study if they were 18 years or older, willing to give written informed consent, including for HIV testing using a rapid test (Abott, Germany) and sample storage. The exclusion criteria included severe anaemia (HB<10g/l), pregnancy, other known diseases such as diabetes mellitus, current anti-TB treatment, anti-TB treatment in the last 90 days, use of quinolone or aminoglycoside antibiotics in the past 60 days, and not been resident in the study area for more than 3 months. A case report form was completed for each participant before the collection of blood, saliva and other intended samples including urine and sputum as required for the main study. Culture of sputum samples was done using the MGIT method (BD Biosciences) and confirmation of isolated *Mtb* complex in all positive cultures was carried out by an *Mtb* complex specific PCR or standard biochemical methods, dependent on the facilities available at the study site [4]. Additionally, 3 ml of blood was collected from the participants for the performance of QFT-IT

assay, which was carried out according to the manufacturer's instruction as previously described [19]. The Human Ethics Research Committee of the University of Stellenbosch gave approval for the study (N10/08/274).

2.3 Reference standard for classification of study participants

Prior to the commencement of recruitment of study participants, harmonized case definitions were established and used for the classification of study participants (presumed TB cases) at all study sites. Participants were classified as having definite TB, probable TB, questionable TB disease status or non TB, using a combination of clinical, radiological, and laboratory findings [45]. The non TB cases were cases had a range of other diagnoses, including upper and lower respiratory tract infections (viral and bacterial infections, although attempts to identify organisms by bacterial or viral cultures were not made), and acute exacerbations of chronic obstructive pulmonary disease or asthma. No participant in the non TB group underwent TB treatment during the 6 month follow up of the study. In assessing the diagnostic accuracy of the markers investigated in the present study, all the definite and probable TB cases were classified as "TB", and then compared to the non TB cases, whereas questionables were excluded (Figure 2.1).

2.4 Whole blood culture assay (WBA)

At enrolment, 10ml of heparinised blood was collected from all participants and transported at ambient conditions within two hours of collection to the laboratory where the WBA was performed. The antigens that were used came from two sources namely: Leiden University Medical Center (LUMC), The Netherlands, and the Statens Serum Institut (SSI), Denmark. ESAT-6 and CFP-10 are two separate antigens, but were measured together as a fusion protein (ESAT-6/CFP-10) in this study. ESAT-6/CFP-10 and RV0081 were selected for the current study because of the promising accuracy shown by host markers elicited by these antigens in our previous studies [16-18] whereas Rv1284 and Rv2034 were selected because of the promise already shown by the antigens as TB diagnostic and vaccine

candidates in previous studies [20, 21]. Prior to their usage the four lyophilised antigens were reconstituted in sterile 1X PBS. The reconstituted antigens were then diluted in sterile 1x PBS, mixed with undiluted whole blood from each study participant at a final concentration of 10µg/ml, and incubated overnight (20-24hours) in 24-well tissue culture plates (Corning Corstar, Sigma) as previously described.[18] Sterile 1x PBS (Lonza, Cat #: 17-517Q) was used as the negative control.

2.5 Luminex multiplex immunoassay

This prospective study included 322 TB and non TB cases and was evaluated using a Luminex multiplex cytokine platform which is based on simultaneous detection and cytometric quantification of different cytokines in a sample. The concentrations of 42 host markers including interleukin (IL)-1β, IL-1Rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, fibroblast growth factor (FGF) basic, granulocyte colony stimulating factor (G-CSF), granulocyte monocyte colony stimulating factor (GM-CSF), IFN-γ, interferon inducible protein (IP)-10, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, platelet derived growth factor BB (PDGF-BB), MIP-1β, RANTES, TNF-α, vascular endothelial growth factor (VEGF), eotaxin-2, BCA-1, 6Ckine, SCF, TRAIL, ENA, ferritin, fibrinogen, procalcitonin, serum amyloid protein A (SAA), tissue plasminogen activator, serum amyloid protein P (SAP), CRP, haptoglobin and α-2 macroglobulin, were evaluated in WBA supernatants of all the study participants. This was done using Milliplex kits (Merck Millipore, St. Charles, Missouri, USA) and Bio-Plex kits (Bio Rad Laboratories, Hercules, CA, USA) on the Bio-Plex™ platform according to the manufacturer's instructions. Standard curves were generated from the serial dilutions that were made from the assay controls supplied and matched against the cytokine concentration for quantification. The concentrations of all the analytes in the quality control reagents were found to be within the ranges as expected. The Bio-Plex manager version 6.1 was used for bead acquisition and analysis of median fluorescence intensity.

2.6 Statistical analysis

Statistical differences in analyte levels were evaluated by the Mann Whitney U test for non-parametric data analysis. The diagnostic accuracies of individual antigen-specific or ustimulated responses for TB disease were ascertained by receiver operator characteristics (ROC) curve analysis. Cut-off levels for estimation of sensitivity and specificity were selected based on the Youden's Index. The predictive abilities of combinations of unstimulated and antigen-specific host markers for TB disease and non TB were investigated by performing best subsets general discriminant analysis (GDA). Data were randomly partitioned into a 70% training data set, which was used for model building and 30% test set, which was used to verify the accuracy of the different models. The leave-one-out cross validation approach was used to test the prediction accuracy of biosignatures after data was stratified according to HIV status, due to the relatively limited number of HIV infected individuals. Data were analyzed using GraphPad prism, version 5.00 for Windows (Graphpad Software, San Diego California, USA) and Statistica (Statsoft, Ohio, USA).

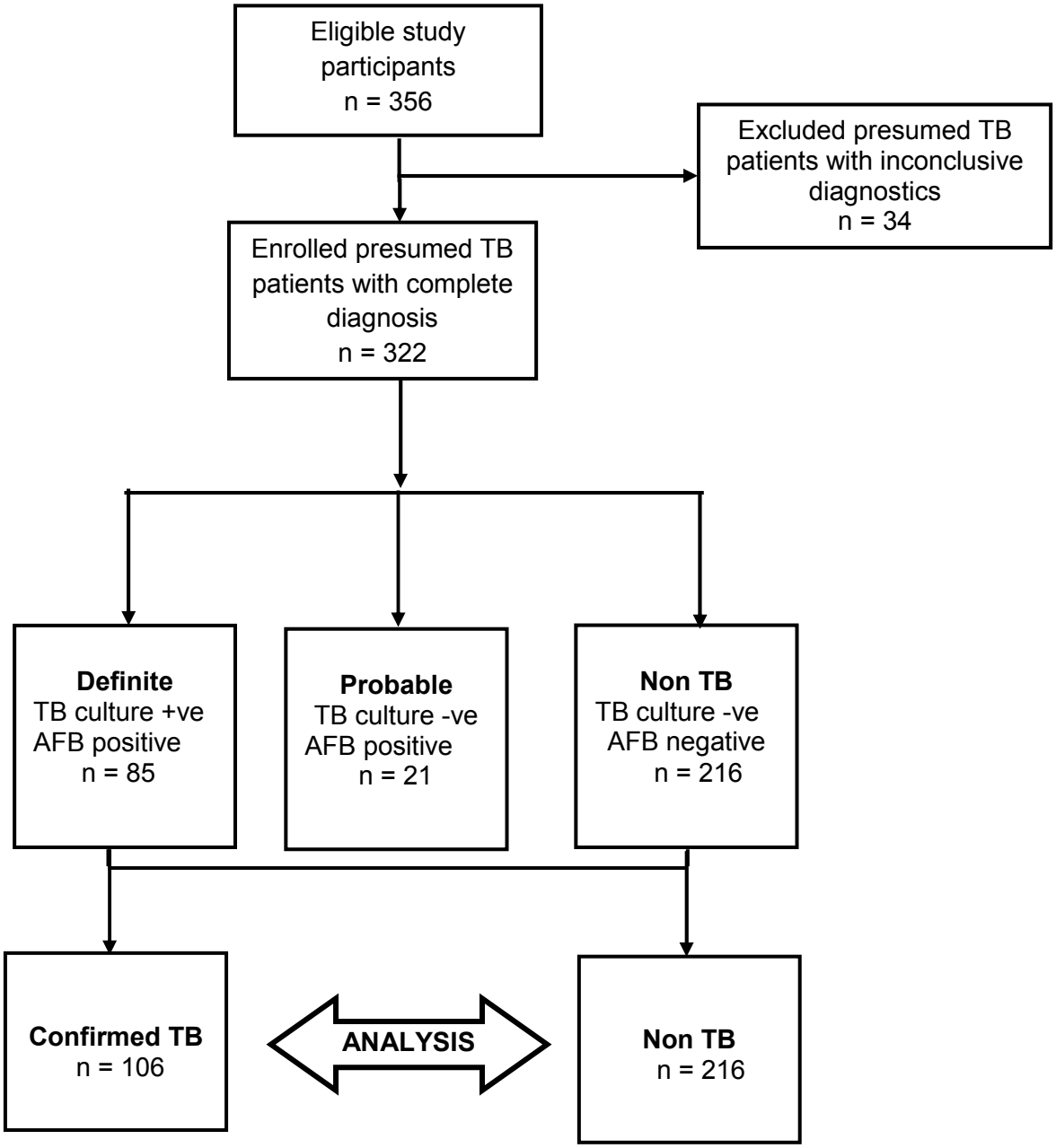

Figure 2.1 Standards for Reporting of Diagnostic Accuracy (STARD) flow diagram for recruitment of participants with presumed TB, enrolment and exclusion. Three hundred and fifty-six individuals presumed to have pulmonary TB were enrolled from different African field sites and WBA performed. Participants were later classified as definite TB cases, probable TB cases and non TB cases using a pre-established diagnostic algorithm. Thirty-four individuals with inconclusive diagnosis were excluded from the analysis. +ve = positive, -ve = negative.

2.7 Results

2.7.1 Study participants

A total of 322 participants were enrolled into this study, 106 (33%) of who were cultures positive TB cases (Figure 2.1). Of the 322 study participants, 168 (52%) were males and 24 (23%) of the 106 TB cases were HIV co-infected. The demographic and clinical information of the participants are shown in table 2.1.

Table 2.1 Clinical and demographic information of study participants.

	All	Pulmonary TB	non TB
Number of participants n, (%)	322	106(33)	216(67)
Male/Female ratio n, (%)	168(52)/154(48)	67(63)/39(37)	97(45)/119(55)
HIV status (pos/neg) n, (%)	54(17)/268(83)	24(23)/82(77)	30(14)/186(86)
QFT-IT result available n, (%)	211(66)	78(37)	133(63)
QFT-IT positive, n (%)	111(53)	56(72)	55(41)

Abbreviation: QFT-IT = Quantiferon TB Gold In Tube; n = number

2.8 Potential of host markers produced by unstimulated supernatants in discriminating between individuals with TB and non TB disease

When the analyte levels detected in the unstimulated control supernatants in TB patients were compared to the levels obtained in the non TB group (50% of this group were QFT-IT positive), the unstimulated levels of 13 out of the 42 host markers evaluated showed significant differences. The concentrations of these markers including CRP, Ferritin, IP-10, IL-6, IL-7, IL-9, IL-13, IFN-γ, VEGF, Haptoglobin, SAP, PCT and SAA were significantly higher in the TB group (Table 2.2). When the diagnostic potentials of these unstimulated host markers were evaluated by ROC curve analysis, four analytes including CRP, IP-10, Ferritin and SAA had an area under the ROC curve (AUC) of ≥ 0.85, ≥ 0.74, ≥ 0.79 and ≥ 0.77 respectively, in unstimulated samples. At their optimal unstimulated cut-off values, SAA

had a sensitivity and specificity of 81% and 72%, ferritin 70% for both sensitivity and specificity, IP-10 had 77% sensitivity and 71% specificity for ascertaining TB disease. The best performance characteristic was with unstimulated CRP with a sensitivity and specificity of 80% (Table 2.2, Figure 2.3). The high AUC recorded for some of these markers support their diagnostic potential.

2.9 Utility of host markers detected in overnight antigen-stimulated culture supernatants in the diagnosis of TB disease

The unstimulated control levels for the different host markers were subtracted from the antigen-stimulated responses for each study participant before the analysis of the data. In response to *Mtb*-specific antigenic stimulation by ESAT-6/CFP-10, median concentrations of IP-10, IFN-γ, IL-1Rα, tPA and TRAIL were significantly higher in the TB group ($p<0.05$) (Table 2.2, Figure 2.2). Following stimulation with Rv2034, IL-2, IL-17 and FGF basic levels were significantly higher in TB cases whereas ferritin was higher in non TB. Rv1284 elicited the production of significantly high levels of IL-2 in the non TB cases, whereas only tPA responses were significantly different between the TB and non TB cases after stimulation with Rv0081 (Table 2.2). When the diagnostic accuracy of individual antigen-specific host markers were investigated by ROC curve analysis, the AUCs for ESAT-6/CFP-10 stimulated IP-10 and IFN-γ were ≥ 0.64 respectively. Antigen-specific level of IP-10 had the best sensitivity of 60% and specificity of 65%. The AUC's of Rv1284-specific and Rv2034-specific markers performed poorly in general. Only Rv2034-specific level of IL-2 attained 0.60 (Table 2.2, Figure 2.3).

Table 2.2 Diagnostic potential of markers detected in overnight culture supernatant for TB disease

Marker/WBA	Median TB	Median non TB	P value	AUC	Optimal cut off	Sensitivity %	Specificity %
CRP_{Nil}	115296 (990-268405)	4903 (0-268405)	0.01	0.85	183	80	80
SAA_{Nil}	1577 000 (0-1577000)	4171 (0-1577000)	0.01	0.79	166	81	72
$Ferritin_{Nil}$	184000 (5668-495381)	84602 (27.74-495381)	0.01	0.74	172	70	70
$IP\text{-}10_{Nil}$	1984 (58.88-3889)	618 (0-3889)	0.01	0.77	170	77	71
$IL\text{-}6_{Nil}$	180.7 (0-1659)	81.7 (0-1659)	0.03	0.57	120	69	51
$IL\text{-}7_{Nil}$	9.44 (0-47.57)	6.04 (0-47.57)	0.01	0.60	114	55	63
$IL\text{-}9_{Nil}$	39.97 (0-128.2)	31.87 (0-128.2)	0.02	0.42	147	46	46
$IL\text{-}13_{Nil}$	6.65 (0-20.06)	2.99 (0-20.06)	0.01	0.39	50	45	42
$IFN\text{-}\gamma_{Nil}$	121.6 (0-396.1)	85.3 (0-396.1)	0.01	0.59	127	58	60
$VEGF_{Nil}$	157.5 (4.1-455.9)	107.5 (0-455.9	0.01	0.60	134	66	50
$Haptoglobin_{Nil}$	92400000 (33178-92400000)	2340000 (0-92400000)	0.01	0.61	158	64	59
SAP_{Nil}	112884 (15819-381489)	83860 (0-381489)	0.01	0.59	172	53	62
PCT_{Nil}	8785 (1567-15324)	8184 (1567-15324)	0.03	0.43	160	39	58
$IP\text{-}10_{Ag\text{-}Nil}$	3943 (0-20816)	1781 (0-20816)	0.01	0.64	174	60	65
$IFN\text{-}\gamma_{Ag\text{-}Nil}$	347.7 (0-1346)	145.5 (0-1346)	0.01	0.64	178	57	70
$IL\text{-}1ra_{Ag\text{-}Nil}$	633.7 (0-2845)	415.2 (0-2845)	0.01	0.59	157	59	56
$tPA_{Ag\text{-}Nil}$	301.6 (0-3908)	0.0 (0-3908)	0.01	0.58	167	55	61
$TRAIL_{Ag\text{-}Nil}$	12.64 (0-144.0)	0.0 (0-144.0)	0.01	0.59	135	57	67
$IL\text{-}2_{Rv1284\text{-}Nil}$	3.30 (0-60.71)	9.34 (0-60.71)	0.03	0.58	151	52	67
$tPA_{Rv0081\text{-}Nil}$	270.1 (0-3699)	0.0 (0-3699)	0.04	0.57	148	58	56
$IL\text{-}2_{Rv2034\text{-}Nil}$	0.0 (0-52.91)	5.46 (0-52.91)	0.01	0.60	96	69	52
$IL\text{-}17_{Rv2034\text{-}Nil}$	12.42 (0-213.4)	26.3 (0-213.4)	0.03	0.58	145	59	59
$FGF\ basic_{Rv2034\text{-}Nil}$	4.35 (0-129.7)	15.51 (0-129.7)	0.03	0.58	166	50	64
$Ferritin_{Rv2034\text{-}Nil}$	7624 (0-110159)	521.5 (0-110159)	0.02	0.58	184	52	66

Median levels of analytes (pg/ml) excluding SAA (ng/ml) and ranges (in parenthesis) showing accuracies in discriminating between active TB and non TB in overnight culture supernatants from all study participants. All analytes that showed significant differences ($p<0.05$) between the TB and non TB cases according to the Mann Whitney U test are shown. Optimal cut off values, sensitivity and specificity were selected based on Youden's index. The levels of the different antigens shown were corrected for background subtraction of the unstimulated levels. AUC= Area under the receiver operator characteristics curve, Nil= unstimulated marker levels and Ag= ESAT-6/CFP-10 stimulated marker levels.

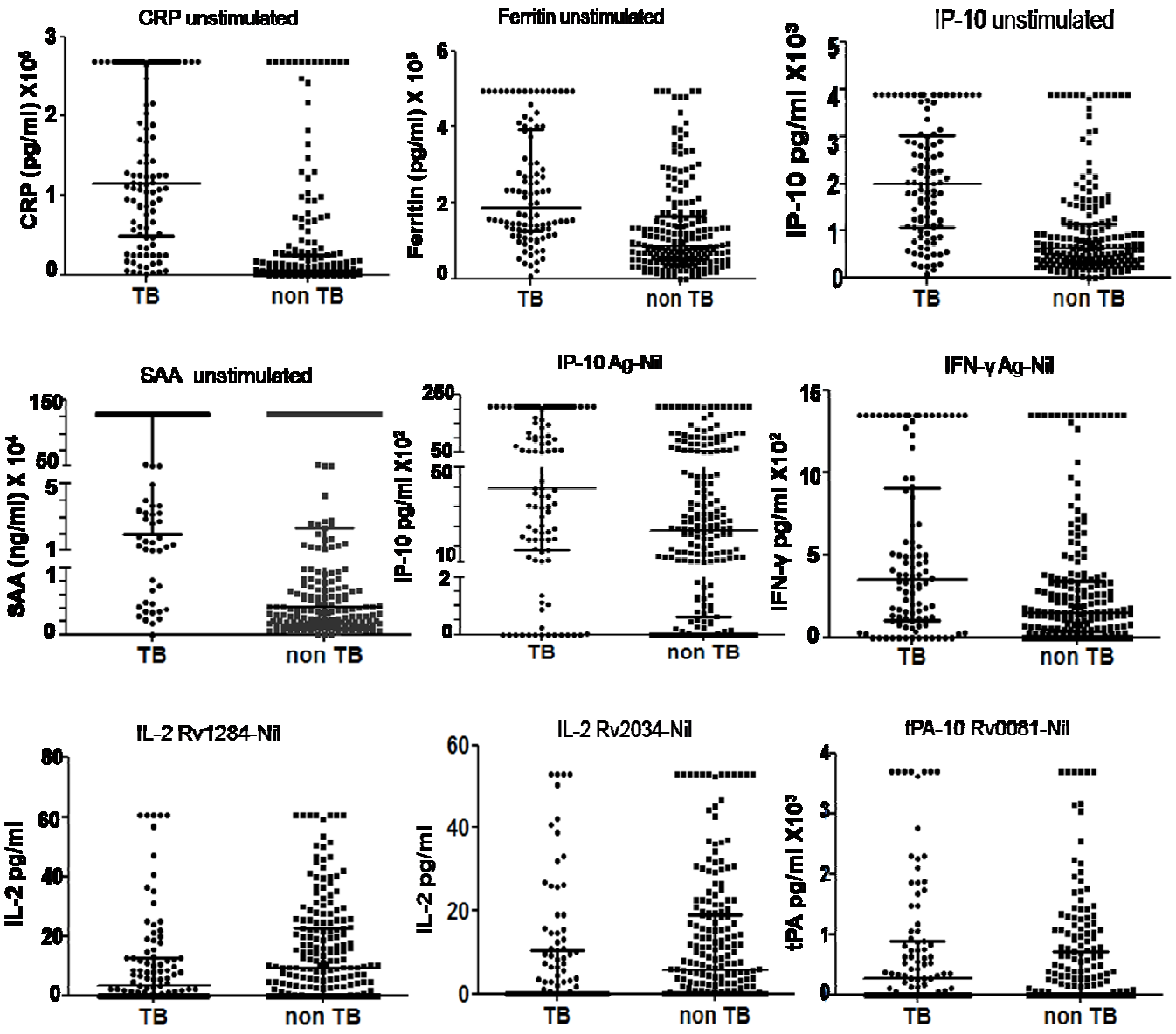

Figure 2.2 Scatter-dot plots of host markers detected in unstimulated and antigen-specific overnight WBA supernatants. Statistical differences in analyte levels were evaluated by the Mann Whitney U test for non-parametric data analysis. Representative plots show the levels of analytes in the overnight whole blood culture supernatants of participants with and without TB disease. Bars in the scatter dot plots represent the median plus interquatile range of the concentration of analyte. Nil= unstimulated marker levels, Ag=ESAT-6/CFP-10 stimulated marker levels CRP=C reactive protein, SAA=Serum amyloid A, IFN=Interferon gamma, IP-10=Interferon-inducible protein-10.

2.10 Ability of cytokine responses to discriminate between LTBI and uninfected controls

When the concentration of host markers detected in QFT-IT positive non TB cases (LTBI) were compared to the levels obtained in the QFT-IT negative non TB cases (uninfected controls), the unstimulated levels of IL-1β, IL-1Rα, IL-6, IL-10, IL-12, MIP-1α, TNF-α and were significantly higher in the uninfected controls. Only unstimulated levels of eotaxin were significantly higher in LTBI subjects. When the host markers elicited after stimulation with the different antigens were compared between the two groups, most of the discriminatory markers were found in ESAT-6/CFP-10 stimulated supernatants. ESAT-6/CFP-10 -specific levels of IL-1Rα, IL-2, IL-4, IL-5, IL-13, IL-15, FGF basic, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α and Eotaxin-2 were significantly higher in the LTBI group. Similarly, Rv2034-specific levels of IL-8, IL-15, MCP-1 and MIP-1α, and Rv1284-specific levels of G-CSF, MCP-1 and PDGF-BB were significantly higher in the LTBI. Stimulation with Rv0081 failed to elicit any response. When the diagnostic accuracies of the markers detected in the culture supernatants were evaluated by ROC curve analysis, only ESAT-6/CFP-10-specific levels of IP-10, IFN-γ, GM-CSF, IL-2 and IL-13 discriminated between the two groups with AUC ≥ 0.70. Out of these five markers, ESAT-6/CFP-10-specific level of IP-10 had the best sensitivity and specificity of 75% and 72% respectively. Although ESAT-6/CFP-10-specific IL-5 and eotaxin-2, Rv2034-specific MCP-1, and Rv1284-specific PDGF-BB all discriminated between the two groups with sensitivities >80%, the specificities of all these markers were poor, ranging between 38-50% (Table 2.3).

Table 2.3 Diagnostic potential of markers detected in overnight culture supernatants in discriminating LTBI from uninfected controls

Marker/WBA	Median LTBI	Median non TB	P value	AUC	Optimal cut off	Sensitivity %	Specificity %
IL-1β $_{Nil}$	2.37 (0-70.54)	6.64 (0-70.54)	0.01	0.65	3.9	64	67
IL-1Rα $_{Nil}$	159.9 (0-2005)	399.0 (0-2005)	0.01	0.62	140.3	75	49
IL-2 $_{Nil}$	6.16 (0-80.04)	13.22 (0-80.04)	0.01	0.62	6.2	70	52
IL-6 $_{Nil}$	19.17 (0-1653)	116.4 (0-1653)	0.01	0.68	51.8	66	74
IL-10 $_{Nil}$	14.20 (0-150.2)	27.01 (0-150.2)	0.01	0.62	26.0	52	78
MIP-1α $_{Nil}$	5.28 (0-89.85)	11.15 (0-89.85)	0.01	0.61	5.5	70	54
TNF-α $_{Nil}$	21.32 (0-198.5)	32.15 (0-198.5)	0.01	0.63	29.5	56	70
Eotaxin-2 $_{Nil}$	484.3 (28.42-1451)	328.1 (0-1451)	0.01	0.61	531.4	76	49
IP-10 $_{Ag-Nil}$	5781 (0-20286)	700 (0-20286)	0.01	0.77	2669	75	72
IFN-γ $_{Ag-Nil}$	376.3 (0-1189)	106.1 (0-1189)	0.01	0.76	196.7	71	74
IL-1Rα $_{Ag-Nil}$	790.0 (0-2614)	323.6 (0-2946)	0.01	0.66	809.7	78	49
GM-CSF $_{Ag-Nil}$	49.39 (0-168.0)	21.51 (0-168.0)	0.01	0.70	32.1	66	70
MCP-1 $_{Ag-Nil}$	4653000 (0-4653000)	27752 (0-4653000)	0.01	0.66	781534	75	56
MIP-1α $_{Ag-Nil}$	281.1 (0-1277)	83.47 (0-1277)	0.01	0.64	151.5	57	67
IL-2 $_{Ag-Nil}$	173.8 (0-357.2)	21.30 (0-357.2)	0.01	0.80	74.8	77	69
IL-4 $_{Ag-Nil}$	2.26 (0-6.52)	1.04 (0-6.82)	0.01	0.64	2.1	70	56
IL-5 $_{Ag-Nil}$	5.72 (0-14.4)	0 (0-14.4)	0.01	0.68	5.7	86	50
IL-13 $_{Ag-Nil}$	22.55 (0-56.09)	2.50 (0-56.09)	0.01	0.76	7.6	71	72
IL-15 $_{Ag-Nil}$	95.74 (0-269.8)	41.06 (0-269.8)	0.01	0.63	78.5	67	60
FGF basic $_{Ag-Nil}$	41.03 (0-137.3)	21.31 (0-137.3)	0.01	0.63	40.9	76	50
Eotaxin-2 $_{Ag-Nil}$	76.15 (0-757.6)	4.27 (0-757.6)	0.01	0.63	252.5	88	38
IL-8 $_{Rv2034-Nil}$	4075 (0-25911)	2467 (0-25911)	0.02	0.59	6111	71	46
IL-15 $_{Rv2034-Nil}$	46.36 (0-245.7)	27.42 (0-245.7)	0.03	0.59	16.5	46	72
MCP-1 $_{Rv2034-Nil}$	30946 (0-178009)	5909 (0-178009)	0.01	0.63	109303	85	40
MIP-1α $_{Rv2034-Nil}$	58.69 (0-391.8)	21.13 (0-391.8)	0.02	0.60	1.8	34	87
G-CSF $_{Rv1284-Nil}$	60.89 (0-343.5)	30.07 (0-343.5)	0.01	0.64	13.6	45	79
MCP-1 $_{Rv1284-Nil}$	91386 (0-608346)	17736 (0-608346)	0.01	0.63	46586	69	57
PDGF-BB $_{Rv1284-Nil}$	88.76 (0-740.5)	0 (0-740.5)	0.01	0.62	144.3	82	44

Median levels of analytes (pg/ml) and ranges (in parenthesis) showing accuracies in discriminating between LTBI and uninfected controls in overnight culture supernatants of all study participants. All analytes that showed significant differences ($p<0.05$) between LTBI and uninfected controls according to Mann Whitney U test are shown. Optimal cut off values, sensitivity and specificity were selected based on Youden's index. The levels of the different antigens shown were corrected for background subtraction of the unstimulated levels. AUC= Area under the receiver operator characteristics curve, Nil= unstimulated marker levels and Ag= ESAT-6/CFP-10 stimulated marker level.

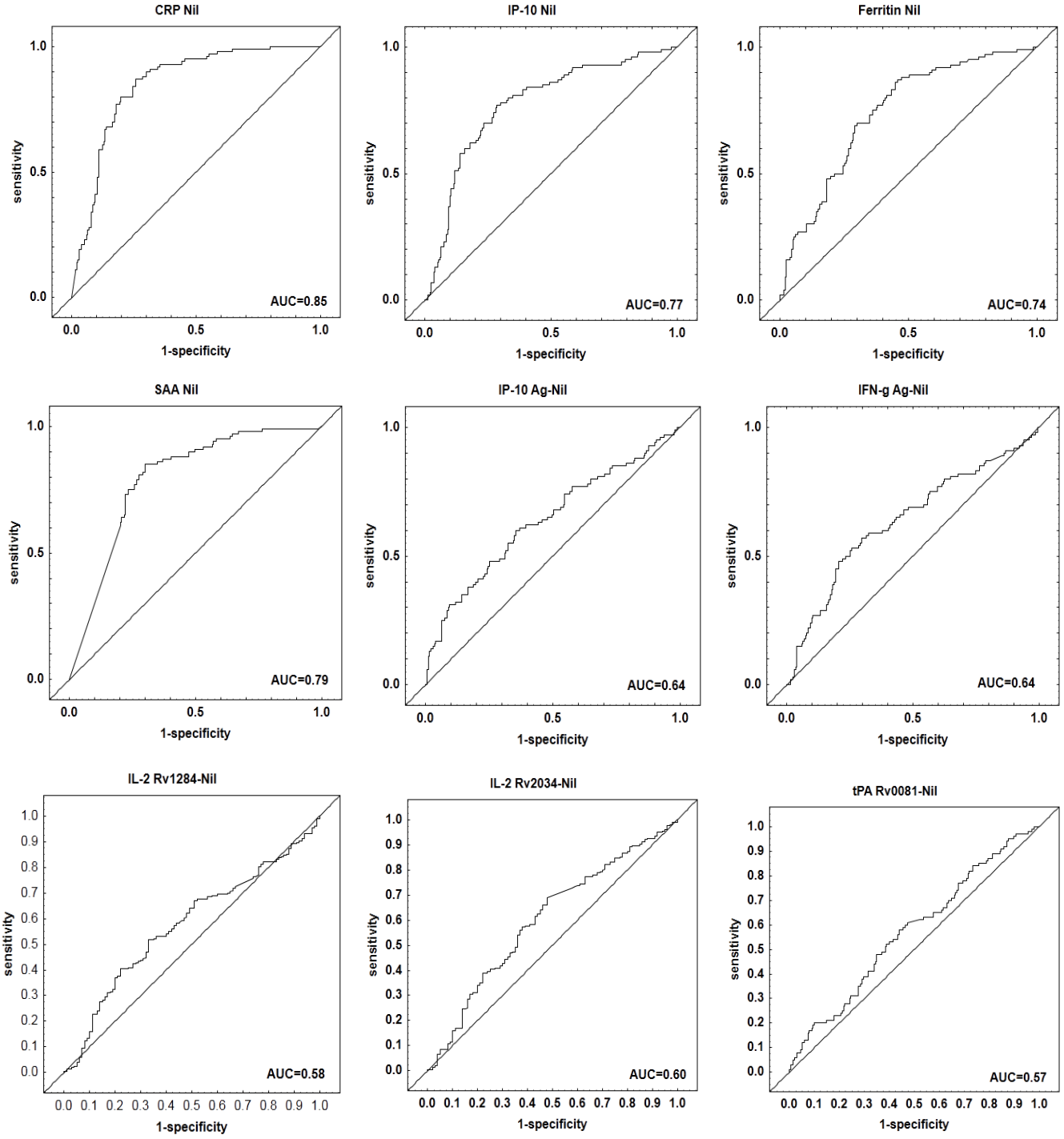

Figure 2.3 Receiver operating characteristic (ROC) curves of host markers detected in stimulated overnight WBA supernatants. Representative ROC curves showing the accuracy of the markers in discriminating between TB and non TB. All markers had area under the curve (AUC) ≥0.70 except IP-$10_{Ag\text{-}Nil}$, IFN-$\gamma_{Ag\text{-}Nil}$, Eotaxin$_{Rv1284\text{-}Nil}$, IL-$2_{Rv2334\text{-}Nil}$ and tPA$_{Rv0081\text{-}Nil}$. CRP=C reactive protein, SAA=Serum amyloid A, IFN-γ=Interferon gamma, IP-10=Interferon-inducible protein-10, IL= (Interleukin)-2, tPA= tissue plasminogen activator.

2.11 Abilities of combinations of analytes in the general discriminant analysis models in discriminating between TB and non TB.

To evaluate the predictive abilities of combinations of analytes for TB and no TB disease data obtained from all study participants were analysed by general discriminant analysis (GDA), regardless of the HIV infection status of the study participants. The unstimulated and antigen-specific responses of each host marker were treated as separate variables, in order to evaluate the contribution of both classes of markers in predictive models. We randomly partitioned all the data from the measurement of the different markers into a 70% training data set for model building, and 30% for a test set for the verification of the models. A combination of six markers IP-10 $_{Ag\text{-}Nil,}$ IFN-γ $_{Ag\text{-}Nil,}$ IP-10$_{Nil}$, Ferritin $_{Nil,}$ SAA $_{Nil,}$ and CRP $_{Nil}$ accurately predicted 77% TB cases and 84% of the non TB cases in the training set, regardless of HIV infection status. In the test set, the six-marker biosignature accurately predicted 83% of the TB cases and 78% of the non TB cases (Table 2.4).

To investigate the influence of HIV infection on the accuracy of the biosignatures, data was stratified according to HIV status, and the GDA procedure repeated. In the HIV uninfected group the six-marker biosignature (IP-10 $_{Ag\text{-}Nil,}$ IFN-γ $_{Ag\text{-}Nil,}$ IP-10$_{Nil}$, Ferritin $_{Nil,}$ SAA $_{Nil,}$ and CRP $_{Nil}$) diagnosed TB disease with a sensitivity of 83% and specificity of 90% in the training data set, and a sensitivity of 88% and specificity of 82% in the test dataset. However, the combination of these analytes performed less well in the HIV infected patients as only 64% of the TB cases and 80% of the non TB cases were correctly classified in the resubstitution classification matrix. After leave-one-out cross validation, the biomarker combination only resulted in the correct prediction of 52% of the TB cases and 76% non TB (Table 4). The frequency of the different analytes in the top 20 models for discriminating between TB disease and non TB in all study participants is shown in figure 2.4.

Table 2.4 Utility of combination of analytes in overnight culture supernatant in the diagnosis of TB

Host marker model	Training classification set			Test classification set		
6 analyte model						
All cases	**Non TB %**	**TB %**	**Total %**	**Non TB %**	**TB %**	**Total %**
IP-10 $_{Ag-Nil,}$ IFN-γ $_{Ag-Nil,}$ IP-10$_{Nil}$ Ferritin $_{Nil,}$ SAA $_{Nil,}$ CRP $_{Nil}$	84	77	82	78	83	80
				PPV:0.65 (95% CI; 0.48-0.79)		
				NPV:0.90 (95% CI; 0.78-0.96)		
6 analyte model						
HIV uninfected	**Non TB %**	**TB %**	**Total %**	**Non TB %**	**TB %**	**Total %**
IP-10 $_{Ag-Nil,}$ IFN-γ $_{Ag-Nil,}$ IP-10$_{Nil}$ Ferritin $_{Nil,}$ SAA $_{Nil,}$ CRP $_{Nil}$	90	83	88	82	88	84
				PPV:0.72 (95% CI; 0.49-0.88)		
				NPV:0.93 (95% CI; 0.76-0.98)		
6 analyte model	**Resubstitution classification matrix**			**Leave-one-out cross validation**		
HIV infected						
	Non TB %	**TB %**	**Total %**	**Non TB %**	**TB %**	
IP-10 $_{Ag-Nil,}$ IFN-γ $_{Ag-Nil,}$ IP-10$_{Nil}$ Ferritin $_{Nil,}$ SAA $_{Nil,}$ CRP $_{Nil}$	80	64	73	76	52	
				PPV:0.64 (95% CI; 0.35-0.86)		
				NPV:0.66 (95% CI; 0.44-0.83)		

Six analyte models generated by general discriminant analysis. Nil=unstimulated, Ag=ESAT-6/CFP-10

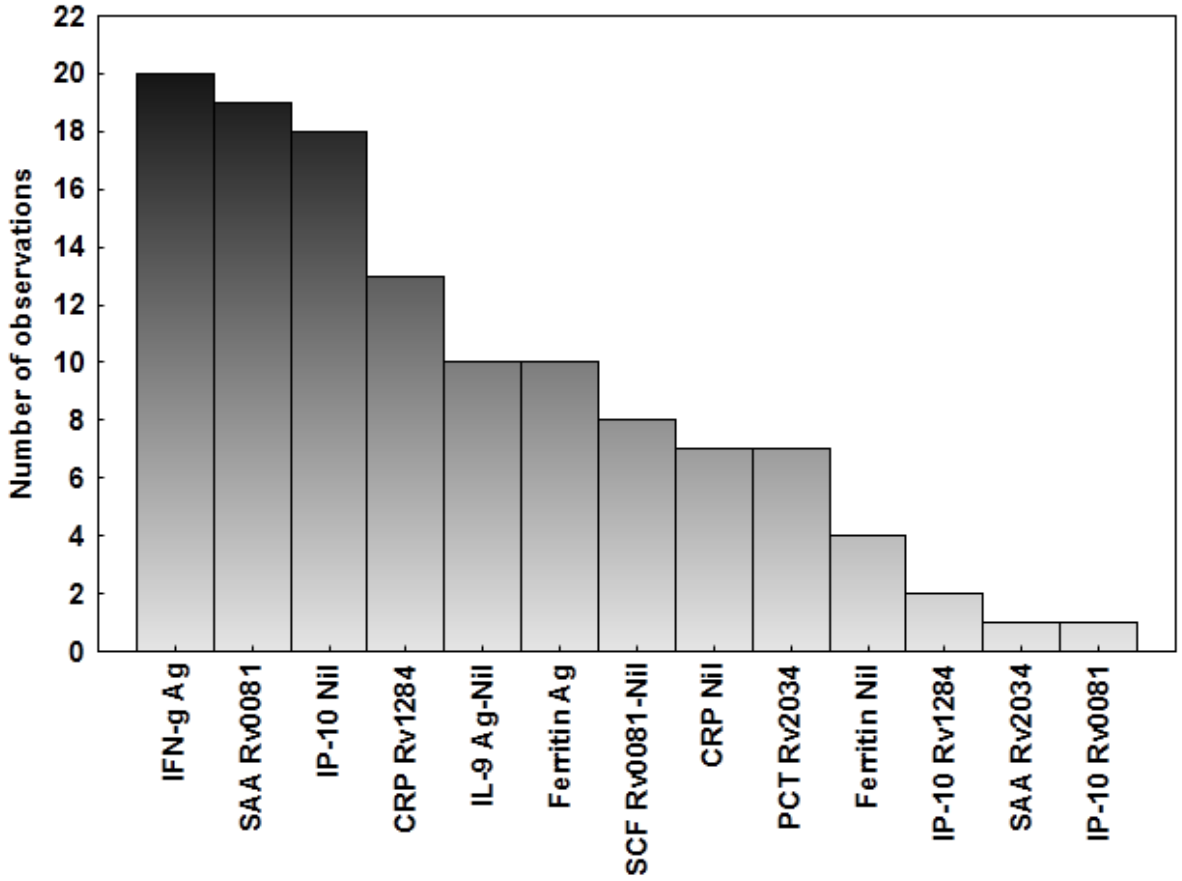

Figure 2.4 Frequency of analytes in the top 20 most accurate GDA predictive models for the classification of study participants as TB disease or non TB. Using best subsets method of variable selection, unstimulated and antigen-specific host markers were fitted into general discriminant analysis prediction models. The columns represent the number of times each analyte occurred in the top 20 general discriminatory models. Nil= unstimulated marker levels, Ag=ESAT-6/CFP-10 stimulated marker levels.

2.12 Discussion

The development of a new, relatively rapid, and accurate test, that does not rely on sputum, which can be difficult to obtain in some patient groups, and which does not reflect the site of infection in extrapulmonary TB, would be a major advance in the TB diagnostic field. The measurement of a small number of analytes that differentiates active TB from LTBI in the blood in a short-term overnight assay, might fulfil this need [19]. Test results would be available within 48 hours, rather than after several weeks as is the case with sputum culture. In this study we investigated the potential accuracy of host markers detected in supernatants, after stimulation of whole blood with *Mtb* infection phase-dependent antigens, in an overnight culture assay. We have shown that multiple biomarkers detected in the antigen-stimulated and unstimulated supernatants can contribute to a diagnostic signature with the ability to discriminate between active TB and non TB. A biosignature of six analytes showed promising results especially in HIV uninfected individuals. We previously reported on the potential of host markers produced after stimulation of blood cells with novel *Mtb* infection phase-dependent antigens, including Rv0081, Rv0867c, Rv2389c, Rv1009 and Rv2032 in the diagnosis of TB disease [16, 17]. However, the 7-day WBA used in that work would not be optimal and useful as a TB diagnostic tool, especially in resource limited settings. Follow-up work evaluated a down selected number of these antigens in the 7-day and overnight cultured assays [18] and the present study is a validation of that pilot data.

We enrolled 322 participants with presumed TB and confirmed active disease in 106, whereas active TB was excluded in 216. Comparison of the levels of markers in these two groups, irrespective of their HIV status, and QFT-IT results was performed. Although a sub-group comparison of these markers in the different *Mtb* infection groups was not our primary objective as we were looking for diagnostic tests suitable for the accurate diagnosis of active TB in high endemic settings, with a high prevalence of LTBI, we evaluated the utility of multiple analyte signatures in the diagnosis of TB disease in different HIV and QFT-IT sub

groups. We identified several markers that discriminated between latently infected individuals and uninfected groups.

Antigen-specific host markers measured in the overnight WBA in this study did not show much diagnostic potential as the top single markers observed; IFN-γ and IP-10, only achieved an AUC of 64% in discriminating between TB disease and non TB. However, unstimulated levels of SAA, ferritin, CRP and IP-10 were the most promising single markers obtained, reaching AUC ≥70%. As observed in our previous studies [16, 17] the predictive abilities of these markers improved when they were used in combinations. Indeed, in this study, a six analyte-model showed an improved diagnostic potential. The results of the acute phase proteins: CRP and SAA, are consistent with the results from the pilot study where these markers also featured strongly and were included in the top four-analyte multi marker models [18]. In contrast to our previous observations none of VEGF, TGF-α or EGF, which was prominent in the best discriminatory marker model in the 7-day assay, was included into the present models. The larger sample size in the present study and the use of the short term assay are probably responsible for the discrepancy.

Rv0081 is a DosR regulon encoded antigen and several studies have shown that the DosR regulon of *Mtb* is associated with latency, nutrient starvation, hypoxia and low nitric oxide or pH [22-27]. Despite the diagnostic potential of this antigen, it did not discriminate between TB and non TB with high sensitivity and specificity and failed to differentiate LTBI from uninfected controls. The evaluation of this antigen in combination with other antigens in previous study did not improve its accuracy [16]. Rv0081 elicited tPA responses that were significantly higher in TB cases, in comparison to the non TB group. In contrast to our previous studies, which were conducted in household contacts (HHC) of TB cases, the present study did not recruit contacts as the control group. DosR regulon antigens might be recognised more frequently by people with recent exposure and infection [23, 28-30]. IFN-γ elicited by ESAT-6/CFP-10 is a commonly used marker for TB infection and although it does not discriminate between active TB and LTBI on its own, it was included most frequently in the GDA models. This classical antigen also elicited SAA, CRP and ferritin responses.

These acute phase proteins are mainly produced in the liver as a result of inflammation and it is not a surprise that these markers, particularly the unstimulated levels, were included in the top analyte models. SAA and CRP are also produced by macrophages and peripheral blood mononuclear cells (PBMCs), respectively [31, 32], are being extensively employed as biomarkers in many disease conditions including pulmonary infections [33, 34]. The potential usefulness of SAA and CRP in serum in the diagnosis of TB has been shown in previous studies [46] although no current TB diagnostic tests use these markers [35, 36]. IP-10 is a chemokine secreted by monocytes with direct interaction with antigen specific T-cells and has been widely researched as an alternative TB immunodiagnostic biomarker [37, 38]. The levels of stimulated IP-10 was higher in TB disease in our study compared to non TB and this is in agreement with other studies where IP-10 differentiated better between active TB cases and unexposed individuals than IFN-γ release assays (IGRA) [39, 40]. Several studies have shown that the combination of both IFN-γ and IP-10 could significantly enhance diagnostic performance [41-44].

The main limitation of our study was the evaluation of fewer antigens than in our previous studies as the down selection of the number of antigens from our pilot work demonstrates the risk for false discovery when a large number of antigens are evaluated in a relatively small number of samples. Antigens can be falsely included or excluded due to insufficient power of the pilot studies. Alternatively, however, the use of shorter term assay here as opposed to the use of long term assays in our previous study might have biased towards responses to a subset of the originally identified antigens only, possibly due to differences in response kinetics. Our results furthermore highlight the fact that multi-marker biosignatures hold promise above the use of single markers. Finally, the results suggest that *ex vivo* samples like plasma and serum may hold promise for the discovery of such biosignatures, as no added accuracy was obtained through stimulation with *Mtb* antigens. We conclude that large future studies should focus on *ex vivo* markers.

2.13 Conclusion

We identified a biosignature of six unstimulated and mycobacteria-specific host markers in antigen-stimulated overnight WBAs that showed potential in the diagnosis of TB disease with an accurate prediction of 77% TB cases and 84% non TB cases. The sensitivity and specificity of this 6-analyte model was better in HIV uninfected patients but as a large percentage of African TB patients have HIV co-infection, this approach has limited future potential. These markers could, however, be adjunctive markers in the diagnosis of TB disease where sputum is difficult to obtain or where extrapulmonary TB is presumed. Future studies in children and extrapulmonary TB patients should evaluate additional novel *Mtb* antigens, ex-vivo unstimulated markers such as in serum and plasma, and host markers possibly using non-biased approaches such as proteomics to improve sensitivity before field-friendly versions of the stimulation assays are developed.

2.14 References

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CHAPTER 3

Evaluation of host markers for tracking early treatment response in newly diagnosed pulmonary TB patients

This chapter will be submitted to a journal for publication

My contribution: Planning of project

Generated data through laboratory experiments

Interpretation of results and data

Writing of manuscript

Abstract

Background: Simple and rapid biomarkers that indicate drug efficacy early would reduce the cost of clinical trials and accelerate TB drug development. We investigated the profiles of several inflammatory markers in TB patients undergoing different treatments for 14 days to identify biomarkers that correlate sputum culture based measures of treatment response.

Method: One hundred newly diagnosed pulmonary TB patients who were taking part in a study of 14-day treatment effects were randomized into six arms receiving experimental treatments while the seventh arm received Rifafour e-275 as positive control. Sputum and serum samples were collected simultaneously before and after completion of treatment (D0 and D14). The sputum bacterial load was determined by time to culture positivity and colony forming units while a total of 33 host markers were measured in the serum using the Luminex platform.

Result: There were significant differences in the degree of change in sputum TTP and CFU between the different treatment arms, ranging from no change (clofazimine) to a significant decline in bacterial numbers in the positive control group (Rifafour e-275). Levels of CRP, IL-6, VEGF, sIL-2Rα, Ferritin, and sTNFRII changed significantly from D0 to D14 in several arms, but no single-marker changes consistently correlated with the decline in bacterial measures in sputum. A four-marker model including IL-2, MMP-9, sCD137 and Granzyme A only accounted for 20% of the variation observed in TTP and the combination of A2M, sIL-2Ra, sIL-6R and sTNFRII predicted only 20% of the variation in CFU. CRP showed the greatest change over the study period.

Conclusion: The findings from our study suggest that the selected host markers, as single markers or as multi-marker combinations, are not suitable to monitor early, day 14, treatment activity. The potential role of host markers to predict treatment outcomes at later stages during or after treatment have to be evaluated in future studies.

3.1 Introduction

Sputum culture conversion from positive at baseline to negative at M2 is considered to be the best available biomarker for the evaluation of TB treatment response with some predictive ability for non-relapse cure but is mainly used in clinical trials, results become available late, and predictive ability at individual level is poor [1, 2]. Host biomarkers that correlate well with a patient's TB treatment response will not only help clinical management but also contribute to the discovery of improved drug regimens and accelerate clinical trials [3]. Such markers should preferably be detectable before the initiation of treatment, ideally representing a measure of disease severity, and following initiation of treatment indicating treatment response and cure.

The efficacy of new drugs and drug combinations in a phase II clinical trial is evaluated by culture conversion at week 8 of treatment on solid (CFU) or liquid (TTP) media. However, the use of a dichotomous approach in determining phase II clinical trials endpoints is not efficient simply because large sample sizes are needed. Furthermore, the culture-dependent techniques, if used beyond the standard EBA observation periods, would require patients to be continuously producing sputum throughout the TB treatment period, which is often a challenge after a few weeks of therapy [4, 5]. The measurement of changes in CFU and TTP provide a continuous variable but require advanced microbiological laboratory expertise and are hampered by bacterial contamination of cultures. Early bactericidal activity (EBA) trials measure the decrease in bacterial load in sputum from patients within the first 14 days of treatment and are the first step in the clinical evaluation of new antituberculosis agents or combinations [6]. The laboratory method for the assessment of treatment activity is the decline of colony forming unit (CFU) counts per volume of sputum or the increase in time to culture positivity in liquid culture

The present study investigated the profiles of inflammatory biomarkers in active TB patients undergoing an EBA trial of seven treatment regimens to identify biomarkers that correlate with early treatment response measured with CFU and TTP.

3.2 Materials and methods

3.2.1 Study participants

The participants who took part in this study were recruited as part of a 14-day phase II EBA clinical trial in newly diagnosed, smear positive (≥1 +; WHO/IUALTD scale [7]) pulmonary TB patients. Participants with any of the following conditions were excluded from the study: HIV positive patients (CD4 count <250 cells/µl) and those on antiretroviral (ARV) treatment, pregnancy, participants with a previous history of TB and those with an evidence of clinically significant diseases other than TB. From October 2012 until May 2013, 100 participants who provided written and informed consent were hospitalized in one of two centres in Cape Town, South Africa (Task Applied Research Centre, Bellville; University of Cape Town Lung Institute, Mowbray). These eligible participants, aged between 18 and 65 years, were randomized to seven parallel groups. The first six equally sized treatment arms received different dosages of a single and/or combinations of different anti-tuberculosis agents and a seventh arm received Rifafour e-275 (standard first line TB treatment as recommended by South African TB guidelines) as control (Figure 1). All the isolated strains were susceptible to the treatment given. An overnight sputum sample and blood for serum was collected simultaneously at the start and end of the EBA study. Although multiple sputum collections were done during the 14-day period, serum was only collected at baseline and at day 14 and our analysis is restricted to baseline and day 14 sputum results. The sputum sample was refrigerated overnight immediately after collection and transported to the central study laboratory (Centre of Clinical Tuberculosis Research, Department of Biomedical Sciences, Stellenbosch University) for processing. Approval for the main study and this sub-study was granted by the Medicines Control Council (MCC) and the health ethics research committee of Stellenbosch University.

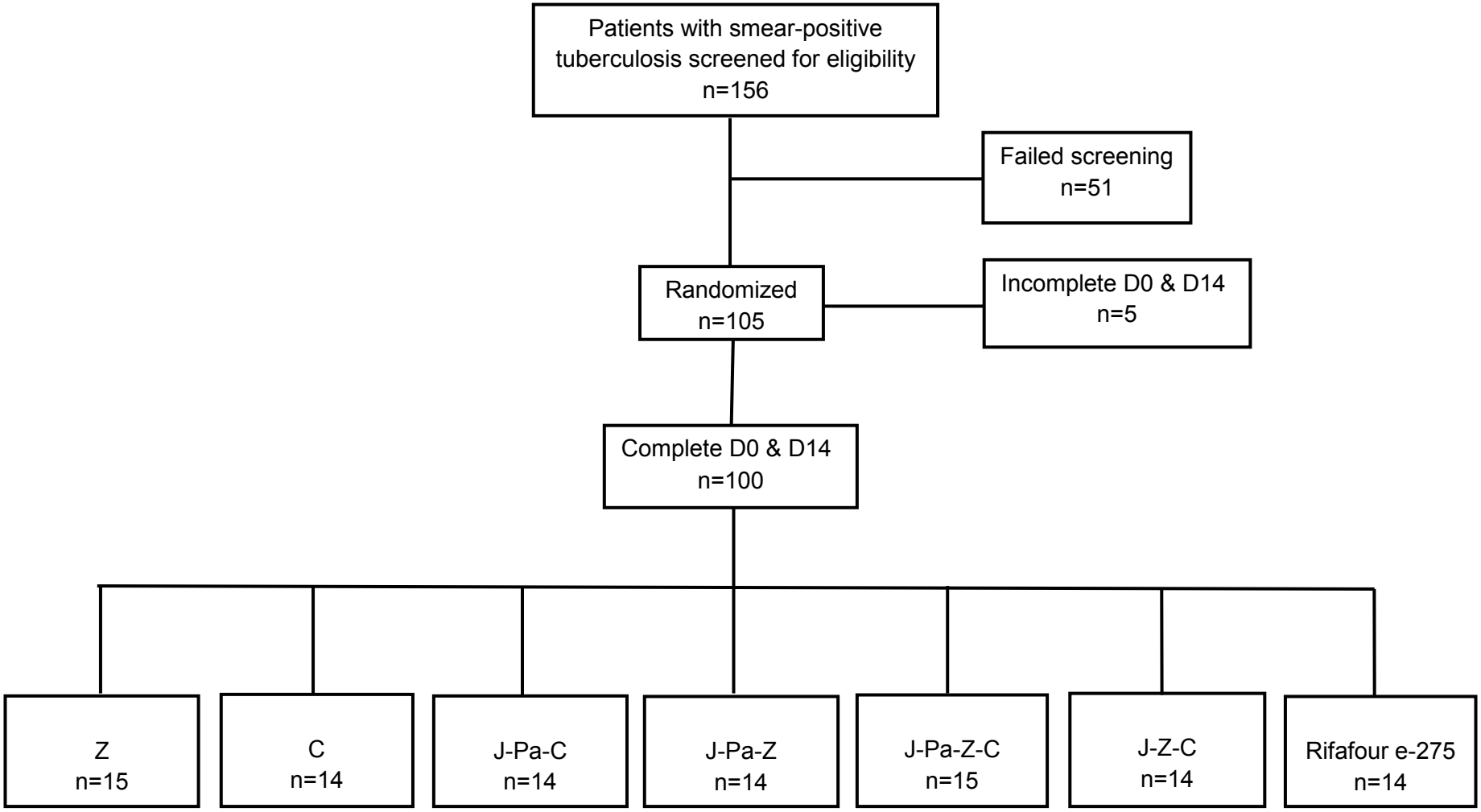

Figure 3.1 Flowchart of patient screening for eligibility and randomization during the 14 day EBA clinical trial. One hundred and fifty-six newly diagnosed pulmonary TB patients were screened for eligibility and fifty-one participants failed screening and were excluded from the study. A total of hundred and five participants were randomized into seven different treatment arms receiving a daily different dosage of a single drug or drug regimen of anti-TB treatment orally for 2 weeks. One hundred participants who had complete baseline and D14 samples were included for analysis. J = TMC207, Pa = PA-824, Z = Pyrazinamide, C= Clofazimine.

3.2.2 Determination of TTP

The method as described by Xavier *et al*; 2013 [8] was used. Briefly, homogenized sputum was digested with 0.1% dithiothreitol (Sputasol; Oxoid, Cambridge, United Kingdom), decontaminated with 2% NaOH (BBL Mycoprep; Becton, Dickinson), this mixture neutralized with sterile phosphate-buffered saline (PBS, pH 6.8; Becton, Dickinson) and centrifuged at 3,000 X g, 4°C for 15 min. The sputum sediment was resuspended in PBS to a final volume of 2 ml. Two BACTEC MGIT 960 media tubes were prepared by adding 0.8 ml of antibiotic mixture containing PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) mixed with OADC (oleic acid-albumin-dextrose-catalase Becton, Dickinson) supplement prior to addition of 0.5 ml resuspended sputum. MGIT tubes were incubated in a BACTEC MGIT 960 instrument at 37°C until either flagged positive or for a maximum period of 42 days, when they were read as negative. Positive tubes were tested for contamination by culturing on blood agar for 48 h. Confirmation of acid fast bacilli (AFB) in positive cultures was done by ZN staining and microscopy. For our analysis, TTPs from acid fast bacilli (AFB) positive and non-contaminated cultures only were used. The data were log transformed (log TTP) prior to analysis.

3.2.3 Log CFU determination

The method described by Xavier *et al*; 2013 [8] was used. Briefly, homogenized sputum was digested with 0.1% dithiothreitol (Sputasol; Oxoid, Cambridge, United Kingdom). Two series of ten-fold serial dilutions (1×10^{-1} , 1×10^{-2} , 1×10^{-3} , 1×10^{-4} , 1×10^{-5}) prepared in sterile saline (0.85%) containing 0.01% Tween were made by inoculating a volume of 1 ml of the digested sputum. For each dilution, two selective OADC supplemented Middlebrook 7H11 agar plates were prepared with the addition of selectatab (Mast; Bootle, Meyerside, UK) containing polymixin B sulphate (200 units/ml), amphotericin B (10 mg/l),ticarcillin (100 mg/l) and trimethoprim (10 mg/l). The plates were incubated for 3–4 weeks at 37°C and CFU were counted at a dilution with a visible count of 20-200 colonies. The average of the two counts was

calculated, corrected for dilution factor to give a CFU/ml sputum result and then log transformed to log CFU.

3.2.4 Serum processing

Blood was collected into a 4 ml red top vacutainer tube (BD, cat. # 369032) and stored overnight in the fridge at 4°C before being transported to the Stellenbosch University Immunology Research Group in a blood transport container at room temperature, on the following day. Upon arrival in the laboratory, the blood tubes were centrifuged at 2,500rpm for 10 minutes and serum harvested and aliquoted (250 µl amounts, four microtubes/per sample) and stored at -80°C until the day of testing using Luminex platform (described below).

3.2.5 Luminex multiplex immunoassay

Using kits purchased from commercial vendors, a total of 33 host markers were evaluated in serum samples from all study participants at the two different time points, baseline (D0) and end of EBA, (D14). The analyte panels that were evaluated included an 11-plex human cytokine customized Milliplex kit (Merck Millipore, St. Charles , Missouri, USA) containing IFN-γ, Interleukin-2 (IL-2), IL-1β, IL-5, IL-6, IL-8, IL-10, IL-13, macrophage derived chemokine (MDC), tumour necrosis factor-α (TNFα), vascular endothelial growth factor (VEGF), a 5-plex human CD8 T cell kit containing soluble CD137(sCD137), Granzyme A, Granzyme B, soluble Fas (sFas), soluble Fas Ligand (sFasL), a 2-plex human Matrix metalloproteinase-2 (MMP-2 and MMP-9), a 6-plex human soluble receptor: gp130, Interleukin-2 receptoralpha (IL-2RA), IL-4R, IL-6R, Tumour necrosis factor-recptor2 (TNF-R2), Vascular endothelial growth factor-receptor3 (VEGF-R3) (all from Merck Millipore, Billerica, MA, USA), a Bio-plex 5-plex human acute phase proteins (Bio-Rad Laboratories, Hercules, USA) Procalcitonin (PCT), Serum amyloid-A (SAA), Tissue plasminogen activator (TPA), Ferritin, Fibrinogen and a Bio-plex human acute phase 4-plex proteins alpha-2-macroglobulin (A2M), C reactive protein (CRP), Haptoglobulin and serum amyloid-P (SAP). Prior to assaying, samples for the 5-plex and 4-plex Bio Plex kits were diluted

1:100 and 1: 10 000 respectively as recommended by the manufacturer, whereas samples for MMP-2 were diluted 1: 500 after previous optimization experiments. All the other markers were evaluated undiluted. All experiments were run on the Bio Plex platform (Bio Plex 200 and Bio Plex Magpix; Bio Rad Laboratories). The values obtained for all host markers were automatically corrected for the dilution by the software used for bead acquisition and analysis of median fluorescence intensity, the Bio Plex Manager Software, version 6.1.

3.3 Statistical analysis

For TTP and CFU responses, the mean of individual patients at baseline and after treatment at D14 were calculated. The difference of the mean at baseline and D14 was then obtained for treatment activity. Analysis for the change in the expression of markers during TB treatment, from D0 to D14, and differences between treatment arms was performed using mixed model repeated measures analysis of variance (ANOVA) with Fisher's Least Significant Difference (LSD) post hoc testing. P-values were considered significant if ≤0.05. Spearman correlations were used to investigate correlations between changes in host marker, CFU and TTP changes (D0 to D14). Multiple Linear regression was used to investigate the predictability of TTP and CFU using host markers. Due to the presence of outliers in the host marker expressions, these variables were winsorized by changing outlier values to the mean ± three times the standard deviation. For this purpose means and standard deviations were calculated using robust methods. The descriptive analysis of the 33 host markers is shown in Table 3.1. STATISTICA software version 12 (Statsoft, USA) was used in the analysis of the data.

3.4 Results

3.4.1 Demographic characteristics

Of the 100 participants enrolled into the present study, 61 (61%) were males. The mean age of study participants was 32.8±11.27 and 10 (10%) were co-infected with HIV.

3.5 Discrimination between EBA treatment arms by log CFU and EBA TTP

There was a significant increase ($p<0.01$) in TTP in all the treatment arms with the exception of the Clofazimine arm. The differences in the J-Pa-Z, J-Pa-Z-C and Rifafour e-275 treatment arms were more pronounced than the other treatment arms (Figure 3.1). Similarly, there was a significant decrease ($p<0.01$) in the bacterial load (log CFU) at D14 in all treatment arms, with the exception of the Clofazimine treatment arm, with the most pronounced differences observed for the J-Pa-Z- and Rifafour e-275 treatment arms (Figure 3.1).

Table 3.1. Descriptive analysis of the 33 host markers that were investigated

Host markers	Valid N	Mean	Minimum	Maximum	Standard deviation
A2M	200	$2.30x10^{7}$	0	$3.56x10^{7}$	$1.20x10^{7}$
Haptoglobin	200	$1.67x10^{9}$	0	$2.46\ x10^{9}$	$1.15\ x10^{9}$
CRP	200	$6.86x10^{5}$	0	$3.19\ x10^{6}$	$7.07x10^{5}$
SAP	200	$1.62x10^{6}$	0	$4.69x10^{6}$	$1.17x10^{6}$
PCT	200	$6.76x10^{3}$	0	$5.38x10^{4}$	$7.0x10^{3}$
Ferritin	200	$1.25x10^{5}$	0	$7.22x10^{5}$	$1.13x10^{5}$
tPA	200	$6.63x10^{3}$	0	$2.93x10^{4}$	$6.97x10^{3}$
Fibrinogen	200	$1.0x10^{4}$	0	$7.65x10^{4}$	$1.56x10^{4}$
SAA	200	$2.77x10^{3}$	0	$1.16x10^{4}$	$2.58x10^{3}$
IL-13	200	$8.99x10^{1}$	0	$3.33x10^{3}$	$3.67x10^{2}$
IL-5	199	$8.63x10^{1}$	0	$5.58x10^{3}$	$5.57x10^{2}$
MDC	199	$2.29x10^{3}$	188.65	$1.11x10^{4}$	$2.26x10^{3}$
IL-8	200	$6.89x10^{2}$	1.54	$5.05x10^{4}$	$4.25x10^{3}$
TNF-a	200	$5.66x10^{1}$	0	$1.0x10^{3}$	$1.29x10^{2}$
VEGF	199	$1.55x10^{3}$	0	$9.28x10^{3}$	$2.02x10^{3}$
IL-6	200	$6.12x10^{1}$	0	$3.84x10^{3}$	$2.90x10^{2}$
IL-1b	200	$3.29x10^{1}$	0	$1.32x10^{3}$	$1.33x10^{2}$
IL-10	200	9.07	0	$2.62x10^{2}$	$2.89x10^{1}$
IFN-g	200	$2.46x10^{2}$	0	$4.78x10^{3}$	$5.48x10^{2}$
IL-2	200	$7.39x10^{1}$	0	$3.34x10^{3}$	$3.85x10^{2}$
sgp130	200	$8.96x10^{4}$	0	$1.89x10^{5}$	$3.75x10^{4}$
sIL-2Rα	199	$2.17x10^{3}$	0	$9.99x10^{3}$	$1.58x10^{3}$
sIL-4R	198	$2.52x10^{2}$	0	$1.82x10^{3}$	$1.92x10^{2}$
sIL-6R	199	$1.44x10^{4}$	2.29	$3.08x10^{4}$	$5.36x10^{3}$
sTNFRII	198	$1.01x10^{4}$	0	$3.47x10^{4}$	$4.86x10^{3}$
sVEGFR3	197	$3.37x10^{3}$	0	$1.44x10^{4}$	$2.57x10^{3}$
MMP-2	200	$4.12x10^{5}$	0	$2.0x10^{6}$	$4.76x10^{5}$
MMP-9	200	$1.18x10^{6}$	0	$6.01x10^{1}$	$1.02x10^{6}$
sCD137	152	$6.74x10^{-2}$	0	2.12	$1.87x10^{-1}$
Granzyme A	152	$2.03x10^{-1}$	0	7.22	$8.19x10^{-1}$
Granzyme B	152	$8.36x10^{-3}$	0	$3.7x10^{-1}$	$3.52x10^{-2}$
sFas	152	$2.27x10^{1}$	0	$2.52x10^{2}$	$2.63x10^{1}$
sFasL	152	$1.64x10^{-3}$	0	$1.3x10^{-1}$	$1.11x10^{-2}$

The low number of observations recorded for sCD137, Granzyme A, Granzyme B, sFas and sFasL were due to low detection. Valid N = valid number of observations

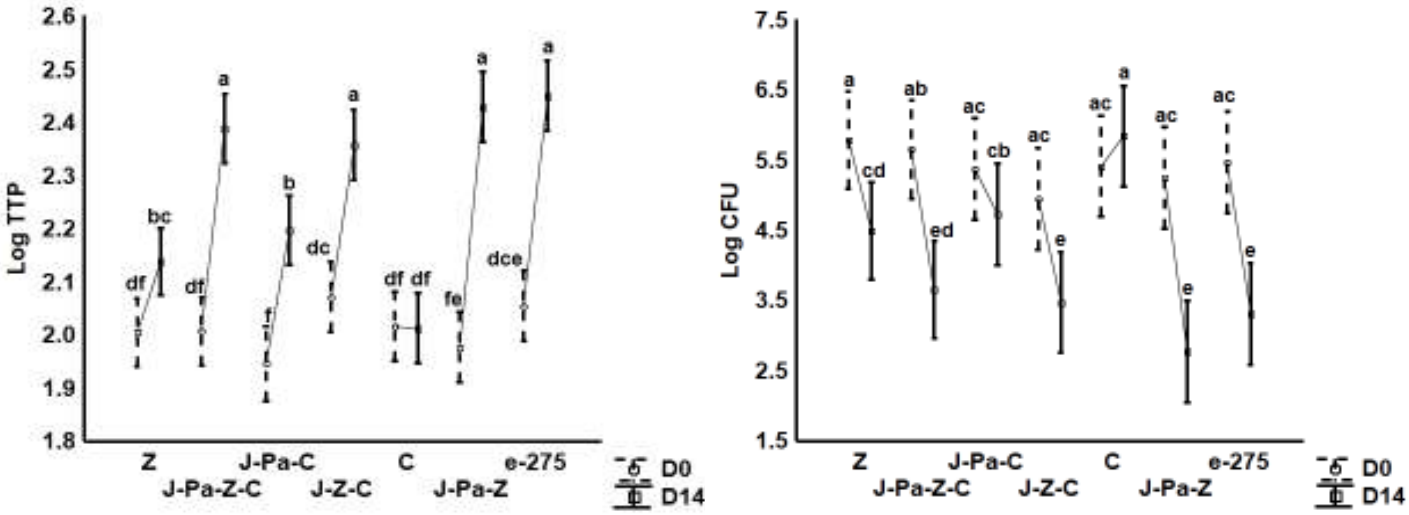

Figure 3.2 Early bactericidal activity of single or drug combinations as determined by time to positivity (TTP) and colony forming unit (CFU). The vertical bars denote 95% confidence intervals. Alphabetical letters were assigned to the measures of the treatment arms and at the time point to indicate significant difference. Occurrence of any of the same letters on different treatment arms or time points indicates no significant difference between groups ($p>0.05$), whereas the absence of the same letter on different treatment arms or time points indicates significant differences. J = TMC207, Pa = PA-824, Z = Pyrazinamide, C= Clofazimine.

3.6 Differences in serum concentrations of host markers at D0 and D14

Out of the 33 host markers that were measured, the changes in the expression of six markers, namely CRP, Ferritin, VEGF, sTNFRII, sIL-2Rα and IL-6 between D0 and D14 were significant in several of the different treatment arms. However, none of the markers adequately mirrored the changes in bacterial load in the sputum during the observation period. The only markers whose change over the 14 days correlated significantly with changes in TTP regardless of treatment arm were sIL-2Rα (Spearman -0.36, $p<0.01$), sTNFRII (Spearman -0.31, $p<0.01$) and Granzyme A (Spearman -0.32, $p<0.01$) and for CFU the markers were VEGF (Spearman -0.30, $p<0.01$), sIL-2Rα (Spearman -0.36, $p<0.01$) and sTNFRII (Spearman -0.32, $p<0.01$). Of the six markers that showed changes over time the baseline levels of CRP, VEGF and sTNFRII were similar in all the treatment arms. Six

treatment arms showed significant baseline differences for Ferritin and two for sIL-2Rα while one treatment arm had significantly higher IL-6 levels than the other arms (Figure 3.2).

The concentration of CRP decreased significantly by D14 in five treatment arms with the exception of the Rifafour e-275 and Clofazimine arms. Ferritin levels dropped significantly in the Pyrazinamide, J-Pa-Z and J-Pa-Z-C arms. A significant decrease in the expression of VEGF was found in the J-Pa-Z-C, J-Pa-Z and Rifafour e-275 treatment arms and the concentration of sTNFRII decreased significantly in the J-Pa-Z-C, J-Pa-C and Rifafour e-275 groups. sIL-2Rα showed a significant drop in the J-Pa-Z-C, J-Pa-C, J-Pa-Z and Rifafour e-275 groups. Levels of IL-6 decreased significantly in J-Pa-Z-C and Rifafour e-275. Out of all these markers, CRP appeared to follow the changes in bacterial load in sputum most closely (Figure 3.2).

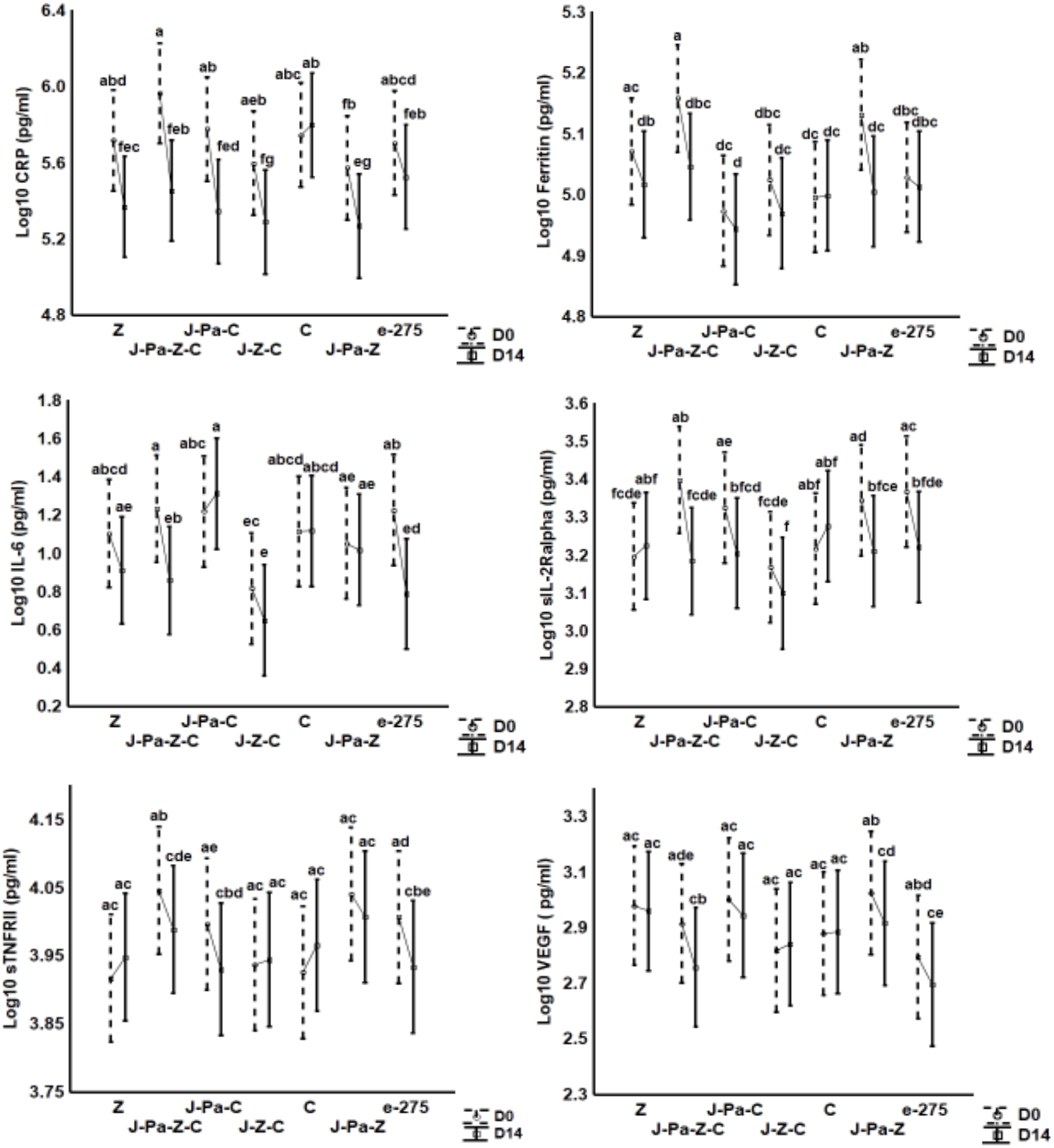

Figure 3.3 Changes in serum concentrations of six host markers in TB patients during a short period of TB treatment. Concentrations of serum host markers were measured in 100 TB patients at baseline (D0) and at end of EBA (D14) using Luminex platform for all the seven treatment arms. The vertical bars denote 95% confidence intervals. Alphabetical letters were assigned to the measures of the treatment arms and at the time point to indicate significant difference. Occurrence of any of the same letters on different treatment arms or time points indicates no significant difference between groups ($p>0.05$), whereas the

absence of the same letter on different treatment arms or time points indicates significant differences. J = TMC207, Pa = PA-824, Z = Pyrazinamide, C= Clofazimine

3.7 Relationship between host marker combinations and log CFU or log TTP

To evaluate the predictive abilities of combinations of markers as potential candidates and/or adjunctive markers in EBA assessment using best subset multiple linear regression analysis, we found that the combination of IL-2, MMP-9, sCD137 and Granzyme A were the best host markers that predicted TTP with an R^2 value of 0.18. Similarly, a four-marker biosignature including A2M, sIL-2Ra, sIL-6R and sTNFRII predicted CFU with an R^2 value of 0.18. The predictive variables were winsorized to reduce the effect of large outliers.

3.8 Discussion

We have investigated the changes in levels of several blood-based biomarkers that have previously shown potential in monitoring TB treatment response [1, 9] and evaluated them directly against culture based sputum markers in the first 14 days of treatment with seven drugs or drug combinations whose activity varied between zero and that of the current standard drug combination. To our knowledge this is the first report of a direct comparison of serum biomarkers with EBA results conducted during a 14-day clinical trial.

Out of the thirty-three host markers that were measured a significant change was observed only in the expression of CRP, Ferritin, VEGF, sTNFRII, sIL-2Rα and IL-6 concentrations by the end of the D14 EBA observation period. As a wide range of changes in immune responses during TB therapy has previously been reported [10-13] the modest number of markers with changes was surprising. Compartmentalized immune responses in the blood versus the lung cannot account for the lack of more changes found in the present study in view of the multiple studies referenced above. We found that only CRP and sIL-2Rα showed statistically significant changes in serum concentrations from D0 to D14 in more than three treatment arms. High concentrations of CRP and sIL-2Rα were reported in patients who responded poorly to treatment and treatment failures in previous studies [14, 15].

CRP is an inflammatory marker that is produced by a variety of cells including hepatocytes [14] and peripheral blood mononuclear cells (PBMCs) [16]. We recently showed the potential of this acute phase protein in TB diagnosis [17] and other studies have also shown its usefulness as biomarker in many disease conditions including pulmonary infections [18, 19], diabetes mellitus [20] and asthma [21]. Thus the elevated expression of CRP concentration reflects disease activity in a non-specific manner. In monitoring response to TB treatment, Siawaya *et al* [22] found a significant decrease in the concentration of CRP after the initiation of TB therapy with a further gradual decline during treatment. The role of CRP as biomarker for cancer treatment has also been described. Elevated expression of serum CRP levels was associated with poor treatment response in patients who received chemoradiotherapy [18]. Moreover, decrease in the expression of CRP after anti-TB therapy has been shown [23]. Our data confirm that the concentration of CRP decreases significantly at the end of D14 EBA in most of the groups where CFU and TTP decline and this might be an early indication of successful chemotherapy. The lack of changes in the Clofazimine group, where CFU and TTP did not change, suggests a potential for CRP in detecting poor response.

The sIL-2Rα is formed as a result of proteolytic cleavage of an α-chain [24] and high levels of sIL-2Rα in serum have been shown in many disorders including autoimmune disease and mycobacterial infection [25]. Previous studies have also reported an increase of serum sIL-2Rα in smaller number of patients with pulmonary TB and sarcoidosis [26-28] and high levels of sIL-2Rα might indicate *in vivo* immune activation [15]. An association between activation of the immune system by mycobacterial antigens and an increase in the levels of sIL-2Rα has previously been found [29]. In the present study, the serum level of sIL-2Rα measured at D14 at end of EBA evaluation deceased significantly in four treatment arms and supports the previously reported correlation with treatment response [15]. A significant decrease in the level of sIL-2Rα was also found in another study after 4 weeks of TB treatment in the sera of slow treatment responders [30].

Clofazimine is a lipophilic antibiotic with antimycobacterial and anti-inflammatory activities and has been in use since 1962 for treating leprosy [31]. However, an anti-inflammatory

effect of Clofazimine on the measured host markers was not evident in this 14 day EBA study, although this does not exclude potential longer-term effects or that such effects are missed by the selected markers in this study. We observed an improved EBA when Clofazimine was used in combination with other drugs and this was also mirrored by a change in cytokine levels.

A four-analyte model predicted 20% of the variation in both TTP and CFU data. Granzyme A one of the markers that were included in the four marker model had a low concentration in the samples. This is not a surprise as low concentration of this serine protease has been shown in other studies [32]. In view of the limited number of individual host markers that showed significant changes during the study, the low percentage of variation explained by a combination of markers may not be unexpected. This low percentage of variation in TTP and CFU that is explained by the host marker combination is not sufficient for them to be considered as a suitable replacement for CFU in a 14 day EBA study for clinical trials of anti-TB drugs.

Besides the small number of participants that were randomised into the different treatment arms, the main limitation of our study was that host marker responses and clinical outcomes beyond 14 days were not determined. However, the strength of the study lies in the fact that multiple regimens were tested with a wide range of activities, that all participants were recruited in the same geographical location and that all the samples were analysed at the same laboratory.

In summary, we did not find a specific host marker or host marker combination that correlated well with sputum culture based markers of 14-day treatment activity, although CRP, sIL-2Ra, Ferritin, sTNFRII, IL-6 and VEGF followed the decline in bacterial numbers in the different treatment arms most closely. It remains unclear if host markers can have a role in early treatment evaluation as a lack of correlation with changes in sputum bacterial numbers does not necessarily preclude a potential to provide important information about the effects of individual treatment regimens on early changes in parameters other than the sputum mycobacterial load or the ultimate treatment outcomes. Further studies should

expand the range of host markers in early treatment response evaluation, the duration of observation and correlations with ultimate treatment outcome.

3.9 References

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CHAPTER 4

Combined specific IgG and IgA based diagnosis of tuberculosis in African primary healthcare clinic attendees with signs and symptoms suggestive of TB

This chapter has been submitted for publication

My contribution: Planning of project

Generated data through laboratory experiments

Interpretation of results and data

Writing of manuscript

Abstract

Background: IgG-based tests for the diagnosis of active tuberculosis (TB) disease often show a lack of specificity in TB endemic regions, which is mainly due to a high background prevalence of latent TB infection (LTBI). Here, we investigated the combined performance of the responses of different Ig classes to selected mycobacterial antigens in primary healthcare clinic attendees with signs and symptoms suggestive of TB.

Methods: We evaluated the sensitivity and specificity of IgA, IgG and/or IgM to LAM and 7 mycobacterial protein antigens (ESAT-6, Tpx, PstS1, AlaDH, MPT64, 16kDa and 19kDa) and 2 antigen combinations (TUB, TB-LTBI) in the plasma of 63 individuals who underwent diagnostic work-up for TB after presenting with symptoms and signs compatible with possible active TB. Active TB was excluded in 42 individuals of whom 21 has latent TB infection (LTBI) whereas active TB was confirmed in 21 patients of whom 19 had a follow-up blood draw at the end of 6-month anti-TB treatment.

Results: The leading single serodiagnostic markers to differentiate between the presence or absence of active TB were anti-16 kDa IgA, anti-MPT64 IgA with sensitivity and specificity of 90%/90% and 95%/90%, respectively. The combined use of 3 or 4 antibodies further improved this performance to accuracies above 95%. After successful completion of anti-TB treatment at month 6, the levels of 16 kDa IgA and 16 kDa IgM dropped significantly whereas LAM IgG and TB-LTBI IgG increased.

Conclusions: These results show the potential of extending investigation of anti-tuberculous IgG responses to include IgM and IgA responses against selected protein and non-protein antigens in differentiating active TB from other respiratory diseases in TB endemic settings.

4.1 Introduction

Tuberculosis (TB) still remains a global threat to mankind and although the millennium development goals target of halting and reversing the increasing incidence of TB globally was achieved, TB still killed 1.5 million people in 2014 [1]. The currently available diagnostic tools have many limitations including poor sensitivity (smear microscopy), long turn-around time (culture), the use of expensive tools and the difficulty to develop these tests into point-of-care (POC) tests [2]. The high prevalence of LTBI in addition to high TB and HIV co-infection in resource-poor settings such as in Africa, calls for the development of rapid diagnostic tools, especially ones that discriminate between active TB and LTBI. The use of commercial serological tests for diagnosing active TB has been strongly criticised, as a result of the poor accuracy of commercial tests in TB endemic settings [3], which is largely due to a high prevalence of LTBI [4-6]. However, further research in the field of antibody-based tests has been encouraged, as serological tests lend itself to the development of POC tests. Furthermore, it needs to be ascertained to what degree the lack of serological test specificity is due to a subgroup of LTBI with high risk for progression to active TB [4].

The standard strategy for TB treatment, directly observed treatment short course (DOTS) consists of a two month period of four drugs followed by another four months of two anti-TB drugs [7]. The necessity of a treatment period of six month regimen is largely due to persistent bacilli that are not rapidly killed [8] and these persister organisms can be the cause of treatment failure and relapse [9]. The identification of better surrogate markers of treatment response than sputum culture would be a major boost towards enhancing treatment monitoring [10] and the potential role of serologic tests needs to be evaluated [11].

The main purpose of the present study was to evaluate the potential of IgG, IgA and IgM serodiagnostic markers for the diagnosis of active TB disease among people presenting with presumed TB at primary health care clinics and to explore their potential as treatment response markers.

4.2 Material and methods

4.2.1 Study population

Participants included in the present study were individuals presenting with signs and symptoms requiring investigation for TB, and were recruited as part of the recently concluded EDCTP-funded African European Tuberculosis Consortium (AE-TBC) study [12]. All study participants were recruited from a peripheral level health care centre, Fisantekraal, situated in the outskirts of Cape Town, South Africa. All participants presented with persistent cough lasting for more than 2 weeks and one of the following: fever, recent loss of weight, night sweats, haemolysis, chest pain or loss of appetite. The eligibility criteria for the study included age of between 18 and 65 years, and willingness to give written informed consent including for HIV testing. The exclusion criteria included severe anaemia (HB<10g/l), current anti-TB treatment, anti-TB treatment in the last 90 days, or taking quinolone or aminoglycoside antibiotics in the past 60 days, and not being resident in the study area for more than 3 months at presentation. Sputum samples were collected from all study participants and cultured using the MGIT method (BD Biosciences). Confirmation of the isolation of organisms of the *M.tb* complex in all positive cultures was carried by the Capilia TB test (TAUNS, Numazu, Japan). Additionally, 3 ml of blood were collected from the participants for the performance of Quantiferon-in Tube (QFT-IT) assay (Qiagen), which was carried out according to the manufacturer's instruction as previously described [13]. For the current study, we included 21 patients with culture positive TB and 42 with other lung diseases of which 21 had LTBI as defined by a positive QFT test and of which 21 were QFT-negative. These participants were randomly selected from Stellenbosch set of samples from the main study and according to availability of baseline and month 6 samples. All active TB patients received standard TB treatment according to South African National Tuberculosis Program and samples were collected from 19 of the TB participants at the end of TB treatment at month 6 (M6). None of the non-TB patients (LTBI and the QFT-negative) received anti-TB treatment. Ethical approval for the study was obtained from the Health

Research Ethics Committee of the University of Stellenbosch (reference number N10/08/274) and written informed consent was obtained from each participant before the study.

4.2.2 Sample collection and preparation

At enrolment, 10ml of whole blood were collected from all study participants directly into heparinized BD vacutainer tubes (BD Biosciences), and transported at ambient conditions within two hours of collection to the laboratory. The tubes were then centrifuged at 1200xg for 10 minutes, and plasma harvested and stored at -80°C until further use. Sample collection was repeated for 19 TB patients at months 6 following anti-TB treatment.

4.2.3 Antigen preparation

Seven cloned and purified recombinant proteins of *M. tuberculosis* (Table 4.1) were used in the present study. For MPT64 expression, recombinant *M. smegmatis* mc^2 155 cells containing the MPT64 expression plasmid (with C-terminal histidine tag) were grown in animal source-free medium at 37°C overnight. The recombinant MPT64 was secreted into the culture medium by constitutive expression. The medium was harvested by centrifugation followed by buffer exchange on a Sephadex G25 column (GE Healthcare) into 10 mM NH_4HCO_3 buffer. The protein containing solution was applied onto Ni-NTA Superflow resin (Qiagen). MPT64 was eluted in a linear imidazole gradient. Highly pure MPT64-containing fractions were pooled and underwent a final buffer exchange on a Sephadex G25 column (GE Healthcare) into 10 mM NH4HCO3, pH 8,0. Aliquots of the protein solution were freeze-dried and stored below -20 °C. The production of the remaining protein antigens has been described previously: 19 kDa [4], AlaDH [14], ESAT-6 [11], 16 kDa [6], PstS1 [15], and Tpx [11]. A sample of highly purified *M. tuberculosis* LAM was kindly provided by Dr. Arend Kolk, Amsterdam. We also evaluated two multiple antigen cocktails in this study. The first multiple antigen cocktail TUB contains PstS1, 16kDa and APA while the second TB-LTBI is

composed of Tpx and L16. All the single antigens and multiple antigen cocktails were supplied by LIONEX Diagnostics and Therapeutics, Braunschweig, Germany.

Table 4.1 Recombinant antigens of M. tuberculosis used in this study

Antigens of *M. tuberculosis*	**Rv no.**	**Mol.mass (kDa)**	**Reference(s)**	**Ig class**
19 kDa glycolipoprotein, LpqH	Rv3763	16	[4]	IgA, IgG
AlaDH	Rv2780	38.7	[14]	IgA, IgG
ESAT-6	Rv3875	9.9	[11]	IgA, IgG
HSP16.3, HSPX, 14 kDa, 16 kDa, ACR	Rv2031c	16.3	[6]	IgA, IgG, IgM
LAM	–	–		IgA, IgG
MPT64	Rv1980c	24.8	[47]	IgA, IgG
PstS1, 38 kDa	Rv0934	38.2	[15]	IgG
Tpx, CFP20	Rv1932	16.9	[11]	IgA, IgG

4.2.4 Enzyme-linked immunosorbent assay

All pre-coated IgG, IgA and IgM ELISA test kits and reagents against *Mtb* antigens were provided by LIONEX Diagnostics and Therapeutics, Braunschweig, Germany. Human plasma was diluted 1:200 in PBS pH 7.5/0.05% BSA buffer. One hundred µl of the diluted plasma and ready-to-use standards were pipetted into the antigen-coated wells of the microtiter plate in duplicate. After 60 minutes incubation while shaking (45 minutes incubation for Immunoglobulin (Ig) M) at 37°C, the well contents were emptied and plates washed three times with 300 µl/well PBS-T (0.15 M PBS, pH 7.5/0.05% Tween-20). 100 µl/well of ready to use anti-human-IgG-conjugate (diluted 1:40 000), anti-human-IgA (1:12 000) or anti-human-IgM (1:12 000) antibodies were added to the wells and the plates incubated for 30 minutes at 37°C while shaking. After a second washing step, the enzyme activity was assayed by rapidly adding 100 µl/well of substrate tetramethylbenzidine (TMB) with a further incubation for 20 minutes at 37°C in the dark. The colour development was ended by the rapid addition of 100 µl/well 0.2 M H_2SO_4 stop solution. The absorbance was measured at 450 nm (OD_{450}) with a 620 nm (OD_{620}) reference filter using an automatic microplate reader (iMark™ Microplate absorbance reader, BIO RAD, USA).The mean OD of the blank wells was subtracted from the sample values.

4.2.5 Statistical analysis

For the evaluation of the diagnostic potential of serodiagnostic markers, statistical differences in the concentrations of markers between active TB and individuals with other respiratory diseases were analysed by analysis of variance (ANOVA) with Fisher Least Significant Difference (LSD) *post hoc* test or Mann-Whitney U test depending on the normality of the distribution. In cases where Levene's test rejected the assumption of homogeneity of variance, weighted means were reported and the Games-Howell post-hoc test conducted. Receiver operating characteristics (ROC) curve analysis was used in evaluating the accuracy of the different markers. To investigate the predictive abilities for the optimal combination of serodiagnostic markers for differentiating TB disease and individuals with ORD, general discriminant analysis (GDA) was performed. The best subsets method was employed in determining optimal subsets of variables that gave the best prediction. Using the best variables that were included in the optimal classification model, a leave-one-out cross validation table was constructed. For the investigation of markers that could be useful in monitoring of the response to TB treatment, the change in the concentrations of markers during TB treatment was analyzed using mixed model repeated measures analysis of variance (ANOVA) with Fisher Least Significant Difference (LSD) post hoc test. A level of 5% significance was used as a guideline for the determination of significance associations. All statistical analysis except ROC analysis was performed using Statistica software (Statsoft, Ohio, USA). ROC analysis was done using R program language.

4.3 Results

4.3.1 Clinical and demographic characteristics of study participants

A total of 63 participants were included in the study. Out of these 63 individuals, 33 (52%) were females. The mean age of all study participants was 34.1±11.3 years and only one of the study participants was HIV positive. Using a pre-established diagnostic algorithm, 21 patients were classified as having TB disease. Of the 42 patients with other respiratory

diseases (ORD), 21 were QFT-negative (*M.tb* uninfected) while 21 individuals were latently infected (LTBI), as defined by a positive QFT-test using the manufacturer's recommended cut-off value (≥0.35 IU/ml). Table 4.2 shows the demographic and baseline characteristics of the study participants.

Table 4.2 Demographic characteristics of study participants

	All	TB	LTBI	QFT-negative ORD
Participants no.	63	21	21	21
Age, yr	34.1±11.3	41.0±10.5	26.0±3.6	35.2±12.2
M/F no. (%)	30(48)/33(52)	8(38)/13(62)	13(62)/8(38)	9(43)/12(57)
HIV status pos/neg	1/62	0/20	0/20	1/20
QFT-IT positive	42	21	21	Nil

Values are mean (±SD) unless indicated otherwise.

4.3.2 Evaluation of antibodies for the diagnosis of active TB disease

We measured the titres of IgG, IgA and/or IgM antibodies against LAM and 7 mycobacterial protein antigens (ESAT-6, Tpx, PstS1, AlaDH, MPT64, 16kDa and 19kDa) and 2 antigen combinations (TUB, TB-LTBI) in plasma samples obtained from all 63 study participants (Table 4.2). When the antibody titres in the TB patients were compared to the titres obtained in all individuals with other respiratory diseases ORD (regardless of QFT results), IgA antibodies against 16 kDa, AlaDH, ESAT-6, MPT64, IgG antibodies against 16 kDa, 19 kDa, LAM, TB-LTBI and IgM antibodies against 16 kDa antigen were significantly higher in TB patients compared to non-cases (Figure 4.1). ROC curve analysis indicated that, anti-LAM IgG, anti-TB-LTBI IgG, anti-MPT64 IgA, and anti-16 kDa IgA antibodies were the leading single serodiagnostic markers (Table 4.3). Anti-MPT64 and 16 kDa antigen IgA ascertained TB disease with areas under the ROC curves (AUC) of 0.96 (95% CI, 0.92-1.00) and 0.93 (95% CI, 0.87-0.99), respectively. The corresponding sensitivities and specificities were 95% / 90% and 90% / 90%, respectively (Table 4.3 and Figure 4.3).

Table 4.3 Sensitivities and specificities of single serodiagnostic markers in differentiating active TB (n=21) from LTBI (n=21) and ORD (n=42)

Antigen	Ig class	AUC (95% CI)	TB vs ORD Sensitivity/specificity (%)	AUC (95% CI)	TB vs LTBI Sensitivity/specificity (%)
AlaDH	A	0.73(0.59-0.86)	0.76/0.69	0.73(0.57-0.89)	0.76/0.71
AlaDH	G	0.63(0.49-0.76)	0.62/0.64	0.65(0.47-0.83)	0.76/0.57
ESAT-6	A	0.73(0.60-0.86)	0.67/0.79	0.75(0.61-0.89)	0.67/0.81
ESAT-6	G	0.46(0.27-0.65)	0.52/0.60	0.45(0.25-0.64)	0.52/0.52
LAM	A	0.64(0.49-0.79)	0.52-0.76	0.59(0.41-0.76)	0.52/0.76
LAM	G	0.91(0.83-0.98)	0.86/0.90	0.95(0.89-1.00)	0.86/0.95
MPT64	A	0.96(0.92-1.00)	0.95/0.90	0.97(0.91-1.00)	0.95/0.90
MPT64	G	0.69(0.56-0.82)	0.76/0.60	0.71(0.54-0.88)	0.76/0.67
PstS1	G	0.43(0.28-0.58)	0.52/0.52	0.51(0.32-0.70)	0.62/0.62
TB-LTBI	A	0.76(0.63-0.88)	0.67/0.76	0.76(0.61-0.91)	0.67/0.86
TB-LTBI	G	0.90(0.83-0.98)	0.90/0.79	0.89(0.78-0.99)	0.90/0.71
Tpx	G	0.54(0.39-0.68)	0.55/0.57	0.52(0.34-0.70)	0.57/0.52
TUB	A	0.74(0.62-0.87)	0.81/0.60	0.79(0.65-0.92)	0.81/0.62
16kDa	A	0.93(0.87-0.99)	0.90/0.90	0.99(0.98-1.00)	0.95/0.95
16kDa	G	0.77(0.66-0.89)	0.81/0.67	0.78(0.63-0.93)	0.95/0.62
16kDa	M	0.84(0.74-0.94)	0.71/0.86	0.80(0.67-0.94)	0.71/0.81
19kDa	A	0.60(0.49-0.72)	0.33/0.88	0.61(0.49-0.73)	0.33/0.90
19kDa	G	0.76(0.63-0.88)	0.86/0.67	0.74(0.58-0.90)	0.86/0.67

Receiver operator curve (ROC) analysis was used in determining the accuracy (sensitivity/specificity) of each serodiagnostic marker. ORD individuals comprised 21 LTBI and 21 QFT-ve ORD. Abbreviations: Ig = Immunoglobin; vs = versus; AUC = Area under the curve; CI = Confidence Interval

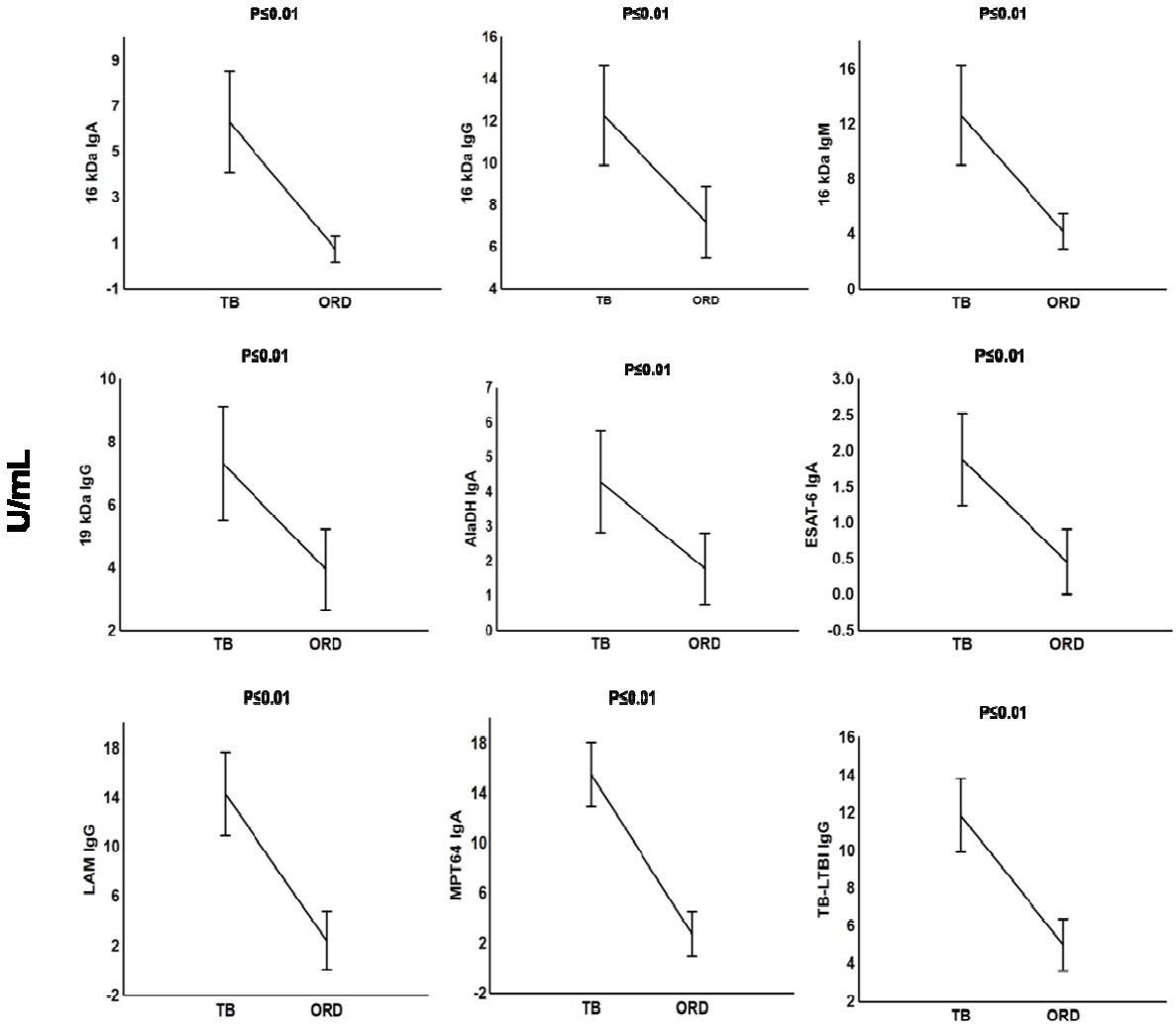

Figure 4.1 Plasma concentrations of serodiagnostic markers in TB and ORD. Concentrations of plasma serodiagnostic markers were measured in 21 TB patients, and 42 ORD cases. The ORD group comprised 21 LTBI individuals and 21 QFT-negative ORD. Representative plots are shown for diagnostic markers showing significant differences between groups and the vertical bars denote the mean and 95% confidence intervals.

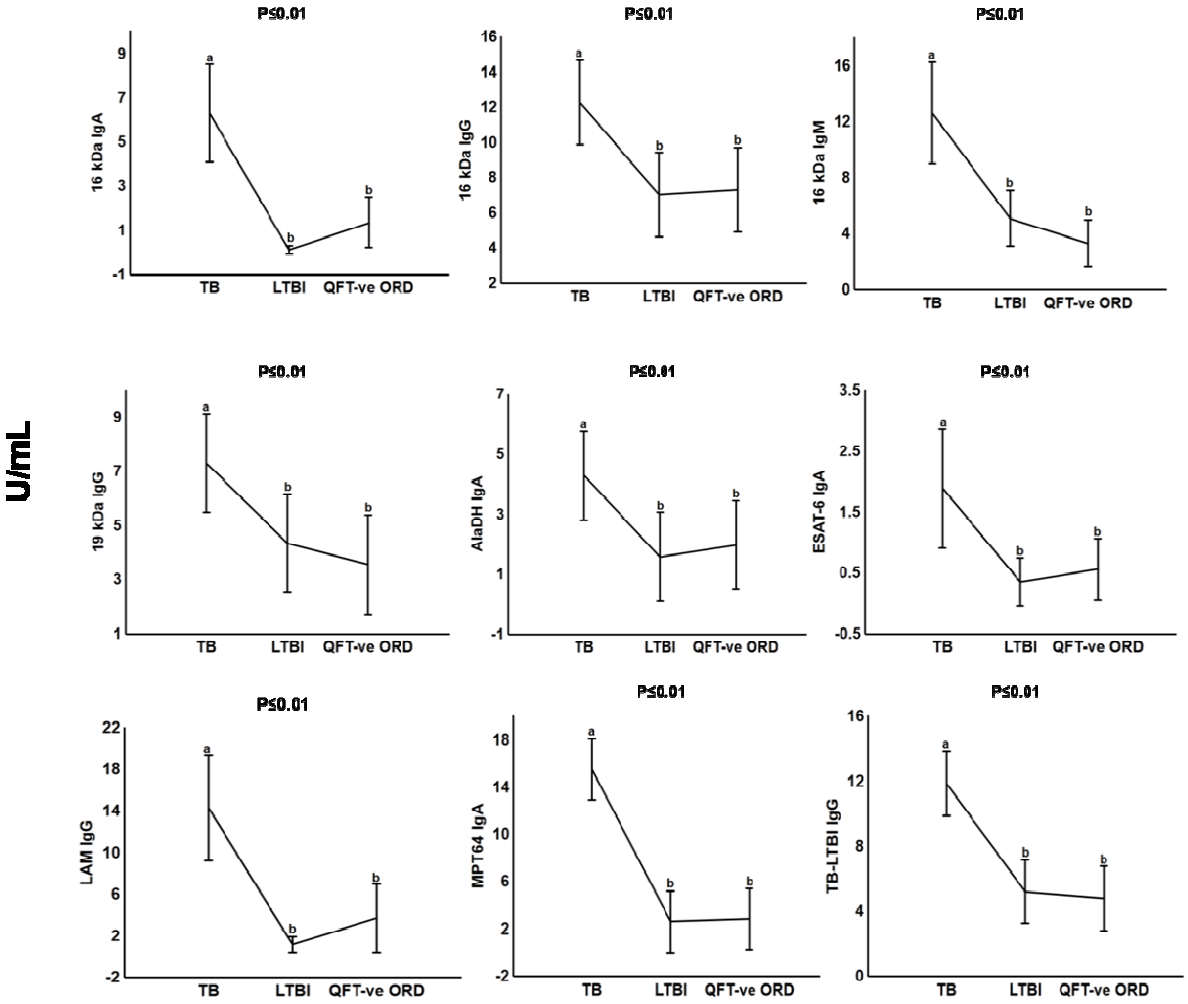

Figure 4.2 Plasma concentrations of serodiagnostic markers in individuals with tuberculosis, latently infected tuberculosis and QFT-negative other respiratory diseases. Concentrations of plasma serodiagnostic markers were measured in 21 tuberculosis patients, 21 latently infected tuberculosis individuals and 21 QFT-negative other respiratory diseases. Representative plots are shown for diagnostic markers showing significant differences between groups and the vertical bars denote 95% confidence intervals. Significant difference between different groups $p<0.05$ is shown with the different alphabetical letters. The same alphabetical letters are used when there is no significant difference $p>0.05$ between the different groups.

4.3.3 Evaluation of antibodies in discriminating between active TB disease and LTBI

We performed a subgroup analysis and compared the antibody titres of the TB patients to those with other respiratory diseases who were QFT-positive. The IgA, IgG and IgM levels against 16 kDa, 19 kDa, AlaDH, ESAT-6, LAM, MPT64 and TB-LTBI protein antigens were

significantly higher ($p < 0.01$ in all cases) (Figure 4.2). When the discriminative abilities of the different antibodies were evaluated by ROC curve analysis, the four antibodies anti-LAM IgG, anti-TB-LTBI IgG, anti-MPT64 IgA, and anti-16 kDa IgA were the most accurate. The AUC for anti-16 kDa IgA was 0.99 (95% CI, 0.98-1.00), and that for anti- MPT64 IgA was 0.97 (95% CI, 0.91-1.00) (Figure 4.4). Both antibodies differentiated between active TB and LTBI with a sensitivity of 95% and specificity ≥90%, respectively (Table 4.3).

4.3.4 Utility of multi-marker combinations to diagnose active TB

We evaluated the diagnostic abilities of combinations between the different antibodies in differentiating active TB from non-TB, or, alternatively from LTBI, using general discriminant analysis (GDA) models. For differentiating TB disease from other respiratory diseases, the most optimal biosignature was a three-marker serodiagnostic model comprising anti-TB-LTBI IgG, anti-Tpx IgG and anti-MPT64 IgA. This model correctly classified 95.2% (20 from 21) of the TB patients and 97.6% (40 from 41) of the ORD cases in the resubstitution classification matrix, with an overall accuracy of 96.8%, with the same level of accuracy after leave-one-out cross validation (Table 4.4). For discriminating between active TB and LTBI, the before-mentioned 3 markers plus anti-LAM IgA (a four-marker signature), classified both groups (TB disease or LTBI) with an accuracy of 100% in the resubstitution classification matrix, and an accuracy of 95.2% after leave-one-out cross validation (Table 4.4).

TB vs ORD

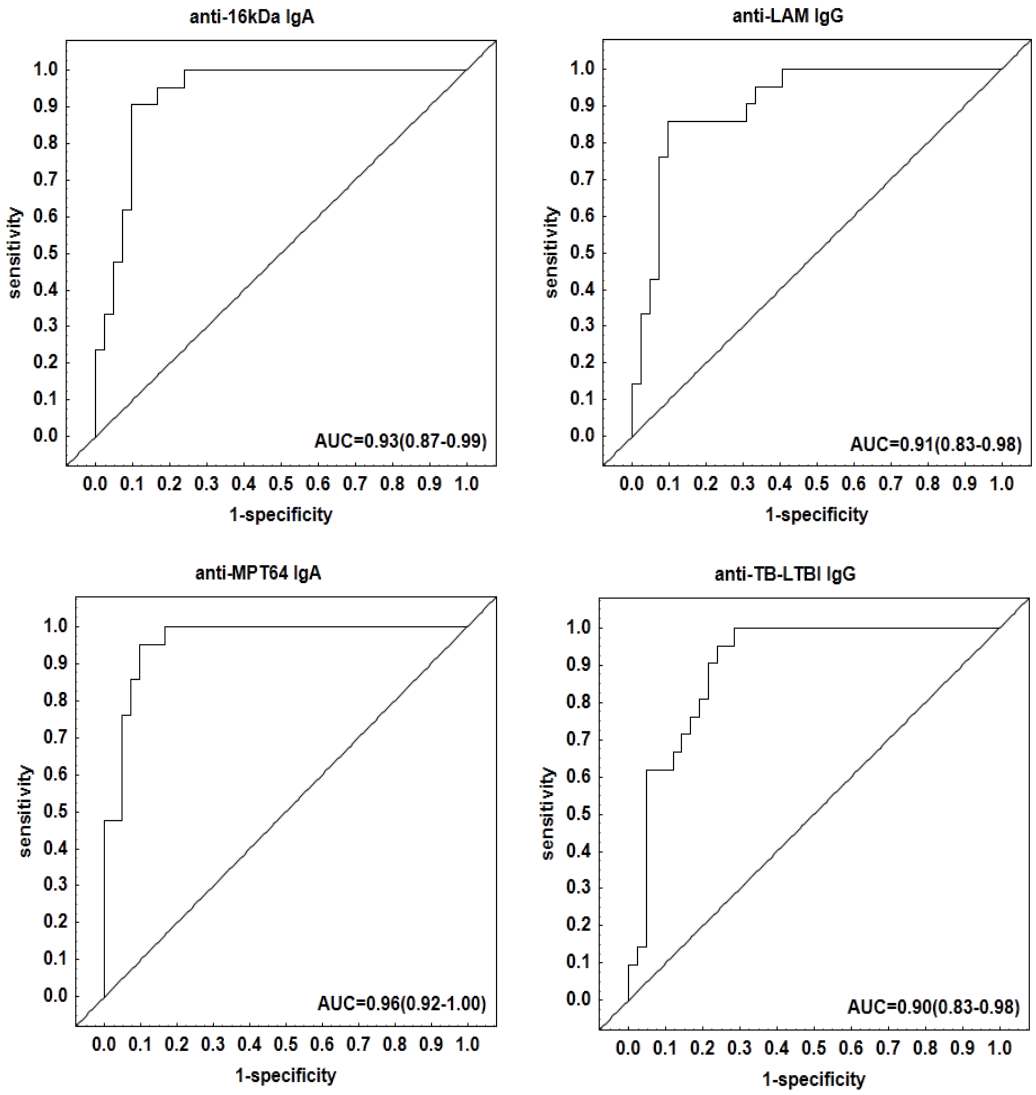

Figure 4.3 Receiver operator characteristics (ROC) curves of top single serodiagnostic markers for discriminating 21 active tuberculosis patients from 42 other respiratory disease cases.

TB vs LTBI

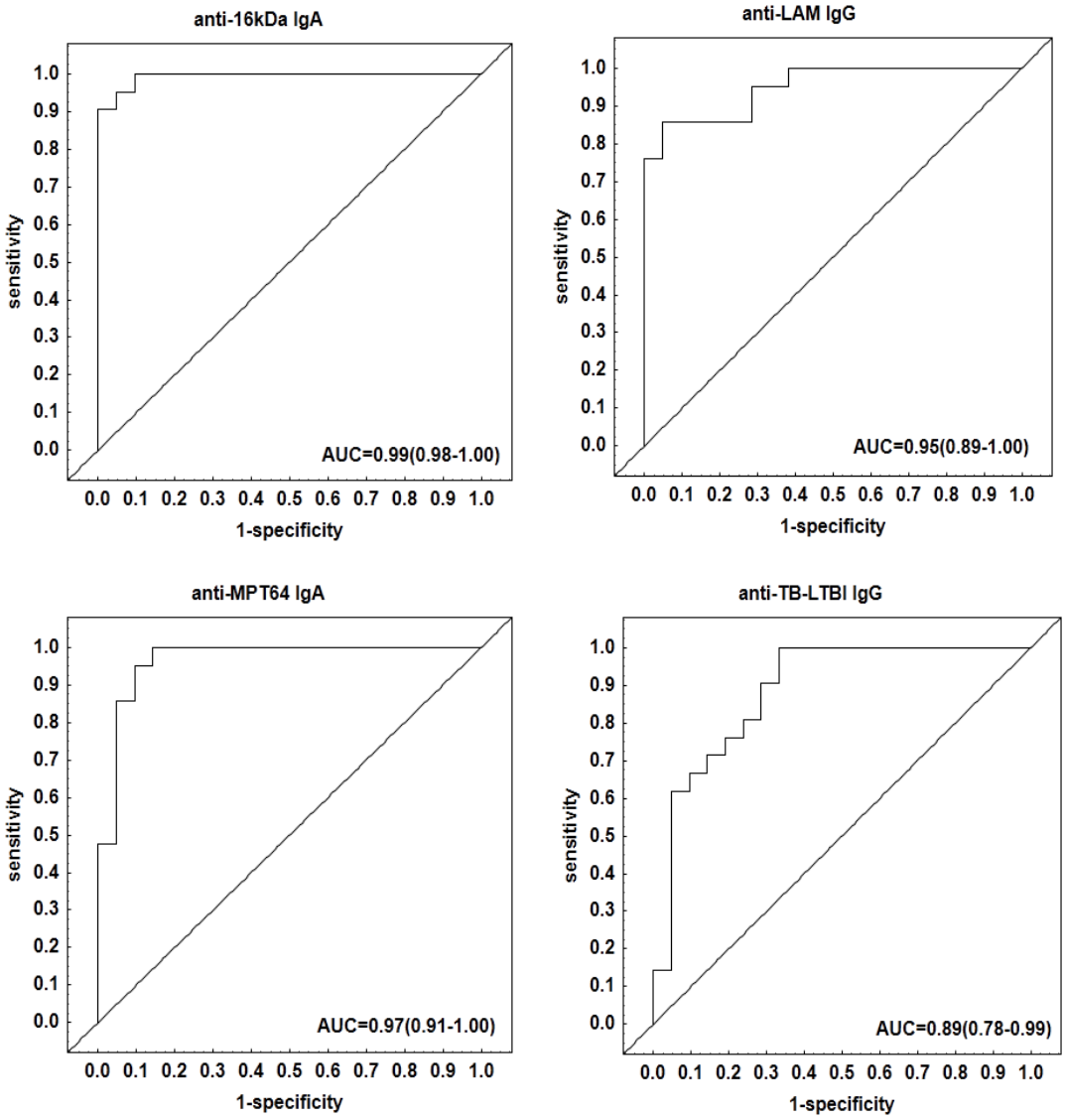

Figure 4.4 Receiving operating characteristics (ROC) curves of top single serodiagnostic markers for discriminating 21 active tuberculosis patients from 21 latently infected individuals.

Table 4.4 Accuracies of seroantigen combinations to distinguish between TB and ORD, or LTBI, after general discriminant analysis

TB vs ORD					
Antigen combination	Resubstitution classification matrix			Leave-one-out cross-validation	
	%TB	% ORD	% Accuracy	% TB	% ORD
Anti-TB-LTBI IgG Anti-Tpx IgG Anti-MPT64 IgA	95.23 (20/1)	97.61 (1/41)	96.8	95.23 (20/1) PPV: 0.95 (95% CI; 0.74-0.99) NPV: 0.97 (95% CI; 0.85-0.99)	97.61 (1/41)
TB vs LTBI					
Antigen combination	Resubstitution classification matrix			Leave-one-out cross-validation	
	%TB	% LTBI	% Accuracy	% TB	% LTBI
Anti-LAM IgA Anti-TB-LTBI IgG Anti-Tpx IgG Anti-MPT64 IgA	100.0 (21/0)	100.0 (0/21)	100.0	95.23 (20/1) PPV: 0.95 (95% CI; 0.74-0.99) NPV: 0.95 (95% CI; 0.75-0.99)	95.23 (1/21)

The predictive abilities of the optimal combination of serodiagnostic markers to differentiate between active TB (n=21), definite LTBI (n=21) ($IGRA^+$) or ORD individuals ($IGRA^+$ and $IGRA^-$ combined) (n=41) was investigated using best subsets general discriminant analysis and a leave-one-out cross-validation table was constructed using the variables that were included in the optimal classification model. PPV=Positive predictive value, NPV=Negative predictive value

4.3.5 Differential antibody responses in TB patients undergoing anti-TB treatment

Next, we investigated whether antibodies reflect the response to TB treatment. This analysis was only done in the 19 out of the 21 active TB patients for whom samples were available at the end of anti-TB treatment. We found significant low pre-treatment IgG responses to LAM and the TB-LTBI antigen combination compared to M6 TB treatment. However, we observed decreased significant levels of anti- anti-16 kDa IgA and anti-16 kDa IgM after successful anti-TB treatment at M6. (Figure 4.5).

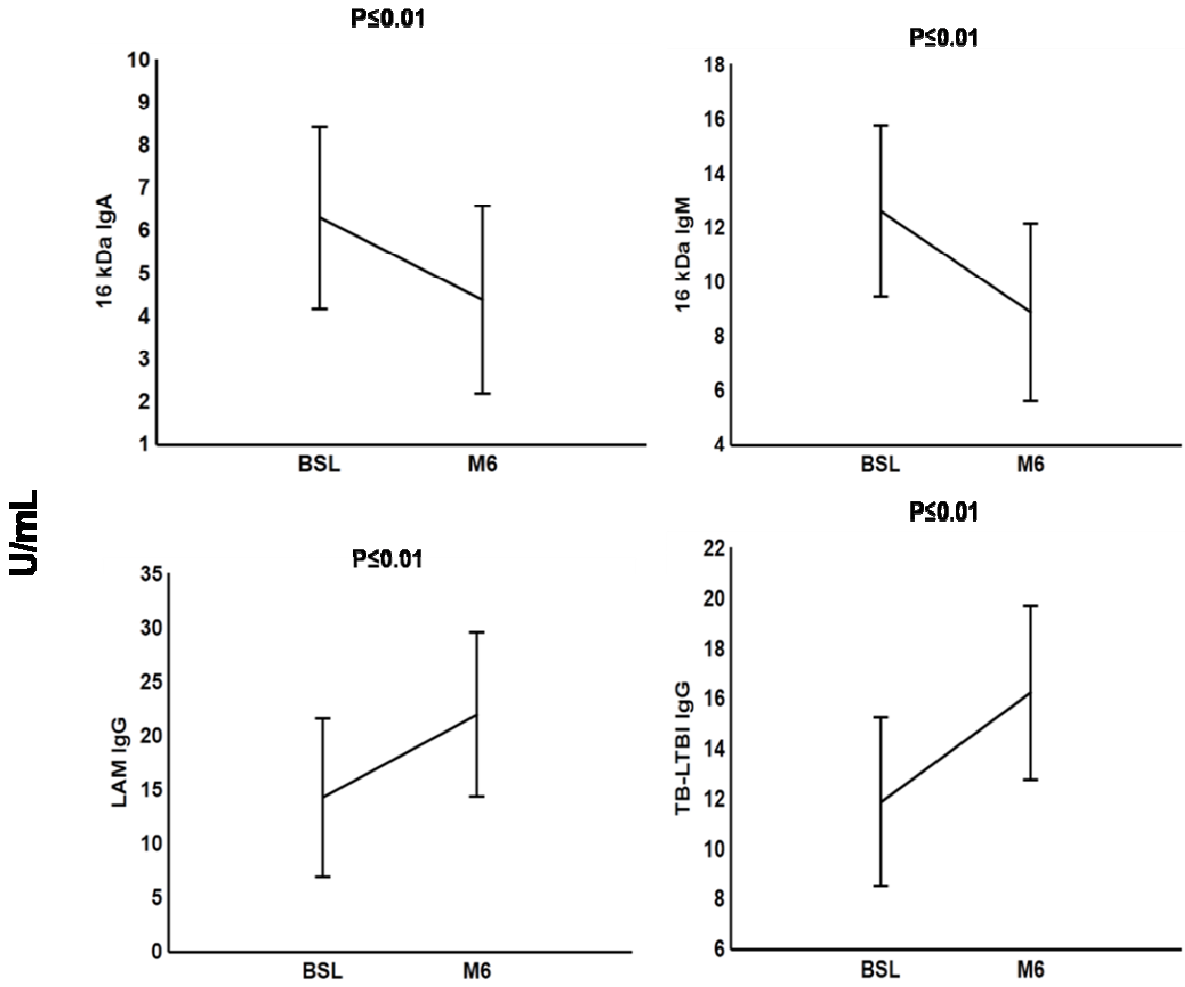

Figure 4.5 Plasma concentrations of serodiagnostic markers of tuberculosis patients during anti-TB treatment. Concentrations of plasma serodiagnostic markers were measured in 21 TB patients at baseline (BSL) and in 19 TB patients who were followed up at end of month 6 (M6) anti-TB treatment. Representative plots are shown for diagnostic markers showing significant differences between the two time points and the vertical bars denote 95% confidence intervals.

4.4 Discussion

The WHO does not support currently available commercial serologic tests for TB diagnosis but rather encourages further research to develop new tests with improved performance [3]. Performance targets for point-of-care tests in adults with smear-positive pulmonary TB include a sensitivity and specificity of 95%, while sensitivity in smear-negative cases should attain 60-80% and specificity of 95%. In extrapulmonary TB, a sensitivity of

80% and specificity of 90% have been proposed [16]. The results of this current study suggest that IgA and IgG responses against highly purified known and also relatively novel mycobacterial antigens, are promising due to the accuracies of individual and combination of marker above 95%. However, this is a small pilot study that requires further validation.

We have previously tested similar antibodies (anti-LAM IgG, anti-Tpx IgA). Results from the study showed that anti-LAM IgG was the best single marker discriminating TB from non-TB subjects and was also included in a five-marker combination with an improved performance. However, at that time, most of the controls were household contacts of active TB cases [6].

Previous studies have also found increased anti-TB antigen antibody levels in LTBI participants [4, 5]. In contrast, in this study, we found that the inclusion of the LTBI group did not affect test performance. Future research should investigate, whether recent household contacts of active TB cases affects serology compared to non-household contacts with LTBI. It is unknown whether increased anti-TB antigen antibody levels characterize a subgroup of LTBI individuals with high risk for progression to active TB, whose identification via antibodies would urgently be desirable [4, 17-19].

Much emphasis has been placed on the evaluation of IgG in most serological studies [5, 20, 21] with less attention directed towards IgA or IgM [22, 23]. IgA is produced particularly at mucosal sites, and enters the host via such sites. IgM is associated with acute infection. Like IgG, both mucosal and systemic IgA have protective effects and can trigger pro-inflammatory response [24, 25]. Therefore, the evaluation of these antibodies makes sense. In the present study, IgA were in part more accurate than their IgG counterparts. In a previous study by Legesse *et al*., [26] IgA responses to protein antigens (ESAT-6/CFP-10 and Rv2031) were also found to be more accurate for diagnosis of active TB than IgG responses in TB endemic settings.

The immunodiagnostic potential of several antigens has been reported [6, 27-29]. Out of all the antigens tested, LAM, the two proteins 16kDa and MPT64, and the antigen combination TB-LTBI best discriminated active TB from the other two groups with high

sensitivity and specificity. The 16 kDa polypeptide is a member of the low molecular weight α-crystallin heat shock proteins. It is a dominant protein produced under oxygen starvation or passive growth phase and also important for the replication of bacteria in macrophages [30, 31]. In the present study, 16 kDa elicited IgA, IgG and IgM responses in the plasma of the TB patients. Similarly, measuring 16 kDa antigen against all three isotypes, Raja and colleagues [32] found a combined specificity of 93% for serological detection in sputum and culture confirmed pulmonary TB patients. LAM is a component of the *Mtb* cell wall [33] and it is a well researched *Mtb* antigen for TB diagnosis in serological studies [6, 34, 35]. LAM elicited pronounced IgG responses in TB patients in this study and this shows its potential in TB serodiagnostic even in endemic settings. Similarly, the serodiagnosis of active TB patients through LAM achieved a high degree of specificity [34]. In contrast to our previous study, IgG responses were only elicited by LAM in LTBI subjects compared to healthy controls. Antibodies against MPT64 a highly specific protein that is secreted by *M. tb, M. bovis and M. africanum* [36-38] could be specific in the detection of TB especially as its expression is less in BCG vaccines [39]. An IgA response to MPT64 discriminated TB from QFT negative non-cases and LTBI with sensitivity of 95%. An MPT64 antibody aptamer, showed serological potential in the diagnosis of pulmonary TB in sputum smear positive as well as sputum smear negative patients [40].

The combination of IgA and IgG responses may help to increase accuracy of serodiagnostic tests for active TB disease in TB endemic settings, as previously suggested [6]. Accuracies of combinations of antibodies revealed that the combined IgG responses to Tpx and TB-LTBI and IgA response to MPT64 best discriminated active TB from ORD with a positive predictive value of 0.95 (95% CI; 0.74-0.99) and negative predictive value of 0.97 (95% CI; 0.85-0.99). The same antibody combinations with the inclusion of anti-LAM IgA were found to give the best discrimination between active TB and LTBI with positive and negative predictive values of 0.95(95% CI; 0.74-0.99) and 0.95(95% CI; 0.75-0.99) respectively. The multivariate analysis in our previous study with mainly TB household contacts also [6] revealed that both Tpx and LAM antigens also featured prominently in

discriminating TB from non-TB with an accuracy of 86.2%. Also the serologic responses to these two consistent antigens should be further investigated in a well-designed cohort study. It has been suggested that using multi-antigen cocktails will increase the sensitivity of TB serodiagnosis above that of single antigens [41, 42]. This might be due to the differential expression of certain antigens during the stages of TB development [43, 44].

We found a significant decrease in the levels of anti-16 kDa IgM and anti-16 kDa IgA after a successful completion of anti-TB treatment. Imaz and Zerbini [45] also reported decreased levels of antibodies, however, only three years after the start of anti-TB chemotherapy. A study on humoral response of TB patients undergoing anti-TB treatment indicated that antibody levels against other antigens had no association with anti-TB chemotherapy [46].

4.5 Conclusion

In summary, this study has shown that IgG and IgA antibody responses against single and multiple-antigen cocktails as well as multi-marker serologic models differentiated active cases from non-cases amongst people presenting with presumed TB regardless of LTBI status. Furthermore, our results suggest that the antibodies against the specific *Mtb* antigens tested in this study may be more useful for TB diagnosis than for monitoring treatment response. This result may encourage additional future efforts to investigate serologic responses in TB diagnostics research as such tests would be amenable to the development of rapid lateral flow-based test formats with application in field settings in a laboratory-free manner. However, future large scale prospective studies to include immunocompromised HIV co-infected patients are needed to further evaluate the validity of these results.

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CHAPTER 5

General discussion, main study implications and future studies

5.1 Overview

TB is a preventable and curable disease and despite the advances of the WHO in curbing this pandemic especially with the introduction of DOTS, this disease still continues to kill millions of people every year. The difficulty in diagnosing TB disease and monitoring of TB treatment response are two main challenges that are working against the elimination of the disease [1, 2]. The limitations of the current diagnostic tests have been extensively discussed in the previous chapters of this thesis. Therefore, there is a need for the development of simple, rapid and easy-to-use tests for TB, especially tests that could be performed in resource-limited settings. Furthermore, identification of host markers that can be easily measured in the blood for the monitoring of TB treatment response will accelerate clinical trials of new drugs for TB treatment. Thus, in the present thesis, the diagnostic potentials of antigen-stimulated and unstimulated host markers, as well as newly described serodiagnostic markers were studied as tools for the future development of field-friendly screening tests for the diagnosis of TB disease in resource limited settings. Additionally, the potential of host markers in tracking early treatment response in active TB patients as a replacement for EBA was also explored.

5.2 Summary of study findings and discussion

In chapter two of this thesis, the diagnostic potential of Rv0081 and three other antigens (Rv1284, Rv2034 and ESAT-6/CFP-10) were evaluated in an overnight whole blood stimulation assay of active TB and non TB participants. The ability of different antigens to elicit host responses was previously explored in our laboratory. In this discovery phase, Chegou *et al*., [3] employed a 7 day whole blood assay to evaluate the diagnostic utility of

118 antigens and found Rv0081 amongst these antigens as the most promising antigen with a diagnostic potential. The performance of this antigen might be associated with its involvement in regulating the bacterial response to hypoxia by regulation of several *Mtb* genes [4]. The potential attraction of an Mtb antigen-stimulated test is that it could theoretically provide more specificity than unstimulated marker measurements, as infection stage specific-antigens could elicit discriminatory immune responses. As immune responses towards individual bacterial antigens varies inter-individually, combination of these proteins holds more potential than using a single marker [5-7]. Of importance in this thesis is the promising ability of the unstimulated host markers, particularly the acute phase proteins, in differentiating active TB from non TB. The short term assay revealed that unstimulated host markers hold more promise for the development of a rapid test for TB. Such a test could be used as a "rule in" or "rule out" test for TB screening in a laboratory-free manner if such tests could be performed on finger-prick blood and on a hand-held device. Such a test will particularly be suitable for the resource limited settings where around 60% of people who develop active TB live with no access to routine sputum microscopy [8].

The serological approach for the diagnosis of TB is premised on the fact that during TB infection there is a strong production of antibody and this has made assays that detect immunologic responses to TB an attractive option to the current diagnostic tools for active TB [11]. Various *Mtb* antigens such as lipoarabinomannan [12], MPT64 [13], 38 kDa antigen [14] and several others [15-18] are being evaluated by these assays in body fluids including serum, plasma and pleural fluids of TB patients in an effort to detect antibodies against these antigens. The assay formats come in varying forms such as rapid immunochromatographic tests and the standard ELISA procedure. LIONEX Diagnostics and Therapeutics, Braunschweig, is a German company that develop such serological tests that is designed solely for use in the rapid detection of active TB. These LIONEX TB diagnostic tests that are available in ELISA forms and rapid immunochromatographic tests measure antibodies against *Mtb* antigens in serum, plasma or pleural fluids. The ELISA test format measure IgA, IgG and IgM. Unpublished data by the manufacturers according to the package insert

showed good results but previous unpublished evaluation of the accuracies of the LIONEX ELISAs by our laboratory in a case control pilot study reported poor sensitivities and specificities. However, improved performances were reported for these ELISAs in other studies [17, 18] mentioned earlier in chapter four of this thesis. This study employed the use of multiple antigens and multiple antibody classes in the evaluation of the accuracies of these ELISAs in African patients who presented with signs and symptoms of TB for the diagnosis of TB. Most serological approaches for the diagnosis of active TB have produced poor and unsatisfactory results [13, 19] while others have reported better performances [20-22]. The result in chapter four of this thesis showed the ability of combination of antibodies particularly IgG and IgA responses against single and multiple-antigen cocktails in correctly classifying the participants into their different respective groups. The performance of the multiple-antigen cocktails used in this study is encouraging and this further demonstrates their usefulness in obtaining higher accuracies in serological assays for the detection of TB. Poor sensitivities and specificities are the hallmark of studies that used single TB antigens especially in smear negative TB compared to higher accuracies in studies that employed antigen cocktails [13, 16]. Of note in this thesis, is the potential usefulness of combining IgG and IgA for TB detection which was highlighted by the identification of a multi-marker model including these two classes of immunoglobulin that resulted in a higher sensitivity and specificity for the correct classification of the participants. The high sensitivity and specificity is a huge diagnostic potential especially when compared to urine LAM test with a poor specificity. As a result of the advances made on T-cell based research and the not too convincing results on serology, one could be tempted to conclude that focus on TB immunodiagnostics research should be on the former.

The main limitation reported in chapter three and chapter four of this thesis is the use of small number of participants although both studies are designed as a pilot study. The accuracy of tests tends to be over or under estimated when small number of samples is used for studies. Future investigations to validate the results should be carried out in a larger group of participants.

Chapter three of this thesis demonstrated changes in the immune profiles of TB patients during 14 days of treatment as part of an EBA study. From the findings in this study, the differential response to single or drug combinations in the 14-day phase II EBA randomized clinical trial resulted in significant changes in the expression of six host markers from D0 to D14 in several treatment arms with CRP showing the greatest difference. The study showed that host marker levels and their changes do not correlate well with CFU and TTP. However, CRP followed changes in microbiological readouts more closely than the other markers. The role of immune markers in assessing ultimate treatment outcome remains uncertain. There is a need to develop new strategies to test new anti-TB drugs and to monitor TB treatment. Currently, the standard TB treatment plan does not allow patient stratification into different treatment groups and as a result patients who are at an increased risk of poor treatment outcome may be receiving insufficient treatment regimens while others who are not at risk receive prolonged treatment regimens. Stratifying TB patients into different risk groups at diagnosis according to their risk for poor treatment response could allow treatment shortening in a substantial percentage of patients even on current drugs, but we have currently no way of identifying such patients [9]. Although EBA plays an important role in the evaluation of new TB drugs, EBA does not predict sterilizing activity and merely represents drug action on a subpopulation of bacteria that are rapidly killed [10]. The effect on populations with slower replication is not captured and it is unknown if host markers can play an informative role in this regard.

5.3 Main study implications and future studies

The findings in this thesis that evaluated the abilities of host markers and *Mtb* specific-antigens in discriminating between active TB and non TB showed that the combination of markers had an improved performance compared to single markers. The serological evaluation used antigen-cocktails in differentiating active TB from LTBI and ORD with a promising result. This is a further prove that optimal sensitivity and specificity could only be attained when markers or antigens are combined. Considering the fact that serological

assays could easily be developed into a rapid, low cost and simple tests that are suitable for use in poor resource settings it is pertinent for more work on the development of such tests. These should include evaluation of more novel antigen candidates and particularly multiple-antigen cocktails. There is a growing body of evidence to support this concept [3, 5-7, 18, 23]. Although unable to discriminate between active TB and LTBI, the commercially available IGRAs still uses a synthetic cocktail of mycobacteria antigens. The implication of this is that in our search towards developing a simple, rapid test to discriminate TB from LTBI emphasis should be placed on using combined markers or antigens rather than employing single ones. The conventional ELISA procedure can be simplified further into a rapid strip test based on lateral flow technology suitable for resource-constraint settings using harvested overnight cultured supernatants or *ex vivo* samples such as serum or plasma. The development of such tests into a rapid field-friendly by combining markers is gradually gaining attention as described by Corstjens *et al*; [24] and Bobosha *et al*; [25] where they demonstrated the usefulness of a robust, low tech upconverting phosphor lateral flow assay (UCP-LFA) technology for the detection of TB and leprosy respectively. The performance of this assay in comparison with the ELISA look promising and this can be further developed for suitability in resource limited settings. Additionally, ScreenTB is a consortium of six African countries that will also be carrying out a large prospective evaluation of a six-marker signature UCP-Transdot test for point-of-care (POC) TB diagnosis. Their mission is to develop a POC, hand-held, rapid screening test for TB conducted on finger-prick blood in a laboratory free manner. Developing such a test could revolutionize the landscape of TB diagnosis as many of the current and novel diagnostic tools are still sputum-based and have a poor sensitivity in paucibacilliary TB, sputum-negative pulmonary and extrapulmonary TB [26-28]. According to WHO in an attempt to define the needs for TB assays for the future, a non sputum-based test besides a triage, rapid and biomarker test needs to be given more attention.

Future studies should explore the possibility of combining the promising antibodies and host markers results as a means of enhancing the performance of this test and this prospect is presently being investigated by an MSc student in our group. Previous study combining

cytokine and antibody diagnostic algorithm for the detection of TB showed an enhanced diagnostic accuracy compared to other conventional methods for diagnosis [29]. Besides protein multiplex assays, further investigations should evaluate potential biomarkers at the gene expression level for the discrimination of TB in the different infection states. A group of Scientists have shown that blood transcriptional signatures differentiated TB from other diseases in African adults who are HIV-infected and uninfected [30]. Supernatants or whole blood from stimulation with *Mtb* specific-antigens and/or PBMC stimulated with *Mtb* specific-antigens might help in identifying candidate biosignature genes. The differential expression of these genes might reflect the degree of changes in the *Mtb* infection state. This approach is being undertaken by a student in our group for her MSc degree.

The field of biomarker is growing very fast and it offers the opportunity to improve on patients' health. Furthermore, in the search for new biomarkers in infectious diseases, there is necessity to embrace the use of non-biased approaches such as the mass spectrometry based proteomics, the leading technology in the field of "omics" approaches including genomics, lipidomics, metabolomics and transcriptomics. The high throughput "omics" technologies can change the face of the rapidly growing biomarker field.

5.4 General conclusion

The results presented in this thesis indicate that host biomarkers have potential as diagnostic tools for TB disease. Although there was no added accuracy achieved with *Mtb* antigen stimulated host markers, these markers could serve as adjunctive markers in TB diagnosis. Serodiagnostic markers showed promise against novel *Mtb* antigens but such markers have to be adapted into simpler formats such as the lateral flow technology for suitability at point-of-care testing especially in resource poor settings. Additionally, CRP could be a valuable adjunct in monitoring TB therapy if measured early. However, further evaluation of the validity of these results are needed in large-scale studies to include children, extrapulmonary TB and immunocompromised patients before field-friendly

diagnostic tests in formats designed for use in high TB burden and poor resource settings are developed.

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