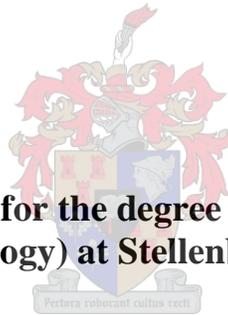


Development of immune-based TB tests suitable for resource limited settings

By

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**Dissertation presented for the degree of Doctor of Philosophy
(Molecular Biology) at Stellenbosch University**

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December 2014

DECLARATION

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Abstract

Background

Tuberculosis (TB) is still one of the leading causes of death in poor socio-economic settings. This situation is encouraged by the lack of simple and rapid tests suitable for rapid diagnosis. The newly developed Interferon-gamma Release assays (IGRAs) can detect *Mycobacterium tuberculosis* (*M.tb*) infection but fail to discriminate active TB from latently infected individuals.

Objectives

The present thesis aims to develop a rapid and simple test for the diagnosis of active TB disease. This objective was divided into four sub-objectives: 1) identification of potential *M.tb* antigens and host markers suitable for a TB test using a 7-day whole blood assay (WBA), 2) validate the promising results in an overnight WBA for a rapid, albeit not *ex vivo*, test, 3) evaluate the diagnostic utility of a two colour ELISpot test, 4) use an unbiased approach to discover multiple new host markers with diagnostic utility using mass spectrometry.

Methods and results

Participants were recruited from the Ravensmead/Uitsig community and day clinics. Stimulated and unstimulated analyte levels in 7-day and overnight WBA supernatants from active TB cases were compared to analyte levels in controls. The results of these experiments showed that Rv0081-stimulated levels of IP-10, IL-12p40, TNF- α and IL-10 were the most promising diagnostic markers in the long term assay as they could correctly classify 100% of the study participants in this assay. Acute phase proteins mainly CRP and SAA were the best diagnostic antigens in the short term assay. The diagnostic utility of these markers was greater in Quantiferon Nil supernatants compared to the stimulated samples.

IFN- γ and IL-2 ELISpot was performed where it was found that single cytokine measures could not discriminate active TB to latent infection. When single and double secreting cell populations were taken into consideration, a combination model of ESAT6/CFP10-stimulated single IFN- γ , single IL-2 and IFN- γ /IL-2 double secreting cells could classify participants into their clinical groups with good accuracy.

In a pilot study for future discovery of diagnostic markers by mass spectrometry, three depletion methods (ProteoSpin column, Heparin column and ProteoPrep 20) were assessed to identify the most appropriate depletion method for high abundant proteins from serum. The depleted serum samples were analysed in Orbitrap Velos. The antibody based method, ProteoPrep 20, was the best depletion method as it led to the visualisation of a larger number of proteins on Orbitrap.

Conclusion

M.tb antigen-stimulated host markers hold promises in diagnosis of active TB disease. The excellent accuracy observed in the long term assay could not be repeated in the short term assay. Acute phase proteins are the most promising but perform better in unstimulated than in stimulated supernatants and should be evaluated in *ex vivo* samples like serum or plasma. However, it is likely that further unbiased proteomic approaches, like mass spectrometry, will identify additional promising markers that will allow the development of *ex vivo*, accurate, point-of-care tests for TB.

Abstrak

Agtergrond

Tuberkulose (TB) is steeds die hoof oorsaak van meeste sterftes in behoeftige gebiede wêreldwyd. Hierdie situasie word aangemoedig deur die gebrek aan 'n eenvoudige en vinnige diagnostiese toets wat spesifiek toepaslik is vir hierdie gebiede. Die nuut ontwikkelde 'Interferon Gamma-Release' (IGRA) toetse kan met uitstekende akkuraatheid 'n *Mycobacterium tuberkulose* (*M.tb*) infeksie opspoor, maar is ondoeltreffend om tussen aktiewe TB en sluimerende *M.tb* infeksie in die mens te onderskei.

Objektiewe

Die huidige tesis het ten doel om 'n vinnige en eenvoudige toets vir die diagnose van aktiewe TB te ontwikkel. Hierdie doelwit is in vier sub-doelwitte verdeel: 1) identifikasie van potensiële *M.tb* antigene en gasheer merkers wat geskik vir 'n TB-toets deur gebruik te maak van 'n 7-dag vol bloed toets (WBA), 2) evaluasie van die toepaslikheid van hierdie potensiële merkers en antigene in 'n oornag WBA, vir die ontwerp van in 'n direkte toets, 3) beoordeling van die diagnostiese waarde van die twee-kleur ELISpot, 4) Assessering van die diagnostiese doeltreffendheid van verskeie gasheer merkers deur gebruik te maak van massaspektrometrie.

Metodes en Resultate

Deelnemers is gewerf vanuit die Ravensmead / Uitsig gemeenskap en klinieke. Gestimuleerde en ongestimuleerde analiet vlakke in 7-dag- en oornag WBA supernatante van aktiewe TB-pasiënte is vergelyk met analiet vlakke in ooreenstemmende kontrole groepe. Resultate van hierdie eksperiment het getoon dat vlakke van IP-10, IL-12p40, TNF- α en IL-10 in antigeen Rv0081 gestimuleerde supernatante, die mees belowende diagnostiese merkers in die lang termyn toets is. Hierdie merkers kon met 100% akkuraatheid die studie deelnemers klassifiseer. Akute fase

proteïene, hoofsaaklik CRP en SAA, is aangewys as die beste diagnostiese merkers in die kort termyn toets. Die diagnostiese waarde van hierdie merkers was meer omvangryk in Quantiferon Nil supernatante in vergelyking met dié van WBAs.

IFN- γ /IL-2 twee-kleur ELISpot is uitgevoer volgens die vervaardiger se instruksies. Direkte vergelyking het aangetoon dat die kol-vormende eenhede vanaf individuele sitokien produserende selle, nie kan diskrimineer tussen aktiewe TB te latente *M.tb* infeksie nie. Alhoewel, indien beide enkel-en dubbel sitokien produserende sel populasies in ag geneem word, kan 'n kombinasie van die model ESAT6/CFP10-stimuleerde enkel IFN- γ , enkel IL-2 en IFN- γ /IL-2 dubbel produserende selle, deelnemers klassifiseer in hul kliniese groepe met goeie akkuraatheid.

Drie metodes (ProteoSpin kolom Heparien kolom en ProteoPrep 20) is gebruik om oorvloedige serum proteïene te vernietig, waarna die diagnostiese nut van sirkulerende serum merkers deur middel van massaspektrometrie bepaal is. Analise van die serum monsters met behulp van die Orbitrap Velos, het aangetoon dat die teenliggaam metode, ProteoPrep 20, die mees suksesvolle metode is, aangesien dit gelei tot die visualisering van 'n groter aantal proteïene op die Orbitrap.

Gevolgtrekking

M.tb antigeen gestimuleerde gasheer merkers toon groot potensiaal in die diagnose aktiewe TB. Die uitstekende diagnostiese akkuraatheid wat waargeneem is in die lang termyn toets kon egter nie met dieselfde graad van akkuraatheid in die kort termyn toets herhaal word nie. Akute fase proteïene is bewys as die mees belowende ongestimuleerde merkers in die kort termyn toets. Daarbenewens verhoog die diagnostiese waarde van akute fase proteïene aansienlik wanneer gemeet word in Quantiferon supernatant in vergelyking met vol bloed supernatant.

ACKNOWLEDGEMENTS

The studies reported in this thesis would not have been possible without the contribution of many individuals. I am grateful to our clinical team including the study nurses for recruitment and characterization of the participants enrolled in the studies described in this thesis. I am grateful to my supervisor, Prof. Gerhard Walzl for the chance he has gave me, all the support, encouragement and guidance throughout all the years that I have been in the laboratory. I am also grateful to all the members of the Immunology Research Group for the friendliness, co-operation and their support in difficult time: André Loxton, Khutso Phalane, Browyn Smith, Andrea Gutschmidt, Nelita Du Plessis. I am deeply grateful to Novel Chegou my co-supervisor for all the useful technical support.

I am grateful to our collaborators in Bostel (Germany) Dr Barbara Kalsdorf and Pr Christoph Lange for all the help and support during the evaluation of the two colour ELISpot. I also want to thank AID Autoimmun Diagnostika GmbH, and in particular the late Dr Volkmar Schoellhorn, for his enthusiastic support, including the financial, equipment and reagents provided during the ELISpot study.

Finally, I am grateful to my family both here in South Africa (my lovely daughter “Faith Annie Essone” and my wife “Grace Essone” and back in Gabon (especially all my sisters and brother, my father and mother) for all the encouragement and support during difficult times.

During the course of my studies at universities (in Gabon as well in South Africa), I received financial assistance from “Agence National des Bourses du Gabon (ANBG)”, I am also thankful for the financial support I received from my laboratory during the course of this PhD. The antigens used in Chapter III and IV were part of the African European Tuberculosis Consortium (AE-TBC). This consortium was sponsored by EDCTP. With Exception of the dual colour ELISpot study, this thesis was financially supported by AE-TBC.

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LIST OF ABBREVIATIONS

ACN	:	Acetonitrile
AE-TBC	:	Africa European- TB Consortium
AFB	:	Acid Fast Bacilli
AID	:	Autoimmun Diagnostika
APCs	:	Antigen presenting cells
AUC	:	Area Under the operating Characteristic Curve
BCG	:	Bacillus Calmette–Guérin
CFP10	:	10 kDa culture filtrate antigen
CRP	:	C-reactive proteins
Da	:	Daltons
DCs	:	Dendritic cells
DosR	:	Dormancy-related
DOTS	:	Direct Observed Treatment, Short-course
EGF	:	Epidermal Growth Factor
ELISA	:	Enzyme-Linked Immunosorbent Assay
ESAT6	:	6 kDa Early Secretory Antigenic Target
GDA	:	General Discriminant Analysis
HHCs	:	Household Contacts
IFN- γ	:	Interferon-gamma
IGRAs	:	Interferon Gamma Release Assays
IL	:	Interleukin
IP-10	:	Interferon-gamma-inducible protein 10
IQR	:	Interquartile Ranges
LTBI	:	latent TB infection

LUMC	:	Leiden University Medical College
<i>M.tb</i>	:	<i>Mycobacterium tuberculosis</i>
MARS	:	Multi Affinity Removal Systems
MCP -1	:	Monocyte Chemotactic Protein-1
MHC II	:	Major Histocompatibility Complex Class II
MIP-1 α	:	Macrophage Inflammatory Protein-1 α
MMP-2	:	Matrix Metalloproteinase-2
MS	:	Mass Spectrometry
MTP-65	:	Microsomal Triglyceride transfer Protein
MWCO	:	Molecular-Weight Cutoff
NTB	:	Non-Tuberculosis diseases
OD	:	Optical Density
PBMCs	:	Peripheral Blood Mononuclear Cells
PHA	:	Phytohaemagglutinin
PI	:	Isoelectric Point
PPD	:	Purified Protein Derivative
QFT IT	:	QuantiFERON® TB Gold In Tube
RD1	:	Region of Difference-1
ROC	:	Receiver Operating Characteristics
RPF	:	Resuscitation-Promoting Factors
SAA	:	Serum Amyloid A
SAP	:	Serum Amyloid P
sCD40L	:	Soluble CD40 ligand
SFCs	:	Spot forming cells
SSI	:	Statens Serum Institute

TB	:	Tuberculosis
T _{CM}	:	Central memory T cells
T _{EM}	:	Effector memory T cells
T _{ET}	:	Effector T cells
TGF- α	:	Transforming Growth Factor-alpha
TNF- α	:	Tumor Necrosis Factor-alpha
TST	:	Tuberculin Skin Test
VEGF	:	Vascular Endothelial Growth Factor
WBA	:	Whole Blood Assay
WHO	:	World Health Organisation

1. Chapter I

Toward the development of immune diagnostic test for the diagnosis of active TB

1.1. Background

Introduction and expansion of the Direct Observed Treatment, Short-course (DOTS) strategy worldwide has been a successful public health intervention. Implementation of this policy has permitted millions of tuberculosis (TB) patients to receive correct treatment saving millions of lives [1]. DOTS has been implemented in several countries and permitted many of them to reach the 70% case detection and 85% of cure rate. Although implementation of DOTS guidelines could claim a number of advantages for the management of this pandemic, TB remains a serious public health problem mainly in developing countries. The year 2011 alone had registered an estimate number of 8.7 million new cases and 1.4 million people died from this infection [2]. *Mycobacterium tuberculosis* (*M.tb*) is the causative agent of this disease. The host is generally infected through inhalation of bacteria-containing droplets, which are liberated by individuals with active pulmonary TB after coughing [3]. Most infected individuals will contain the infection as latent TB infection (LTBI) with no clinical sign of the disease. Diagnosis of LTBI remains challenging with the absence of a sensitive reference standard test. Moreover, sputum based tests including Acid Fast Bacilli staining (AFB), *M.tb* culture methods and GeneXpert are totally inappropriate for LTBI as this infection state does not yield bacteria in sputum samples. The diagnosis of LTBI completely relies on immune challenge tests namely the Tuberculin Skin Test (TST, an *in vivo* test) and Interferon Gamma Release Assays (IGRAs) (*ex vivo*). Sensitivity and specificity of these immune tests are particularly difficult to estimate with the absence of a gold standard test for LTBI as mentioned earlier.

Among the LTBI infected individuals, only a reduced fraction (5 to 10%) will develop active TB. These new cases will represent new sources of infection for their community members.

Early, accurate and affordable point of care test could represent a vital improvement in the management of this disease and considerably reduce TB incidence. The current review aims to discuss the different limitations of the tests currently in used before discussing the potential of host marker-based tests and the different techniques employed towards developing such tests.

1.2.Sputum based tests

Sputum microscopy is the oldest test for active TB and has been used for more than 100 years. It had contributed to the case detections of actively infected patients worldwide and is still the main test in low-income and some middle-income countries, where 95% of TB cases and 98% of death due to this disease occur [4]. In socio-economically poor areas, the direct smear method with Ziehl-Neelsen staining is mostly employed [5]. It is a special bacteriological stain method used to identify acid-fast bacilli. Acid fast organisms like *M.tb* contain large amounts of mycolic acids within their cell walls. Acid-fast bacilli will be bright red after staining and stand out against the blue background of the smear. This method is relatively inexpensive, fast and simple to perform. In addition, this test has been proven by many independent studies to be highly specific to *M.tb* even in high endemic areas, although non-tuberculous bacteria and some other acid-fast bacteria cannot be distinguished from *M.tb*. Unfortunately, this high specificity is usually followed by a moderate sensitivity [6]. Alternative sample processing methods like bleach sedimentation, bleach centrifugation and the introduction of fluorescence microscopy have been proposed to improve the sensitivity of this rapid test. Unfortunately, their early promising results have not been confirmed and they may constitute a drawback for this simple test due to their cost and technical requirement [6-8].

M.tb culture has been endorsed by the World Health Organisation (WHO) as a gold standard in TB diagnostic [2]. Indeed, this method has an excellent sensitivity and specificity and could lead to the detection of drug resistance TB through the implementation of drugs susceptibility testing after bacteria growth on solid or liquid culture medium [9, 10]. The implementation of this method at clinical level faces a number of challenges: the main challenge is the time-lapse between the test and the results (up to 6 weeks); this important window does not permit the management of the disease in a time-effective manner and therefore favours the spread of the

disease. The second important limitation of this test is the requirement of a specialized and dedicated microbiology laboratory that is not always available in resource-limited settings [11, 12]. The mere growth of bacteria on either agar or in liquid culture does not necessarily mean the presence of *M.tb* but requires that contamination by other bacteria has to be ruled out and that speciation with antibody-based tests like Capilia or by PCR is required [13].

Nucleic acid amplification assays are commonly used in developed countries for a rapid identification of *M.tb* complex in clinical specimens [14]. Although this method provided satisfactory results in these countries, it did not gain popularity in low-income and middle-income countries mainly due to the high risk of cross contamination, as well as the cost and technicality associated to this test [15]. The recent automated real-time sputum processing molecular beacon assay, XpertMTB/RIF (Cepheid Inc., CA, USA) had been developed and constitutes a breakthrough in the TB diagnostic field. This test can be performed with minimum training [14, 16] and does not require a special *Mycobacteria* laboratory. More importantly, XpertMTB/RIF could potentially allow a single visit test approach due to its rapid performance (results within 2 hours) with sensitivity and specificity above 90% in smear positive TB cases [17]. The introduction of this instrument has been welcomed in the field and was endorsed by WHO with rapid implementation in 2010. The enthusiasm surrounding the introduction of this new tool has led to the discovery of its limitations: the cost-effectiveness constitutes the major drawback of this technique; in some laboratories in high endemic areas where thousands of samples are received daily it may require significant investments on buildings, instruments and maintenance. A South African study showed a 50% increase on TB diagnostic cost when sputum smear microscopy was replaced by XpertMTB/RIF [18]. It has also been reported by WHO that around 60% of people developing active TB lives in areas where laboratory infrastructures or laboratory budgets do not allow the routine performance of simple and relatively inexpensive tests like sputum smear microscopy [19] therefore, implementation of a new method at higher cost and technically more challenging may not have a bright future. This lack of cost effectiveness may explain the slow implementation of this technique even in the developed world: 53% of cartridges procured globally are found in South Africa [2].

A combination of smear microscopy and XpertMTB/RIF could be an interesting diagnostic approach; it will save extra XpertMTB/RIF cost for smear positive patients. But the low sensitivity of XpertMTB/RIF (69-72%) on smear negative patients may complicate the

interpretation of XpertMTB/RIF negative results [19] in this type of setting. Studies evaluating the accuracy of XpertMTB/RIF on children have also reported a moderate sensitivity of this method [20] on this particular population. Owing to these limitations, implementation of a rapid, cheap and affordable test is needed. A serum based test could be advantageous due to the numerous benefits offered by this body fluid.

1.3. Granuloma formation and persistence

TB patients transmit the bacilli through minute droplets by cough, expectoration and even during speaking [21]. The households or community members are infected by the aerosol route where the lung becomes the favoured site of disease manifestation [21].

Once in the lung, *M.tb* is taken up by alveolar macrophages through a mechanism named phagocytosis. This mechanism will lead to the formation of a new intracellular organelle called phagosome. Fusion of the phagosome with a lysosome will lead to the formation of the phagolysosome and *M.tb* antigen presentation on the major histocompatibility complex Class II (MHC II) [21, 22] molecules. The first hours following monocytes/macrophages infection by *M.tb* is characterized by increased levels of cytokines and chemokines, eventually resulting in the formation of a dynamic multicellular aggregate named granuloma [23, 24].

The newly formed granuloma will essentially be composed of monocytes/macrophages, dendritic cells (DCs), B cells and T cells [22, 25]. The inner cellular layer of granuloma will be dominated by infected and uninfected monocytes/macrophages, DCs, neutrophils and CD4⁺ T cells [25]. This inner structure will play an important role in immune defence against TB as antigen presenting cells (APCs) including monocytes/macrophages and DCs will continuously interact with CD4⁺ T cells and permanently enrich their surroundings and the bloodstream with newly made analytes, like mediators of inflammation. The specific immune response to *M.tb* is primary directed by CD4⁺ T cells, particularly the cells in the CD4⁺ T cell/APCs-rich inner layer of the granuloma. The typical granuloma could be described as a well-organized rim of APCs, T cells and B cells surrounding a central necrotic core. Some bacteria may be localised in the necrotic core but most bacteria are located between the CD4⁺ T cells-APCs rich inner layer and the necrotic core [22, 26-28]. It has been shown that as the disease progresses, the granulomas tend to lose their organized structure with the formation of cavities [26] (Figure 1.1). These changes at

tissue level might be accompanied by measurable changes and unique cytokine bio signatures that might be relatively specific to TB disease.

While research on granulomas reveals interaction between *M.tb* and immune cells, identification of stage-specific peripheral blood immune markers suitable for diagnostic should be considered. Thus, it is important to understand the correlation between the infection site and the peripheral blood. Granulomas are highly vascularised structure, creating a reliable connection between the infection site and the blood stream [27, 29, 30]. Thus, newly synthesized markers might easily be measurable in circulating plasma or serum.

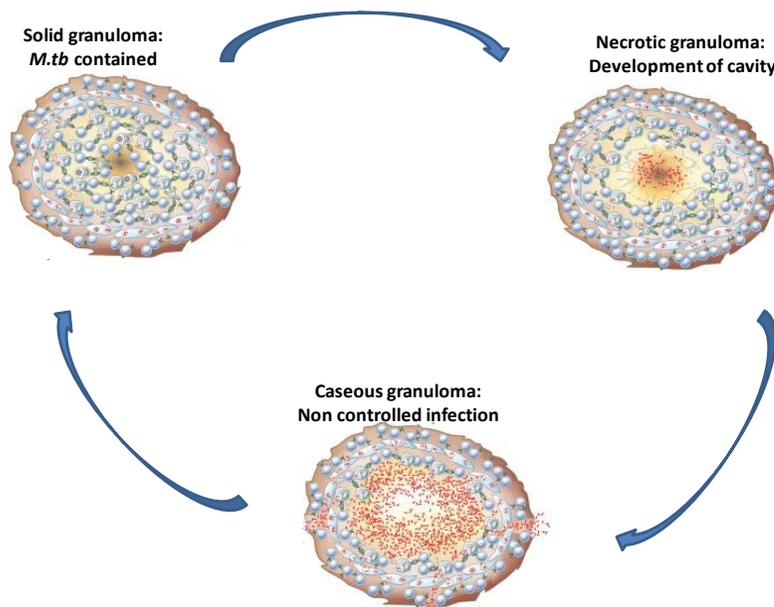


Figure 1.1: Representation of three granuloma steps

Granuloma is the principal milieu of *M.tb*-T cells interaction. Three phases of granuloma are represented here. **Solid granuloma:** is a structured granuloma with the absence of central necrosis and may represent the ability to contain the infection. **Necrotic granuloma:** this stage is characterized by the development of a necrotic cell death region where bacteria and macrophages are usually detected in close proximity and may represent the development of disease. **Caseous granuloma:** Destruction of granuloma organization with liberation of bacteria in the lung resulting in clinical manifestation of the disease. This figure was adapted from reference [26].

1.4. Immune markers for TB diagnosis

Each CD4⁺ T lymphocyte has a unique T cell receptor capable of recognising a unique antigen fraction presented on MHC II molecule. This specific recognition is obtained after VDJ genes rearrangement [31]. After the first antigen-naïve T cell encounter event, T cells undergo rapid proliferation leading to the formation of multiple daughter cells. These newly generated daughter cells will soon release a number of surface and soluble markers in response to antigens. Many of these markers could be disease state specific.

Immunological markers should best discriminate the 3 disease states: uninfected, latently infected and the active disease state. Immunological events are central for the disease outcome since they are responsible of tissue damage and protection. Thus, a specific immunological event may be associated to a specific *M.tb* infection state. Investigation of *M.tb* antigen-stimulated T cell marker secretion associated to a unique disease state in this thesis has been largely dominated by *ex vivo* interrogation of peripheral blood immune cells [32]. These interrogations were done following 3 main study designs: i) **Case-control study**: a primary approach considered as a discovery stage where TB cases and their matched or unmatched controls are recruited from the same ethnic group and community [33], ii) **Cross sectional study**, which presents many similarities to the case-control approach but includes all TB cases diagnosed at a specific point of care(s) during a determined period of time [34]. In both studies, controls could represent household contacts (person living in the same household with a TB patient for a prolonged period of time) or a community member, and iii) **Prospective study** is considered as the most appropriate diagnostic study design. All participants are enrolled as TB suspects at the same point of care(s). Samples are processed and stored as a uniform group reducing any biased results. When sputum, X ray and *M.tb* culture results are made available, samples are divided into two distinct groups: confirmed active TB cases and Non-Tuberculosis diseases (NTB), also called alternative pulmonary diseases as all these participants had cough for more than two weeks.

During the *ex vivo* interrogation, the whole blood or the peripheral blood mononuclear cells (PBMCs) are incubated overnight (short-term assay) or for 7 days (long-term assay) in presence of *M.tb* bacteria (live or attenuated) or *M.tb* antigen(s) [35]. Long-term assays have been used in

a number of diagnostic studies with promising results. Indeed, Shuck and colleagues have demonstrated that a “7-day re-stimulation assay” could lead to the detection of *M.tb* antigen-stimulated IFN γ previously undetected in short-term assay using flow cytometry [36]. By measuring *M.tb* antigen-stimulated IFN γ levels in blood supernatant, our group has also shown the importance of long-term assay in antigen selection [37].

Long-term assays may improve antigen recognition by the host and antigen-stimulated T cells characterisation but may not be suitable in the diagnostic field. A good diagnostic test must yield results in a relatively short time frame to facilitate early treatment and limit the spread of the disease. For these reasons the long-term assay was only used in a discovery setting to select promising antigens or promising host markers in the present thesis. These promising diagnostic biomarkers must subsequently be evaluated in a shorter-term assay for the development of a diagnostic test. The following sections will concentrate on antigen-stimulated and unstimulated marker research and the different techniques employed in biomarkers research.

1.5. Diagnostic utility of T cell sub-populations

Flow Cytometry has been a useful platform to characterise immune cells in response to bacterial and viral infections. Using this platform, many studies have successfully classified antigen-experienced and naïve cells according to their cells surface markers [38, 39]. The earlier stage of differentiation (or naïve stage) is associated with the expression of the majority of the following surface markers: CD45RO/RA, CD62L, CD127, CD27, CD28, CD7 and CCR7 and the advanced stage of differentiation (antigen-experienced T cell) loses the expression of a number of surface markers including CD62L, CD127, CD28, CD7 and CCR7[40-42]. Antigen-experienced T cells are usually labelled memory cells or effector cells according to their cell surface markers and secreted cytokines. The term “memory cells” is usually attributed to any antigen-specific T cells that remain after complete removal of an infectious agent and retraction of immune response [43]. In the case of persistent infections like TB, where the immune system fails to eradicate the infection, the antigen-experienced T cells are often labelled memory cells [43]. In these conditions, memory cells have been further divided into Central memory T cells (T_{CM}), and Effector memory T cells (T_{EM}). Researchers mostly use CD45A, CCR7 and CD127 to identify the three antigen-experienced T cells sub-populations: T_{CM} ($CD45RA^{-}CD127^{+}CCR7^{+}$),

T_{EM} ($CD45RA^-CD127^{+/-}CCR7^-$) and Effector T cells (T_{ET}) ($CD45RA^+CD127^-CCR7^-$) [43-45] (Figure 1.2, Table 1.1).

A series of functions are associated with T cell sub-populations and can be used together with phenotypic markers for a better characterisation. Indeed, cell secretions have also been adopted by many authors to classify T cells populations [43, 46]. Pantaleo and colleagues have shown that IFN- γ and IL-2 are the most relevant secreted cytokines to define antigen-experienced T cell sub-populations [47]. By classifying cell populations according to their ability to produce these cytokines, three major clusters of antigen-experienced T cells can be distinguished: single IFN- γ , single IL-2 and dual IFN- γ /IL-2 producing cells [48, 49].

IFN- γ plays a crucial role in effector response to *M.tb* infection due to its involvement in key functions like macrophage activation [50, 51]. Single IFN- γ secreting cells have been associated with effector cell phenotypes with poor proliferative ability. The secretion of IL-2 by antigen-experienced T cells in situations with low or reduced bacterial load may reflect its role in the termination of T cell responses. Single IL-2 producing cells have been associated with central memory phenotype and dual IFN- γ /IL-2 secreting cells have been associated with the effector-memory phenotype [48, 49, 52]. Investigations in the TB field have shown that antigen-experienced T cells are mostly effector cells in actively infected patients whereas the immune response in LTBI infected individuals is dominated by central memory cells [25, 32, 53, 54]. The phenotypic distribution of antigen-experienced T cells populations in actively infected individuals is considerably reversed during the course of TB therapy. Indeed, several authors have shown an increase of central memory cells and decrease of effector cells in successfully treated patients [32, 52, 53]. These results suggest that the relative frequency of memory and effector cells could be a reliable indication of antigen burden and consequently be the source of a TB diagnostic or treatment monitoring test. Although flow cytometry is highly informative, the technicality and the cost associated to this method do not allow its introduction in the diagnostic field mainly at peripheral health post level. A simple method capable of detecting distinct T cells population is needed.

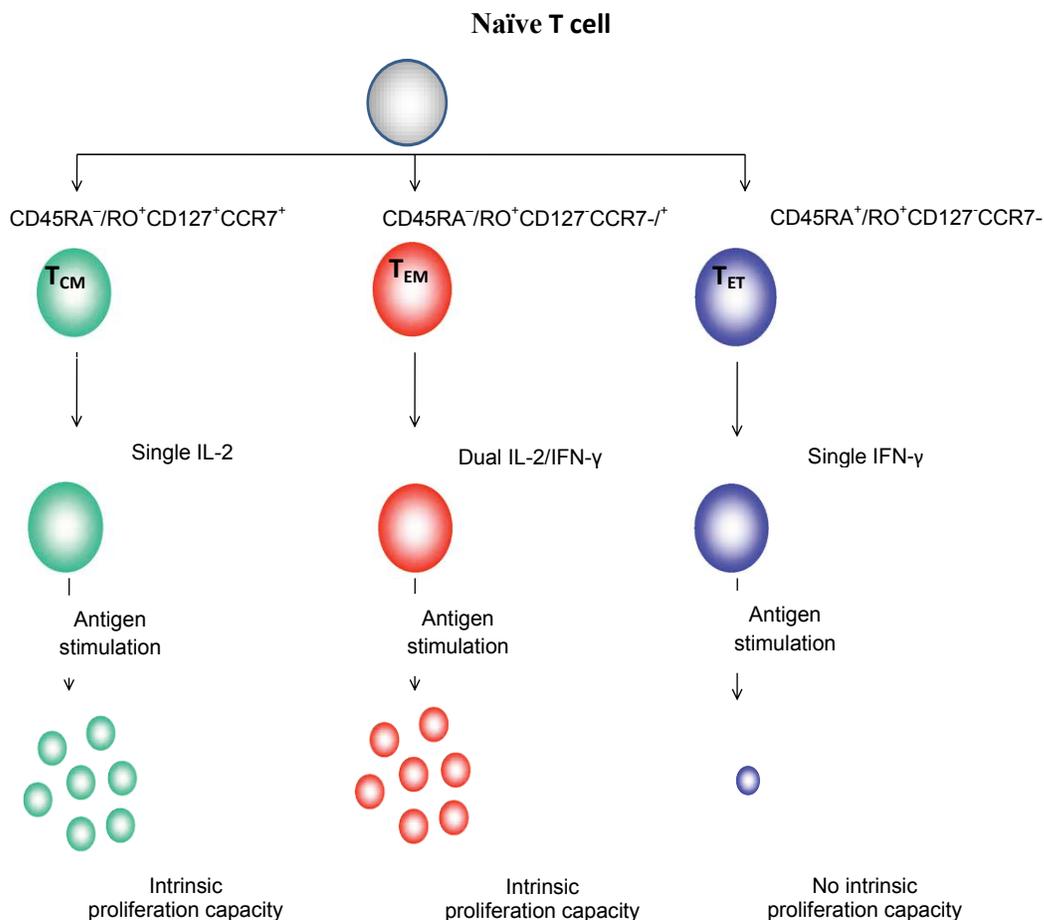


Figure 1.2: Phenotypically and functionally distinct antigen-experienced T cell populations

Circulating Naïve T cells have the ability to differentiate to three principal antigen-experienced T cell populations after their antigens encounter event. These three populations are phenotypically and functionally distinct: T-central memory cells (T_{CM}) are characterized by their CD45RA⁻/RO⁺CD127⁺CCR7⁺ phenotype and are mostly IL-2 single producing cells. T-effector memory cells (T_{EM}), CD45RA⁻/RO⁺CD127⁻CCR7^{-/+}, are mainly dominated by IL-2/IFN- γ double producing cells. T_{CM} and T_{EM} maintain their proliferation capacity compared to the terminally differentiated T-effector cells (T_{ET}) (CD45RA⁺/RO⁺CD127⁻CCR7⁻).

Table 1.1: Surface markers and infection

Circulating Naïve T cells have the ability to differentiate to three principal antigen-experienced T cell populations after their antigens encounter event. These three populations are phenotypically and functionally distinct. The following markers are usually used for their identification: CD45RA, CD45RO, CD127, CCR7, IL-2 and IFN- γ . This table summaries cells secreted cells and functions of these markers.

Marker	Class	Secreted cell	Function
CD45	Cell surface marker	leucocytes	Immune defence
CD45RA	Cell surface marker	naive T cells (CD4+ or CD8+)	Specific immune defence
CD45RO	Cell surface marker	Memory T cells (CD4+ or CD8+)	Specific immune defence
CD127	Cell surface marker	T cells	Cell activation and proliferation
CCR7	Cell surface marker	T cells	control the migration of memory T cells home to secondary lymphoid organs
IL-2	Cytokine	T cells	Promote growth, proliferation, and differentiation of effector T cells
IFN- γ	Cytokine	T cells	Activation of macrophages

1.6.Dual colour ELISPOT

The enzyme-linked immunospot (ELISPOT) assay is a simplified assay able to identify specific cytokine secreting cells. In the particular field of TB, the assay is generally associated with IFN- γ secreting cells. Introduction of IGRAs has improved the screening of latently infected individuals. The success observed in LTBI has encouraged researchers to investigate the utility of these techniques in active TB. Even though some studies have shown a higher IFN- γ level in active TB cases, many discordance results have been demonstrated [55-58]. These results could be improved through the analysis of specific cell populations. In this way, effector cell populations in TB cases could be compared to the same population in LTBI. ELISPOT has become a powerful instrument for the analysis of T cell responses in diseases as well as in vaccine trials [59]. This method is relatively simple to perform with high sensitivity, high reproducibility and a very low background. Its performance could be compared to competing

methods including intracellular cytokine staining. ELISPOT is limited by the fact that only one protein secreting cell could be analysed per assay.

The dual colour ELISPOT opens the opportunity to assessed more than one distinct cell population simultaneously with the same accuracy obtained with ELISPOT [59]. The dual colour ELISPOT is an ELISPOT based technique, but facilitates the analysis of several cell populations from the same sample. The most standardized and optimized dual colour ELISPOT is the FluoroSpot assay (AID, Germany). The FluoroSpot is further simplified by the use of an automatic reader system (EliSpot reader) that reduced considerably variations between readers; AID EliSpot software is an automated counting program optimized to detect, quantify and attribute a specific colour to a specific cell population. In the case of IL-2/IFN- γ FluoroSpot, the AID reader captures the Cy-3/IL-2 signal and the FITC/IFN- γ signal and overlays both images to create an artificial image highlighting the double-stained spots (Figure 1.3). To differentiate between the single producing cells and the double producing cells, several parameters including size, intensity and circularity could be adjusted. By colour labelling of the individual cytokines and dual cytokine producers, different cell population could be analysed and therefore the diagnostic utility of different T lymphocyte populations could be assessed. In this model and as reviewed above, effector cells will be referred to as IFN- γ single producing cells (total IFN- γ producing cells – double cytokine producing cells), central memory cells will be referred as IL-2 single producing cells (total IL-2 producing cells – double cytokine producing cells) and effector memory cells will be represented by the double producing cells.

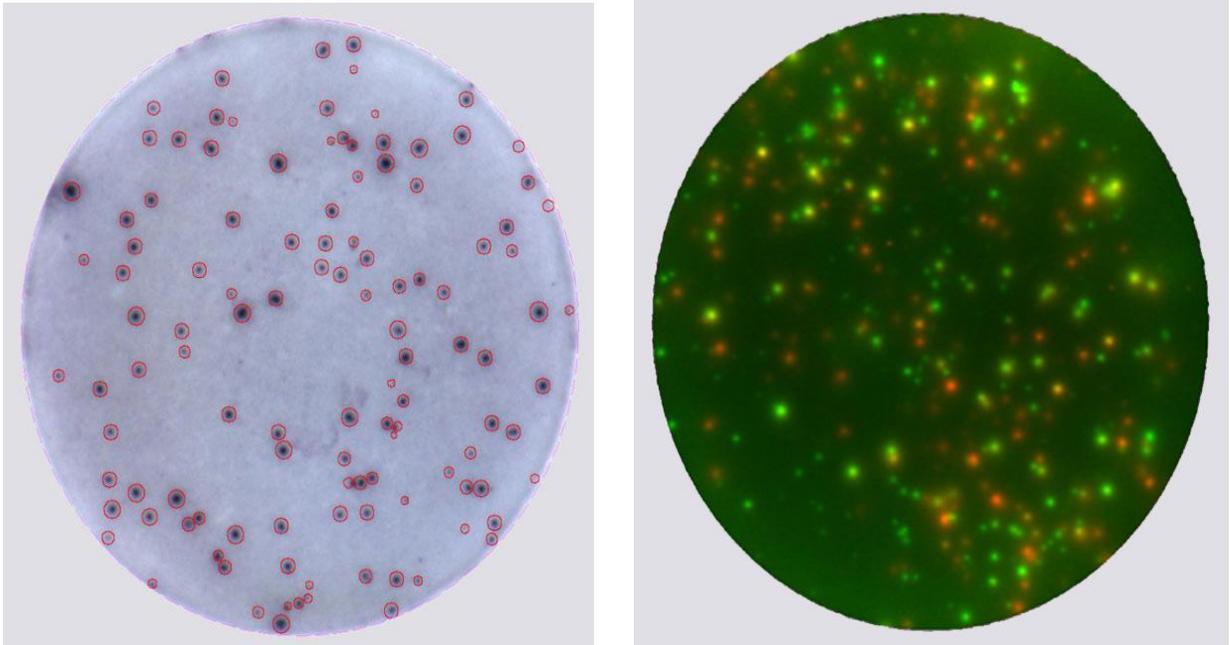


Figure 1.3: Images obtained after the traditional IFN- γ ELISPOT assay (left) and fluorospot assay (right) analysed with the AID EliSpot software

1.7. Diagnostic potential of antigen-stimulated soluble makers

M.tb has the ability to reside inside its host for a prolonged period of time in a non-replicative or slow replicative state. During this period, the host is latently infected and does not develop any TB symptoms. Tubercle bacilli are typically contained within immune-mediated granulomas [28]. In their new habitat, tubercle bacilli are exposed to difficult conditions including: hypoxia, low nitric oxide, low pH, nutrient and oxygen deprivation mounted by their host [60-62]. By creating *in vivo* and *ex vivo* models that mimic the conditions encountered by the bacilli in the

granuloma as infection progresses from latency to active disease, investigators have identified infection phase dependent genes. A number of these genes encode proteins with diagnostic potential [63, 64]. These proteins have been classified according to the infection state during which they are predominantly expressed. Dormancy-related (DosR) antigens or latency antigens are upregulated in response to the particularly hostile environment during latent infection [65] and Resuscitation-Promoting Factors (RPF) are thought to be upregulated during the active phase of the disease [66, 67]. Because these proteins are expressed in different growth phases, they may be excellent diagnostic candidates. Many studies have investigated the diagnostic utility of these novel *M.tb* antigens [60, 68]. In a recent study conducted in our laboratory, we have investigated the immunogenicity of 118 antigens [37]. Among the 51 DosR antigens evaluated in this study, 5 discriminated TB from latent infection with area under the operating characteristic curve (AUC) above 0.70. The RPFs were the most promising antigens in this experiment; the 5 known RPFs were evaluated and each individual RPF could separately discriminate the 2 clinical states of the study participants.

Interrogations of the immunogenicity of the newly identified *M.tb* antigens have been dominated by IFN- γ responses in presence of these stimuli in long or short term assays [37, 36, 69].

Whereas short-term assays detect recent *M.tb* infection, prolonged stimulation of whole blood increases the sensitivity of the assay [36]. Some innovative work has demonstrated that IFN- γ may not be the best antigen-host marker in the diagnostic or biomarker field. Indeed, Harari and colleagues have shown that TNF- α could better discriminate active TB from a latent infection [70]. Another line of investigation has also proved that multifunctional T cells (TNF- α , IFN- γ , and IL-2 secreting cells) have a better diagnostic value than the traditional IFN- γ measurement [71, 72]. Many soluble antigen-stimulated and unstimulated cytokines have shown promising diagnostic values.

IFN- γ -inducible protein 10 (IP-10) is one of the promising immune marker to measure immune sensitisation of *M.tb* protein. It is a member of the CXC chemokine family and is expressed by a number of innate cells including monocytes, macrophages, neutrophil, fibroblasts and endothelial cells. This chemokine plays an important role during infection as a chemoattractor to monocytes and lymphocytes at inflammatory foci [73]. The diagnostic utility of *M.tb* antigen-stimulated IP-10 has been reported promising in a number of studies [74-76]. This maker has several advantages compared to IFN- γ : its secretion is less affected by immune suppression and

is age independent [77]. IP-10 has been proposed as a replacement marker of latent infection in paediatric or in immunodeficient patients [78]. Although IP-10 could discriminate active TB from latent infection in some studies, discordant results have been published [75, 79, 80]. Alternative cytokines other than IFN- γ and IP-10 may be considered as biomarkers for active TB. Host marker like growth factors, including vascular endothelial growth factor (VEGF), Epidermal Growth Factor (EGF) and Transforming Growth Factor (TGF), and acute phase proteins could be excellent candidates to indicate tissue damaged in active TB. Indeed, EGF is a growth factor that stimulates cell growth, proliferation and differentiation [81] whereas VEGF is a signal protein important for angiogenesis (formation of new blood vessels from pre-existing vessels) [82]. All these functions could be important during active TB compared to latent TB due to the high presence of bacilli leading to tissues damaged and a subsequent need for repair processes. EGF and VEGF levels have been reported higher in active TB compared to latent TB and may be candidates for a rapid TB immune diagnosis test [83, 33]. Acute phase proteins including serum amyloid A (SAA), C-reactive proteins (CRP) and serum amyloid P (SAP) are mainly produced in the liver and may play a role in immune defence to *M.tb*. CRP and SAA levels increase in patients with active TB compared to latently infected individuals or successfully treated TB cases [84, 85]. Soluble TNF- α , IL-12 and IL-17 have also been proposed as diagnostic candidates. Research on soluble biomarkers for TB is dominated by two principal detection methods: enzyme-linked immunosorbent assay (ELISA) and multiplex assays like Luminex.

1.8. ELISA

IGRAs have been developed to diagnose latent TB and includes ELISPOT (explained above) and ELISA. IGRAs tests are based on the discovery of ESAT6 and CFP10. The lack of these two *M.tb* antigens in the BCG vaccine strains and in most of the Non-Tuberculous Mycobacteria (NTMs) provides high specificity for *M.tb* to IGRAs. Indeed, ESAT6 and CFP10 are contained within the region of difference-1 (RD1) of the genome of mycobacteria. This region is absent in BCG vaccine strains, and is also absent in most of the NTMs, with the exception of *M. kansasii*, *M. szulgai* and *M. marinum* [86]. To perform the IGRA ELISA, whole blood is stimulated overnight with *M.tb* specific antigens. Analytes secreted by the sensitized T cells are then

directly measured in the supernatant using specific antibodies. The QuantiFERON[®] TB Gold In Tube (QFT IT) provides an optimal assay where *M.tb* antigens are directly coated into Quantiferon tubes and the whole blood is directly collected and incubated in the same tube [33]. Apart from measuring IFN- γ this assay system also allows the measurement of multiple other biomarkers in culture supernatant. The QFT tubes and related procedures may improve stability of a number of proteins and also provides a highly standardized sample collection and stimulation method for biomarker discovery. Research on biomarkers for diagnostic or treatment response through ELISA had important limitations. The main limitation is the amount of supernatant needed to measure the circulating quantity of single analytes. This required amount of supernatant does not always allow evaluation of multiple analytes. The time, energy and consumables required to evaluate a small set of analytes constitute a drawback of this method in the research field.

1.9. Luminex

Luminex technology provides an excellent platform for biomarkers research. It allows evaluation of multiple analytes in a small volume of sample based on the principle of double interrogation as employed in flow cytometry.

Luminex technology uses micro beads or micron polystyrene microspheres internally dyed with two fluorosphores namely red and green dye. Each single bead will have a particular fluorescence obtained from the mixture of the two dyes. Thus, some beads will be red dye fluorescence dominated and others will be infrared dye fluorescence dominated. The unique fluorescence in a single bead obtained from a specific mixture of the two dyes is called region (unique and different from bead to bead). Each region is attributed to a unique capture antibody. Following this process, up to 100 captured antibodies could be used to coat each single well plate. A small volume of sample is then added and incubated in the wells plate. During the incubation time, the targeted analytes interfere with captured antibodies coated on the bead. Detection antibodies and streptavidin-PE are added for the detection of newly formed complexes (bead-captured antibody coupled to detection antibody- streptavidin).

The Luminex reader includes three main components: a fluidics system, lasers and detectors. As observed in flow cytometry, the fluidic system aligns the beads into single file as they enter a stream of sheath fluid. Once the beads are in single file, they can be individually interrogated by the two lasers. The green laser (532nm) excites the streptavidin-PE dye for the fluorescence intensity and the red laser (635nm) excites the dye inside the bead to determine the bead colour or region. All these information are recorded by the four detectors shown on Figure 1.4 More details on the function of this technology can be assessed on:

<http://www.luminexcorp.com/Products/>.

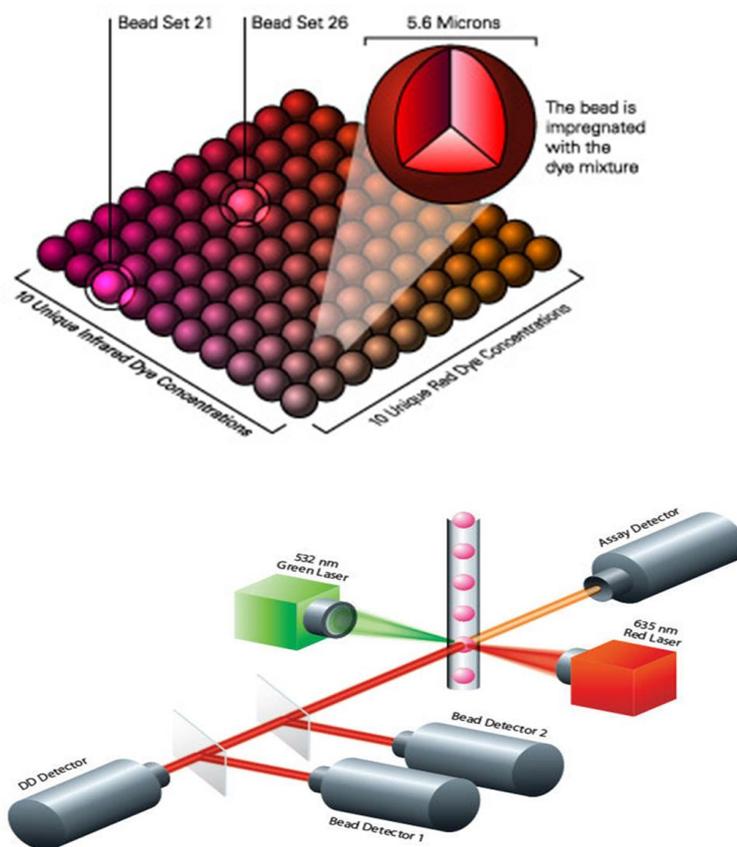


Figure 1.4: Luminex technology

Luminex technology uses micro beads. Each bead is situated in a specific fluorescence region and each bead/analyte/antibodies complex be detected by the 4 detectors after excitation by the combination of red and green lasers. The green laser collects information on the fluorescence intensity whereas the red laser interrogates the bead region.

Although Luminex allows evaluation of up to 100 proteins simultaneously in a limited volume of serum, this number represents less than 10% of the total number of serum proteins (more than a thousand serum proteins) [87]. An alternative method like the mass spectrometry (MS) to evaluate a larger number of biomarkers could be beneficial in this field.

1.10. Mass Spectrometry

Serum is a sample of choice in biomarkers research. This preference is driven by the fact that serum is easily accessible, non-invasively obtainable and widely collected in clinics. In addition, serum proteins originate from almost every tissue and cell type, including infected tissues or organs and therefore any quantitative or qualitative change in protein profiles at the site of infection could affect serum proteins profile [88]. Serum samples are particularly rich in proteins; they contain thousands of circulating biomarker candidates [89]. Accessing the diagnostic, prognostic and treatment monitoring utility of these proteins through ELISA, ELISPOT or even Luminex could be time consuming, extremely costly and particularly challenged by the availability of antibodies. Proteomic methods based on new advances in MS appear as a preferred strategy in unbiased biomarker discovery. Indeed, introduction of MS in biomarker studies has led to a simultaneous evaluation of the diagnostic utility of hundreds or thousands of markers with an antibody free approach [90, 91]. The number of serum proteins identified with MS varies from study to study and can be related to the sample preparation methods [92]. Indeed, the complexity of serum samples often necessitates a pre-fractionation (high abundant proteins depletion) step followed by a fractionation step for maximum protein identification on MS.

1.10.1 High abundant proteins depletion

Although evaluation of serum proteins on MS presents some advantages, the dynamic range of serum proteins constitutes a serious technical challenge in serum proteomic analysis; serum

albumin and immunoglobulins represent around 90% of the serum protein content [93]. The 20 most abundant proteins represent around 99% of the serum protein content [94]. The remaining 1% of serum proteins is constituted by thousands of proteins with another complex dynamic range. Without any pre-fractionation and fractionation (on solid agarose gel or in solution), the complexity of the serum could be overwhelming and important biomarker candidates could be lost in the background noise [95]. Different techniques have been proposed to deplete high abundant proteins from serum.

Solid phase extraction methods are depletion techniques that use solid phase to isolate a specific protein, a specific group of targeted proteins or a group of proteins with common feature (s). Solid phase extraction columns are the most widely used approach to deplete high abundant proteins from serum. Different types of solid phase extraction columns have been developed including ion-exchange [96] based depletion and antibodies based depletion [97].

Ion-exchange based depletion methods could be very attractive as they offer a number of advantages including high sample capacity and low cost. The ProteoSpin column (Norgen, Canada) is an excellent example of an ion-exchange column as it can deplete high abundant proteins from 100 to 500 ul of serum in a single run [98, 96] for a total cost of R2000/ 25 sample kit. Nevertheless, this method presents a major inconvenient that makes it less suitable in biomarker studies. Indeed the lack of specificity in ion-exchange depletion methods render their quantification results less reliable as many differential expression results could be a result of partial loss during the depletion step rather than a biological change.

Antibody based methods are the preferred approach in biomarkers discovery studies for several well defined reasons; these methods have a good reproducibility across reactions, they are efficient and particularly selective [99]. The targeted proteins can be simultaneously depleted in a reduced period of time [94] with a single run. Antibodies are relatively robust so that the same column can be used in 100 serum depletion cycles without compromising on the capacity or specificity of the column [99]. Antibodies based depletion methods lead to a minimum loss of nontargeted proteins [100]. The ProteoPrep 20 from Sigma is the most complete antibody depletion based method; it can simultaneously deplete the 20 most abundant serum proteins in a single reaction in less than 20 minutes [91]. Yadav and colleagues [94] have evaluated the number of untargeted serum proteins systematically removed during the depletion process using the ProteoPrep 20 and other antibody columns. They have discovered a total of 101 low

abundant proteins systematically depleted along with high abundant proteins during the depletion process. This number is relatively small if compared to alternative depletion methods but it also implies that researchers should consider both fractions (flowthrough and bound fraction) as relevant in biomarkers discovery. The high cost and low capacity represent the main disadvantages of antibody base depletion methods.

1.10.2 Fractionation

Antibody columns have been shown to be effective in removing up to the 20 most abundant serum proteins. Even after this effective depletion, the dynamic range of serum proteins may not be totally resolved as the abundance of the remaining proteins varies to such an extent that it is still difficult to identify the proteins with the lowest abundance. An extensive fractionation step is usually required to identify a maximum number of proteins on MS.

This extensive fractionation can be done through agarose gels or in solution. Agarose gel lanes are usually cut into five fractions but this number does not lead to maximum serum proteins identification and some researchers have called for further fractionation of their gel lanes. Eric Thouvenot and colleagues [101] have systematically cut their gel lanes into 68 fractions and separately analysed each fraction on MS with satisfactory results. A number of questions arise with a gel approach in quantitative proteomic: protein patterns may not be exactly the same across the gel lanes challenging the cutting process. More importantly, after in-gel digestion, peptides must be removed from the gel for MS analysis and the percentage of peptide recovery for each peptide across samples in this process may not be guaranteed. These two important gel steps can compromise the discovery of biomarkers and introduce false discovery results. Recent works have employed in solution fractionation (gel-free approaches) [102].

1.11. Hypothesis for the present study

Diagnosis of active TB represents one of the main drawbacks of current TB management. This situation is caused by the poor performances of some of the most widely used methods or, on the other hand, high cost and requirement for relatively advanced infrastructures of newer tests.

The present study explores the utility of host-based biomarker as new host diagnostic candidates.

Hypothesis 1

M.tb antigens are believed to be differentially regulated during different infection phases in the host (e.g. DosR antigens are expressed at higher frequency during latent TB infection whereas RPFs levels increase with the development of the disease). Stimulation of whole blood with *M.tb* antigens will induce different immune responses with potential diagnostic application.

Objective 1

To assess the diagnostic utility of the promising *M.tb* antigens by comparing immune responses from active TB cases to health controls.

Hypothesis 2

Whole blood stimulation with *M.tb* antigens in the 7-day whole blood assay has shown excellent diagnostic potential. These results will be validated in overnight assay for the development of a rapid diagnostic test.

Objective 2

To compare the diagnostic utility, the quality and the intensity of immune responses in three whole blood assays.

Hypothesis 3

Introduction of the Dual colour ELISpot has allowed evaluation of effector, effector memory and memory cell populations. We hypothesise that dual colour ELISpot can detect disease activity, based on differential expression of IFN- γ and IL-2.

Objective 3

To compare the diagnostic and treatment monitoring utility of the different cell populations using dual colour ELISpot.

Hypothesis 4

Alteration in proteins concentration at the infection site could be observed in whole blood. Mass spectrometry offers an excellent platform to assess changes in proteins expression in serum or plasma samples. Unfortunately, the presence of high abundant proteins in serum sample makes the evaluation of serum proteins expression particularly challenging. We hypothesised that depletion of high abundant proteins in serum with different depletion methods will lead to the identification of different number of proteins on mass spectrometry.

Objective 4

To develop an optimal sample preparation protocol for MS analysis and evaluate the diagnostic utility of an optimal number of human proteins

1.12. Study design

A direct head to head comparison was used to test different hypotheses. The first experimental chapter (chapter III), developing hypothesis 1, was divided into three parts. The first part evaluated the diagnostic utility of 24 recombinant stress induced antigens with in-house ELISA and QFT ELISA for IFN- γ . The second part validated the promising RPFs and DosR antigens with IFN- γ QFT ELISA whereas the third part concentrated on alternative RPFs and DosR antigen-stimulated and unstimulated host markers other than IFN- γ in a long term assay. Results

from this study have shown the promising potential of a number of antigen-stimulated markers confirming the initial hypothesis.

Chapter IV validated the long term assay results in a shorter assay (overnight WBA) with additional host markers. This study could not confirm our initial hypothesis as many of the promising results in the 7-day WBA could not be validated here but new promising biomarkers were discovered.

Chapter V focused on the development of the most appropriate methods to deplete high abundant proteins in serum. Antibody based column method has shown to be the optimal method in this exercise confirming our original hypothesis.

The last experimental chapter of this thesis (Chapter VI) evaluated the diagnostic utility of the newly developed dual colour ELISpot. This study confirmed our hypothesis on the diagnostic suitability of host cell populations.

1.13. References

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2. Chapter II

Materials and Methods

2.1 Study setting

The present thesis consists of four studies based on four distinct assays: the 7-day whole blood assay (WBA), overnight WBA, Dual colour ELISpot and Mass spectrometry (MS). The 7-day WBA was employed in the discovery step (chapter III). This discovery step was further divided into two parts where the first part aims to select the most promising antigens as measured by Interferon-gamma (IFN- γ) enzyme-linked immunosorbent assay (ELISA) and the second part was designed to select the most promising antigen-stimulated and unstimulated host markers using the Luminex platform.

The promising results were then validated in chapter IV with a shorter stimulation assay (overnight WBA) together with additional host markers for the development of a rapid test. The Luminex platform led to the simultaneous evaluation of the diagnostic utility of 73 host markers. This number may appear important when compared to ELISA (single marker) but very limited when the number of circulating analytes in plasma (more than 1 000 analytes) [1] is taken into account. Diagnostic evaluation of this important number of circulating plasma markers through Luminex is limited by antibody availability.

MS was introduced in chapter VI as an antibody-free method to evaluate the diagnostic utility of a maximum number of host markers. This work introduces our laboratory to proteomics for the first time and the focus of this chapter was shifted to optimization of sample preparation and the development of the most suitable protocol for the evaluation of optimal numbers of circulating host markers. Thus, three serum depletion methods including a ProteoSpin column from Norgen (Canada), ProteoPrep 20 from Sigma (Germany) and a heparin column from Sigma were evaluated for their accuracy to deplete high abundant proteins in human serum.

The diagnostic utility of the Dual colour ELISpot was evaluated in chapter V. This technique allows the simultaneous evaluation of the production of two cytokines by specific cell populations (single IFN- γ and single IL-2 secreting and dual cytokine secreting cells in our setting).

The work presented in the present thesis was carried out at the Immunology Research Laboratory of the Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences of the University of Stellenbosch situated in Tygerberg district (Western Cape, South Africa).

2.2 Study population

All study participants were recruited in the same district, namely in the Ravensmead/Uitsig day hospitals (for tuberculosis (TB) patients or TB suspects) and Ravensmead/Uitsig communities (for household contacts or HHCs and community controls). The Ravensmead/Uitsig community has been well studied. A two year (1998 – 1999) survey conducted by Desmond Tutu TB Centre showed that this community had an annual risk of TB infection of 3.5%. This rate progressed to 4.1% in 2005. TB disease incidence, defined by smear positivity, was estimated at 341 per 100 000 in 2002. Bacillus Calmette–Guérin (BCG) vaccination (Danish strain, 1331, Statens Serum Institute [SSI], Copenhagen, Denmark) is routinely administered at birth in this community.

Study participants could be divided into four groups at recruitment: active TB cases, people with suspected TB, household contacts of TB cases and community controls.

Active TB cases were recruited in case control studies. The TB case classification for individuals was obtained after different investigations at the clinic including symptoms screening, smear microscopy, *Mycobacterium tuberculosis* (*M.tb*) culture and chest X-ray. These subjects were recruited in the different studies after their clinical classification as TB cases and just before initiation of TB therapy. Sputum samples were collected together with the blood samples and the TB disease status was confirmed in our laboratory by culture and PCR.

People with suspected TB were recruited in a prospective study (Africa European- TB Consortium [AE-TBC]) and some of the confirmed TB cases and non-TB were used in chapter

IV. This group included all individuals coughing for at least two weeks and on whom the clinic was willing to administer routine TB tests for the presence of *M.tb*. Blood and sputum samples were collected from these individuals. TB disease was confirmed by culture in our laboratory, our results were then used in conjunction with clinic results.

A HHC was defined as a person sharing the same household with a diagnosed TB cases at least for the last two months before enrollment of the HHC in the study. This group is believed to be constituted by newly infected individuals who may present a unique TB pattern different from the active disease and also different from uninfected people.

The community controls are study controls living in the same community where TB cases were recruited. Due to the high level of TB incidence and prevalence in Ravensmead/Uitsig community, as mentioned above, this population is expected to have been sensitized to *M.tb* antigens. Blood and sputum samples were collected from all controls and the absence of active TB was confirmed by chest X-ray and *M.tb* culture. Latent infection was confirmed by Tuberculin Skin Test (TST) or by Quantiferon test.

2.3 Inclusion criteria

Participants were enrolled in one of the studies if: i) they were willing to undergo a HIV rapid test (Abbot Determine™ HIV 1/2; Abbott, Wiesbaden, Germany) or present a previous HIV result (from the last three months), ii) they permanently reside within the study area, iii) they were willing to sign informed consent for participation in a specific study, iv) they were adults (18 years old and above).

Specific inclusion criteria were also applied for each group. Participants were enrolled in the control group if they were available for TST reading 48 to 72 hours after the test (if TST is required in the concerned study) and their chest X-ray as well as their culture results confirmed the absence of TB disease. Active TB cases were included if they were willing to receive TB therapy in one of day hospital within the study area.

2.4 Exclusion criteria

Participants presenting the following criteria were excluded from the concerned study: i) Second episode of TB, ii) already receiving TB treatment, iii) HIV infected (not applicable for chapter

IV), iv) participation in a drug or a vaccine trial at the time of recruitment or six months before, v) presence of a chronic condition like cancer or diabetes mellitus, vi) presence of alcoholism.

2.5 Ethical approval

All studies were approved by the Committee for Human Research of the Faculty of Medicine and Health Sciences, University of Stellenbosch. All participants signed an informed consent form before enrollment in the study.

2.6 Samples processing

Participants were enrolled in a specific study after satisfaction of all inclusion and exclusion criteria. At enrolment, sputum and blood samples were collected from each participant. Participants were tested with different tests according to the study group (TB or control) and according to their specific study requirements. Thus, people with suspected TB were tested by sputum acid fast bacilli staining (AFB) and study controls had either a TST test using 2 tuberculin units of PPD-RT23 (SSI, Denmark) by the Mantoux method or blood was collected in the QFT tubes for Quantiferon testing. The TST test result was read by an experienced study nurse 48 to 72 hours after the test was administered. The blood and sputum samples were processed in the laboratory within two hours of the sample collection. Sputum samples were cultured and blood was prepared for the WBAs or Peripheral Blood Mononuclear Cells (PBMC) isolation. Serum, plasma and PBMCs were stored at -80°C in our sample bank. The day of the assay, the required number of TB cases was randomly selected from the TB case list in the sample bank. The same process was also performed for the selection of study controls.

2.7 Seven-day WBA

2.7.1 Antigens in the 7-day WBA experiment

The immunogenicity of 118 phase dependent *M.tb* antigens was previously evaluated in our laboratory using in-house ELISA [2]. Five dormancy regulated antigens (DosR antigens) (recombinant proteins) including Rv2032, Rv2625c, Rv1996c, Rv0081 and Rv2624 as well as the five known resuscitation promoter factors (RPF) including Rv0867c, Rv2389c, Rv1009, Rv2450c and Rv1884c were selected as the most promising antigens. RPFs discriminated TB disease from latent infection with similar diagnostic accuracy. Only three of the five RPFs (Rv0867c, Rv2389c and Rv1009) together with the five promising DosR antigens were selected for further evaluation in the present thesis (chapter III).

All 118 lyophilized antigens were reconstituted and coated on plates in the previous study. Sample recruitment as well as the 7-day WBA were processed during the same study. Aliquots of the stimulated and unstimulated supernatant from the selected antigens were collected for evaluation in the present study (chapter III). Details on the selected RPFs and DosR antigens are summarized in (Table 2.1) and more details can be obtained in reference number [2].

A number of 24 stress induced recombinant antigens (Rv0244, Rv0352, Rv0353, Rv0711-N, Rv0711-N, Rv0767, Rv0839, Rv1528, Rv1908-C, Rv1908-N, Rv1909, Rv1945, Rv2382, Rv2383-C, Rv2383-N, Rv2745, Rv2913, Rv2987, Rv2988, Rv2989, Rv3229, Rv3654, Rv3710 and Rv3840) were not properly evaluated in the previous study and were not included in the analysis of the 118 antigens. To analyze these antigens in the present study, additional study participants were recruited and the 7-day WBA was performed before the in-house ELISA and the QFT ELISA. The characteristics of these antigens are shown in (Table 2.2). All stress induced antigens, RPFs and DosR antigens were provided by Leiden University Medical College (LUMC), The Netherlands.

2.7.2 Performance of 7-day WBA

Antigens were previously reconstituted, coated on plates and stored at -80° as previously mentioned. To assess the diagnostic utility of the 24 stress induced antigens, the 7-day WBA was performed on samples from all additional study participants.

On the day of the assay, plates were taken out of the freezers and brought to room temperature. Each plate was randomly assigned a study participant number. After labeling, RPMI 1640 and Phytohaemagglutinin (PHA) ($20 \mu\text{g/ml}$) were added to the plate as negative and positive controls respectively.

Blood samples were diluted 1 in 5 with pre-warmed RPMI 1640 (pre-warmed in 37°C water-bath) in 15 ml polypropylene tubes. $100 \mu\text{l}$ of the diluted bloods were added to each well plate. Each participant was tested with 27 different conditions in duplicate (1 negative control + 2 positive controls + 24 antigens = 27 conditions). The total volume in each well was $200 \mu\text{l}$ ($100 \mu\text{l}$ of antigen + $100 \mu\text{l}$ of diluted blood). The five times diluted blood was now diluted again to achieve a final dilution of 1:10 and the final concentration of antigen in each well was $10 \mu\text{g/ml}$. Plates were then incubated for 7 days in a humidified, 5% CO_2 37°C incubator.

2.7.3 Harvesting and storage of the 7-day WBA supernatants

After seven days, plates were taken out of the incubator. $150 \mu\text{l}$ of supernatant was harvested from each well. Thus, a total of $450 \mu\text{l}$ were harvested for each antigen. $210 \mu\text{l}$ were aliquoted into plates and the remaining volume was aliquoted into 2 Eppendorf tubes.

The $210 \mu\text{l}$ aliquots stored in plates were used for IFN- γ ELISA and QFT ELISA and supernatants stored in tubes were used for Luminex experiment.

Table 2.1: Details of RPFs and DosR antigens evaluated during the present study

Class of <i>M.tb</i> antigen	Study code	Rv number	Description	<i>M.tb</i> region	Protein size (aa)	Batch No
DosR Antigens	17	Rv0081	Probable transcriptional regulatory protein		114	50212
	38	Rv1996	Hypothetical protein (USP)		317	030311
	70	Rv2032	Conserved hypothetical protein Acg (nitroreductase)	Acg	331	20919
	71	Rv2624c	Conserved hypothetical protein – USPA		272	030308
	72	Rv2625c	Probable conserved trans-membrane protein		393	050610
RPFs	84	Rv0867c	Possible conserved trans-membrane protein (tryglycosylase)	rpfA	401	51106
	86	Rv1884c	Transglycosylase	rpfC	176	51107
	87	Rv2389c	Probable resuscitation-promoting factor rpfD (transglycolase)	rpfD	154	51108

Table 2.2: Details of the stress induced antigens

Study code	Rv number	Description	<i>M.tb</i> region	Protein size (aa)	Batch No
N1	Rv0244	Probable Acyl-coA dehydrogenase	<i>fadE5</i>	611	061002
N2	Rv0352	Probable chaperone protein	<i>dnaJ1</i>	395	061013
N3	Rv0353	Probable heat shock protein transcriptional repressor	<i>hspR</i>	126	061005
N4	Rv0711	Possible arylsulfatase	<i>atsA</i>	787	061014
N5	Rv0711-N	Possible arylsulfatase			061001
N6	Rv0767	Conserved hypothetical protein	<i>Rvo767</i>	213	061010
N7	Rv0839	Conserved hypothetical protein		270	060604
N8	Rv1528	Probable conserved polyketide synthase	<i>papA4</i>	165	060606
N9	Rv1908-C	Catalase-peroxidase-peroxynitritase T katG	<i>KatG</i>	740	060705
N10	Rv1908-N	Catalase-peroxidase-peroxynitritase T	<i>KatG</i>		060704
N11	Rv1909	Ferric uptake regulation protein	<i>furA</i>	150	060702
N12	Rv1945	Conserved hypothetical protein		454	060902
N13	Rv2382c	Polyketide synthase	<i>mbtC</i>	444	060703
N14	Rv2383c-C	Phenylloxazoline synthase	<i>mbtB</i>	1414	061006
N15	Rv2383c-N	Phenylloxazoline synthase			060903
N16	Rv2745c	Possible transcriptional regulatory protein		112	061103
N17	Rv2913	Possible D-amino acid amonohydrolase		611	061007
N18	Rv2987c	Probable 3-isopropylmalate dehydratase	<i>leuD</i>	198	060605
N19	Rv2988c	Probable 3-isopropylmalate dehydratase	<i>leuC</i>	473	060404
N20	Rv2989	Probable transcriptional regulatory protein		233	061009
N21	Rv3229	Possible lenoleoyl-coA desaturase	<i>desA3</i>	427	061106
N22	Rv3654	Conserved hypothetical protein		84	061104
N23	Rv3710	2-isoprolmalate synthase	<i>leuA</i>	644	060904
N24	Rv3840	Possible transcriptional regulatory protein		137	061003

2.8 In-house IFN- γ ELISA

The in-house IFN- γ ELISA was performed in all additional study participants involved in the evaluation of stress-induced antigens. This ELISA was performed in 3 different days using commercial pairs of monoclonal antibodies (BD Pharmingen™, BD Biosciences).

On the first day, IFN- γ was coated on 96-well flat-bottomed micro plates as followed: undiluted IFN- γ capture antibody (1mg/ml) was 1 in 500 diluted with coating buffer (0.1M NaHCO₃). Plates were labelled with study participant number, antigen names and date. 50 μ l of the diluted IFN- γ capture antibody was added into each well plate at a final concentration of 2 μ g/ml. Plates were sealed and incubated overnight at 4⁰C.

On the second day, plates were washed four times with washing buffer (1 X PBS + 0.05% Tween 20). 150 μ l of 10% heat-inactivated foetal calf serum (in PBS) was added to plates and incubated for two hours at room temperature to prevent unspecific binding. After the blocking step and serial dilutions of recombinant human IFN- γ standard (BD Pharmingen™, BD Biosciences), plates were washed again with washing buffer. 100 μ l of standards and samples were added into each wells according to the ELISA plate templates. Plates were sealed and overnight incubated at 4⁰C.

On the third day, 0.5 mg/ml biotinylated mouse-anti-human IFN- γ detection antibody (BD Pharmingen™, BD Biosciences) was 1 in 500 diluted with blocking buffer. Plates were washed four times and 100 μ l of the diluted detection antibody was added to plates at a final concentration of 1 μ g/ml. Plates were sealed and incubated at room temperature for one hour. During the incubation step, 1 mg/ml Avidin Peroxidase (Sigma, Germany) was 1 in 400 diluted with blocking buffer. After the incubation step, plates were washed with washing buffer and 100 μ l of Avidin Peroxidase was added to each well. Plates were sealed and incubated for another hour. Urea hydrogen peroxide tablets (Sigma, Germany) were dissolved in distilled water during incubation time. After the incubation time, plates were washed and OPD tablets (Sigma, Germany) were added to the urea hydrogen solution. 200 μ l of this solution was added to each well and plates were incubated in the dark at room temperature for 25 minutes. Plates were read at 450 nm on the Bio-Rad Bench Plate reader (Bio-Rad laboratories Inc). IFN- γ concentrations were automatically calculated from the optical densities by the micro plate manager software version 5.2, Build 103 (Bio-Rad Laboratories Inc). The standard curve range was 31 – 4000 pg/ml.

2.9 QuantiFERON-TB GOLD in tube (QFT) ELISA

The QFT ELISA (Cellestis, Australia) was employed in this study as a validation method because of its high sensitivity (minimum limit detection: 0.2pg/ml). It could detect low concentrations of protein previously undetected by our in-house ELISA (minimum limit detection: 31pg/ml). The most promising RPFs, DosR antigens and the stress induced antigens were validated using this method. In addition to its high sensitivity, this method is more standardized and commercially available.

The QFT ELISA was conducted according to the manufacturer instructions. Briefly, all reagents (except for 100X concentrated conjugate) and samples were brought to room temperature before use. The freeze dried kit standard and the freeze dried Conjugate were reconstituted with appropriate volume of distilled water. The reconstituted kit standard was used to produce 1 in 4 dilution series of IFN- γ (4.0 IU/ml, 1.0 IU/ml, 0.25 IU/ml and 0.0 IU/ml) and working strength conjugate was prepared by diluting 60 μ l of 100X concentrated Conjugate in 6 ml of Green Diluent. 50 μ l of the freshly prepared Working Strength conjugate was added to all wells using a multichannel pipette. 50 μ l plasma samples and 50 μ l of standards were added to the appropriate wells. The standards were added in triplicate. The ELISA plate was incubated in the dark for 120 minutes. During the incubation time, 20X concentrated Wash Buffer was diluted 20 times. After the incubation step, the plate was manually washed for at least 10 cycles. 100 μ l of Enzyme Substrate solution was added to each well before incubation for 30 minutes in the dark. After this second incubation step, 50 μ l of Enzyme stopping Solution was added to each well and the plate was immediately read (within 5 minutes) at 450nm filter. Optical Density (OD) was read using a microplate reader. IFN- γ concentrations were calculated from the OD values.

2.10 Overnight WBA

2.10.1 Antigens in overnight WBA

The Promising results obtained in the 7-day WBA were validated in a shorter assay (overnight WBA) for the development of a rapid test. To validate these results, eight recombinant antigens were received in our laboratory from collaborators. Six antigens including Rv0081, Rv1733,

Rv2029, LUCO1, LUCO2 and ESAT6/CFP10 were provided by LUMC and Statens Serum Institute (SSI, Denmark) provided PPD and TB18.2.

LUCO1, LUCO2 and TB18.2 are recently identified *M.tb* antigens and their immunogenicity was unknown (Table 2.3).

Table 2.3: Lyophilized recombinant proteins reconstituted for overnight WBA

Study Code	Antigen name	Batch number	Amount Received
3	ESAT-6/CFP-10	100802	1.5mg
4	Rv1733	091102	0.75mg
5	Rv2029	080901	0.75mg
6	Rv0081	080514	1.5mg
7	LUMC01	111101	1.5mg
8	LUMC02	111103	1.5mg
2	PPD	224	1mg/ml (1.4 ml)
10	TB18.2	11-01-2	1.5 mg
9	PHA	L-1668	5mg

2.10.2 Negative control (sterile 1x PBS)

10x PBS (Lonza, USA) was diluted to a 1x PBS using autoclaved distilled water. The diluted PBS was autoclaved and allowed to cool to room temperature before use.

2.10.3 Positive control (PHA)

A stock solution of 1mg/ml was made by adding 5ml sterile 1 X PBS directly to the original vial. One ml of this stock solution (1mg/ml) was aliquoted into five Eppendorf tubes. Four aliquots were stored at -80°C and the last one was further aliquoted into 20µl amounts in 50x 0.5ml Eppendorf tubes before storage at -80°C. On the day of the assay, after thawing, the 20µl was further diluted to 100 µg/ml by addition of 180µl of sterile 1x PBS directly into the thawed tube.

50µl of the diluted PHA (100µg/ml) was added to the appropriate PHA wells into the plate. The final PHA concentration in the plate was 10µg/ml.

2.10.4 Reconstitution and dilution of antigens

All antigens were reconstituted as instructed by respective manufacturers (LUMC and SSI). All antigens are shown on Table 2.3 and controls (PBS and PHA) were evaluated on each study participant in overnight WBA as well as in 7-day WBA. Each antigen was assigned a unique study code. The study code was written on each original antigen vial, tubes and plates (where the antigens will be stored) before reconstitution.

Some of the antigens (ESAT-6/CFP-10, Rv0081, PPD, PHA, TB18.2, LUMC01 and LUMC02) were evaluated at a final concentration of 10µg/ml (group 1) while Rv2029 and Rv1733 (group 2) were evaluated at 5µg/ml. Antigens evaluated at a final concentration of 10 µg/ml were reconstituted and stored at 100 µg/ml and antigens that were evaluated at 5 µg/ml, at 50 µg/ml. All steps were carried out in a class II laminar flow hood.

All provided lyophilized recombinant proteins were first reconstituted to 0.5mg/ml (500µg/ml) with 1x PBS or sterile water as directed by the manufacturer. The sterile 1x PBS or autoclaved distilled water was added to the antigens using their original containers in multiple steps of 250 µl. The reconstituted and diluted tubes were vortexed and mixed very well in between the PBS/water addition steps. Three aliquots were made from each diluted 0.5mg/ml antigen.

Antigens were further diluted to a final concentration of 100µg/ml (group 1) or 50µg/ml (group 2) and aliquoted in a volume of 50µl. On the day of WBA, 50µl of each antigen (100µg/ml or 50µg/ml) was added to each well containing 450µl of blood and the final concentration was then be 10µg/ml or 5µg/ml in each well. The remaining 50µl aliquots (100µg/ml or 50µg/ml) were stored at -80°C.

2.10.5 PPD

PPD was provided in a total volume of 1.4ml, it was diluted directly from the stock 2ml vial when required for use. On the day of whole blood assay, a 10x dilution was made by adding 5µl of the stock PPD (in the original 2ml vial) to 45µl of sterile 1xPBS. The new PPD concentration was 100µg/ml.

2.10.6 Performance of overnight WBA

All antigens and PHA were now at 100µg/ml except for Rv1733 and Rv2029, which were at 50µg/ml. On the day of the assay and according to the number of participants bled that day, a corresponding number of aliquots from each antigen were thawed and plates were labelled with appropriate study participant number and antigen codes. Heparinized venous blood was used for the WBAs. The 7-day WBA was processed as indicated in the 7-day WBA section above. The overnight WBA was processed as follow: 450µl of undiluted blood was added in each well of the 48-well plates. 50µl of each antigens and controls was added to appropriate well-plate. ESAT-6/CFP-10, Rv0081, LUMC01, LUMC02, PPD and PHA were now at a final concentration of 10µg/ml whereas Rv1733 and Rv2029 were at a final Concentration of 5µg/ml in the well. Plates were incubated at 37°C, 5% CO₂ for 24 hours. After the incubation time, plates were removed from the incubator and around 200µl supernatant was harvested from each well-plate. The harvested supernatants were aliquoted (50µl) in 0.5ml micro tubes and stored at -80°C.

2.11 Luminex

Luminex technology was employed in this study to evaluate the diagnostic utility of alternative stimulated and unstimulated host markers other than IFN-γ. Whole blood (7-day and overnight WBAs) supernatant as well as the Quantiferon supernatant (for chapter IV) from the selected participants were thawed at room temperature and cytokines levels were measured using Milliplex kits (Millipore, St. Charles, Missouri, USA) according to the manufacturer's

instructions. Briefly, plates were pre-wet with washing buffer. Standards and assay buffer were added to appropriate wells before addition of samples and internal controls. Beads were mixed in the mixing bottle and added into all well-plates. Plates were sealed and incubated with agitation on a plate shaker for 1 hour at room temperature. The fluid was removed by vacuum and plates were washed twice before addition of detection antibodies and 30 minutes incubation. After the 30 minutes incubation, Streptavidin-phycoerythrin was added into each well-plate and incubated for 30 minutes with agitation in a plate shaker. Plates were washed twice and Sheath Fluid was added into all wells before incubation for 5 minutes with agitation. Plates were run on Bio Plex Manager Software, version 4.1.1.

2.12 Evaluation of two color ELISpot

2.12.1 Peripheral Blood Mononuclear Cell (PBMC) Isolation

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from participants according to our laboratory protocol; Blood was 5 X inverted in its tube and 1 X diluted with Phosphate PBS. The diluted blood was slowly pipetted onto Histopaque (SIGMA, Germany) and centrifuged at room temperature for 25 minutes with brake and accelerator off. The PBMC layer was aspirated using a plastic Pasteur pipette and dispensed into a 50 ml tube. PBMC's were first washed in 50ml PBS then in 10ml. The pellet was re-suspended in 1ml. 10 µl of the cell suspension was mixed with 40 µl of Trypan blue in 0.5 ml Eppendorf tube and 10 µl was added into a Neubauer Chamber before PBMC's were counted using a microscope (Wirsam).

2.12.2 Antigens and Antibodies

Monoclonal anti-CD28 was obtained from Autoimmun Diagnostika (AID) GmbH, (Straßberg, Germany) and diluted 1:1000 with the cell suspension. Recombinant ESAT-6/CFP-10 and microsomal triglyceride transfer protein (MTP)-65 from AID were used as *M. tuberculosis* specific antigens in a ready to use format (no specific information concerning ESAT-6/CFP-10

and MTP-65 concentration was provided by the manufacturer). PPD (Statens Serum Institute, Copenhagen, Denmark) was used at 10µg/mL. Five µg/mL pokeweed mitogen (Biochrom AG, Berlin, Germany) and 10 ng/mL anti-CD3 (clone X35, Beckman Coulter GmbH, Krefeld, Germany) were used as positive controls. 96 well plates had been pre-coated by AID with the two capture antibodies anti-human IL-2 and anti-human IFN-γ. Concentrates of biotin-conjugated anti-human IL-2 and fluorescein isothiocyanate-conjugated anti-human IFN-γ secondary antibodies as well as the streptavidin RED-(Cy3)-conjugate and anti-fluorescein isothiocyanate green were provided from AID and diluted as recommended by the manufacturer.

2.13 Serum proteomic study

Serum samples were treated with three different high abundant protein depletion kits including ProteoSpin columns, Heparin columns and ProteoPrep 20. Details on these depletion methods can be found in the proteomic chapter (chapter V). The total protein concentrations of the depleted and non-depleted samples were measured by the Bradford method as follows: all reagents and samples were brought to room temperature. The Bradford standard (provided in the kit) was diluted with PBS in seven serial dilution points according to the manufacturer's protocol. 5µl of each standard and sample were added to appropriate wells in triplicate. 250µl of dye reagent was added to all wells and mixed. The plate was incubated at room temperature for ten minutes in the dark and read at 595 nm.

The depleted and non-depleted samples were denatured at 95°C for five minutes and loaded onto an agarose gel. The gel was submitted to the proteomic unit manager in our department who provided the detailed protocol below.

2.13.1 In gel trypsin digestion

Proteins were run on agarose gel, cut into five pieces and washed twice with water followed by 50% (v/v) acetonitrile for 10 minutes. The acetonitrile was replaced with 50 mM ammonium

bicarbonate, incubated for 10 minutes and the wash repeated two more times. All the gel pieces were then incubated in 100% acetonitrile until they turned white, after which the gel pieces were dried *in vacuo*. Proteins were reduced with 10 mM DTT for 1 h at 57 °C. This was followed by brief washing steps of ammonium bicarbonate followed by 50% acetonitrile before proteins were alkylated with 55 mM iodoacetamide for 1 h in the dark. Following alkylation the gel pieces were washed with ammonium bicarbonate for 10 minutes followed by 50% acetonitrile for 20 minutes, before being dried *in vacuo*. The gel pieces were digested with 100µl of a 10 ng/µl trypsin solution at 37°C overnight. The resulting peptides were extracted twice with 70% acetonitrile in 0.1% trifluoroacetic acid for 30 minutes and then 100% acetonitrile for 30 minutes, and then dried. The dried peptides were then dissolved in 5% formic acid and cleaned using Stage Tips (Thermo Scientific) according to the instructions. The peptides were again dried and stored at -20°C. Dried peptides were dissolved in 5% acetonitrile in 0.1% formic acid and 10µl injections were made for nano-LC chromatography.

2.13.2 Mass spectrometry

All experiments were performed on a Thermo Scientific EASY-nLC II connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray source. For liquid chromatography, separation was performed on an EASY-Column (2 cm, ID 100µm, 5µm, C18) pre-column followed by XBridge BEH130 NanoEase column (15 cm, ID 75µm, 3.5µm, C18) column with a flow rate of 300nl/minute. The gradient used was from 5-17 % B in 5 min, 17-25% B in 90 min, 25-60% B in 10 min, 60-80% B in 5 minute and kept at 80% B for 10 min. Solvent A was 100% water in 0.1 % formic acid, and solvent B was 100 % acetonitrile in 0.1% formic acid.

The mass spectrometer was operated in data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Data were acquired using the Xcaliber software package. The precursor ion scan MS spectra (m/z 400 – 2000) were acquired in the Orbitrap with resolution $R = 60000$ with the number of accumulated ions being 1×10^6 . The 20 most intense ions were isolated and fragmented in linear ion trap (number of accumulated ions 1.5×10^4) using collision induced dissociation. The lock mass option (polydimethylcyclsiloxane; m/z

445.120025) enabled accurate mass measurement in both the MS and MS/MS modes. In data-dependent LC-MS/MS experiments, dynamic exclusion was used with 60 s exclusion duration. Mass spectrometry conditions were 1.8 kV, capillary temperature of 250 °C, with no sheath and auxiliary gas flow. The ion selection threshold was 500 counts for MS/MS and an activation Q-value of 0.25 and activation time of 10 ms were also applied for MS/MS.

2.13.3 Data analysis

MaxQuant 1.2.2.5 was used to identify proteins via an automated database of all tandem mass spectra against the Uniprot Homo sapiens database. Carbamidomethyl cysteine was set as fixed modification, and oxidized methionine, N-acetylation and deamidation (NQ), Pyro-Gln, Pyro-Glu was used as variable modifications. The precursor mass tolerance was set to 20 ppm, and fragment mass tolerance set to 0.8 Da. Two missed tryptic cleavages were allowed. Proteins were considered positively identified when they were identified with at least 1 tryptic peptides per proteins, and protein and peptide FDR of 0.01. Statistical analysis was done using Perseus. Data were transformed (\log_2) and imputed (width 0.3, Down shift 1.8) to replace missing values. ANOVA testing were performed using Permutation based FDR with threshold of 0.05. Z-scoring normalisation was also done.

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3. Chapter III

Diagnostic utility of newly identified *Mycobacterium tuberculosis* antigens in the diagnosis of TB disease in long term assay

3.1 Introduction

The discovery of the 6 kDa early secretory antigenic target (ESAT6) and 10 kDa culture filtrate antigen (CFP10) from *M.tb* region of difference 1 (RD1)[1], marked a new era in TB T cell immunology, especially in investigations towards the development of improved TB immune-based tests. One of the most important applications of these antigens was in the development of the Interferon-gamma release assays (IGRAs) [2]. The most widely available commercial IGRAs include the Quantiferon TB Gold tests (QFT) and the T SPOT.TB. The development of IFN- γ based tests (QFT and T SPOT.TB) has improved the specificity of immune test compare to the traditional Tuberculin Skin Test (TST) based on PPD antigen [3]. These new antigens still do not allow discrimination of latently infected individuals from active TB cases, which is important in resource limited areas [4]. This therefore calls for investigation into new antigens that could allow for discrimination between TB disease and LTBI.

In a previous study from our group, the immunogenicity of 118 newly identify infection phase-dependent *M.tb* antigens were assessed using a 7-day diluted whole blood culture assay (WBA) and IFN- γ ELISA. Among the 118 antigens tested, only 5 Dormancy Regulon antigens (DosR) and the 5 known Resuscitation Promoter Factors (RPFs) could significantly discriminate active from latent TB [5]. The detection range of the in-house IFN- γ ELISA was between 31 and 4000pg/ml but many antigens discriminating study groups had IFN- γ below this range. Therefore the present study was designed to investigate whether the diagnostic potential of the promising antigens could be confirmed by the use of a more sensitive, highly standardized and validated commercially available QFT ELISA. According to the manufacturer, it could detect analyte at lower concentration (detection limit around 2pg/ml).

A number of stress induced antigens were not evaluated during the previous study. The diagnostic utility of 24 stress induced antigens was first evaluated with IFN- γ ELISA before evaluating all promising antigens by QFT ELISA.

3.2 Evaluation of the diagnostic utility of *M.tb* stress induced antigens in a 7-day whole blood assay

3.2.1 Evaluation of *M.tb* stress induced antigens with an in-house IFN- γ ELISA

3.2.1.1 Aims of the study

We aim to investigate whether stimulation of whole blood with *M.tb* stress induced antigens and measurement of IFN- γ using an in-house ELISA holds promise as new diagnostic tool.

3.2.1.2 Materials and methods

3.2.1.2.1 Study participants and sample collection

We have previously investigated the diagnostic utility of 118 *M.tb* antigens, including 24 stress induced antigens. The number of stress induced antigen-stimulated samples was low and the results could not be analysed. These stress induced antigens included: Rv0244, Rv0352, Rv0353, Rv0711-N, Rv0711-N, Rv0767, Rv0839, Rv1528, Rv1908-C, Rv1908-N, Rv1909, Rv1945, Rv2382, Rv2383-C, Rv2383-N, Rv2745, Rv2913, Rv2987, Rv2988, Rv2989, Rv3229, Rv3654, Rv3710 and Rv3840. The first part of this thesis evaluated the diagnostic potential of these antigens. To evaluate these antigens, first episode, HIV negative, sputum culture positive pulmonary tuberculosis (TB) cases were recruited together with recently exposed healthy (sputum culture negative) household contacts (HHCs) of TB cases. After written consent was obtained, blood was collected from all participants and directed to the laboratory. Upon arrival in

the laboratory, blood samples were 5 times diluted with RPMI1640 medium containing glutamine (Sigma Aldrich, Johannesburg, South Africa) in 96-well plates and incubated for 7 days with *M.tb* antigens as previously described [5]. Plasma were harvested and stored at -80°.

3.2.1.2.2 Data analysis

M.tb stress induced antigen-stimulated (after negative control subtraction) and unstimulated IFN- γ levels were compared between TB cases and HHCs using Mann Whitney U test for nonparametric data analysis. Sensitivity and specificity were ascertained by receiver operating characteristics (ROC) analysis. The predictive abilities of combinations model of antigens was investigated by performing general discriminant analysis (GDA). The data were analysed using GraphPad prism, version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

3.2.1.3 Results

Participants enrolled into this study were originally recruited for the on-going Bill & Melinda Gates Foundation-funded Grand Challenges in Global Health (GC6-74) study (<http://www.biomarkers-for-tb.net/>). A total of 42 participants were randomly selected from the sample bank including 23 TB cases and 19 HHCs. All TB cases had QFT tested at recruitment. HHCs received a Tuberculin Skin Test (TST) or QFT test (Table 3.1).

Table 3.1: Demographic and clinical characteristics of participants in the stress induce antigen study

A total number of 42 participants were evaluated in this case control study: 23 TB cases and 19 household contacts. They were randomly selected from our samples bank. Household contacts received a TST test (9) or QFT test (10). All active TB cases received a QFT test.

	All	TB	Household contacts
Number of participants(n)	42	23	19
Mean age (years)	28	30	27
Age range (years)	10 - 57	10 - 57	10 - 57
Male/female ratio	20/22	17/6	3/16
Participants with TST test	9	0	9
Participants with QFT test	33	23	10
QFT or TST positive	39/42 (62.86 %)	22/23 (96%)	16/19 (84%)
QFT positive (%)	94	91	90
TST positive (%)	N/A	N/A	79

QFT = QuantiFERON TB Gold in Tube, TB = active tuberculosis, TST = tuberculin skin test, N/A = Not Applicable

When antigen-stimulated and unstimulated IFN- γ levels were compared using Mann Whitney U test (non parametric), antigen-induced IFN- γ levels from 10 different stress induced antigens were found statistically different between the cases and non-cases with a p value ≤ 0.05 . The discrimination utility of 6 of the 10 antigens was confirmed by using the receiver operating characteristics (ROC) analysis with area under the curve (AUC) ≥ 0.70 (Table 3.2).

Table 3.2: Utility of stress induced *M.tb* antigens in differentiating TB infection states

Median levels of IFN- γ (pg/ml) and ranges (in parentheses) detected in supernatants from TB cases (n = 24) and household contacts (n = 19) using in-house IFN- γ ELISA 7-day WBA with 23 *M.tb* ' antigens. IFN- γ values were corrected for the unstimulated control levels. P value and AUC are in bold when significant. Sensitivity and specificity are reported with 95% confidence interval.

Antigens	Median TB	Median HHC	P value	AUC	Cut-off value	Sensitivity	Specificity
Rv0244	10 (0 - 91)	0 (0 - 5)	0.23	0.57	< 9.7	100	26
Rv0352	3 (0 - 56)	0 (0 - 4)	0.37	0.54	< 4.8	100	13
Rv0353	5 (0 - 74)	0 (0 - 6)	0.4	0.54	< 3.2	95	13
Rv0711-N	7 (0 - 59)	2 (0 - 28)	0.004	0.73	< 0.6	90	57
Rv0767	7 (0 - 44)	2 (0 - 35)	0.010	0.71	< 0.5	84	57
Rv0839	73 (0 - 1539)	0 (0 - 1)	0.679	0.52	< 9.6	100	14
Rv1528	64 (0 - 1435)	1 (0 - 24)	0.493	0.54	< 5.8	95	17
Rv1908-C	5 (0 - 81)	2 (0 - 25)	0.318	0.56	< 1.4	90	23
Rv1908-N	8 (0 - 89)	0 (0 - 3)	0.023	0.66	< 0.8	95	41
Rv1909	11 (0 - 187)	0 (0 - 6)	0.022	0.65	< 0.2	95	36
Rv1945	31 (0 - 386)	0 (0 - 0)	0.003	0.7	< 0.4	100	39
Rv2382	52 (0 - 752)	0 (0 - 4)	0.007	0.69	< 0.3	95	44
Rv2383-C	37 (0 - 797)	1 (0 - 25)	0.203	0.58	< 0.2	90	26
Rv2383-N	57 (0 - 1268)	0 (0 - 2)	0.010	0.68	< 0.1	95	39
Rv2745	6 (0 - 41)	2 (0 - 46)	0.001	0.75	< 0.6	95	59
Rv2913	45 (0 - 901)	2 (0 - 37)	0.005	0.71	< 1.1	95	52
Rv2987	16 (0 - 112)	2 (0 - 45)	0.000	0.81	< 0.1	84	73
Rv2988	7 (0 - 77)	2 (0 - 43)	0.088	0.62	< 0.1	90	35
Rv2989	1 (0 - 21)	3 (0 - 56)	0.547	0.53	< 0.6	95	17.
Rv3229	3 (0 - 42)	2 (0 - 35)	0.350	0.56	< 0.1	90	22
Rv3654	2 (0 - 39)	0 (0 - 7)	0.139	0.6	< 0.1	90	30
Rv3710	88 (0 - 1889)	25 (0 - 467)	0.159	0.59	< 1.1	90	26
Rv3840	85 (0 - 1929)	2 (0 - 29)	0.74	0.52	< 1.2	84	22

The major draw backs of these results are the low IFN- γ levels and the low number of participants responding to each antigen. Indeed, none of the antigens induced a mean IFN- γ response ≥ 90 pg/ml. For the development of a field-friendly test like a lateral flow detection method, concentration > 100 pg/ml are generally required. The worst antigens included: Rv0244, Rv0352, Rv0353, Rv1908-C, Rv1908-N, Rv2988, Rv2989, Rv3229 and Rv3654. These antigens could not induce IFN- γ mean level ≥ 10 pg/ml. The highest response was observed by Rv3710 and Rv3840 with IFN- γ mean level ≥ 80 pg/ml in TB group (Table 3.2). The low IFN- γ production was more pronounced in the HHCs group. Indeed, none of the antigen induced IFN- γ mean levels ≥ 10 pg/ml in this specific group; furthermore, each antigen induced responses in less than 20% of participants in this group (Figure 3.1).

Although 6 stress induced antigens could discriminate between TB disease and non-disease, the low IFN- γ mean levels and the overlap between groups constitute important limitations in these results; examination of the dot plot (Figure 3.2) demonstrates the lack of clear separation between the study groups. While the p value is statistically significant for a number of markers, most of the values fall between 0 and 40 pg/ml for all antigens. The promising antigens may need a further evaluation with a more standardized and more sensitive ELISA. It is important to mention that the statistical analysis provided in this section may not be accurate due to the number of extrapolated values but could be helpful for antigens selection for further assessment.

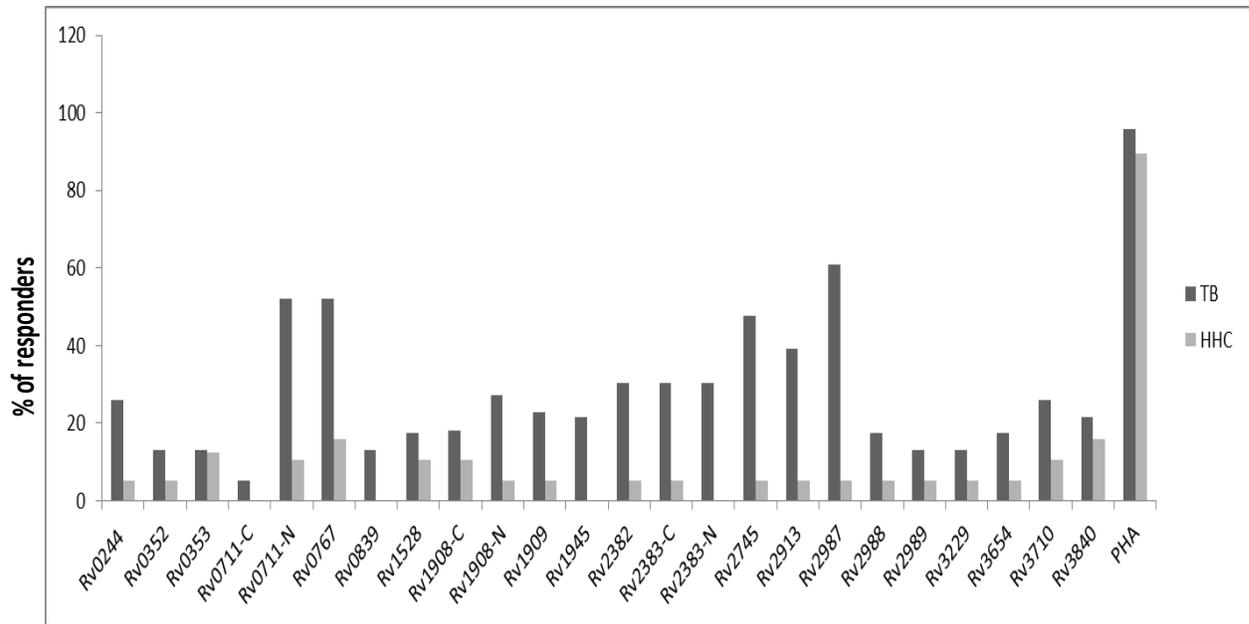


Figure 3.1: Percentage of responders

Percentage of participants responding to stress induced antigens with IFN- γ level ≥ 2 pg/ml (PHA has been used as a positive control). Percentage of responders was low, mainly in household contacts. PHA = Phytohaemagglutinin

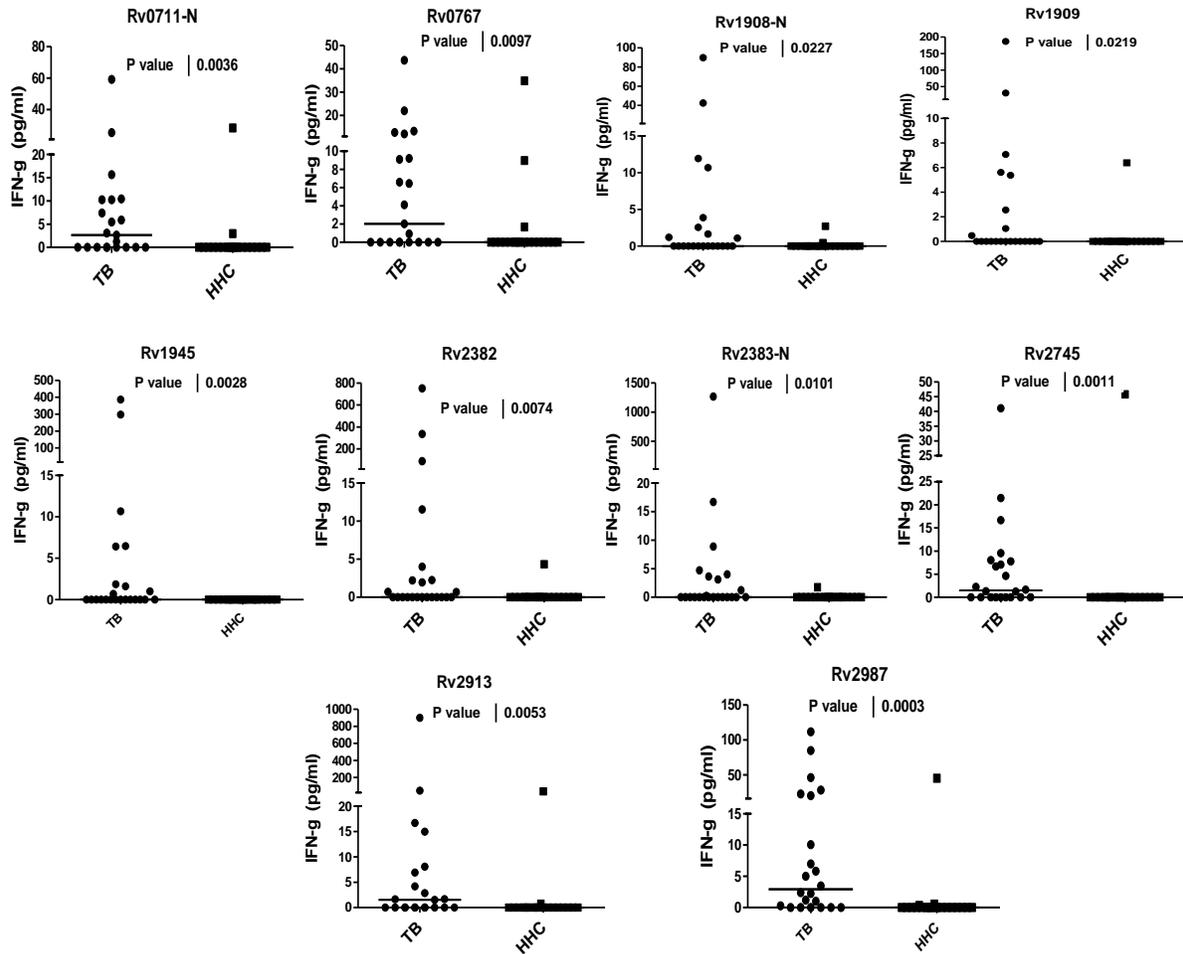


Figure 3.2: IFN- γ (pg/ml) levels

Levels of IFN- γ (pg/ml) detected in TB cases (n = 23) and household contacts (n=19) after in-house IFN- γ ELISA 7-day WBA with stress induced antigens. A total of 23 antigens were tested in this study; only 10 have a statistically significant P value. These discriminative antigens are remarkable by their low IFN- γ levels.

3.2.2 Evaluation of diagnostic utility of IFN- γ in whole blood cultures stimulated with *M.tb* stress induced antigens by QFT ELISA

3.2.2.1 Aims of the study

The aim of this study was to validate the promising stress induced antigens obtained with an in-house IFN- γ ELISA with the more sensitive QFT ELISA.

3.2.2.2 Materials and methods

The diagnostic utility of 24 stress induced antigen were evaluated in the first part of the study. Among the 24 antigens, six had a significant P value in the comparison between TB and non-TB cases. These antigens included: Rv0711, Rv0711-N, Rv0767, Rv2745c, Rv2913 and Rv2987c but some of the median IFN- γ levels were below the lower limit of detection of the in-house ELISA assay. To confirm these results, these antigens were evaluated with the QFT ELISA, which has a wider dynamic range. Samples from Active TB cases and HHCs were collected, processed and analysed as explained above.

3.2.2.3 Results

A total number of 30 participants were randomly selected from the sample bank. This number included 16 TB cases and 14 HHCs. All TB cases had QFT tested at recruitment. HHCs received a TST test or a QFT test at recruitment. The 2 groups had a similar mean age and most of the controls were latently infected (Table 3.3).

Table 3.3: Demographic and clinical characteristics of participants in the validation of the promising stress induced antigen study

The discriminative utility of the most promising stress induced antigens ($AUC \geq 0.70$) evaluated with in-house ELISA (in the first part of this study) was validated here with QFT ELISA. TB (n=16) cases and household contacts (n=14) were randomly selected from our sample bank. Household contacts received a TST test (7) or QFT test (7). All active TB cases received a QFT test.

	All	TB	HHCs
Number of participants	30	16	14
Mean age (years)	29.1	31	26
Age range (years)	10 - 57	10 - 57	10 - 48
Male/female ratio	17/13	12/4	5/9
Participants with TST test	7	N/A	7
Participants with QFT test	23	16	7
QFT or TST (positive/negative)	26/4	15/1	11/3
QFT positive, %	87	94 (15/16)	71 (5/7)
TST positive, %	N/A	N/A	85.7 (6/7)

QFT = QuantiFERON TB Gold In Tube, TB = active tuberculosis, TST = tuberculin skin test, N/A = Not Applicable

ESAT6 was used as *M.tb* control protein in this experiment and was the only marker that discriminated TB disease from non-disease with $AUC = 83$. None of the 6 promising stress induced antigens could separate the study groups when $IFN-\gamma$ levels were compared between groups.

Although the QFT ELISA used in this part of the study had a lower detection limit, antigen-stimulated $IFN-\gamma$ levels could not be detected in most of the study participants. Rv2745c and Rv2913 had the worst performance with their mean levels equal to zero after subtraction of negative control levels (Table 3.1).

As observed with the in house- $IFN-\gamma$ ELISA, the antigen-stimulated $IFN-\gamma$ detection levels were lower in HHCs. None of the participants in this group showed a positive response after negative control subtraction toward any of the stress induced antigens (0% responders for each stress induced antigen) (Figure 3.3). Although $IFN-\gamma$ levels were higher in TB cases compared to HHCs, these levels were also very low for a diagnostic test. Rv0711 and Rv2987c had the highest $IFN-\gamma$ response in the TB group with mean levels ≤ 5 pg/ml and only around 20% responders.

After validation, these results had demonstrated that none of the stress induced antigens were promising in the diagnostic field and that validation of all promising antigens with the QFT ELISA or similar sensitive ELISA is warranted.

Table 3.4: Diagnostic utility of stress induced *M.tb* antigens QFT ELISA

Mean levels of IFN- γ (pg/ml) and ranges (in parentheses) detected in supernatants from TB cases (n = 16) and HHCs (n = 14). The discriminating IFN- γ levels after stress induced antigen stimulation in 7-day whole blood culture supernatants were detected with an in-house IFN- γ ELISA and subsequently validated in a different aliquot of the same sample with a more standardized and sensitive assay (QFT ELISA). IFN- γ antigen-stimulated median levels after the QFT ELISA are shown in this table. IFN- γ values were corrected for the unstimulated control levels. Sensitivity and specificity are reported with 95% confidence interval.

Antigens	Mean TB	Mean HHC	P value	AUC	Cut-off value	Sensitivity	Specificity
Rv0711	4 (0 - 68)	0 (0 - 1)	0.519	0.57	<0	33	73
Rv0711-N	1 (0 - 1246)	0 (0 - 1.6)	0.520	0.57	<0	60	67
Rv0767	0 (0 - 11)	0 (0 - 1)	0.950	0.51	> 0	100	40
Rv2745c	0 (0 - 2)	0 (0 - 1)	0.350	0.6	> 0	93	53
Rv2913	0 (0 - 3)	0 (0 - 0.8)	0.787	0.53	>0	80	47
Rv2987c	4 (0 - 61)	0 (0 - 1)	0.950	0.51	< 1.1	100	47
ESAT6	341 (0 - 696)	89 (0 - 385)	0.003	0.83	< 2.7	64	93

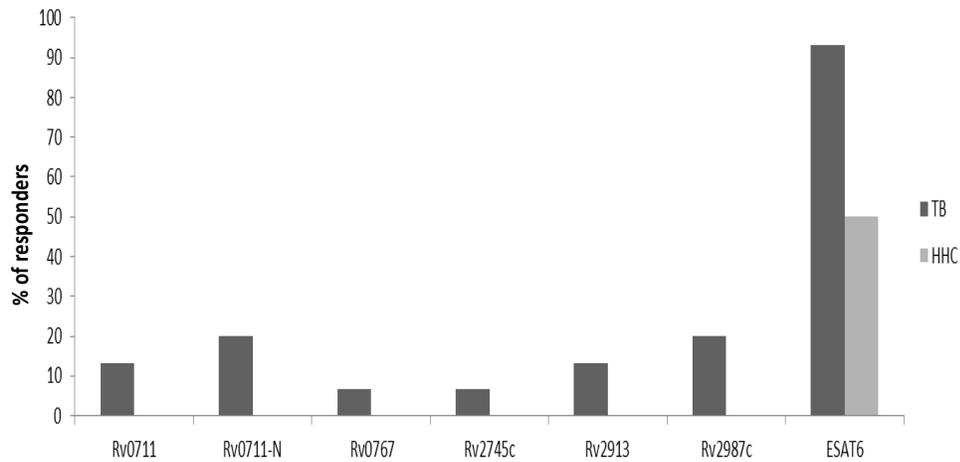


Figure 3.3: Percentage of responders

Percentage of responders to promising stress induced antigens with IFN- γ level ≥ 2 pg/ml. None of the household contacts recognized any of the stress induced antigens.

3.3 Validation of the diagnostic utility of promising DosR and RPFs antigens tested in a previous study

3.3.1 Declaration:

Data presented for this study was part of the publication: “Potential of novel *Mycobacterium tuberculosis* infection phase-dependent antigens in the diagnosis of TB disease in a high burden setting (Chegou, N. N. *et al* [5])

3.3.2 Aims of the study:

The aim of this study was to validate the diagnostic potentials of promising *M.tb* infection phase-dependent antigens that were identified in previous work, by the use of a more sensitive and commercially available IFN- γ ELISA (QFT ELISA kit).

3.3.3 Materials and methods

3.3.3.1 Study participants and sample collection

The first discovery stage of this study was previously conducted in our laboratory and evaluated the diagnostic utility of 118 infection phase-dependent antigens including: resuscitation promoter factors (RPFs) (n=5), dormant regulon (DosR) antigens (n=51), classical antigens (n=8), reactivation antigens (n=24) and other stress induced antigens (n=24) together with 6 peptide pools (Figure 3.4). Among the 118 antigens and peptide pools evaluated, IFN- γ levels induced by the 5 RPFs and 5 of the 51 DosR antigens could discriminate active TB from latent infection with a significant P value and $AUC \geq 0.70$. The present study was designed to validate these promising results. To conduct this experiment, first episode HIV negative, untreated TB cases and recently exposed HHCs were recruited. HHCs were HIV and *M.tb* culture negative, had normal chest X-rays and had been living with a TB case in the same household for at least 2 months. Active TB was confirmed by X-ray, clinical symptoms and sputum culture result. Whole blood was collected from participants in heparinized tubes. Upon arrival in the laboratory, blood samples were 5 times diluted with RPMI1640 medium containing glutamine, aliquoted into 96-well plates and incubated for 7 days with *M.tb* antigens as previously described (see Methods chapter and reference [5]). Plasma were harvested and stored in 3 aliquots at -80°C. The first aliquot was used during the primary stage of the evaluation where IFN- γ levels from each study participant was assessed using an in-house IFN- γ ELISA.

Evaluation of the 118 antigens had identified 10 new promising antigens for the diagnostic field (Table 3.5). Aliquots from 15 TB cases and 27 HHCs were randomly selected from the sample

bank and IFN- γ levels in supernatants for the selected antigens were retested with the QFT ELISA according to the manufacturer instructions. According to the manufacturer, the detection limit of the QFT ELISA was 0.05IU/ml, corresponding to 2 pg/ml. Unstimulated-IFN- γ responses were considered as background and subtracted from antigen-induced IFN- γ responses before further analysis.

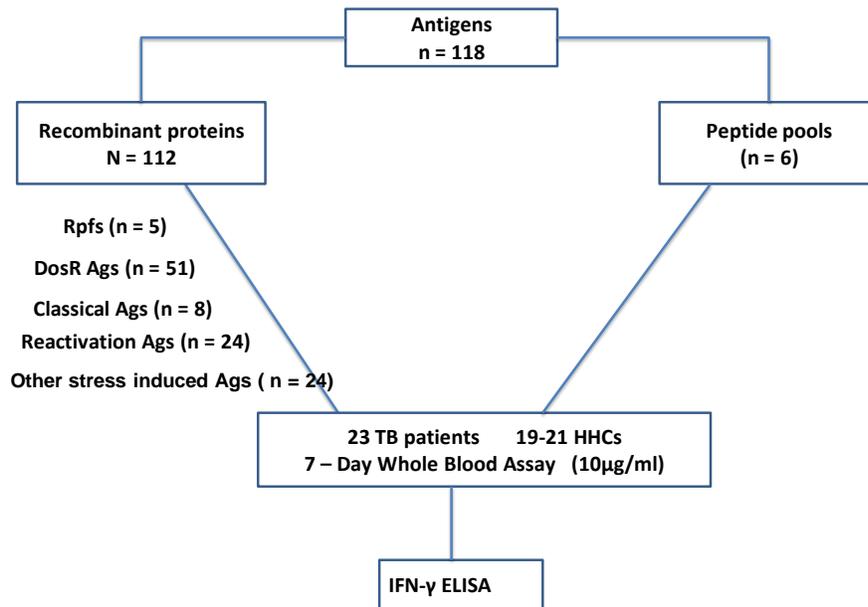


Figure 3.4: Total number of antigens evaluated

A total number of 118 *M.tb* infection phase-dependent antigens were previously evaluated in our laboratory for their ability to induce IFN- γ in a 7-day WBA by employing an in-house IFN- γ ELISA. RPFs: Resuscitation promoter factor, DosR: Regulon antigens

Table 3.5: Most promising infection phase-dependent antigens as assessed with in-house IFN- γ ELISA

Among the 118 antigens previously tested in our group, 5 DosR antigens and the 5 tested RPFs antigens discriminated best between the active TB and latent TB participants with $AUC \geq 0.70$. These antigens were selected for the present study and evaluated in 7-day WBA QFT ELISA. The present table shows the sensitivity, specificity and AUC of the most promising antigens during the evaluation of the 118 antigens with the in-house ELISA. Sensitivity and specificity are reported with 95% confidence interval.

Antigens	Sensitivity	Specificity	AUC
DosR antigens			
Rv2032	70.0	82.6	0.77
Rv2625c	70.0	82.6	0.74
Rv1996c	76.2	82.6	0.75
Rv0081	50.0	95.7	0.71
Rv2624	55.0	91.3	0.73
RPF antigens			
Rv0867c	85	78.2	0.80
Rv2389c	80	78.2	0.78
Rv2450c	80	73	0.76
Rv1009	80	78.3	0.79
Rv1884c	60	87.0	0.72

3.3.3.1 Data analysis

IFN- γ levels elicited by *M.tb* antigens-stimulated and unstimulated samples were compared between TB cases and HHCs using the Mann Whitney U test for nonparametric data analysis. Sensitivity and specificity were ascertained by receiver operating characteristics (ROC) analysis. The predictive abilities of antigen combination models was investigated by performing general discriminant analysis (GDA). The data were analysed using the Statistica 8 software, (Statsoft, Ohio, USA) and GraphPad prism, version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

3.3.4 Results

To confirm the diagnostic utility of the promising antigens observed in the primary stage of this study, supernatants from 15 TB cases and 27 HHCs (as all the antigens were not evaluated in all HHCs at recruitment) were randomly selected from our samples bank (Table 3.6). According to results reported on Table 3.5, Rv0867c, Rv2389 and Rv1009 were the best RPF antigens. They discriminated active TB from latent infection with the same accuracy. The three RPFs could be a good representation of immune response to RPFs. For financial constraints, only the three cited RPFs were selected for further evaluation together with the five discriminative DosR antigens. The selected antigens were re-evaluated using the commercial QFT ELISA. ESAT6/CFP10 was evaluated in this study as *M.tb* control.

Table 3.6: Demographic and clinical characteristics of participants in the validation of promising DosR and RPFs antigens study.

The discriminative utility of the most promising DosR and RPFs antigens previously evaluated in our laboratory was validated in the present study using 7-day WBA QFT ELISA. Plasma from 15 TB cases and 27 household contacts were randomly selected from our sample bank. Household contacts had a skin test at recruitment.

	All	TB	HHCs
Number of participants	42	15	27
Mean age (years)	37	35	37
Age range (years)	13 - 74	13 - 57	16 - 74
Male/female ratio	25/17	13/2	12/15
Participants with TST test	N/A	N/A	20
Participants with unknown TST status	N/A	N/A	7
TST positive (%)	N/A	N/A	63.2

TB = active tuberculosis, TST = tuberculin skin test, N/A = Not Applicable

Of the 9 antigens (5 DosR antigens, 3 RPFs, ESAT6/CFP10) re-evaluated in the present study, only the 3 RPFs confirmed their discriminative utility. Rv0867c and Rv1009 confirmed their discriminative utility reproducing the exact AUC (0.80) observed in the primary study after the ROC analysis (Table 3.5 and Table 3.7). The discriminative utility of Rv2389c slightly decreased

from 0.80 to 0.78. Although these RPFs had satisfactory AUCs, their promising diagnostic utility is limited by the overlap between the two study populations. Indeed, examination of the dot plot figure (Figure 3.5) indicated that RPFs-induced IFN- γ response levels were higher in HHCs than in TB cases. The overlap in IFN- γ values between the two groups makes diagnostic application unlikely at individual participant level. None of these individual antigens could clearly separate the two study groups with AUC above 0.80.

Table 3.7: Diagnostic utility of DosR and RPFs antigens using QFT ELISA

Median levels of IFN- γ (pg/ml) and ranges (in parentheses) detected in plasma from TB cases and household contacts after evaluation with the 7-day WBA QFT ELISA. IFN- γ values were corrected for the unstimulated control levels. The 10 most promising antigens tested in the previous study were evaluated in the present study. All antigens were evaluated in all active TB cases and 15 TB cases were randomly selected from our samples bank. All antigens were not evaluated in all household contacts and 27 HHC samples were selected to allow all antigens to be tested in 15 household contacts. Sensitivity and specificity are reported with 95% confidence interval.

Antigen	Median TB	Median HHC	Cut-off Value	AUC	Sensitivity	Specificity	PPV	NPV
Rv0867 (RPF)	0.9 (0–4)	26 (0–258)	6.2	0.80	0.87	0.73	0.76	0.85
Rv1009 (RPF)	0.6 (0–27)	10 (0–273)	4.5	0.79	0.80	0.67	0.71	0.77
Rv2389c (RPF)	0.4 (0–30)	24 (0–297)	4.9	0.80	0.73	0.80	0.79	0.75
Rv0081	0.2 (0–558)	1 (0–97)	1.6	0.63	0.67	0.73	0.71	0.69
Rv1996 (DosR)	0.8 (0–34)	0 (0–195)	2.7	0.58	0.47	0.80	0.70	0.80
Rv2032	0.5 (0–21)	1 (0–434)	2.6	0.62	0.50	0.80	0.70	0.63
Rv2624 (DosR)	0 (0–4)	0 (0–2)	1.2	0.54	0.33	0.87	0.71	0.57
Rv2625 (DosR)	0.3 (0–31)	1.3 (0–4)	2.3	0.58	0.53	0.67	0.62	0.59
ESAT-6/CFP10	305 (0–666)	308 (0–452)	107	0.46	0.71	0.47	0.71	0.47

AUC = Area under the ROC curve, PPV = Positive predictive value, NPV = Negative predictive value

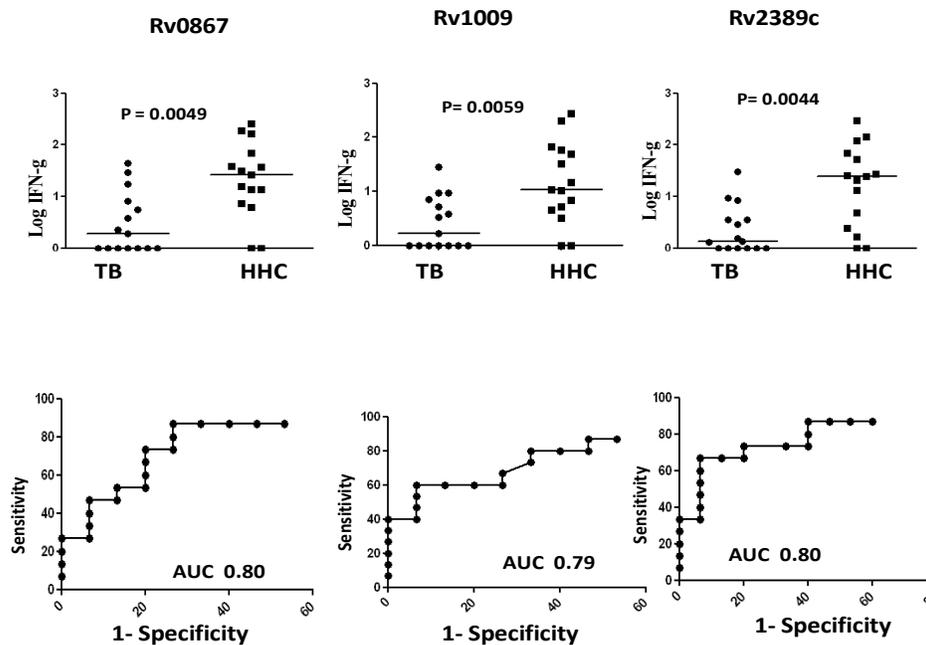


Figure 3.5: IFN- γ levels and AUCs

Log of IFN- γ (pg/ml) levels and the receiver operator characteristics plots (AUC) (shown under each dot plot) showing the accuracies of the resuscitation promoter factor antigens to discriminate between active TB (n=15 patients) and no TB (n=15).

None of the DosR antigens could confirm their diagnostic utility in this validation study. The lack of discrimination of these antigens was accompanied by their poor recognition by study participants mainly in the HHCs group; DosR antigens were only recognized by 40% (or less) of participants. Rv2624 was recognized by none of the HHCs and only 7% of TB cases recognized this antigen (Table 3.8). Contrary to DosR antigens, RPFs were more recognized by HHCs; more than 70% of this group recognized these antigens. RPFs were less recognized in active TB cases (30 to 47%) but contrary to DosR antigens, their average percentage of responders in all samples (TB cases + no TB) was always above 50%.

Table 3.8: Percentage of participants responding to DosR and RPFs antigens in 7-day WBA ELISA

Only RPF could discriminate between TB cases and non TB disease. This table shows AUC, sensitivity and the percentage of participants responding to individual antigen. The percentage of participants responding to distinct antigens was low in this setting mainly with DosR antigens: none of the DosR antigens reached 50 % responders in any of the participant groups. ESAT6/CFP10 was used as *M.tb* control.

	ESAT6/ CFP10	Rv0081	Rv1996	Rv2032	Rv2624	Rv2625c	Rv0867c	Rv1009	Rv2389c
Responders (%) TB	87	26	33	26	7	33	40	47	33
Responders (%) HHCs	82	47	20	43	0	47	87	87	73
Responders (%) TB + HHCs	84	37	27	34	3	40	63	67	53
Sensitivity	71	67	47	50	33	53	87	80	73
Specificity	47	73	80	80	87	67	73	67	80
AUC	46	63	58	62	54	58	80	79	80

When the diagnostic utility of RPFs and DosR antigens was evaluated by General Discrimination Analysis (GDA), ESAT6/CFP10 and 2 of the DosR antigens (Rv2624 and Rv2032) were the most useful markers in GDA models. Each of these antigens was entered into 7 of the 10 best 3-antigen models (Figure 3.6). The most accurate multi-antigen model (ESAT6/CFP10+Rv2624+Rv0867c) accurately classified 83% of study participants into their respective groups. After leave-one-out cross validation the predictive accuracy was 73% for TB cases and 80% for HHCs (Table 3.9).

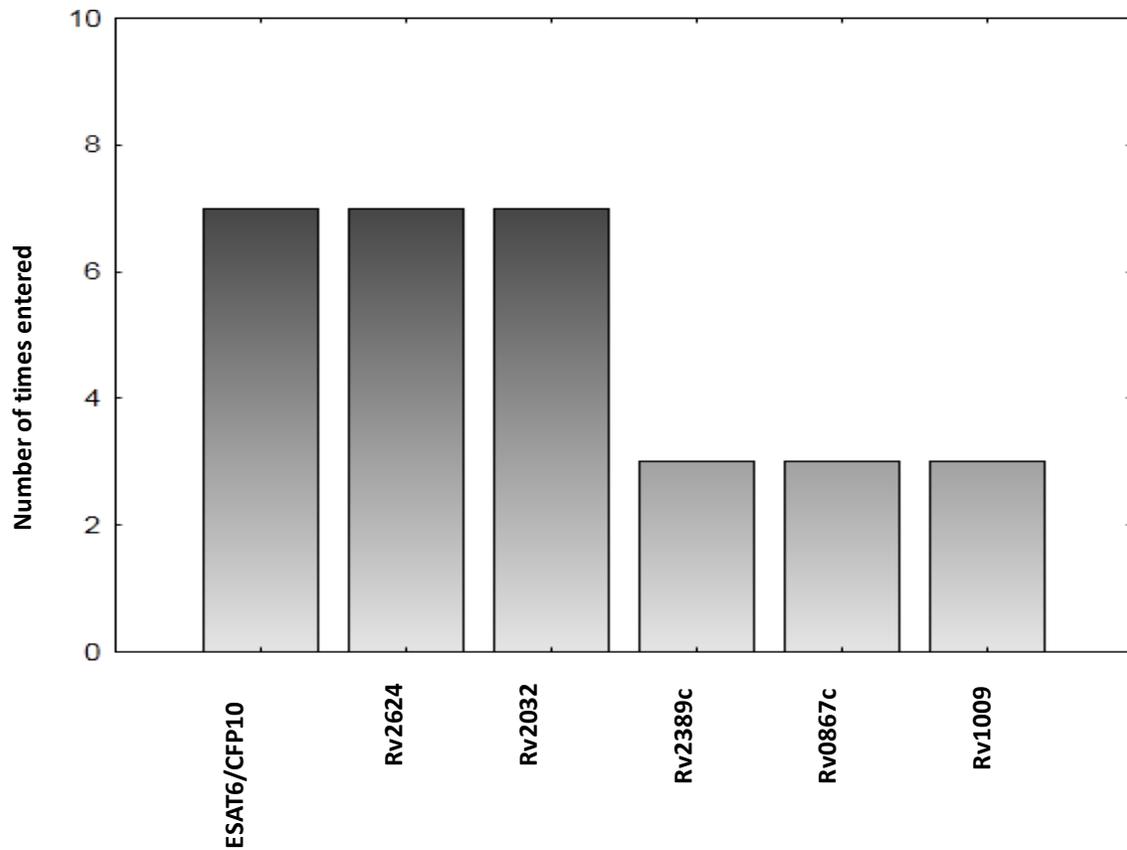


Figure 3.6: Number of inclusions of antigens into the 10 most accurate general discriminant analysis models that most accurately predicted the presence or absence of TB disease

Six *M.tb* antigens were included in different combination models that best discriminated TB from latent infection. The antigens included: ESAT6/CFP10, two DosR antigens (Rv2624, Rv2032) and three RPFs (Rv0867c, Rv1009 and Rv2398c).

Table 3.9: Accuracy of antigen combination models in diagnosing TB disease

The diagnostic utility of antigens was assessed in General discriminant analysis models. Ten different 3-antigen models classified participants into their correct group with reasonable accuracy.

Antigen combination	Resubstitution Classification Matrix			Leave-one-out Cross validation		Wilks lambda	P-value
	% TB cases	% HHCs	Total %	% TB cases	% HHCs		
ESAT6/CFP10, Rv2624, Rv2389c	87	73	80	73	73	0.59	0.0002
ESAT6/CFP10, Rv2624, Rv0867c	87	80	83	73	80	0.60	0.0002
ESAT6/CFP10, Rv2624, Rv1009c	73	73	73	67	67	0.62	0.0004
Rv2032, Rv2624, Rv0867c	80	79	79	80	71	0.39	<0.0001
Rv2032, Rv2624, Rv2389	87	79	83	87	64	0.39	<0.0001
Rv2032, Rv2624, Rv1009	87	71	79	87	64	0.41	<0.0001
ESAT6/CFP10, Rv2032, Rv2389	80	71	76	80	64	0.61	0.0005
ESAT6/CFP10, Rv2032, Rv0867c	80	79	79	73	71	0.62	0.0005
ESAT6/CFP10, Rv2032, Rv1009	73	71	72	73	64	0.63	0.0008
ESAT6/CFP10, Rv2032, Rv2624c	60	64	62	53	64	0.67	0.0015

3.4 Diagnostic potential of alternative antigen-stimulated host markers other than IFN- γ in a long term stimulation assay

3.4.1 Declarations

Data presented in this part has been published in an international, peer reviewed journal:

Potential of host markers produced by infection phase-dependent antigen-stimulated cells for the diagnosis of tuberculosis in a highly endemic area. Chegou *et al*, PLoS ONE [6].

3.4.2 Aims of the study

We aim to investigate whether stimulation of whole blood with promising *M.tb* antigens, results in the production of alternative host markers (other than IFN- γ) which could be used as a diagnostic tool.

3.4.3 Background

By IFN- γ measurement, we have shown that the 5 known *M.tb* RPFs and 5 of the 51 DosR antigens evaluated in the primary study were suitable TB diagnostic candidates. However, none of these antigens was able to diagnose TB disease alone or in GDA model with accuracy above 85%. Works done in our group and subsequently by other investigators have shown that stimulation of whole blood samples with *M.tb* specific antigens results in the production of many host markers, some of which have potential in discriminating between latent TB and TB disease[7][8][9]. However, most of these studies were only based on ESAT6/CFP10 and/or PPD not the new *M.tb* infection phase dependent antigens evaluated in the current study. We assessed the diagnostic utility of other host markers produced in response to these antigens in stimulated and unstimulated samples using the Luminex platform. The study was a case control study where whole blood was stimulated for 7 days with *M.tb* antigens and stimulated and unstimulated levels of each host markers were compared between the study groups.

3.4.4 Material and methods

This part of the study was a multi-analytes case control study where analyte levels from TB cases were compared to levels in healthy controls. Six antigens were selected for the experiment due to the cost of the Luminex platform: ESAT6/CFP10, three DosR antigens (Rv0081, Rv2032 and Rv1737) and two RPFs (Rv2389 and Rv0867). Rv1737 was newly obtained from our

collaborator and was first tested in this section. The diagnostic potential of 12 *M.tb* antigen-stimulated and unstimulated host markers was assessed for each individual antigen. These host markers included: RANTES, Tumor necrosis factor-alpha (TNF- α), Transforming growth factor-alpha (TGF- α), Interferon gamma-induced protein 10 (IP-10), Vascular Endothelial Growth Factor (VEGF), Fractalkine, Epidermal Growth Factor (EGF), Interleukin 12 (IL-12) P40, IL-10, IL-4, IFN-alpha 2, IFN- γ . The classes and functions of these selected markers are shown in Table 3.10 TB cases and HHCs were randomly selected in our sample bank and analysed as explained above.

Table 3.10: classes and functions of the selected markers

Markers	Classes	Functions
RANTES	Chemokine	Leukocytes recruitment
TNF- α	Cytokines	Involve in the inflammation and activation of acute phase reaction
TGF- α	Growth factor	Promote cells proliferation
IP-10	Chemokine	chemoattraction for immune cells and promotion of T cell adhesion to endothelial cells
VEGF	Growth factor	create new blood vessels
Fractalkine/ CX3CL1	Cytokine	Soluble CX3CL1 chemoattracts T cells and monocytes, while the cell-bound promotes strong adhesion of leukocytes to activated endothelial cells,
EGF	Growth factor	Promote cells proliferation
IL-12	Cytokine	Involved in the differentiation of naive T cells into Th1 cells
IL-10	anti-inflammatory cytokine	Downregulates the expression of Th1 cytokines
IL-4	anti-inflammatory cytokine	Downregulates the expression of Th1 cytokines
IFN-alpha 2	Cytokine	mainly involved in innate immune response
IFN- γ	Cytokine	type II interferon, is a cytokine that is critical for innate and adaptive immunity

3.4.5 Results

Participants enrolled into this study were recruited as part of the on-going Bill & Melinda Gates Foundation-funded Grand Challenges in Global Health (GC6-74) study (<http://www.biomarkers-for-tb.net/>). All antigens were tested in all TB cases at recruitment but as not all HHCs were evaluated for each antigen, several groups of 15 HHCs per antigen had to be evaluated. A total number of 39 participants, 15 TB cases and 24 HHCs, were randomly selected. TB cases were all TST positive, only 65% HHCs were TST positive (Table 3.11).

Table 3.11: Demographic and clinical characteristics of participants

Thirty nine participants originally recruited from the on-going Bill & Melinda Gates Foundation-funded Grand Challenges in Global Health (BMGF GC6-74) study (<http://www.biomarkers-for-tb.net/>) were randomly selected for the present study. Plasma from 15 TB cases and 24 HHCs (as all antigens were not tested in all No TB participants) were used for the present study. Each antigen was tested in 15 TB cases and 15 HHCs. All participants had a Tuberculosis Skin Test (TST) at recruitment.

	All	TB	No TB
Number of participants(n)	39	15	24
Mean age (years)	19	31	10
Age range (years)	1 – 59	13 – 59	1 – 59
Male/female ratio	22/14	13/2	9/12
Participants with TST test	35	15	20
Participants with positive TST	28 (80%)	15 (100%)	13 (65%)

3.4.5.1 Diagnostic potential of unstimulated host markers in 7-day culture supernatant

When levels of the 12 cytokines was compared between cases and non-cases in unstimulated supernatants using the Mann Whitney U test (non-parametric), significant differences were observed with EGF, IFN- α 2 and IL-4 (P value ≤ 0.05). Only EFG and IL-4 confirmed their discrimination utility with receiver operating characteristics (ROC) analysis with AUC ≥ 0.70 . These analytes were produced at higher levels in TB cases (Table 3.12).

When these unstimulated analytes were examined in GDA, 20 different combination models of 4 markers could classify participants into their respective groups. The discrimination accuracy of GDA models varied from 85% to 88%. EGF, IL-4, IFN- α 2 and Fractalkine were the most promising markers in combination; they were entered 18, 16, 13, and 9 times, respectively, into the 20 best combinations (Figure 3.7 and Table 3.13).

Sensitivity, specificity and median levels (with ranges in parenthesis) reported in this study were obtained with Prism software. The unstimulated control levels were subtracted from each stimulated host markers for all study participants prior to further analysis of the data. The discriminative utility of each analyte was found to be stimulus dependent.

Table 3.12: Potential of antigens-stimulated and unstimulated host markers to differentiate TB disease from No TB

The whole blood was stimulated with *M.tb* specific antigens (ESAT6/CFP10, Rv0081, Rv2032, Rv1737, Rv2389, and Rv0867) in 7-day WBA. The antigen-stimulated (unstimulated values subtracted) and unstimulated marker levels were measure with the multiplex immunoassay (Luminex). The median levels (pg/ml) and ranges (in parenthesis), AUC, sensitivity and specificity of the most promising host markers (AUC >0.70) for each antigen are shown in this table. Sensitivity and specificity are reported with 95% confidence interval.

Antigens	Markers	Median all	Median TB	Median HHC	AUC	Sensitivity (%)	Specificity (%)	Cut off value
Unstimulated	EGF	2 (2.2 - 18)	12 (2 - 19)	2 (2 - 16)	0.73	53.3	95.8	> 11
	IL-4	8 (2.2-16)	9 (2 - 16)	5 (2 - 16)	0.79	86.7	70.8	> 7
ESAT6/CFP10	EGF	9 (0 - 48)	26 (0 - 48)	0 (0 - 36)	0.8	80	70.8	> 11
	TGF-alpha	1 (0 - 7)	3 (0 - 6)	0 (0 - 7)	0.73	67	79	> 2
	TNF-alpha	79 (0 - 278)	118 (27 - 276)	64 (0 - 276)	0.71	73.3	70.8	> 95
Rv0081	IFN-alpha 2	147 (0 - 205)	1 (0 - 14)	12 (1 - 47)	0.87	78.5	84.6	< 5
	IL-12 P40	0.0 (0 - 20)	0 (0 - 0)	78 (28 - 521)	1	100	100	< 14
	IP-10	139 (0 - 515)	8 (0 - 256)	882 (262 - 5515)	1	100	100	< 2578
	TNF-alpha	50 (1 - 62)	17 (1 - 50)	224 (18 - 621)	1	100	100	< 60
	VEGF	147 (0 - 205)	0 (0 - 740)	315 (0 - 1205)	0.84	80	92.9	< 90
	IFN-γ	0 (0 - 209)	0 (5 - 9)	8 (0 - 209)	0.84	80	76.9	< 1
	IL-10	2 (0 - 129)	0 (0 - 2)	48 (20 - 129)	1	100	100	< 11
	RANTES	366 (0- 2827)	0 (0 - 708)	963 (0 - 708)	0.86	80	78.6	< 393
Rv2032	Fractalkine	6 (0 - 111)	19 (0 - 111)	0 (0 - 26)	0.8	86.7	61.5	> -10
	IL-12p40	3 (0 - 75)	9 (0 - 76)	0 (0 - 27)	0.75	60	84.6	> 7
	TGF-alpha	2 (0 - 14)	7 (0 - 14)	0 (0 - 3)	0.92	80	100	> 3
	TNF-alpha	68 (0 - 468)	135 (49 - 468)	16 (0 - 61)	0.99	93.3	100	> 68
	VEGF	87 (0 - 1057)	168 (0 - 1057)	54 (0 - 226)	0.75	73.3	69.2	> 72
	IL-10	4 (0- 25)	12 (0 - 24.6)	0 (0 - 12)	0.90	93.3	92.3	> 4
	RANTES	195 (0 - 7355)	1699 (0 - 7355)	0 (0 - 1809)	0.81	86.7	84.6	> 20

Rv1737	EGF	14 (0 - 67)	29 (0 - 68)	0 (0 - 39)	0.72	53.3	92.3	> 29
	IL-12	24 (0 - 251)	60 (0 - 251)	18 (0 - 62)	0.78	66.7	92.3	> 30
	TGF-alpha	3 (0 - 20)	10 (0 - 20)	2 (0 - 6)	0.88	80	92.3	> 4
	TNF-alpha	191 (17 - 1715)	291 (117 - 762)	57 (17 - 1715)	0.87	100	69.2	> 97
	IL-10	18 (0 - 150)	41 (14 - 150)	3 (0 - 36)	0.95	100	84.6	> 12
Rv2389	TGF-alpha	5 (0 - 20)	10 (2 - 20)	0 (0 - 5.8)	0.98	100	85.7	> 1
	TNF-alpha	234 (30 - 979)	342 (92 - 979)	167 (31 - 373)	0.86	86.7	78.6	> 200
	VEGF	218 (0 - 999)	394 (0 - 999)	22 (0 - 523)	0.82	80	64.3	> 100
	IFN- γ	11 (0 - 319)	6 (0 - 81)	32 (0 - 319)	0.73	66.7	71.4	< 9
	IL-10	35 (8 - 222)	52 (9 - 222)	26 (8 - 79)	0.73	66.7	85.7	> 42
	RANTES	1413 (0 - 11900)	3992 (0 - 11900)	319 (0 - 3418)	0.74	73.3	71.4	> 934
Rv0867	IFN- γ	18 (0 - 534)	5 (0 - 162)	40 (0 - 534)	0.74	80	71.4	< 23
	TGF-alpha	3 (0 - 21)	8 (0 - 22)	2 (0 - 4)	0.90	80	92.9	> 3

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Table 3.13: Diagnostic potential of unstimulated host markers in GDA models of 4 markers

Negative Control	Resubstitution Classification Matrix			Leave-one-out Cross validation		Wilks lambda	P-value
	% TB cases	% HHCs	Total %	% TB cases	% HHCs		
EGF, IFN- α 2, VEGF, IL-10	80	92	88	73	85	0.53	0.000
EGF, IFN- α 2, IL-4, IL-10	80	92	88	73	85	0.43	0.000
EGF, IFN- α 2, IL-4, IP-10	80	92	88	67	88	0.24	0.000
Fractalkine, TGF- α , IL-4, RANTES	80	92	88	53	92	0.74	0.001
EGF, IFN- α 2, IL-4, TNF- α	80	92	88	73	89	0.24	0.000
EGF, IFN- α 2, IL-4, INF- γ	80	92	88	73	89	0.24	0.000
EGF, IFN- α 2, RANTES, IL-10	80	88	85	73	85	0.50	0.000
EGF, Fractalkine, IFN- α 2, IL-10,	80	88	85	73	85	0.59	0.000
EGF, Fractalkine, IFN- γ , IL-4	73	92	85	66	89	0.39	0.000
EGF, IFN- α 2, IL-4, VEGF	80	88	85	67	89	0.34	0.000
EGF, Fractalkine, TNF- α , IL-4	73	92	85	67	92	0.40	0.000
Fractalkine, TGF- α , IL-12p40, IL-4	73	92	85	67	92	0.71	0.000
EGF, TGF- α , IL-4, IFN- α 2	80	88	85	67	85	0.44	0.000
EGF, Fractalkine, TGF- α , IL-4	80	88	85	67	89	0.62	0.000
EGF, IFN- α 2, IL-4, RANTES	80	88	85	67	85	0.27	0.000
EGF, Fractalkine, IP-10, IL-4	73	92	85	67	92	0.39	0.000
EGF, IFN- α 2, IL-12p40, IL-4	80	88	85	67	85	0.26	0.000
EGF, Fractalkine, IFN- α 2, IL-4	80	88	85	80	89	0.31	0.000
EGF, Fractalkine, IL-4, IL-10	73	92	85	67	89	0.69	0.000
EGF, IFN- α 2, VEGF, IFN- γ	80	88	85	73	89	0.35	0.000

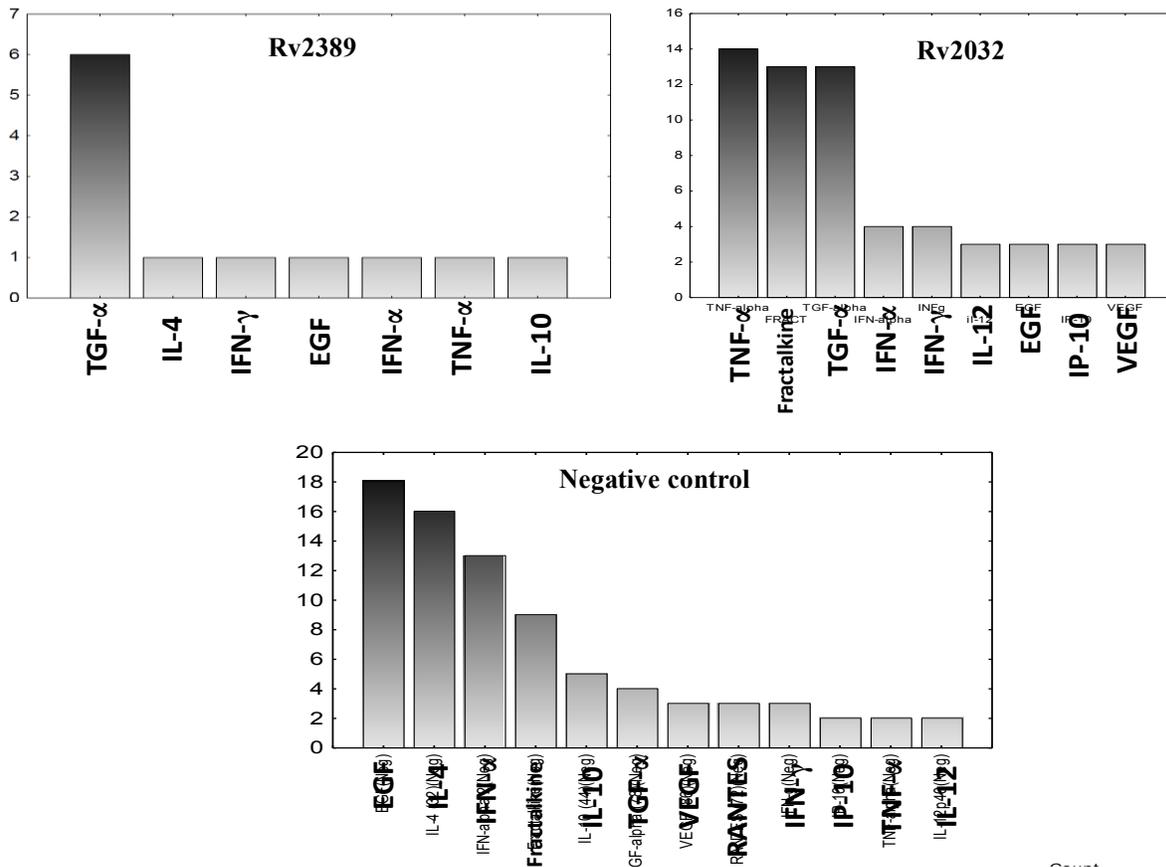


Figure 3.7: Number of inclusions of antigen-stimulated or unstimulated markers into the 20 most accurate GDA models for prediction of the presence or absence of TB disease obtained

3.4.5.2 Diagnostic potential of ESAT6/CFP10 stimulated host markers in 7-day WBA supernatant

When ESAT6/CFP10 stimulated-analyte levels were analysed using the Mann Whitney U test (non-parametric), significant differences were obtained with EGF ($p= 0.001$), TGF- α ($p= 0.014$) and TNF- α ($p= 0.03$). The discriminatory utility of these markers was confirmed with ROC analysis ($AUC \geq 0.70$) (Table 3.12).

3.4.5.3 Diagnostic potential of DosR antigen-stimulated host markers in 7-day WBA supernatant

Three DosR-regulon-encoded antigens (Rv2032, Rv1737c and Rv0081) were evaluated in this study. The diagnostic potential of IFN- γ secretion as measured by Luminex in response to these antigens was poor. Although Rv0081-induced IFN- γ has a significant p value and AUC above 0.70, the low median level measured in both groups makes Rv0081-stimulated IFN- γ unsuitable for a diagnostic test confirming the results obtained with QFT ELISA above.

Rv0081-stimulated host markers (n=12) were evaluated in 7-day culture supernatant in this case-control study. Of the 12 markers, eight (IFN- α 2, IL-12p40, IP-10, IFN- γ , TNF- α , VEGF, IL-10 and RANTES) could discriminate TB disease from the disease-free state with AUC above 0.84. Rv0081-stimulated IP-10, IL-10, TNF- α and IL-12(p40) perfectly classified all the participants (100%) into their respective group with AUC=1 (Table 3.12). These analytes were produced at higher levels by HHCs than TB cases. IFN- α 2, IL-12(p40), IFN- γ and IL-10 have a low median level and would be difficult to measure using most rapid tests.

Rv2032-stimulated analyte levels were also compared between TB cases and HHCs. A number of host markers (n=7) (fractalkine, IL-12(p40), TGF- α , TNF- α , VEGF, IL-10, RANTES) were able to discriminate active TB from no disease with AUC above 0.70. Among these seven host markers, TNF- α , VEGF, and RANTES could be excellent candidates for a diagnostic test. These markers were produced at higher levels by TB cases at a concentration that can be easily measured by available field-friendly tests like lateral flow tests (concentration above 100ng/ml).

When Rv2032-stimulated marker data were analysed in GDA, 20 different models of 3 markers could classify more than 86% of TB cases and 100% of HHCs. After leave-one-out cross validation, these models classify 73 to 100% of TB cases and more than 92% of HHCs (Table 3.14). TNF- α , TGF- α and fractalkine were the most present markers in different models; they respectively entered 12, 11, and 11 times into the top 20 Rv2032- host marker models used in this study (Figure 3.7).

A total number of five Rv1737-stimulated host markers (IL-10, TGF- α , TNF- α , IL-12(p40) and EGF) were significantly higher in the TB cases in comparison to HHCs with AUC above 0.72. Rv1737-stimulated TNF- α could be considered as the best diagnostic marker (among all Rv1737-stimulated host markers) due to the low median levels found in others markers.

Table 3.14: Diagnostic potential of Rv2032-stimulated host markers in GDA models of 3 markers

Rv2032	Resubstitution Classification Matrix			Leave-one-out Cross validation		Wilks lambda	P-value
	% TB cases	% HHCs	Total %	% TB cases	% HHCs		
Fractalkine, TGF- α , TNF- α	93	100	96	80	100	0.2	0.000
Fractalkine, IL-12p40, TGF- α	93	100	96	80	100	0.2	0.000
EGF, Fractalkine, TGF- α	87	100	93	87	100	0.3	0.000
Fractalkine, TGF- α , IFN- α 2	87	100	93	87	100	0.2	0.000
Fractalkine, TGF- α , IP-10	87	100	93	87	92	0.2	0.000
EGF, Fractalkine, TNF- α	87	100	93	73	100	0.3	0.000
Fractalkine, TNF- α , IFN- α 2	87	100	93	80	100	0.2	0.000
Fractalkine, TNF- α , IL-12p40	87	100	93	80	100	0.2	0.000
Fractalkine, TNF- α , IP-10	87	100	93	87	92	0.2	0.000
EGF, TNF- α , TGF- α , IFN- α 2, TNF- α , TGF- α ,	87	100	93	80	100	0.2	0.000
IL-12p40, TNF- α , TGF- α ,	87	100	93	80	100	0.2	0.000
IP-10, TNF- α , TGF- α ,	87	100	93	87	92	0.2	0.000
Fractalkine, TGF- α , VEGF	87	100	93	87	100	0.2	0.000
TGF- α , TNF- α , VEGF	87	100	93	80	92	0.2	0.000
Fractalkine, TNF- α , VEGF	87	100	93	87	100	0.2	0.0000
Fractalkine, TGF- α , IFN- γ	87	100	93	87	92	0.2	0.000
Fractalkine, TNF- α , IFN- γ	87	100	93	87	100	0.3	0.000
IFN- γ , TNF- α , IFN- α 2	87	100	93	80	100	0.3	0.000
TGF- α , TNF- α , IFN- γ	87	100	93	87	100	0.2	0.000

3.4.5.4 Diagnostic potential of RPF-stimulated host markers in 7-day WBA supernatant

Rv0867c and Rv2389c were evaluated in this study as two of the five known RPFs. The diagnostic potential of IFN- γ was confirmed with both RPFs. Both RPF-induced IFN- γ tests discriminated TB cases from HHCs with AUC above 0.72 confirming the results obtained with the in-house IFN- γ ELISA and QFT ELISA. The low IFN- γ levels measured in both groups makes Rv0867c and Rv2389c-stimulated IFN- γ less suitable candidates for diagnostic tests (Table 3.12). IFN- γ was produced at higher levels in HHCs than in TB cases.

TGF- α followed the same pattern as IFN- γ with both RPFs: it was produced at higher levels in TB cases than in HHCs and discriminated TB disease from no TB. As IFN- γ , the TGF- α level was low. None of the other Rv0867c stimulated host markers could discriminate between TB disease and no disease.

Rv2389c could discriminate TB disease from no disease with four others host markers (TNF- α , VEGF, IL-10 and RANTES) in addition to IFN- γ and TGF- α . Like TGF- α and IFN- γ , these markers were produced at higher levels in TB cases. Rv2389c-stimulated TNF- α , VEGF and RANTES could be more suitable diagnostic candidates due to their high levels in supernatants that could be accessed by available rapid tests.

When Rv2389c-stimulated analytes were analysed in GDA, models of two markers could classify 100% of HHCs and 86 to 100% of TB cases. After leave-one-out cross validation, these models classified 80 to 100% of TB cases and 92 to 100% of HHCs (Table 3.15). TGF- α was entered into all six two-marker models (Figure 3.7).

Table 3.15: Diagnostic potential of Rv2389-stimulated host markers into the best six two-marker GDA models.

Rv2389	Resubstitution Classification Matrix			Leave-one-out Cross validation		Wilks lambda	P-value
	% TB cases	% HHCs	Total %	% TB cases	% HHCs		
TNF- α , IL-4	100	100	100	100	100	0.12	0.000
TGF- α , IFN- γ	93	100	96	93	100	0.25	0.000
EGF, TGF- α	93	100	96	93	100	0.28	0.000
TGF- α , IFN- α 2	87	100	93	87	93	0.18	0.000
TGF- α , TGF- α	87	100	93	80	100	0.19	0.000
TNF- α , IL-10	87	100	93	87	100	0.18	0.000

3.5 Discussion

ESAT6/CFP10 and PPD remain the main *M.tb* antigens for immune diagnosis of *M.tb* infection. This study has demonstrated the possible use of alternative *M.tb* antigens and subsequently alternative host markers. Little is known about the immunogenicity of these antigens in active TB cases or in latently infected individual. The few studies that have investigated the immunogenicity of these antigens have found a correlation between IFN- γ production and latent infection [10,11]. Stress antigens-induced IFN- γ and DosR antigens-induced IFN- γ , as a single marker, could not discriminate active TB from latent infection. Alternative antigen-induced host markers like Rv0081-induced IP-10 and Rv0081-induced TNF- α are promising markers in the immune diagnostic field.

Stress induced antigens and DosR antigens were poorly recognized in this study as measured by IFN- γ ELISA. Stress induced antigens are recombinant proteins usually associated with stress conditions [12]. DosR antigens are associated to latent infection and are upregulated under certain conditions including hypoxia, nutrient starvation, low nitric oxide or low pH in the macrophage[10-15]. IFN- γ responses elicited by stress induced antigens and DosR antigens were not significantly different between TB cases and HHCs. This lack of discrimination could be explained by the low IFN- γ detection levels in response to these antigens. The inclusion of some of the DosR antigens-induced IFN- γ into models that best predicted active TB may indicate their utility in the TB diagnostic field.

RPFs were the only antigens to confirm their discrimination utility with a strong concurrence with previous results. They are growth-promoting proteins; they resuscitate dormant bacteria and enhance their growth [16]. Like the DosR antigens, they were more recognized by HHCs than TB cases. RPFs are believed to be upregulated during the replication phase of the bacteria in the host [16, 17]. These data support the belief that *M.tb* can live inside the host in a slow replicating form [18]. Indeed, after the first contact with *M.tb*, the bacteria can replicate inside the host with or without invasion of its host immune system [19].

The last part of this study concentrated on alternative antigen-induced host markers other than IFN- γ . The results have demonstrated that IFN- γ may not be the most suitable host marker to discriminate infection states: None of the antigen-induced IFN- γ (alone or in combination models) could discriminate more than 85% active TB cases from latently infected individuals. IP-10, TNF- α , VEGF and RANTES could be more suitable in a long term assay. When Rv0081 stimulated IP-10 and TNF- α were evaluated, each of these individual stimulated markers had excellent stand-alone diagnostic ability in the long term assay. To the best of our knowledge, this was the first study indicating a perfect discrimination of active and latent infection using newly identified *M.tb* antigens.

The host markers evaluated in the present study include pro-inflammatory and anti-inflammatory molecules. The presence of these host markers during infection may not be specific to TB. Indeed, the host produces a number of cytokines and chemokines in order to eradicate different infectious agents including *M.tb* [20-22]. Although the host proteins measured in this work may also be expressed in other diseases the present work evaluated *M.tb* antigen-specific production of these effector molecules and the biosignatures that were found may be able to differentiate between TB and other lung conditions but this would have to be evaluated in future studies where other conditions are included. Such a unique pattern could be quantitative or qualitative and could lead to rapid and field-friendly tests of TB disease. Diagnosis of TB disease is further complicated by the presence of a subclinical form of infection, so-called latent infection. This state is frequently found in high endemic areas, often in low or middle income countries with no treatment policy for latent infection due to limited resources [24]. The challenge is not the diagnosis of infection, as it is successfully accomplished by IGRAs, but to identify a host marker that could discriminate active TB case from latently infected individuals. Researchers have

recently identified novel *M.tb* infection phase-dependent antigens including DosR antigens and RPFs. Data evaluating the discriminative utility of *M.tb* infection phase-dependent antigen-stimulated host markers are very limited, conflicting and have only been performed on small studies with limited number of study participants due to the high cost of multiplex immunoassay kits [23, 25, 26]. A few studies that have investigated the diagnostic utility of *M.tb* antigen-stimulated markers, mostly including ESAT6/CFP10, are in agreement with the present study regarding a number of promising diagnostic markers like IP-10, IL-12p(40) and TNF- α [27, 28, 22].

Although these results are novel and promising, the study has many limitations. The major limitations are the small sample size and the use of a 7-day whole blood assay instead of an overnight or an ex vivo assay, which would be more suitable for a diagnostic test. This is a pilot study; results will be validated in a shorter-term (overnight) culture assay in chapter 4.

3.6 Conclusion

This study has shown the promising diagnostic values of RPFs antigens certainly due to the high frequency of responders to these antigens. This result was obtained with a small population, further studies should include some DosR antigens.

3.7 References

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4. Chapter IV

Evaluation of alternative host markers in a short term assay

4.1 Introduction

The diagnosis of tuberculosis (TB) is still a major challenge in the control of this pandemic [1]. Sputum based tests and serological tests have many well-known limitations. These limitations call for the development of a new TB test.

In view of all the limitations of sputum based tests, urine based tests and serological tests, immune based tests might be a promising approach for the development of a rapid, simple and cost effective test.

IGRAs have improved the immune diagnostics of latent TB [2]. This development is mainly due to the introduction of *M.tb* specific antigens: ESAT6 and CFP-10. The absence of these antigens in most of the non-tuberculosis Mycobacteria and in Bacille Calmette-Guérin (BCG) has increased IGRAs specificity. The discovering of novel *M.tb* phase dependant antigens has opened a new area in TB research.

The use of alternative *M.tb* antigens other than ESAT6 and CFP10 should be mirrored by the search for alternative host markers other than IFN- γ . Indeed, in the previous chapter, we have shown that alternative antigen-induced host markers like Rv0081-induced IP10 and Rv0081-induced TNF- γ could correctly discriminate between latent TB and active TB [3]. However, this study was conducted in a long term stimulation assay (7-day WBA) less suitable for a diagnostic test. Furthermore, healthy individuals were recruited as controls instead of people with alternative pulmonary disease in the present study.

In this chapter the diagnostic utility of alternative antigen-induced host markers other than IFN- γ was assessed in a short term assay and compared to the long term assay. Analyte levels in each individual assay were correlated across assays.

4.2 Diagnostic utility of antigen-stimulated and unstimulated in long and short term assays

4.2.1 Declaration

Data presented in this part has been published in an international, peer reviewed journal:

Essone PN, Chegou NN, Loxton AG, Stanley K, Kriel M, van der Spuy G, Franken KL, Ottenhoff TH, Walzl G. Host cytokine responses induced after overnight stimulation with novel *M. tuberculosis* infection phase-dependent antigens show promise as diagnostic candidates for TB disease. *PLoS One*. 2014 15;9(7):e102584.

4.2.2 Aim of the study

We aim to validate, in a short term stimulation assay, promising alternative host markers that were found in the previous chapter and compare the marker levels in the 3 different assay formats.

4.2.3 Materials and Methods

4.2.3.1 Study population

Participants were recruited from the Ravensmead/Uitsig community in the Western Cape Province of South Africa. The study was performed as a pilot study to an ongoing larger investigation, the EDCTP-funded AE-TBC project, that was conducted in 7 African and 5 European institutions (www.ae-tbc.eu). The study population consisted of newly diagnosed first time pulmonary TB cases who were enrolled before the initiation of anti-TB therapy (n=10), and 20 participants who presented with a cough for more than two weeks at the health care facility and were suspected of having TB disease. All study participants underwent a thorough clinical

workup including chest x-rays and HIV testing using a rapid test (Abott, Germany). Blood and sputum samples, along with other samples needed for routine clinical investigations were collected from all study participants. Sputum samples were cultured using the MGIT method (BD Biosciences). Isolation of *M.tb* complex organisms was confirmed in all positive cultures by means of an *M.tb* complex specific PCR [4]. Participants were eligible for the study if they were ≥ 18 years old, had no previous history of TB, were not pregnant, were not involved in a drug or vaccine trial and if they had no other known chronic diseases like diabetes mellitus. All participants provided written informed consent for participation in the study including for HIV testing, storage and use of the samples for immunological biomarker discovery purposes. Ethical approval for the study was obtained from the Human Research Ethics Committee of the University of Stellenbosch (N10/08/274).

Pulmonary TB disease was confirmed in five of the 20 participants suspected of having TB, as well as all the 10 already known first-time TB cases by means of culture as described above. These 15 individuals constituted the “TB disease” group as described in this study. The “no TB” disease group comprised of the 15 remaining participants who had negative cultures and no other features suggestive of active pulmonary or extra-pulmonary TB disease, including negative chest X rays. None of the participants in the no TB group was treated for TB by the national TB control program.

4.2.3.2 Antigen and cytokines selection and samples processing

The diagnostic utility of three newly identified *M.tb* antigens (Rv2029c, Rv2032 and Rv2389c) together with ESAT6/CFP10 was evaluated in two assays (overnight WBA and 7-day WBA) and marker expressions were also compared in Quantiferon supernatants.

A total number of 26 host markers were selected for the present study including EGF, Fractalkine, Interferon-gamma induced protein-10 (IP-10), monocyte chemotactic protein-1 (MCP-1), Macrophage inflammatory protein-1 α (MIP-1 α), MIP-1 β , soluble CD40 ligand (sCD40L), TGF- α , Tumor Necrosis factor alpha (TNF- α), Vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 (MMP-2), MMP-9, RANTES, C-reactive protein (CRP),

Serum amyloid A (SAA), Serum amyloid P (SAP), IFN- α 2, IFN- γ , Interleukin-1alpha (IL-1 α), IL-12p40, IL-15, IL-17, IL-4, IL-10, IL-1 β and IL-12p70.

Whole blood was collected from each participant and delivered to the laboratory within 2 hours. Upon arrival to the laboratory, the whole blood from each participant was divided for the overnight and 7-day assay. Whole blood was 5 times diluted with RPMI 1640 (containing L-glutamine) (Sigma, Germany) in the 7-day assay. The undiluted (overnight WBA) and diluted blood was mixed with the different *M.tb* antigens and placed in the incubator (5% CO₂, 37°C) for overnight and 7 days respectively. RPMI 1640 was used as negative controls when Phytohaemagglutinin (PHA) (Sigma, Germany) was used as positive control at a final concentration of 0.5 μ g/ml. The Quantiferon tubes (Nil, Antigen, Mitogen) were also incubated overnight under the same conditions. Supernatants from individual assays were aliquoted into microcentrifuge tubes and frozen at -80⁰.

4.2.3.3 Luminex multiplex immunoassay

The 26 host marker levels were measured in overnight, 7-day and QFT-IT culture supernatants from all study participants, using customized Milliplex kits (Merck Millipore, St. Charles, Missouri, USA). Assays were performed on the Bio-Plex™ platform (Bio-Rad Laboratories, Hercules, USA) according to the instructions of the kit manufacturer (Merck Millipore). Prior to assay, samples for the detection of CRP, SAA and SAP were diluted 1 in 8000 using the assay buffer provided in the kit, following optimization experiments. To enable the accurate detection of all the other host markers evaluated, the overnight WBA and QFT-IT culture supernatants were diluted 1 in 2 using the kit serum matrix as previously described [5], whereas the 7-day WBA supernatants were tested neat (undiluted). Samples were evaluated in a blinded manner. All analyte levels in the quality control reagents provided by the kit manufacturer were within the expected ranges. 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 (Bio-Plex Manager™ Software, version 4.1.1).

4.2.3.4 Statistical analysis

Comparison between groups (for example TB vs. no TB) was done using the Mann Whitney U test for non-parametric data analysis. The accuracy of all antigen-induced host markers for the diagnosis of TB disease was estimated by performing receiver operator characteristics (ROC) curve analysis. Optimal cut-off values were selected based on the Youden's index, defined as the largest difference between the sensitivity and 1-specificity, taken over all points on the ROC curve. The predictive abilities of combinations of analytes for TB disease were investigated by performing best subsets general discriminant analysis (GDA), with leave-one-out cross validation as previously described [5]. Data were analysed using the Statistica software (Statsoft, Ohio, USA) and GraphPad prism, version 5.0 (GraphPad Software, San Diego, CA, USA).

4.2.4 Results

4.2.4.1 Study participants

Of the 30 participants enrolled into this study, 6 (25%) were males. The mean age of all study participants was 29.9(\pm 12.5) years. All the 15 study participants with TB disease had culture and *M.tb*-PCR-confirmed pulmonary TB. Of the 27 participants in whom QFT-IT testing was done 19 (70.4%) were positive using the manufacturer's recommended cut-off value (\geq 0.35IU/ml). The clinical and demographic characteristics of all study participants are shown in Table 4.1

Table 4.1: Demographic and clinical characteristics of participants

Participants (n=30) were recruited in the African European tuberculosis consortium (AE_TBC) study.

	All	TB	NO TB
Number of participants	30	15	15
Mean age, years (SD)	29.9(\pm 12.5)	26(\pm 14.2)	33.5(\pm 10.2)
Male/female ratio	6/24	4/11	2/13
HIV status, (positive/negative)	3/30	0/15	3/15
Participants with QFT-IT test	27	13	14
QFT-IT positive, n(%)	19(70.4)	12(92.3)	7(50)

4.2.4.2 Utility of host markers detected in 7-day antigen-stimulated culture supernatants in the diagnosis of TB disease

The unstimulated or antigen-specific levels of 8 of the 26 host markers evaluated (fractalkine, IFN- α 2, SAA, IP-10, EGF, IFN- γ , MMP-2, MMP-9) were significantly different ($p < 0.05$) between the TB cases and controls (50% of whom were QFT-IT positive) either in the 7-day unstimulated supernatants, or following 7-day stimulation with at least one of the four antigens evaluated.

The unstimulated levels of fractalkine and SAA were significantly higher in TB cases whereas the unstimulated levels of IFN- α 2 and MMP-2 were higher in the non TB cases (Table 4.2).

When the antigen-specific responses were calculated by subtraction of the unstimulated marker levels from the levels obtained after stimulation with the respective antigens, ESAT-6/CFP-10-specific levels of EGF, IP-10 and MMP-9 were significantly higher ($p < 0.05$) in TB cases (Table 4.2). Following stimulation with Rv2029c, only MMP-9 responses were significantly different between the TB and no TB cases (higher in the TB cases) (Table 4.2). Similarly, Rv2389c elicited the production of higher levels of MMP-9 and EGF in the TB cases. Only EGF levels were significantly different between the TB and no TB cases (higher in TB cases) following stimulation with Rv2032 (Table 4.2, Figure 4.1).

When the diagnostic accuracy of the markers obtained in supernatants after 7-day stimulation of whole blood with the antigens was evaluated by ROC curve analysis, the area under the ROC curve (AUC) was above 0.70 for ESAT-6/CFP-10 specific levels of EGF, IP-10 and MMP-9, Rv2029c-specific levels of MMP-9, Rv2389c-specific levels of EGF and MMP-9, Rv2032-specific levels of EGF, and the unstimulated levels of fractalkine, IFN- α 2, MMP-2 and SAA (Table 4.2). Using the cut-off values derived after ROC analysis, the unstimulated levels of fractalkine and Rv2389c-specific levels of MMP-9 ascertained TB disease with sensitivities of 100% and 93% respectively, but with lower specificities (53% and 79% respectively). ESAT-6/CFP-10 specific MMP-9 was the only marker that ascertained TB disease with both sensitivity and specificity $\geq 80\%$ (Table 4.2).

When the accuracy of combinations of markers was assessed by general discriminant analysis (GDA), optimal prediction of TB disease was achieved if markers were used in combinations of four. Seven different GDA prediction models accurately classified all the study participants (100%) into their appropriate clinical groups. After leave-one-out cross validation, these models could still correctly classify 100% of the non-TB cases, but only $\leq 80\%$ of the individuals with active TB. The most accurate prediction model (ESAT-6/CFP-10-specific EGF, Rv2032-specific VEGF, Rv2029C-specific IL-1beta and Rv2389c-specific MMP-9) accurately classified all participants (100%) after leave-one-out cross validation (Table 4.3. 1). The most frequently occurring analytes in the 20 most accurate predictive models included ESAT-6/CFP-10 specific EGF, Rv2029c-specific SAP and Rv2029c-specific SAA (Figure 4.2).

Table 4.2: Diagnostic potential of antigen-stimulated and unstimulated host markers in 7-day WBA

Median levels (and ranges in parenthesis) of analytes detected in 7-day whole blood culture supernatants and accuracy in the diagnosis of TB disease are shown. P-values were calculated using the Mann Whitney U test. Only analytes with $AUC \geq 70\%$ are shown. Cut-off values, sensitivity and specificity (at 95% confidence interval) were selected based on the Youden's index. AUC= Area under the receiver operator characteristics curve. All analyte levels are in pg/ml except for SAA (ng/ml).

7-day WBA	Markers	Median TB	Median NO TB	P value	AUC	Sensitivity (%)	Specificity (%)	Cut off value
ESAT6/CFP10	EGF	6.2 (0 – 13)	0 (-2.3 – 4.8)	0.004	0.80	67	80	> 1.5
ESAT6/CFP10	IP-10	4015 (334 - 15943)	1664 (0 - 9999)	0.05	0.71	73	53	> 1675
ESAT6/CFP10	MMP-9	77644 (33259-205268)	32611 (0 - 272113)	0.006	0.80	87	80	> 61832
Rv2029c	MMP-9	83925 (23153-234362)	36556 (0 - 120001)	0.014	0.77	71	60	> 46418
Rv2032	EGF	8 (0 – 25)	5 (0 – 23)	0.023	0.74	67	60	> 4.3
Rv2389c	EGF	10 (0 – 27)	1 (0 – 37)	0.023	0.74	73	79	> 8.7
Rv2389c	MMP-9	69513 (37074-960090)	17719 (382 -83527)	0.000 4	0.89	93	79	> 50285
Unstimulated	Fractalkine	33 (15 - 33)	3.5 (2 - 432)	0.046	0.71	100	53	> 9.5
Unstimulated	IFN- α	2 (2 - 6)	10 (2 -19)	0.038	0.72	87	60	< 7.4
Unstimulated	MMP-2	251 (112-40475)	5805 (112 -66578)	0.023	0.74	80	73	< 4359
Unstimulated	SAA	10006 (0-90178)	560 (560–34274)	0.041	0.72	73	69	> 630

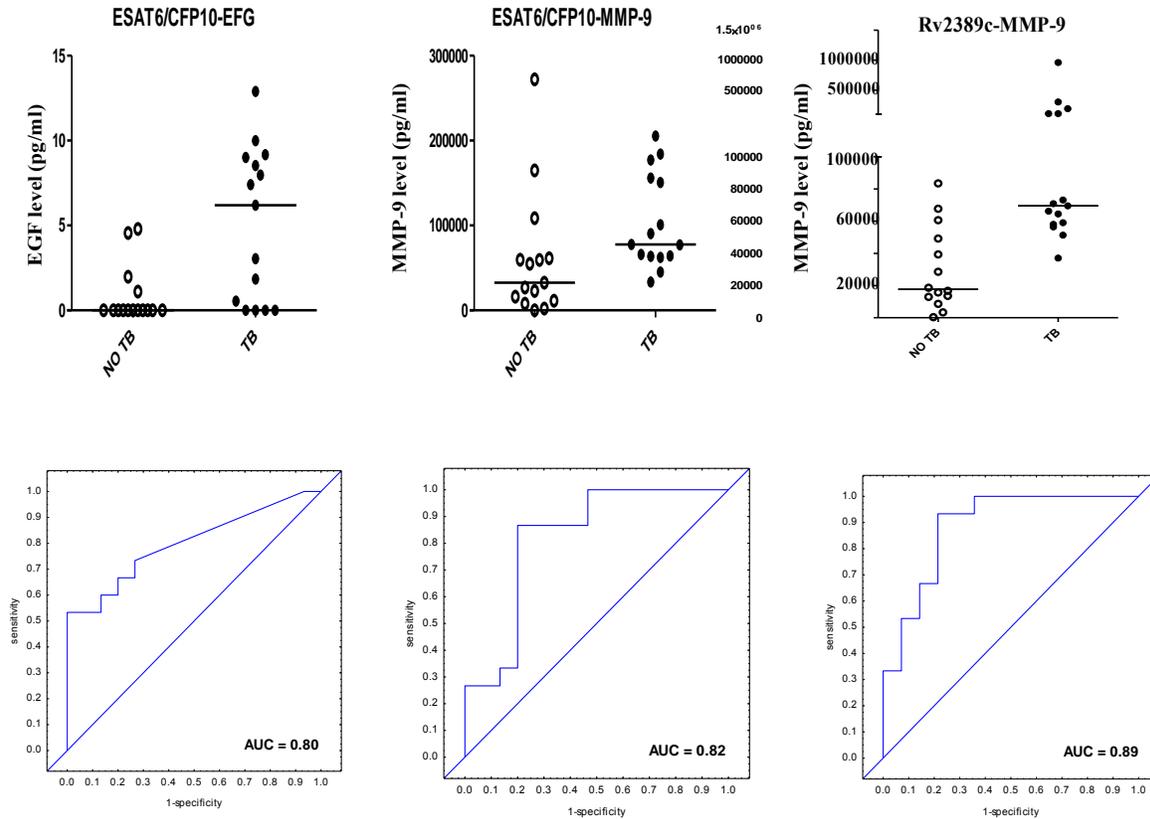


Figure 4.1: The top discriminative host markers in the 7-day WBA culture supernatant

Whole blood from TB cases and controls were stimulated with *M.tb* antigens and several host marker levels were measured with Luminex. The scatter dot plot and ROC plots shown here demonstrate the diagnostic accuracies of the best three markers in the 7-day whole blood supernatant. Bars in the scatter dot plots represent the median analyte levels.

Table 4.3. 1: Utility of combination of analytes in 7-day culture supernatants in the diagnosis of TB disease

The 4-analyte models were generated by general discriminant analysis. In each case, effect df = 1, HHC: Household contact

Host marker model (7-day WBA)	Resubstitution Classification Matrix			Leave-one-out Cross validation		Wilks lambda	f	Error df	P- value
	% TB cases	% HHCs	Total %	% TB cases	% HHCs				
EGF(ESAT6/CFP10), VEGF(Rv2032), MCP-1(Rv2389c), MMP-9(Rv2389c)	93	100	97	93	100	0.862	3.8	24	0.062
EGF(ESAT6/CFP10), VEGF (Rv2032), sCD40L(Rv2389c), MMP-9(Rv2389c)	93	100	97	87	100	0.939	1.6	24	0.22
EGF(ESAT6/CFP10), VEGF (Rv2032), MMP-9(Rv2389c), SAP(Rv2029c)	100	100	100	87	100	0.975	0.57	23	0.459
EGF(ESAT6/CFP10), VEGF(Rv2032), MCP-1(Rv2032), MMP-9(Rv2389c)	93	100	97	93	100	0.866	3.7	24	0.066
EGF(ESAT6/CFP10), VEGF(Rv2032), IP-10(Unstimulated), MMP-9(Rv2389c)	93	100	97	87	100	0.851	4.2	24	0.052
EGF(ESAT6/CFP10), VEGF(Rv2032), Rantes(Unstimulated), MMP-9(Rv2389c)	93	100	97	87	100	0.833	4.8	24	0.038
EGF(ESAT6/CFP10), VEGF(Rv2032), MMP-2(ESAT6/CFP10), MMP-9(Rv2389c)	93	100	97	93	100	0.972	0.7	24	0.420
EGF(ESAT6/CFP10), VEGF(Rv2032), IL-1beta(Rv2029c), MMP-9(Rv2389c)	100	100	100	100	100	0.996	0.1	23	0.768
EGF(ESAT6/CFP10), VEGF(Rv2032), MMP-9(Rv2389c), MCP-1(ESAT6/CFP10)	93	100	97	93	93	0.942	1.5	24	0.239
EGF(ESAT6/CFP10), VEGF(Rv2032), MMP-9(Rv2389c), Fractalkine(Unstimulated)	93	100	97	93	93	0.866	3.7	24	0.066
EGF(ESAT6/CFP10), VEGF(Rv2032), MMP-9(Rv2389c), IL-12p40(Rv2029c)	93	100	97	93	100	0.966	0.8	23	0.384
EGF(ESAT6/CFP10), VEGF(Rv2032), MMP-9(Rv2389c), MIP-1α(Rv2029c)	100	100	100	86	100	0.995	0.1	23	0.757
EGF(ESAT6/CFP10), VEGF (Rv2032), MMP-9(Rv2389c), SAP(Rv2032)	100	100	100	87	100	0.988	0.3	24	0.596
EGF(ESAT6/CFP10), VEGF(Rv2032), MMP-9(Rv2389c), VEGF(Unstimulated)	93	100	97	80	100	0.871	3.4	24	0.072
EGF(ESAT6/CFP10), VEGF(Rv2032), MMP-9(Rv2389c), TNF-α(Rv2029c)	93	100	97	93	100	0.994	0.1	23	0.729
EGF(ESAT6/CFP10), VEGF(Rv2032), MMP-9(Rv2389c), IL-12p40(Rv2032)	100	100	100	80	100	0.980	0.5	24	0.492
EGF(ESAT6/CFP10), VEGF(Rv2032), MMP-9(Rv2389c), CRP(Unstimulated)	93	100	97	80	100	0.913	0.2	24	0.145
EGF(ESAT6/CFP10), VEGF(Rv2032), MMP-9(Rv2389c), EGF(Rv2389c)	93	100	97	80	100	0.912	2.3	24	0.143
EGF(ESAT6/CFP10), VEGF(Rv2032), MMP-9(Rv2389c), MIP-1β(Rv2389c)	100	100	100	93	100	0.998	0.04	23	0.832
EGF(ESAT6/CFP10), VEGF(Rv2032), MMP-9(Rv2389c), IL-1α(Rv2032),	100	100	100	80	100	0.996	0.08	24	0.786

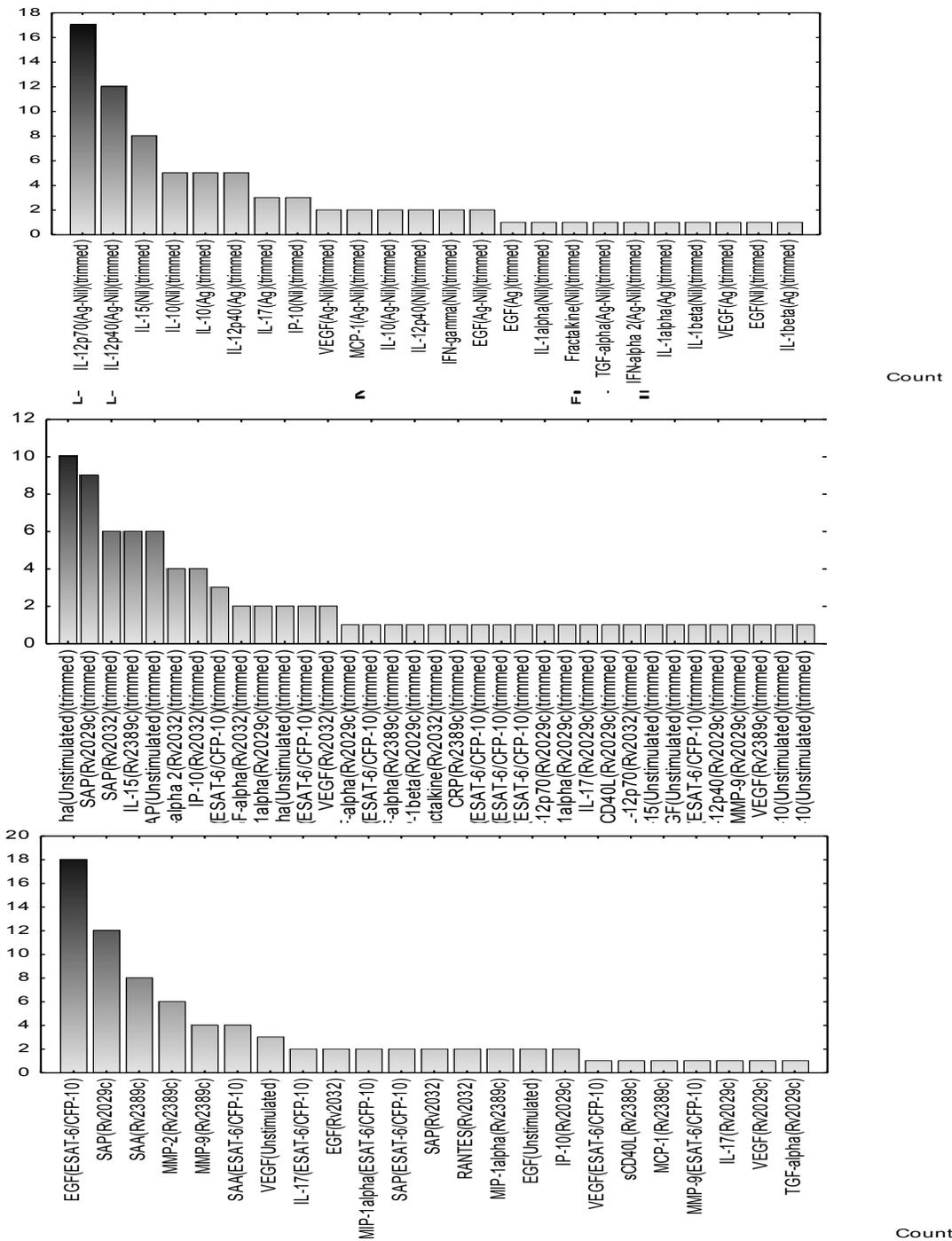


Figure 4.2: Number of inclusions of antigen-induced markers into the most accurate general discriminant analysis (GDA) models in different assays.

GDA of 4 unstimulated and stimulated-host marker models was generated for each individual assay. The column represents the frequency of an inclusion of unstimulated or stimulated-host marker in the top models for each assay. NC: unstimulated, Ag: antigen EC: ESAT6/CFP10

4.2.4.3 Utility of host markers detected in overnight culture supernatants (in-house assay) in the diagnosis of TB disease

The unstimulated or antigen-specific levels of 8 markers (MMP-2, sCD40L, IP-10, IFN- γ , IL-1 β , SAA, SAP and CRP) showed significant differences between the TB cases and controls. Unstimulated CRP and SAA levels were significantly higher in the TB cases (Figure 4.3). ESAT-6/CFP-10-specific levels of IP-10, sCD40L, IFN- γ and SAP were significantly higher in TB cases. Similarly, Rv2389c-specific levels of SAP and CRP and Rv2032-specific levels of SAP were higher in the TB cases whereas Rv2032-specific MMP-2 was higher in the non TB cases (Table 4.4). When the diagnostic accuracy of the markers detected in overnight culture supernatants was assessed by ROC curve analysis, all the markers that showed significant differences between groups had AUC > 0.70 (range, 0.71 to 0.83). ESAT-6/CFP-10-specific IP-10 and IFN- γ , Rv2029c-specific IL-1 β and Rv2032-specific MMP-2 all diagnosed TB disease with sensitivity $\geq 93\%$, but specificity was low for some markers (e.g. 67% for ESAT-6/CFP-10-specific IP-10). Contrarily, unstimulated CRP levels ascertained TB with low sensitivity (60%) but with very high specificity (100%) (Table 4.5).

When the data obtained from overnight culture supernatants were analysed by the GDA procedure, optimal prediction of TB or no TB disease was achieved if markers were used in combinations of four. The most accurate 4-analyte model (Rv2029c-specific TNF- α + Rv2032-specific IFN- $\alpha 2$ + Rv2032-specific SAP + Rv2389c-specific IL-15) accurately classified 92% of the TB cases and 85% of non TB cases after leave-one-out cross validation (Table 4.5). The most frequently occurring analytes in the top 20 predictive models included unstimulated TGF- α and Rv2029c-specific SAP (Figure 4.2).

Table 4.4: Diagnostic potential of antigen-stimulated and unstimulated host markers in the overnight assay

Median levels (and ranges in parenthesis) of analytes detected in overnight (*our in-house*) whole blood culture supernatants and accuracy in the diagnosis of TB disease. All analyte levels are in pg/ml except for CRP, SAA and SAP (ng/ml). P-values were calculated using the Mann Whitney U test. AUC = Area under the receiver operator characteristics curve. 95% CI = 95% confidence interval. Sensitivity and specificity are reported with 95% confidence interval.

Overnight WBA	Markers	Median TB	Median NO TB	P value	AUC	Sensitivity (%)	Specificity (%)	Cut off value
Unstimulated	SAA	705597 (560-4000000)	15641 (560-309939)	0.013	0.76	73	56	> 20389
ESAT6/CFP10	IP-10	16811 (8176-19512)	1826 (0-19353)	0.042	0.72	93	67	> 4499
ESAT6/CFP10	sCD40L	252 (0-2539)	0 (0-435)	0.015	0.78	79	60	> 15.01
ESAT6/CFP10	IFN- γ	339 (8-3578)	8 (0- 1558)	0.047	0.72	93	67	> 59.81
ESAT6/CFP10	SAP	4481 (0-22380)	0 (0-17026)	0.022	0.75	86	67	> 8.650
Rv2029c	IL-1 β	0 (0-18.64)	0 (0-24)	0.037	0.71	100	25	> -3.260
Rv2032	MMP-2	0 (0-7236)	12379 (0-20606)	0.002	0.83	100	75	< 9147
Rv2032	SAP	5529 (0-24063)	0 (0-20031)	0.034	0.75	73	73	> 2842
Rv2389c	CRP	6317 (0-442665)	12.3 (0-116862)	0.050	0.72	69	67	> 1922
Rv2389c	SAP	6509 (0-22648)	0 (0-10267)	0.032	0.74	69	60	> 1216

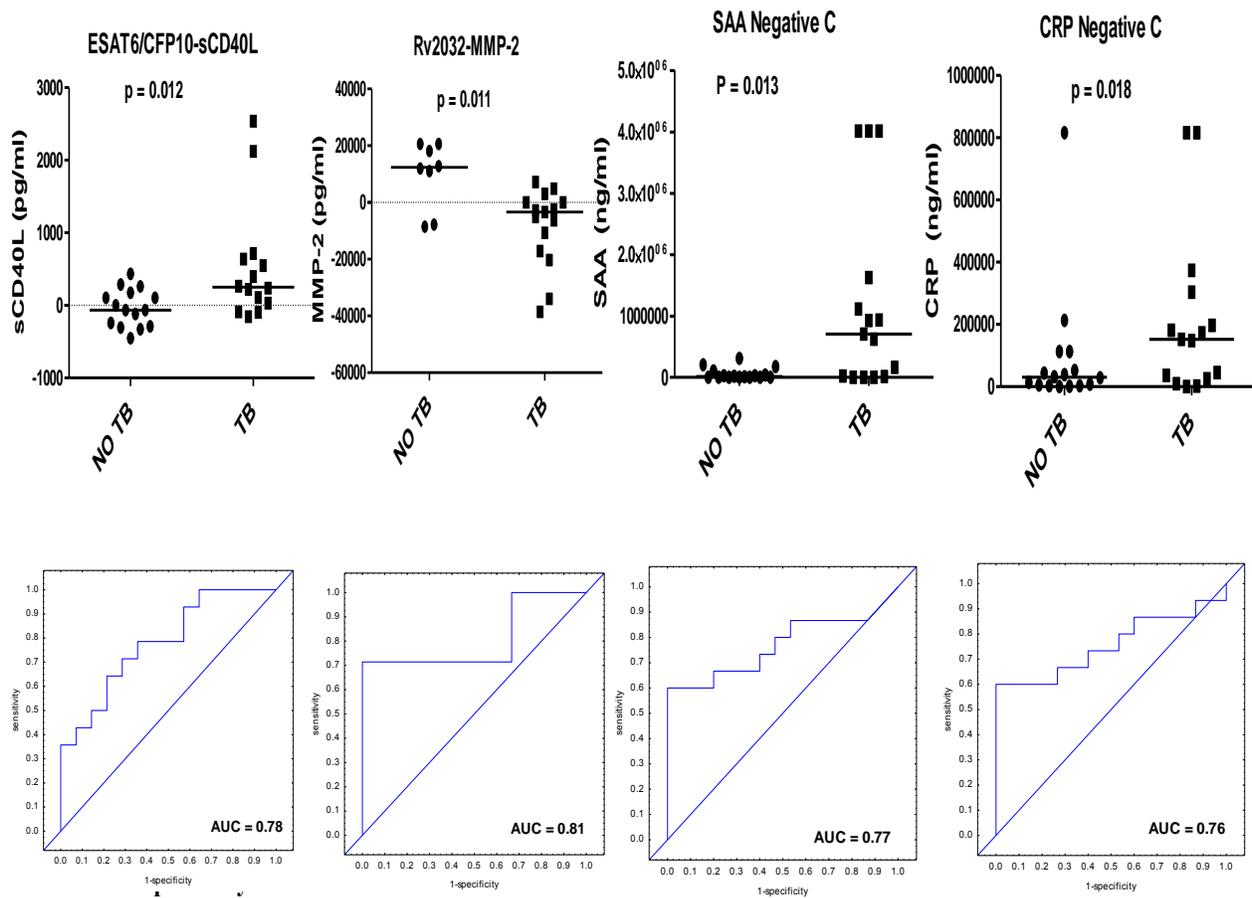


Figure 4.3: The top discriminative host markers in the overnight WBA culture supernatant

Whole blood from TB cases and controls were stimulated with *M.tb* antigens and several host marker levels were measured with Luminex. The scatter dot plot and ROC plots shown here demonstrate the diagnostic accuracies of the best four markers (with $AUC \geq 0.76$) in the overnight whole blood supernatant. Bars in the scatter dot plots represent the median analyte levels.

Table 4.5: Accuracy of combinations of analytes detected in overnight culture supernatants

Accuracy of 4-analyte models generated for markers detected in overnight (*our in-house*) whole blood culture supernatants in the diagnosis of TB disease. In each case, effect df = 1

Host marker model	Resubstitution Classification Matrix			Leave-one-out Cross validation		Wilks lambda	f	Error df	P-value
	% TB cases	% HHCs	Total %	% TB cases	% HHCs				
TNF- α (Rv2029c), IFN- α 2(Rv2032), SAP(Rv2032), IL-15(Rv2389c)	100	85	92	92	85	0.799	5.0	20	0.037
SAP(Rv2029c), TGF- α (Rv2032), SAP(Unstimulated), TGF- α (Unstimulated)	92	79	85	92	64	0.949	1.1	21	0.302
IL-1 α (ESAT6/CFP10), SAP(Rv2029c), TGF- α (Rv2389c), SAP(Unstimulated)	100	79	88	92	79	0.909	2.1	21	0.163
fractalkine(ESAT6/CFP10),SAP(Rv2029c), TGF- α (Unstimulated), SAP(Unstimulated)	92	71	80	92	71	0.986	0.3	21	0.602
MIP-1 β (Rv2029c), IFN- α 2(Rv2032), SAP(Rv2032), IL-15(Rv2389c)	100	85	92	92	77	0.814	4.6	20	0.045
IL-1 α (Rv2029c), SAP(Rv2029c), IL-15(Rv2389c), IL-1 α (Unstimulated)	92	71	81	75	64	0.493	21.6	21	0.000
SAP(Rv2029c), fractalkine(Rv2032), TGF- α (Unstimulated), SAP(Unstimulated)	100	86	92	83	71	0.910	2.1	21	0.166
sCD40L(ESAT-6/CFP-10),IL-1 α (Rv2029c),TGF- α (Rv2032), CRP(Rv2389c)	92	92	92	92	92	0.623	12.1	20	0.002
IL-12p40(ESAT-6/CFP-10), IP-10(Rv2032), VEGF(Rv2032), TGF- α (Unstimulated)	64	54	59	64	54	0.578	16.0	22	0.000
fractalkine(ESAT-6/CFP-10), IL-1 β (ESAT-6/CFP-10), SAP(Rv2029c), TGF- α (Unstimulated)	94	64	77	92	57	0.451	25.5	21	0.000
fractalkine(ESAT-6/CFP-10), IL-10(ESAT-6/CFP-10), SAP(Rv2029c), TGF- α (Unstimulated)	100	64	81	100	57	0.522	19.2	21	0.000
IL-12p70(Rv2029c), IP-10(Rv2032), VEGF(Rv2032), TGF- α (Unstimulated)	92	36	62	58	29	0.632	12.2	21	0.002
MIP-1 α (Rv2029c), IFN- α 2(Rv2032), SAP(Rv2032), IL-15(Rv2389c)	100	77	88	92	77	0.862	3.2	20	0.089
sCD40L(ESAT-6/CFP-10),IL-17(Rv2029c), SAP(Rv2032), IL-15(Rv2389c)	100	85	92	92	85	0.504	19.6	20	0.000
sCD40L(Rv2029c), IL-12p70(Rv2032), SAP(Rv2032), IL-15(Unstimulated)	100	86	92	100	79	0.376	34.8	21	0.000
SAP(Rv2029c), EGF(Unstimulated), TGF- α (Unstimulated), SAP(Unstimulated)	100	67	81	92	67	0.965	0.8	22	0.386
IP-10(ESAT-6/CFP-10), IL-12p40(Rv2029c), IP-10(Rv2032), TGF- α (Unstimulated)	100	85	92	83	85	0.795	5.1	20	0.035
MMP-9(Rv2029c), IFN- α 2(Rv2032), SAP(Rv2032), IL-15(Rv2389c)	92	100	94	92	100	0.752	4.3	13	0.059
IP-10(Rv2032), VEGF(Rv2389c),IP-10(Unstimulated), IL-10(Unstimulated)	77	69	73	62	62	0.825	4.5	21	0.047
SAP(Rv2029c), IL-1 α (Unstimulated), TGF- α (Unstimulated), SAP(Unstimulated)	92	73	91	92	73	0.985	0.3	22	0.570

4.2.4.4 Utility of host markers detected in QFT-IT culture supernatants in the diagnosis of TB disease

The unstimulated or antigen-specific levels of ten markers (MCP-1, MIP-1 β , VEGF, IP-10, IL-10, IL-1 β , CRP, SAA, SAP and MMP-9) showed significant differences when evaluated in QFT-IT supernatants, with most of the discriminatory markers being detected in unstimulated supernatants. The unstimulated levels of six markers (MCP-1, MIP-1 β , VEGF, IL-10, IL-1 β and MMP-9) were significantly higher in the non-TB cases while the unstimulated levels of CRP, SAA, SAP and IP-10 were significantly higher in the TB cases. The antigen-specific levels of MCP-1 and IP-10 were significantly higher in the TB cases (Table 4.6, Figure 4.4). When the diagnostic accuracy of the data obtained from QFT-IT supernatants was assessed by ROC curve analysis, AUC was ≥ 0.70 for all the markers showing significant differences between the TB cases and non-cases including unstimulated MCP-1, MIP-1 β , VEGF, IL-10, IL-1 β , CRP, MMP-9, SAA, CRP and SAP (range 0.72-0.84). When the data was analysed by GDA, eight of the 20 most accurate 4-analyte prediction models accurately classified 100% of the TB cases and up to 89% of the non-TB cases after leave-one-out cross validation (Table 4.7). The most frequently occurring analytes in the top 20 models included the antigen-specific levels of IL-12p70, IL-12p40, and unstimulated IL-15 amongst others (Figure 4.2).

Table 4.6: Diagnostic potential of antigen-stimulated and unstimulated host markers in QFT-IT assay

Median levels (and ranges in parenthesis) of analytes detected in QFT-IT supernatants and accuracy in the diagnosis of TB disease. All analyte levels are in pg/ml except for CRP, SAA and SAP (ng/ml). P-values were calculated using the Mann Whitney U test. Cut-off values, sensitivity and specificity were selected based on the Youden's index. AUC= Area under the receiver operator characteristics curve. 95% CI = 95% confidence interval. Nil = unstimulated marker levels, Ag = levels detected in antigen stimulated supernatants, Ag-N = Antigen specific marker levels obtained after subtraction of nil responses. Sensitivity and specificity are reported with 95% confidence interval.

QFT WBA	Marker	Median TB	Median NO TB	P value	AUC	Sensitivity (%)	Specificity (%)	Cut off value
Ag	CRP	188210 (3411-816000)	12918 (120 - 332914)	0.009	0.79	79	53	> 21119
Ag	SAA	579939 (560 - 4000000)	1115 (560 - 222320)	0.004	0.82	79	73	> 22743
Ag	SAP	48247 (24799 - 74998)	35242 (560 - 69282)	0.034	0.73	79	80	> 41151
Ag-NIL	IP-10	15799 (0 - 19426)	1773 (0 - 16430)	0.006	0.80	86	60	> 3011
Ag-NIL	MCP-1	388 (0 - 7881)	0 (0 - 12922)	0.047	0.72	79	67	> -957
NIL	MCP-1	9969 (1909 - 18125)	14912 (4368 - 20001)	0.015	0.77	93	60	< 14413
NIL	MIP-1 β	1096 (74 - 2258)	1792 (548 - 3190)	0.017	0.76	71	60	< 1587
NIL	VEGF	31 (2 - 626)	285 (2 - 727)	0.042	0.72	79	60	< 199
NIL	IL-10	8 (2 - 51)	41 (2 - 86)	0.032	0.74	64	80	< 11
NIL	IL-1 β	108 (2 - 365)	331 (17 - 1957)	0.050	0.72	64	67	< 236
NIL	CRP	457028 (2831 - 816000)	35080 (157 - 816000)	0.032	0.74	71	60	> 38702
NIL	SAA	1979000 (943 - 4160000)	1821 (560 - 173709)	0.002	0.84	79	67	> 19493
NIL	SAP	44639 (23918 - 56777)	32733 (22470 - 79572)	0.026	0.75	79	73	> 38040
NIL	MMP-9	617975 (275049 - 7201000)	1347000 (331645-3605000)	0.018	0.80	79	89	< 741242

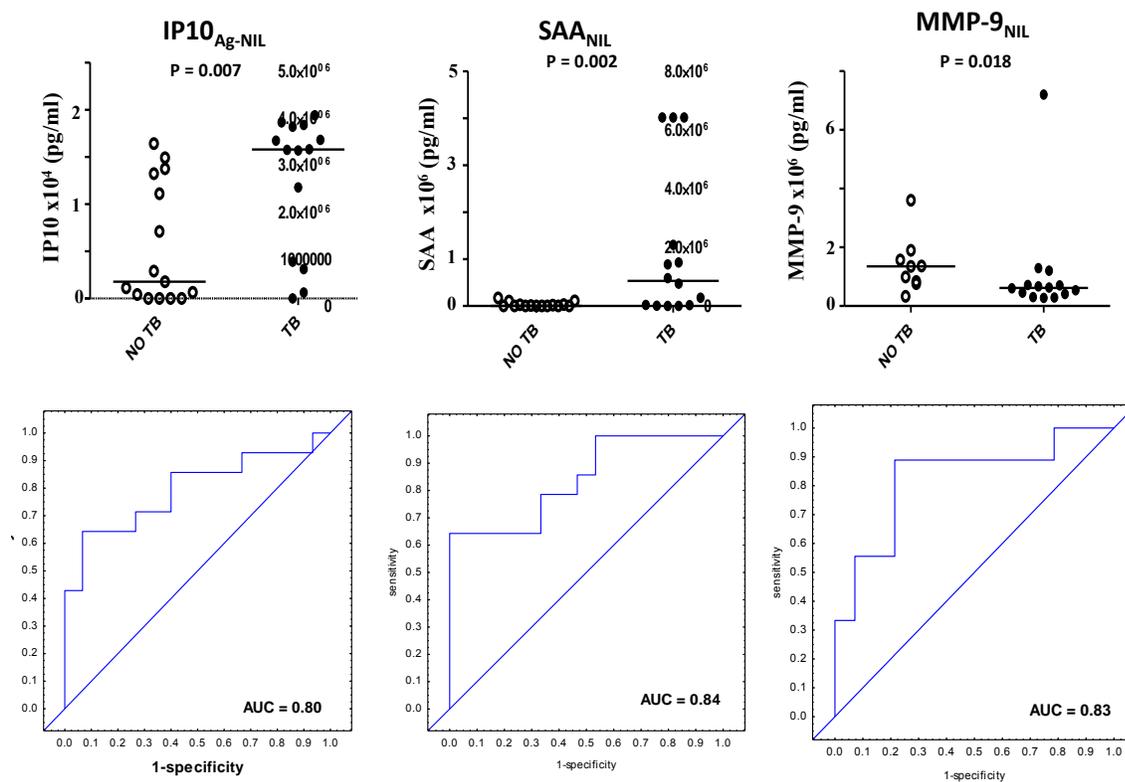


Figure 4.4: The top discriminative host markers in QFT supernatants

Whole blood from TB cases and controls were collected in Quantiferon tubes incubated overnight and several host marker levels were measured with Luminex. The scatter dot plot and ROC plots shown here demonstrate the diagnostic accuracies of the best three markers (with $AUC \geq 0.80$) in quantiferon supernatant. Bars in the scatter dot plots represent the median analyte levels.

Table 4.7: Utility of combinations of analytes detected in QFT-IT supernatants in the diagnosis of TB disease

Accuracy of 4-analyte GDA models in QFT-IT supernatants in the diagnosis of TB disease. Nil = unstimulated marker levels, Ag = levels detected in antigen stimulated supernatants, Ag-N = Antigen-specific biomarker levels obtained after subtraction of Nil responses. In each case, effect df = 1, error df = 18

Host marker model	Resubstitution Classification Matrix			Leave-one-out Cross validation		Wilks lambda	f	P-value
	% TB cases	% HHCs	Total %	% TB cases	% HHCs			
IL-12p40 _{Ag-Nil} , IL-12p70 _{Ag-Nil} , IL-15 _{Nil} , IL-10 _{Nil}	100	100	100	100	89	0.850	3.2	0.092
EGF _{Ag} , IL-12p40 _{Ag-Nil} , IL-12p70 _{Ag-Nil} , IL-15 _{Nil}	100	100	100	100	89	0.844	3.3	0.084
IL-12p40 _{Ag-Nil} , IL-12p70 _{Ag-Nil} , IL-15 _{Nil} , IL-1 α _{Nil}	100	89	96	100	89	0.601	11.9	0.002
IL-17 _{Ag} , VEGF _{Ag-Nil} , fractalkine _{Nil} , IP-10 _{Nil}	100	100	100	100	89	0.433	23.5	0.000
IL-10 _{Ag} , IL-12p40 _{Ag-Nil} , IL-12p70 _{Ag-Nil} , TGF- α _{Ag-Nil}	100	89	96	100	89	0.994	0.1	0.745
IL-17 _{Ag} , MCP-1 _{Ag-Nil} , VEGF _{Ag-Nil} , IP-10 _{Nil}	100	100	100	100	100	0.426	24.2	0.000
IL-12p40 _{Ag} , IL-10 _{Ag} , IL-10 _{Ag-Nil} , IL-12p70 _{Ag-Nil}	100	100	100	100	89	0.812	4.2	0.056
IL-12p40 _{Ag} , IL-12p40 _{Nil} , IL-12p70 _{Ag-Nil} , IFN- γ _{Nil}	93	100	96	93	89	0.886	2.3	0.147
IL-12p40 _{Ag} , IL-10 _{Ag} , IL-12p70 _{Ag-Nil} , IL-10 _{Nil}	100	100	100	100	89	0.794	4.7	0.045
IL-10 _{Ag} , EGF _{Ag-Nil} , IFN- α 2 _{Ag-Nil} , IL-12p70 _{Ag-Nil}	93	100	96	93	89	0.890	2.2	0.152
IL-12p40 _{Ag} , IL-12p40 _{Ag-Nil} , IL-12p70 _{Ag-Nil} , IL-10 _{Nil}	100	100	100	93	100	0.929	1.4	0.256
IL-1 α _{Ag} , IL-12p40 _{Ag-Nil} , IL-12p70 _{Ag-Nil} , IL-15 _{Nil}	100	89	96	100	89	0.571	13.5	0.002
IL-1 α _{Nil} , IL-12p40 _{Ag-Nil} , IL-12p70 _{Ag-Nil} , IL-15 _{Nil}	100	100	100	100	89	0.667	9.0	0.008
IL-17 _{Ag} , EGF _{Ag-Nil} , IL-12p40 _{Nil} , IP-10 _{Nil}	93	100	96	93	89	0.371	30.5	0.000
VEGF _{Ag} , IL-12p40 _{Ag-Nil} , IL-12p70 _{Ag-Nil} , IL-15 _{Nil}	100	89	96	100	89	0.661	9.2	0.007
IL-12p40 _{Ag-Nil} , IL-12p70 _{Ag-Nil} , IL-10 _{Nil} , IFN- γ _{Nil}	93	100	96	93	89	0.921	1.5	0.230
IL-10 _{Ag} , MCP-1 _{Ag-Nil} , IL-12p40 _{Ag-Nil} , IL-12p70 _{Ag-Nil}	100	89	96	93	89	0.995	0.1	0.768
IL-12p40 _{Ag-Nil} , IL-12p70 _{Ag-Nil} , EGF _{Nil} , IL-15 _{Nil}	100	100	100	100	78	0.886	2.3	0.147
IL-12p40 _{Ag-Nil} , IL-12p70 _{Ag-Nil} , IL-1 β _{Ag} , IL-15 _{Nil}	100	100	100	100	89	0.760	5.7	0.028
IL-12p40 _{Ag} , IL-12p70 _{Ag-Nil} , IL-10 _{Nil} , IL-10 _{Ag-Nil}	100	100	100	100	89	0.786	4.9	0.040

4.2.4.5 Correlation between host marker levels detected in QFT-IT, overnight and 7-day whole blood culture assays

The marker levels obtained after stimulation with each antigen (Rv2029c, Rv2032, Rv2389c and ESAT-6/CFP-10) or the unstimulated control were compared between the different techniques. Comparison between all three assay types was only possible with ESAT-6/CFP-10-specific and the unstimulated control responses as these were the only common conditions between all the assay types. Because of the relatively large number of markers evaluated in this study, only analytes that showed significant differences between the TB cases and non-cases in at least one of the assay types were included in the analysis.

In general, the unstimulated and antigen-stimulated analyte levels were lower in the 7-day WBA in comparison to the QFT-IT and our in-house overnight WBA (Figure 4.5 and Figure 4.6). Of the 26 analytes evaluated, 12 (fractalkine, IFN- α 2, MCP-1, MIP-1 β , VEGF, IL-1 β , IL-10, MMP-2, MMP-9, CRP, SAA, and SAP) showed significant differences between the TB cases and non-cases in at least one of the three assays, when evaluated in unstimulated supernatants. The levels of the markers were compared across assays. The unstimulated levels of MCP-1, MIP-1 β and IL-1 β were significantly higher in QFT-IT supernatants compared to the two in-house WBAs. QFT-IT levels of the remaining unstimulated markers were similar to the levels obtained in our in-house WBA (Table 4.8). With exception of fractalkine, all unstimulated analyte levels were significantly lower (2-200 fold) in the 7-day WBA supernatants in comparison to either the QFT-IT or our in-house overnight WBA, although no correction was made for the 10x dilution of whole blood that is performed for the 7-day assay. Similarly, there were generally no significant differences in the levels of markers upon stimulation with ESAT-6/CFP-10 between the QFT-IT and our in-house overnight WBA, but generally lower levels were obtained in the 7-day stimulated supernatants (Table 4.9). ESAT-6/CFP10-specific IP-10 and unstimulated SAA were the only markers that discriminated between the TB cases and non-cases in all three WBA types (Table 4.2, Table 4.4, Table 4.6 and). The levels of the two analytes also correlated positively between all the three assay types ($r^2=1$, $p < 0.0001$ in some cases). The highest significant correlations were observed between the QFT-IT and our in-house overnight WBA responses (Table 4.9, Figure 4.5 and Figure 4.6).

Table 4.8: Comparison of analyte levels in the 3 assays

The diagnostic utility of each analyte were assessed in each individual assay. Only analytes with AUC > 0.70 for at least one assay are reported in this stable. The mean levels of discriminating host markers were compared between the 3 assays. All analytes levels shown are in pg/ml.

	marker	QFT	1 Day	7 Day
Unstimulated	fractalkine	24.44	52.79	22.56
Unstimulated	IFN-alpha2	71.01	88.45	3.710
Unstimulated	MCP-1	11606	773.5	58.39
Unstimulated	MIP-1alpha	1641	14.47	2.200
Unstimulated	VEGF	165.7	130.9	35.14
Unstimulated	IL-1beta	254.0	2.200	2.200
Unstimulated	IL-10	12.56	6.280	2.200
Unstimulated	MMP-2	79865	77647	3421
Unstimulated	MMP-9	711195	292133	52850
Unstimulated	CRP	43188	38419	1083
Unstimulated	SAA	27608	39332	701.0
Unstimulated	SAP	38751	31467	1067
ESAT6/CFP10	IP10	11788	9092	2666
ESAT6/CFP10	MCP-1	0.0	1905	7324
ESAT6/CFP10	IFN- γ	179.8	71.85	0.0
ESAT6/CFP10	EGF	1.000	0.0	0.0
ESAT6/CFP10	sCD40L	859.9	106.6	22.30
ESAT6/CFP10	SAP	1386	1841	88.27

Table 4.9: Correlation of analyte levels in the 3 assays

ESAT6/CFP10-stimulated and unstimulated-analyte levels from the same participants were determined in different assays. The diagnostic utility of each analyte were assessed in each individual assay. The detected levels of these analytes were correlated between the assays. The R square and its P value were computed. Only analytes with AUC > 0.70 for at least one assay are reported in this table. All analytes with R square above 0.5 and a significant P value are in bold.

	QFT vs 1 day		QFT vs 7 day		1 day vs 7 day	
	r^2	P value	r^2	P value	r^2	P value
ESAT6/CFP10 IP10	0.60	0.0004	0.60	0.001	0.78	< 0.0001
ESAT6/CFP10 MCP-1	0.53	0.004	0.35	0.076	0.20	0.31
ESAT6/CFP10 IFN-γ	0.85	1.44	0.84	3.96	0.77	< 0.0001
ESAT6/CFP10 EGF	-0.16	0.41	-0.20	0.31	0.29	0.15
ESAT6/CFP10 MMP-9	0.62	0.002	0.19	0.40	0.19	0.40
ESAT6/CFP10 sCD40L	0.05	0.78	-0.15	0.44	-0.01	0.97
ESAT6/CFP10 SAP	0.74	< 0.0001	0.06	0.75	0.15	0.43
Unstimulated Fractalkine	0.40	0.031	0.42	0.021	0.16	0.41
Unstimulated IFN-α2	0.98	< 0.0001	0.18	0.35	0.16	0.40
Unstimulated MCP-1	0.07	0.73	-0.07	0.72	0.14	0.47
Unstimulated MIP-1β	0.45	0.015	0.17	0.39	0.41	0.03
Unstimulated VEGF	0.32	0.096	0.08	0.68	0.26	0.17
Unstimulated IL-1β	0.65	0.0001	0.12	0.55	0.40	0.03
Unstimulated IL-10	0.63	0.0003	-0.18	0.36	-0.15	0.43
Unstimulated MMP-2	0.73	0.0001	0.41	0.06	0.07	0.76
Unstimulated MMP-9	0.62	0.002	0.19	0.40	0.19	0.41
Unstimulated CRP	0.95	< 0.0001	0.38	0.044	0.40	0.033
Unstimulated SAA	1.0	< 0.0001	0.91	< 0.0001	0.90	< 0.0001
Unstimulated SAP	0.74	< 0.0001	0.06	0.75	0.15	0.43

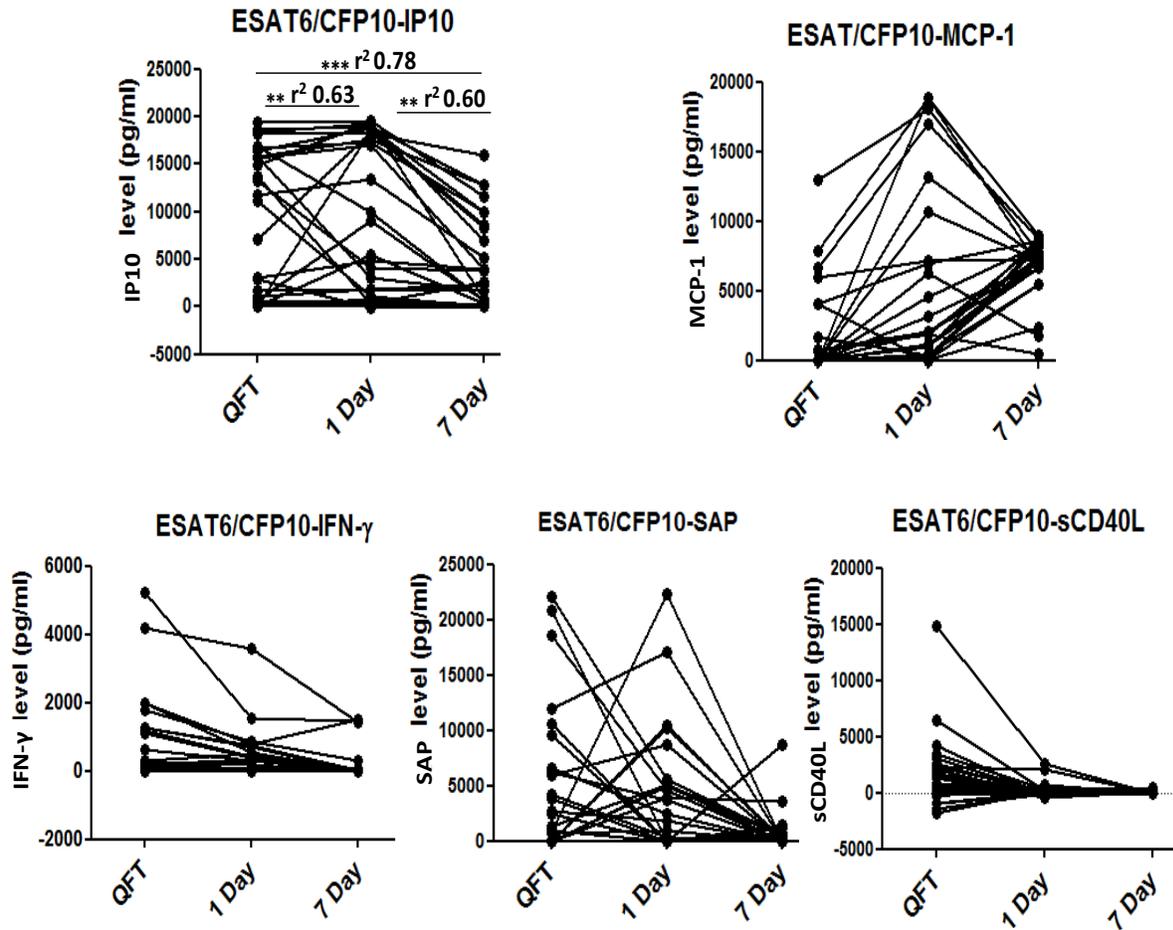


Figure 4.5: Correlation of ESAT6/CFP10-analyte levels in the 3 assays

ESAT6/CFP10-stimulated analyte levels from the same participants were measured in the 3 assays. The diagnostic utility of each analyte was assessed in each individual assay. Correlation of ESAT6/CFP10-analyte levels was done for the top 5 markers between the 3 assays. The R square and its P value were computed. Only the top 5 diagnostic ESAT6/CFP10-induced host markers are shown in this figure. ns = non-significant, * = P value < 0.05, ** = P value \leq 0.001, *** = P value < 0.0001.

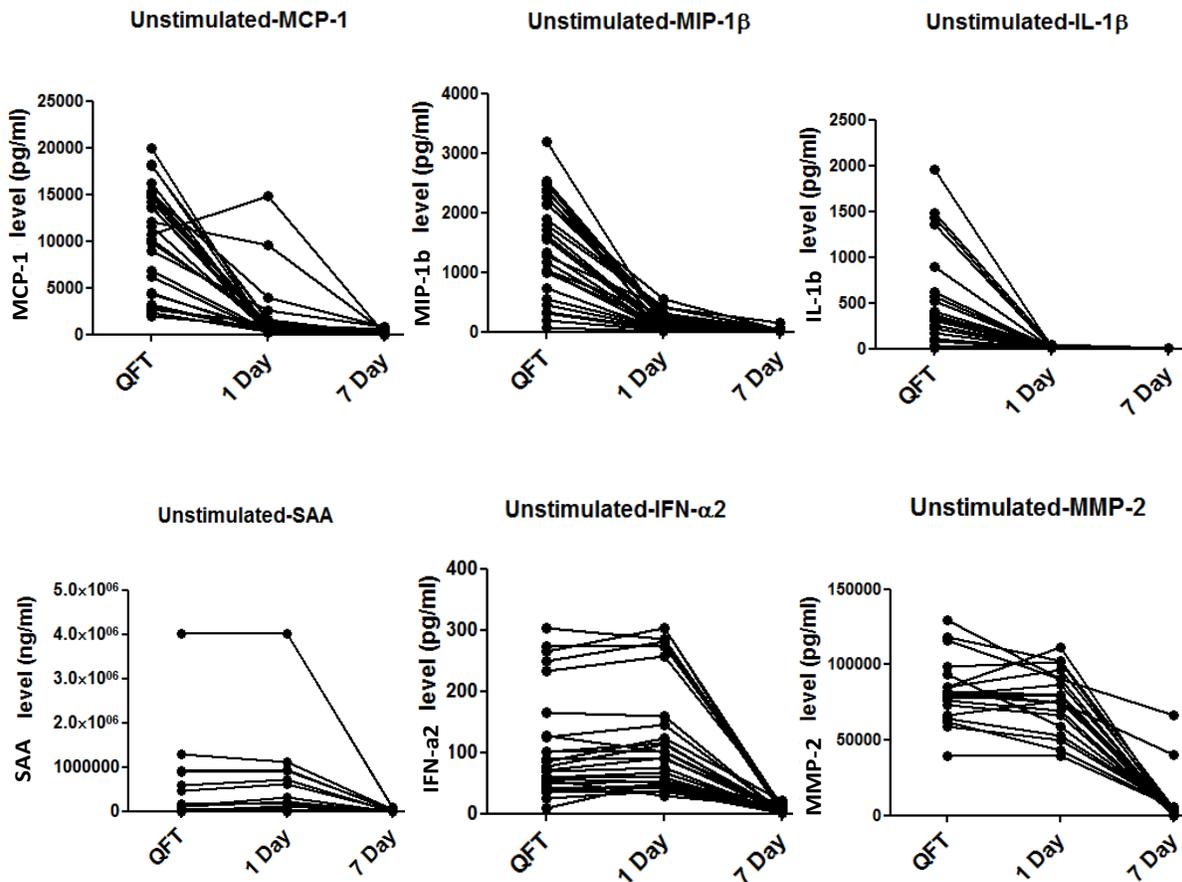


Figure 4.6: Correlation of unstimulated-analyte levels

Unstimulated analyte levels from participants were measured in the 3 assays. The diagnostic utility of each analyte was assessed in each individual assay. The detected levels of these unstimulated-analytes were correlated between the assays. The R square and its P value were computed. Only the 6 top diagnostic host markers are shown in this figure

ns = non-significant, * = P value < 0.05, ** = P value \leq 0.001, *** = P value < 0.0001.

4.3 Evaluation of diagnostic utility of a larger host marker panel in a short term assay

4.3.1 Aim of the study

The present study aims to evaluate the diagnostic utility of 73 host markers in supernatants from a short term assay stimulated with 8 *M.tb* antigens.

4.3.2 Materials and Methods

4.3.2.1 Study population

This study was designed as a pilot study where a large number of markers were evaluated with a reduced number of participants. Sixteen participants were randomly selected from the AE-TBC samples bank. All participants were recruited as TB suspects and there were 8 confirmed active TB cases and 8 alternative pulmonary diseases (sharing TB symptoms). All study participants had a HIV test at recruitment and all were negative.

4.3.2.2 Antigen and cytokine selection and sample processing

The diagnostic utility of 8 different *M.tb* antigens including ESA6/CFP10, LUMC01, LUMC02, PPD, Rv0081, Rv1733c, Rv2029c and TB18.2 were evaluated in this study. The diagnostic utility of these antigens were assessed in the context of the production of 73 different host markers including IFN- γ , IL-10, IL-1b, IL-6, EGF, Eotaxin, G-CSF, GM-CSF, IFN-alpha2, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, IL-1Ra, IL-1a, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IP-10, MCP-1, MIP-1a, MIP-1b, TNF-a, TNF-b, VEGF, MCP-2, MCP-4, ENA-78, SDF-1a+b, BCA-1, I-309, IL-16, MIP-1delta, TARC, 6-Ckine, Eotaxin-2, Eotaxin-3, CTACK, IL-23, LIF, TPO, TRAIL, SCF, TSLP, IL-20, IL-21, IL-28A, IL-33, APOA-1, APOC-3, APOE, HPALBN,

HCFH, HCC3, HA2MG, CRP, SAA, SAP, TIMP-1, TIMP-2, TIMP-3, TIMP-4, IL-17F, IL-22, IL-17E/IL-25, IL-27, IL-31, MMP-2 and MMP-9. Classes and functions of these selected markers are shown in Table 4.10. Whole blood was collected from each participant, processed and analysed as described above.

Table 4.10: Classes and functions of selected markers

Markers	Classes	Functions
Eotaxin, IP-10, MCP-1, MIP-1a, MIP-1b, MCP-2, MCP-4, MIP-1delta, Eotaxin-2 and Eotaxin-3	Chemokines	Chemoattractant
IFN- γ , IL-6, G-CSF, GM-CSF, IFN-a2, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, IL-2, IL-3, IL-5, IL-7, ENA-78, IL-8, IL-16, IL-23, TRAIL, TSLP, IL-21, IL-28A, IL-27 and IL-31	Th1 cytokine	Pro-inflammatory cytokines
IL-10, IL-4, IL-20, IL-33, IL-1b, IL-1Ra, IL-1a and IL-22	Th2 cytokine	Anti-inflammatory cytokines
EGF, TNF-a, TNF-b, VEGF,	Growth factor	Stimulate cellular growth
CRP, SAA and SAP	Acute phase proteins	
MMP-2 and MMP-9	Matrix metalloproteinase	The MMPs play an important role in tissue remodelling
TIMP-1, TIMP-2, TIMP-3 and TIMP-4	tissue inhibitor of metalloproteinases	Inhibit MMPs functions
IL-17F	IL17	Participate in monocytes and neutrophils recruitment

4.3.3 Results

4.3.3.1 Study population

A reduced number of 16 participants were enrolled in this study including 8 TB cases and 8 non TB cases due to financial considerations. TB disease was confirmed by *M.tb* culture and X-ray. All participants were HIV negative according to a HIV rapid test at recruitment (Table 4.11).

Table 4.11: Demographic and clinical characteristics of participants

Participants (n=16) including 8 TB cases and 8 alternative pulmonary diseases were randomly selected from the AE_TBC sample bank. They were all recruited as people with suspected TB.

	All	TB	NO TB
Number of participants	16	8	8
Mean age (years)	36	40	31
Age range (years)	21 - 51	27 - 51	21 - 40
Male/female ratio	8/8	1/7	7/1

4.3.3.2 Diagnostic potential of unstimulated markers

Whole blood was incubated overnight with 9 different antigens. Supernatants were harvested and stored in separate tubes until analysis by Luminex. When the unstimulated levels of markers in supernatant were compared between groups, 9 markers could discriminate between the 2 groups with a significant P value (P value <0.05). Acute phase proteins were the best markers in the unstimulated sample; the 3 acute phase proteins evaluated in this study discriminated TB from alternative pulmonary disease with a P value < 0.005. When these results were analysed using the receiver operating characteristics (ROC) analysis, 2 of the 3 acute phase proteins (CRP and SAP) confirmed their discrimination utility with a perfect AUC (AUC=1). TIMP.1 was the only additional unstimulated marker that could perfectly classify participants into their correct groups with AUC=1 (Table 4.12 and Table 4.13).

4.3.3.3 Diagnostic potential of classical *M.tb* antigens

The diagnostic utilities of 3 classical *M.tb* antigens were evaluated in this study including ESAT6/CFP10, PPD and TB18.2. The number of discriminative markers was stimulus dependant. Only one PPD-induced marker could discriminate study groups (PPD-VEGF) with P value ≤ 0.01 and AUC=0.98. The 2 other classical antigens yielded 5 discriminative markers. The 5 ESAT6/CFP10-markers were different from the 5 TB18.2-markers. These stimulated markers discriminated groups with P value ≤ 0.01 and AUC ≥ 0.94 . Among the classical antigens evaluated in this study, only TB18.2-GM-CSF and TB18.2-IL15 could classify 100% of participants into their respective groups. Although ESAT6/CFP10 and PPD could not correctly classify all participants, 17 ESAT6/CFP10-induced markers and 6 PPD-induced markers discriminated between groups with AUC ≥ 0.95 .

4.3.3.4 Diagnostic potential of newly identified antigens

Five recently identified antigens were selected for the present study. Rv0081, Rv1733c and Rv2029c were selected for their promising results in the 7-day whole blood assay. LUMCO1 and LUMCO2 were just identified by our collaborators in Netherlands and their diagnostic potentials were first evaluated in this study.

The diagnostic potential of LUMCO1 and LUMCO2 were promising with 4 (IL5, ENA.78, X5.Ckine, TIMP2) and 2 (IL1 α and VEGF) markers respectively. The 2 discriminative LUMCO2- markers (IL1 α and VEGF) perfectly separated 100% of study participants with AUC=1. Among the 4 discriminative LUMCO1-markers, only LUMCO1-TIMP2 could correctly classify all participants on their clinical groups.

Rv1733c was the worst performing antigen; none of the 73 Rv1733c-induced markers could discriminate between TB disease and alternative pulmonary disease with a P value \leq 0.01. Rv0081 was the best performing antigen. Six Rv0081-induced markers could correctly classify 100% of participants into their clinical groups with AUC=1. Rv2029c-ENA.78 and Rv2029c-X6.Ckine also classified 100% of participants into their correct clinical groups. ENA.78 and X6.Ckine were evaluated for the first time in this study and they were the only antigens that could correctly classify 100% of participants into their clinical groups with AUC=1 with more than one antigen.

Table 4.12: Diagnostic potential of antigen-stimulated and unstimulated host markers: P values

Sixteen participants were selected for this pilot study: 8 confirmed TB cases and 8 No-TB (alternative pulmonary diseases). The 8 *M.tb* antigens (ESAT6/CFP10, LUMCO1, LUMCO2, PPD, Rv0081, Rv2029c, Rv1733c and TB18.2) used in this study were tested on all participants. Their diagnostic utility was assessed with 73 different host markers using Luminex platform. The table below shows the P value for individual stimulated or unstimulated host marker.

Host	ESAT6/CFP10	LUMCO1	LUMCO2	PHA	PPD	Rv0081	Rv1733c	Rv2029c	TB18.2	Unstim	P < 0.01	P < 0.05
IFN- γ	0.0097	0.2785	0.0202	0.3737	0.4280	0.5671	0.8305	0.7658	0.0187	0.2656	1	3
IL-10	0.8731	0.5096	0.5693	0.0151	0.0450	0.1263	0.3180	0.7047	0.2893	0.3092	0	2
IL-1 β	0.1807	0.6403	0.6728	0.6815	0.7468	0.3721	0.8704	0.9646	0.8195	0.9767	0	0
IL-6	0.8956	0.8686	0.9960	0.3475	0.8315	0.0005	0.0212	0.4221	0.6218	0.4972	1	2
EGF	0.7452	0.4137	0.8214	0.7354	0.8430	0.6037	0.8509	0.4426	0.5774	0.1630	0	0
Eotaxin	0.7066	0.6214	0.6137	0.8962	0.2921	0.2132	0.9964	0.0313	0.0373	0.2037	0	2
G.CSF	0.3759	0.7852	0.4424	0.8066	0.8119	0.0893	0.7553	0.3366	0.7251	0.6283	0	0
GM.CSF	0.0518	0.6085	0.1566	0.0172	0.2705	0.9362	0.2905	0.2641	0.0005	0.7904	1	2
IFN-alpha2	0.1576	0.9632	0.1799	0.5660	0.4347	0.7013	0.3476	0.3086	0.1548	0.8811	0	0
IL-1.p40.	0.0056	0.5319	0.2187	0.4884	0.2318	0.9302	0.4897	0.5780	0.9862	0.5042	1	1
IL-12p70	0.0079	0.2649	0.6311	0.1006	0.5845	0.9687	0.9794	0.6879	0.0120	0.6064	1	2
IL-13	0.0172	0.0120	0.0163	0.0165	0.0144	0.8108	0.7027	0.0319	0.0099	0.5923	1	7
IL-15	0.6350	0.2564	0.2394	0.4448	0.9139	0.1228	0.1947	0.9377	0.0005	0.6021	1	1
IL-17A	0.6195	0.9179	0.8934	0.0114	0.7177	0.8485	0.9578	0.9845	0.1235	0.8498	0	1
IL-1Ra	0.5604	0.4911	0.7764	0.7692	0.9351	0.0258	0.7274	0.9115	0.0273	0.4789	0	2
IL-1a	0.0041	0.7033	0.0005	0.9614	0.9405	0.7895	0.2374	0.0285	0.3768	0.0079	3	4
IL-2	0.2882	0.2078	0.4329	0.4257	0.5091	0.8197	0.2305	0.8538	0.2554	0.9903	0	0
IL-3	0.6002	0.8752	0.5436	0.0219	0.2415	0.3930	0.9220	0.8909	0.3011	0.4962	0	1
IL-4			0.5923	0.0082	0.3904						1	1
IL-5	0.0798	0.0079	0.3374	0.0130	0.0578	0.9174	0.7459	0.1605	0.0079	0.8910	2	3
IL-7	0.8923	0.8923	0.5911	0.5930	0.8799	0.6522	0.8098	0.7776	0.7542	0.8371	0	0
IL-8	0.4236	0.4890	0.8250	0.8799	0.8796	0.1642	0.6566	0.5367	0.8245	0.8184	0	0
IP-10	0.7827	0.7585	0.2244	0.6568	0.8559	0.6139	0.5590	0.6631	0.0848	0.5370	0	0
MCP-1	0.9719	0.6691	0.7682	0.7444	0.6990	0.0005	0.4515	0.2180	0.3990	0.9422	1	1
MIP-1 α	0.0393	0.6420	0.2781	0.0347	0.1825	0.0178	0.2189	0.7264	0.9473	0.3740	0	3
MIP-1 β	0.4620	0.8460	0.9211	0.1054	0.5481	0.0005	0.0741	0.5249	0.0921	0.3165	1	1
TNF- α	0.1682	0.8384	0.9931	0.3151	0.3395	0.0005	0.0481	0.8038	0.4464	0.6904	1	2
TNF- β	0.1035	0.8912	0.8099	0.0135	0.3995	0.8825	0.9422	0.3576	0.5101	0.3494	0	1
VEGF	0.0040	0.7096	0.0005	0.4672	0.0054	0.7493	0.2317	0.3345	0.5692	0.8176	3	3

MCP-2	0.0469	0.7229	0.0139	0.6029	0.1703	0.2091	0.9535	0.4189	0.5951	0.8674	0	2
MCP-4	0.3155	0.6719	0.8617	0.8701	0.6465	0.5949	0.5030	0.0041	0.8083	0.0041	2	2
ENA-78	0.0114	0.0027	0.2161	0.0146	0.0661	0.0005	0.1424	0.0005	0.4921	0.7909	3	5
SDF-1a+b	0.5095	0.9636	0.2395	0.4911	0.5448	0.8361	0.5764	0.9561	0.3441	0.5873	0	0
BCA.1	0.2007	0.1675	0.4078	0.0007	0.7650	0.3192	0.3915	0.7130	0.0379	0.0085	2	3
I-309	0.8186	0.8089	0.7158	0.7833	0.6079	0.7626	0.8499	0.3575	0.0638	0.2859	0	0
IL-16	0.4324	0.2044	0.2657	0.4954	0.8109	0.6201	0.3845	0.2623	0.9970	0.3510	0	0
MIP-1delta	0.5953	0.7495	0.8838	0.6103	0.2636	0.5780	0.7295	0.9398	0.6666	0.8919	0	0
TARC	0.4672	0.3385	0.6024	0.0189	0.0120	0.6479	0.9361	0.8288	0.1107	0.7501	0	2
X6-Ckine	0.0142	0.0023	0.1425	0.1716	0.4656	0.0005	0.0175	0.0005	0.6352	0.7589	3	5
Eotaxin-2	0.0312	0.7013	0.1151	0.2304	0.6678	0.8179	0.5189	0.0689	0.0086	0.1151	1	2
Eotaxin-3	0.5923	NA	0.4305	NA	0.5628	NA	NA	0.5923	NA	NA	0	0
CTACK	0.8955	0.4051	0.6741	0.0590	0.0701	0.7492	0.9330	0.0722	0.6733	0.8576	0	0
IL-23	0.1505	0.6254	0.7478	0.3529	0.2328	0.8976	0.2462	0.5018	0.8195	0.7222	0	0
LIF	0.0141	NA	NA	0.0137	0.3046	NA	NA	NA	NA	NA	0	2
TPO	0.8627	0.8552	0.8245	0.6008	0.1501	0.6216	0.5763	0.9596	0.8362	0.8520	0	0
TRAIL	0.9248	0.4469	0.9923	0.0007	0.1023	0.6314	0.8594	0.6044	0.8781	0.9901	1	1
SCF	0.0141	NA	NA	0.0166	0.0166	0.0141	0.0120	0.0141	0.0120	NA	0	7
TSLP	0.7448	0.6237	0.4969	0.9041	0.9889	0.9907	0.7566	0.3658	0.9846	0.9595	0	0
IL-20	0.5902	0.8147	0.7075	0.7509	0.6448	0.8399	0.7129	0.5923	0.2162	0.4305	0	0
IL-21	0.3862	0.0262	0.2709	0.0722	0.9752	0.8558	0.3930	0.5635	0.7157	0.6873	0	1
IL-28A	0.4658	0.7171	0.4903	0.7070	0.2044	0.9703	0.6522	0.6795	0.7650	0.9973	0	0
IL-33	0.4028	0.8075	0.1995	0.1149	0.9458	0.4280	0.4106	0.7985	0.5581	0.8408	0	0
APOA-1	0.1188	0.1879	0.2079	0.0107	0.2080	0.6158	0.9815	0.5307	0.1715	0.5388	0	1
APOC-3	0.8878	0.7294	0.6824	0.2307	0.5888	0.4436	0.7170	0.8759	0.2231	0.0551	0	0
APOE	0.1237	0.1055	0.4035	0.0007	0.0488	0.5224	0.5436	0.2648	0.0187	0.5715	1	3
HPALBN	0.5961	0.8573	0.6714	0.0007	0.3487	0.9820	0.8569	0.5318	0.0168	0.0143	1	3
HCFH	0.1606	0.4938	0.4961	0.0530	0.2899	0.5622	0.6492	0.3748	0.3282	0.0108	0	1
HCC3	0.1027	0.4616	0.7048	0.0871	0.0890	0.7641	0.8043	0.2384	0.2509	0.2438	0	0
HA2MG	0.4548	0.3955	0.7120	0.0889	0.1723	0.6852	0.9011	0.6159	0.7387	0.4781	0	0
CRP	0.7876	0.5928	0.5700	0.2421	0.2006	0.8040	0.6926	0.9093	0.0301	0.0005	1	2
SAA	0.5274	0.7720	0.4753	0.2981	0.3942	0.7325	0.8710	0.7331	0.0234	0.0046	1	2
SAP	0.1899	0.6593	0.3770	0.1840	0.1335	0.6165	0.5491	0.5370	0.2137	0.0005	1	1
TIMP-1	0.0659	0.0208	0.3830	0.1893	0.3745	0.1760	0.2297	0.1280	0.0498	0.0005	1	3
TIMP-2	0.3911	0.0005	0.2201	0.1764	0.3453	0.5771	0.2557	0.0617	0.8588	0.4855	1	1
TIMP-3	0.8393	0.6944	0.6029	0.0778	0.7931	0.7474	0.2383	0.4364	0.9848	0.8109	0	0
TIMP-4	0.9339	0.0288	0.7585	0.2131	0.5558	0.5714	0.8719	0.5320	0.1731	0.3544	0	1
MMP-2	0.8633	0.0356	0.0764	0.1190	0.5307	0.4858	0.1621	0.1067	0.5867	0.2188	0	1

MMP-9	0.9238	0.2482	0.5793	0.8270	0.9873	0.3600	0.9883	0.8630	0.7198	0.3016	0	0
IL-17F	0.4536	0.5898	0.9128	0.0347	0.0732	0.9590	0.4167	0.7446	0.7224	0.0626	0	1
IL-22	0.0938	0.9709	0.1987	0.0692	0.0313	0.5780	0.0109	0.8265	0.7803	0.8334	0	2
IL-17E/IL-25	0.4824	0.6790	0.3295	0.6188	0.4282	0.1498	0.9745	0.1154	0.1309	0.9444	0	0
IL-27	0.8865	0.4094	0.6876	0.9341	0.9097	0.6318	0.7532	0.4081	0.6106	0.4501	0	0
IL-31	0.5736	0.7147	0.6233	0.7785	0.6519	0.8940	0.3906	0.3844	0.9154	0.2240	0	0
P value > 0.01	5	4	2	5	1	6	0	3	5	7	1	1
P value > 0.05	13	9	5	19	7	9	5	7	16	9	0	0

Table 4.13: Diagnostic potential of antigen-stimulated and unstimulated host markers: AUC values

Sixteen participants were selected for this pilot: 8 confirmed TB cases and 8 No-TB (alternative pulmonary diseases). The 8 distinct *M.tb* antigens (ESAT6/CFP10, LUMCO1, LUMCO2, PPD, Rv0081, Rv2029c, Rv1733c and TB18.2) used in this study were tested in all participants. Their diagnostic utility was assessed with 73 different host markers using the Luminex platform. The table below shows the AUC values for individual stimulated or unstimulated host marker.

	ESAT6/ CFP10	LUMCO1	LUMCO2	PHA	PPD	Rv0081	Rv1733c	Rv2029c	TB18.2	Unstimula ted
IFN- γ	0.97	0.94	0.95	0.86	0.94	0.92	0.89	0.91	0.95	0.94
IL-10	0.89	0.91	0.91	0.98	0.96	0.97	0.92	0.89	0.88	0.89
IL-1 β	0.89	0.91	0.89	0.86	0.94	0.89	0.88	0.89	0.82	0.88
IL-6	0.91	0.94	0.89	0.86	0.86	1	0.97	0.94	0.88	0.91
EGF	0.89	0.89	0.89	0.88	0.84	0.91	0.88	0.89	0.91	0.94
Eotaxin	0.92	0.86	0.94	0.86	0.9	0.92	0.86	0.95	0.91	0.94
G-CSF	0.89	0.91	0.91	0.88	0.82	0.95	0.89	0.9	0.86	0.9
GM-CSF	0.94	0.91	0.92	0.92	0.92	0.89	0.92	0.94	1	0.89
IFN-alpha2	0.91	0.88	0.95	0.88	0.88	0.91	0.94	0.93	0.93	0.82
IL-12.p40	0.97	0.9	0.92	0.91	0.94	0.84	0.88	0.9	0.88	0.84
IL-12.p70	0.97	0.91	0.89	0.9	0.9	0.88	0.89	0.89	0.95	0.88
IL-13	0.95	0.95	0.97	0.92	0.96	0.84	0.91	0.94	0.96	0.83
IL-15	0.89	0.94	0.91	0.86	0.9	0.94	0.89	0.94	1	0.89
IL-17A	0.94	0.97	0.95	0.98	0.9	0.88	0.84	0.84	0.95	0.91
IL-1Ra	0.89	0.91	0.89	0.84	0.86	0.94	0.89	0.91	0.97	0.94
IL-1a	0.98	0.92	1	0.9	0.94	0.85	0.94	0.95	0.92	0.97
IL-2	0.94	0.94	0.92	0.88	0.9	0.86	0.94	0.88	0.95	0.88
IL-3	0.88	0.95	0.91	0.92	0.9	0.97	0.91	0.88	0.97	0.88
IL-4	0.82	0.82	0.83	0.97	0.82	0.82	0.82	0.82	0.82	0.82
IL-5	0.92	0.97	0.92	0.96	0.92	0.92	0.92	0.92	0.97	0.88
IL-7	0.88	0.88	0.89	0.9	0.85	0.88	0.88	0.89	0.88	0.83
IL-8	0.88	0.94	0.89	0.88	0.86	0.95	0.91	0.88	0.86	0.88
IP-10	0.92	0.92	0.95	0.9	0.88	0.86	0.88	0.88	0.95	0.92
MCP-1	0.89	0.92	0.89	0.86	0.88	1	0.86	0.89	0.95	0.88
MIP-1 α	0.94	0.88	0.92	0.9	0.92	0.97	0.91	0.86	0.89	0.92
MIP-1 β	0.89	0.84	0.89	0.86	0.88	1	0.95	0.89	0.95	0.95
TNF- γ	0.94	0.83	0.88	0.86	0.96	1	0.95	0.89	0.92	0.86
TNF- α	0.95	0.88	0.86	0.95	0.91	0.89	0.87	0.91	0.88	0.84
VEGF	0.98	0.86	1	0.9	0.98	0.85	0.91	0.95	0.86	0.93
MCP-2	0.95	0.84	0.98	0.84	0.92	0.94	0.88	0.91	0.94	0.91
MCP-4	0.94	0.91	0.89	0.88	0.88	0.91	0.94	0.98	0.9	0.98
ENA-78	0.95	0.98	0.94	0.94	0.9	1	0.95	1	0.94	0.88
SDF-1a.b	0.91	0.92	0.92	0.88	0.94	0.86	0.88	0.89	0.91	0.89

BCA.1	0.92	0.92	0.89	1	0.8	0.89	0.89	0.88	0.92	0.97
I.309	0.9	0.88	0.88	0.9	0.85	0.88	0.89	0.91	0.93	0.91
IL-16	0.92	0.94	0.95	0.92	0.9	0.92	0.92	0.94	0.88	0.97
MIP-1delta	0.89	0.92	0.86	0.88	0.9	0.92	0.88	0.88	0.89	0.88
TARC	0.91	0.92	0.91	0.94	0.98	0.89	0.86	0.88	0.91	0.92
X6-Ckine	0.97	0.99	0.91	0.88	0.88	1	0.98	1	0.9	0.88
Eotaxin-2	0.92	0.91	0.95	0.92	0.92	0.89	0.91	0.89	0.97	0.94
Eotaxin-3	0.88	0.82	0.84	0.8	0.87	0.82	0.82	0.88	0.82	0.82
CTACK	0.89	0.92	0.89	0.94	0.92	0.88	0.88	0.95	0.89	0.89
IL-23	0.92	0.91	0.89	0.94	0.94	0.86	0.92	0.89	0.86	0.92
LIF	0.95	0.82	0.82	0.95	0.95	0.82	0.82	0.82	0.82	0.82
TPO	0.84	0.89	0.92	0.92	0.92	0.88	0.88	0.88	0.88	0.89
TRAIL	0.84	0.92	0.88	1	0.94	0.91	0.91	0.91	0.88	0.89
SCF	0.95	0.82	0.82	0.94	0.94	0.95	0.95	0.95	0.95	0.82
TSLP	0.88	0.9	0.89	0.9	0.84	0.91	0.88	0.92	0.88	0.86
IL-20	0.84	0.82	0.83	0.82	0.83	0.83	0.83	0.83	0.85	0.84
IL-21	0.88	0.97	0.94	0.86	0.84	0.92	0.95	0.89	0.84	0.9
IL-28A	0.9	0.9	0.91	0.86	0.9	0.88	0.89	0.9	0.91	0.86
IL-33	0.9	0.88	0.91	0.92	0.81	0.91	0.89	0.88	0.89	0.84
APOA-1	0.95	0.94	0.94	0.98	0.94	0.91	0.86	0.94	0.95	0.89
APOC-3	0.88	0.89	0.89	0.9	0.9	0.89	0.88	0.88	0.89	0.92
APOE	0.91	0.91	0.89	1	0.92	0.88	0.89	0.94	0.95	0.89
HPALBN	0.88	0.88	0.88	1	0.9	0.88	0.88	0.91	0.98	0.98
HCFH	0.97	0.91	0.88	0.96	0.92	0.92	0.91	0.94	0.86	0.97
HCC3	0.97	0.88	0.88	0.94	0.94	0.88	0.89	0.91	0.94	0.89
HA2MG	0.89	0.89	0.89	0.96	0.92	0.89	0.88	0.91	0.86	0.88
CRP	0.91	0.84	0.91	0.94	0.92	0.89	0.94	0.84	0.88	1
SAA	0.94	0.91	0.89	0.94	0.94	0.88	0.94	0.91	0.89	0.98
SAP	0.95	0.91	0.91	0.94	0.94	0.89	0.92	0.94	0.89	1
TIMP-1	0.95	0.97	0.92	0.92	0.92	0.94	0.92	0.94	0.91	1
TIMP-2	0.91	1	0.91	0.94	0.88	0.89	0.89	0.92	0.91	0.91
TIMP-3	0.91	0.9	0.9	0.92	0.94	0.88	0.94	0.92	0.88	0.89
TIMP-4	0.84	0.94	0.92	0.94	0.92	0.95	0.83	0.95	0.94	0.91
MMP-2	0.88	0.95	0.97	0.92	0.89	0.92	0.95	0.94	0.88	0.94
MMP-9	0.88	0.94	0.91	0.86	0.88	0.91	0.9	0.9	0.86	0.91
IL-17F	0.89	0.92	0.94	0.92	0.89	0.91	0.94	0.86	0.93	0.97
IL-22	0.88	0.84	0.91	0.86	0.91	0.9	0.96	0.84	0.94	0.89
IL-17E-IL-25	0.88	0.91	0.88	0.86	0.86	0.91	0.87	0.88	0.89	0.91
IL-27	0.88	0.91	0.84	0.86	0.86	0.89	0.85	0.86	0.89	0.91
IL-31	0.89	0.91	0.88	0.86	0.86	0.88	0.93	0.86	0.91	0.92
AUC > 0.94	17	8	11	13	6	13	9	9	18	12
AUC = 1	0	9	2	4	0	6	0	2	2	3

4.4 Discussion

The diagnostic potential of host markers detectable in supernatants after overnight or 7-day whole blood stimulation with recently identified *M.tb* infection-phase-dependent antigens was evaluated in this study. We have shown that the novel *M.tb* infection-phase-dependent antigens evaluated in this study elicit a variety of host responses after short-term incubation with whole blood. This thereby confirms the suitability of these antigens and associated host markers for use as diagnostic candidates for TB disease. The potentially useful diagnostic host markers induced by these antigens include acute phase proteins, cytokines, chemokines, growth factors and matrix metallo-proteinases. The most promising candidate markers are unstimulated SAA, CRP, SAP, IP-10, MMP-9, EGF, MMP-2, MMP-9 and TIMP-1; ESAT-6/CFP10-induced IP-10 and sCD40L; Rv2032-induced MMP-2; Rv0081-specific IL-6, MCP-1, MIP-1 β , TNF- α , ENA-78, X6-Ckine; Rv2029c-specific ENA-78 and X6-Ckine; TB18.2-specific GM-CSF and IL-15.

In chapter III [3], we have shown that stimulation of whole blood with Rv2032, Rv0081, Rv1737c, Rv0867c and Rv2389c for 7 days resulted in the production of multiple host markers, some of which showed potential to diagnose TB disease with 100% accuracy. Because the 7-day whole blood assay technique employed in that study would not be optimal as a diagnostic test, we investigated if the antigens employed in that study as well as other antigens such as TB18.2 had potential to induce the same promising diagnostic host markers in short-term culture assays. This report hereby confirms our previous observations and additionally confirms the suitability of these antigens for use as diagnostic candidates as they elicit the production of multiple host markers in overnight assays. In addition to confirming our previously published observations, we show for the first time that stimulation of whole blood with infection phase-dependent antigens such as ESAT-6/CFP-10, Rv2032, Rv0081, Rv2029c, TB18.2 induces novel potentially highly useful diagnostic host markers such as ENA-78, X6-Ckine, MMP-2, IL-6, MCP-1, GM-CSF and IL-15. As observed in previously published studies including studies based on responses in QFT-IT supernatants in children, many host markers, with potential in the diagnosis of TB disease are detectable in unstimulated supernatants [3, 5-7].

There was generally a good correlation between the markers detected using our in-house overnight WBA with those detected using the QFT-IT assay and this is not surprising. The magnitude of responses obtained with the two overnight assays was consistently higher than the responses obtained in the 7-day WBAs. This could be explained by the amount of sample that was used in the different assay types. The two overnight assays (our in-house overnight WBA and the QFT-IT assay) employed undiluted whole blood while blood samples were diluted five times before being evaluated in the 7-day WBA as is standard practice [5]. The excellent correlation between our in-house assay and the QFT-IT test, a well-standardised assay that is considered in some settings as the “gold standard” for LTBI means that the promising antigens and associated host markers detected using our in-house WBA have excellent potential to be useful in the diagnosis of TB disease. However, direct comparison of performance of the three assay types was only possible for the markers detected in unstimulated samples and those detected in ESAT-6/CFP-10 stimulated supernatants. This comparison showed that the levels of stimulated or unstimulated markers were higher in QFT-IT assay compared to the other two assays: After direct comparison of 18 analyte levels from the three assays, more than 60% (11/18) of analytes have their highest detection levels in Quantiferon assay. The high levels obtained in QFT-IT may be due the assay procedure; the whole blood is directly collected into QFT tubes pre-coated with *M.tb* antigens. These results demonstrate the stability of proteins in this optimized assay. The reduced number of discriminative unstimulated markers in whole blood assays supernatants may be a direct consequence of the higher number of manipulations that are needed for the in-house assays.

IFN- γ release assays (IGRAs) are well established and remain the assays of choice for the diagnosis of LTBI in many settings. However, these assays do not discriminate between LTBI and active disease and are therefore not recommended in high burden settings for the diagnosis of active TB. This is mainly because of the large proportion of LTBI cases in high-burden settings, coupled with limited resources [7]. The current study was performed in a setting with high burden of TB. The results of the current study as well as those obtained in our previous studies raise the hope that diagnostic tests that are based on these antigens and host markers, might be highly suitable in high-burden settings. However, diagnostic tests based on these novel antigens and novel host markers will mostly be beneficial to TB control programs if such antigens

and markers are incorporated into rapid, point-of-care test platforms such as the lateral flow technology. Such testing platforms are currently being investigated as part of our on-going studies. In addition, the diagnostic utility of these antigens and host markers have to be validated in larger sample sets.

4.5 Conclusion

In conclusion, the results of the current study show that the markers reported in our previous investigations continue to show promise in the diagnosis of TB disease. Furthermore, additional markers such as CRP, SAA, SAP, sCD40L, MMP-2, MMP-9, MCP-1 and MIP-1 β , amongst others as shown in Table 4.12 and Table 4.13, might also be very useful in the diagnosis of TB disease if measured in unstimulated supernatants or whole blood culture supernatants after stimulation with infection-phase-dependent antigens including Rv0081, ESAT-6/CFP-10, Rv2029c, Rv1733c and TB18.2 in short-term culture assays. These data could form the basis for the development of a novel immunodiagnostic test for TB disease.

4.6 References

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5. Chapter V

Optimisation of sample preparation protocol to assess the diagnostic utility of circulating host markers by mass spectrometry

5.1 Introduction

Human serum is an important bio-specimen in biomarkers research. It is the sample of choice in proteomic studies. This choice can be explained by a number of advantages: cellular proteomic changes are thought to be represented in serum via leakage from tissues [1][2], it is easily accessible and several standardized protocols have been developed for its analysis [3]. Serum composition can be a direct reflection of the patho-physiological condition of cells, tissues, organs or at the whole organism [4]. This sample type could therefore be a diagnostic goldmine for biomarkers research. It is also one of the most difficult body fluid to work with in proteomic research because of its complexity and the large dynamic range of abundant proteins [2]. The 20 most abundant proteins in serum represent around 99% of its total protein quantity [5] [6]. The remaining serum proteins (1%), more than 1000 proteins, are expressed in low quantity yet a number of them may play an important role in some diseases like cancer, diabetes, cardiovascular disorders and tuberculosis (TB). Unlike targeted biomarker discovery efforts, including ELISA and multiplex cytokine arrays, unbiased discovery offers the opportunity to find biomarkers that are not included in standard kits and is not limited by our current understanding of a biological system. Proteomic analysis represents such an unbiased approach. Identification of these proteins on mass spectrometry is difficult due to the shadow effect of the high abundant proteins. Many techniques have been explored and a number of kits have been commercialised for the removal of the high abundant proteins in serum prior to mass spectrometric analysis.

The ProteoSpin Abundant Serum Protein Depletion Kit (Norgen Biotek, Canada) is an attractive method due to its affordability and the simple procedure for the removal of abundant proteins in

serum [7]. This kit does not require any specific antibody; it is an ionexchange depletion-based method. Albumin can be depleted by 70%, transferrin and haptoglobin by 50% and α -antitrypsin by 90% [7]. The complexity of the sample is thus greatly reduced and less abundant proteins may be detected by mass spectrometry.

Heparin column protocol was not developed for high abundant protein depletion in serum. The idea was proposed by Ting Lei and colleagues [8] where heparin molecules are coated into a column and interact with serum proteins. Heparin is a polysaccharide biological molecule that contains a large number of linear chains. Its molecular weight ranges from 3.000 to 30.000 Daltons (Da). The mayor repeating unit is the trisulfated disaccharide 2-O-sulfo- α -L-iduronic acid 1"4 linked to 6-O-sulfo-N-sulfo- α -D-glucosamine (IdoA2S (1, 4) GlcNS6S) (Figure 5.1) [9]. Heparin is the most negatively charged biological molecule known and can interact with a number other molecules. It is principally an anticoagulant in the blood stream. The anticoagulant activity of heparin derives from its ability to interact with several enzymes involved in blood coagulation [10] [11].

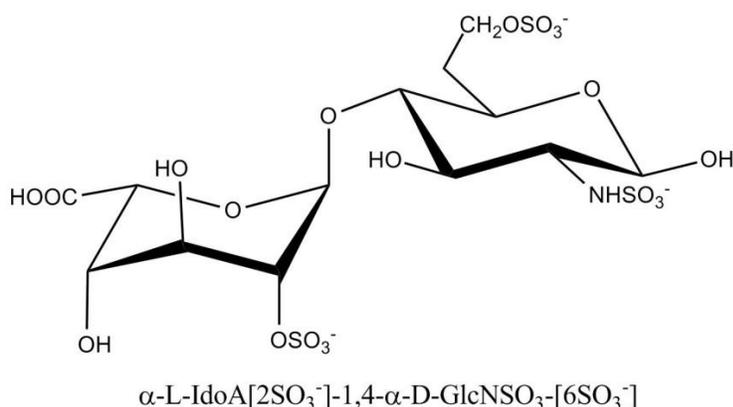


Figure 5.1: Heparin polysaccharide precursor

The main structural components of heparin were established as followed: *N*- and 6-*O*-sulfated α -D-glucosamine, 2-*O*-sulfated α -l iduronic acid along with *N*-acetyl D-glucosamine and β -D-glucuronic acid. It is the most negatively charged biological molecule known with high affinity for antithrombin. Source: http://polysac3db.cermav.cnrs.fr/discover_GAGs.html

Heparin chromatography is a method of affinity chromatography used to fractionate biological molecules that can interact with heparin [12]. Heparin is able to bind to a wide range of low abundant proteins that could be diseases related proteins. These proteins include: enzymes, serine protease inhibitors, growth factors, extracellular matrix proteins, nucleic acid-binding protein 9 initiation factors, elongation factors, restriction endonucleases, DNA ligase, DNA and RNA polymerases, hormone receptors, and lipoproteins [13].

Sample pre-fractionation using a combination of antibodies has shown to effectively and specifically remove targeted high abundant proteins and improve the detection of low abundant proteins in plasma samples [14]. A number of multi affinity removal systems (MARS) have been developed and are commercially available [15]. ProteoPrep 20 (Sigma, Germany) is the most complete MARS depletion method. It removes the 20 most abundant proteins in serum samples [5].

The aim of this study was to develop a serum sample preparation protocol in order to identify a maximum number of low abundant proteins by mass spectrometry.

We have evaluated the three different protocols named above.

5.2 Materials and Methods

5.2.1 High abundant proteins depletion by ProteoSpin columns

All samples used in this section were obtained from healthy volunteers from our laboratory. Samples depletion with ProteoSpin columns was carried out in two steps:

5.2.1.1 Protein concentration:

Total serum protein was first concentrated using “The ProteoSpinTM Total Protein Concentration Micro Kit” (Norgen Biotek, Canada). This kit concentrates the total proteins and peptides in a sample while simultaneously removing salt, detergents and denaturants. The columns do not have any molecular weight cut off; therefore a number of proteins, peptides and

antibodies can be processed. All the reagents used in this process were provided in the kit. Total protein was concentrated according to the manufacturer's instructions. Briefly, serum sample was diluted in an equal volume of PBS (Sigma, Germany) to obtain a total volume of 1 ml. 40µL of pH Binding Buffer was added to the diluted serum sample to regulate the serum pH. 400 ul of column activation and wash buffer (pre-mixed) was added to the column and centrifuged for two minutes to activate the column. The flowthrough was discarded and the activation step was repeated. 500 ul of the diluted serum was added to the activated column and centrifuged at 3300 g for two minutes. After discarding the flowthrough, the remaining 500 ul was added to the same column and centrifuged for two additional minutes at 3300 g. The column was washed twice with the pre-mixed buffer and centrifuged for two minute at 3300 g. Neutralizer (9.3 ul) was added to a fresh Elution tube. 100 ul of Elution buffer was added to the washed and dried column and centrifuged for two minutes. A second 100 ul of Elution buffer was added to the same column and centrifuged. The second elution was collected in a different tube.

5.2.1.2 High abundant proteins depletion

To deplete the high abundant proteins with ProteoSpin columns, the spin column was activated twice by adding 500 ul of the pre-mixed buffer (Column Activation and Wash Buffer) and centrifuged for one minute at 6700 g. The concentrated sample was mixed to the pre-mixed buffer to a total volume of 500 ul. The 500 ul was added to the activated column and centrifuged for one minute. The flowthrough was discarded and the column was washed twice with 500 ul of pre-mixed buffer. 100 ul of Elution buffer was applied to the washed column and centrifuged for 6700 g. An additional 100 ul of Elution buffer was applied to the column and centrifuged at the same speed. The two eluted fractions were collected in the same Elution tube containing 5 ul of Neutralizer.

5.2.2 Heparin column

High abundant proteins were depleted using heparin columns as the second option in the present study. Serum samples from healthy volunteers were used to optimize this protocol. The heparin was pre-load in columns by the manufacturer (Prepack columns) (Sigma, Germany). This experiment was conducted according to Ting Lei and colleagues' protocol. Unspecific bindings (protein to protein) were first destroyed by adding 60µl of pure water and 40µl of acetonitrile (ACN) (20% v/v) and warmed at 40°C for 15 minutes. The samples were spun at 14,000 g for 10 min to precipitate any insoluble material. 180µL of ACN-treated serum was diluted into 1 ml equilibration buffer (10 mmol/l Tris-HCl, 50 mmol/l NaCl, pH 7.0) and applied to the heparin agarose column. 5 ml of equilibration buffer was applied to the column to wash and the bound proteins were eluted by addition of 2.5 ml elution buffer (10 mmol/l Tris-HCl, 1.5 mol/l NaCl, pH 7.0). The eluted fraction was collected and concentrated using a 3 K molecular-weight cutoff (MWCO) spin concentrator (Millipore). The column was cleaned sequentially with 5 ml of cleaning buffer 1 (0.1 mol/l borate, pH 9.8, 1 mol/l NaCl), cleaning buffer 2 (0.1 mol/l borate, pH 9.8), cleaning buffer 3 (Milli Q water), and cleaning buffer 4 (2.0 mol/l NaCl).

5.2.3 ProteoPrep 20

ProteoPrep 20 (Sigma, Germany) was the third method in the present work used to deplete high abundant proteins from serum. This kit is based on an immunodepletion column consisting of a resin bioconjugated with polyclonal IgG antibodies against 20 high-abundance plasma proteins including: albumin, apolipoproteins A1, A2 and B, alpha-1-acid-glycoprotein, alpha-1-antitrypsin, alpha-2-macroglobulin, ceruloplasmin, complements C1q, C3 and C4, haptoglobin, fibrinogen, IgAs, IgDs, IgGs, IgMs, plasminogen, transferrin and transthyretin. These abundant proteins were depleted according to the manufacturer's instructions. All reagents were provided in the kit. The kit could only process a maximum of 8µL of serum (diluted in 100µL equilibration buffer) per aliquot. Briefly, the column was washed with 4 ml of equilibration

buffer, spun at 5000 rpm for 30 seconds and placed in a collector tube. The diluted serum was then loaded to the column and left at room temperature for 20 minutes. After the 20 minutes, the column was spun at 5000 rpm for 60 seconds. The remaining unbound proteins were washed twice by adding 100uL of Equilibration Buffer to the top of the medium bed and centrifuged at 5000 rpm for 60 seconds. The flowthrough fractions were collected in the same tube. The bound fraction was eluted by slowly passing the Elution Solution through the resin. The column was immediately re-equilibrated with 4 ml of equilibration buffer and was ready for a second aliquot. After high abundant protein depletion, all the flows through fractions from the sample were mixed and reconcentrated at 4°C using Vivaspin 500 Centrifugal concentrators.

The total protein concentration was determined either by the Bradford assay (Bio-Rad, USA). After the protein concentration step, samples were run on 1-D electrophoresis agarose pre-casted gel (Bio-Rad, USA) and stained overnight with Coomassie Blue (Bio-Rad, USA). After the destaining step, the gel was cut into 5 pieces and digested with trypsin (Bio-Rad, USA). Each digested sample was separately analysed in Orbitrap Velos Mass Spectrometry.

5.3 Results

5.3.1 ProteoSpin columns

To evaluate the efficiency of this kit, whole blood was collected from two healthy volunteers. The blood samples were centrifuged and the supernatants were harvested and stored at -80°C. On the day of the assay, samples were thawed and the total protein was concentrated. After the total protein concentration procedure, the concentrated protein was eluted in two separated tubes in elution buffer. According to the manufacturer, 90% of the bound proteins are eluted during the first elution (collected here in Elution tube 1). The second elution should only contain approximately 10% of the total protein (Elution tube 2) and could be neglected for the downstream application to avoid further protein dilution. To confirm the total protein content of the two Elution tubes, the total protein concentration for each eluted tube was estimated using the

Bradford method. The protein concentration in the Elution tube 1 was above the maximum value on the standard curve ($>2000\mu\text{g/ml}$) for both samples. The second tubes had more than $1000\mu\text{g/ml}$ protein. Contrary to what was stated in the protocol, the amount of proteins obtained in the second elution tube was substantial and could not be left out of downstream analysis.

According to the manufacturer, a volume as small as 10ul of concentrated protein could be used to deplete high abundant proteins and run the downstream applications with the low abundant proteins. 10ul of concentrated protein (from the first elution tube) was used to deplete high abundant proteins. After the depletion procedure, the sample concentration was measured by the Bradford method. No protein was detected in the depleted samples (Table 5.1). The protein concentration of the concentrated non-depleted samples (elution 1) could not be determined using the values in Table 5.1 as the concentrations were above the maximal value in the standard and the values obtained may not represent the exact values.

Two serum aliquots from our volunteers were thawed at room temperature and the total protein was concentrated using ProteoSpin total protein concentration micro kit. Each sample was eluted in two separate tubes. The depleted samples from the two tubes were mixed into a single tube for each volunteer. Each mixed sample was diluted with different dilutions to estimate the most suitable dilution factor. Dilution factor of five and ten gave protein concentrations within the standard curve range. The dilution factor of ten was selected for future experiments as dilution five may be insufficient for some samples (Table 5.2).

An additional aliquot from each volunteer was thawed at room temperature and the proteins were concentrated. The bound proteins were eluted separately in two different tubes, the two tubes contents were mixed into a single tube. High abundant proteins were depleted from the total protein quantity in the mixed tube instead of the 10ul suggested by the manufacturer. The depleted protein concentration was measured using the Bradford method (Table 5.3).

Table 5.1: Total proteins concentration measured by Bradford method

High abundant proteins were depleted using ProteoSpin columns in two steps: total proteins were first concentrated using “ProteoSpin total proteins concentration Micro kit”. Bound proteins were eluted in two fractions collected in two separate tubes. High abundant proteins were then depleted from 10 ul of the concentrated proteins samples (Elution tube 1) using the “ProteoSpin abundant serum proteins depletion kit”. The total protein concentration after depletion was also measured by the Bradford method.

*Extrapolated values as value fell outside the standard curve.

	Sample ID	OD (mean)	Concentration (ug/ml)
Total proteins concentration: Sample 1	Elution 1	0.79	2003*
	Elution 2	0.44	1102
Total proteins concentration: Sample 2	Elution 1	0.95	2412*
	Elution 2	0.62	1564
Total proteins concentration after depletion of high abundant proteins	Sample 1	-0.04	0
Total proteins concentration after depletion of high abundant proteins	Sample 2	0.04	0

Table 5.2: Serial dilution of serum samples

Total protein concentration was obtained using the Bradford method. This method had a standard curve ranging from 125–2000 ug/ml. Serum samples (n=2) were obtained from volunteers and diluted 01, 05, 10 and 20 times. The most suitable dilution factors are shown in bold.

Dilution factor	Sample ID	OD (mean)	Concentration (ug/ml)
01	Sample 1	0.84	2516
05	Sample 1	0.33	796
10	Sample 1	0.17	248
20	Sample 1	0.10	0
01	Sample 2	0.76	2233
05	Sample 2	0.63	1811
10	Sample 2	0.15	185
20	Sample 2	-0.05	0

Table 5.3: Concentration of serum samples after depletion of high abundant proteins

Serum samples were obtained from two healthy volunteers and proteins were concentrated with ProteoSpin. After protein concentration, each sample was eluted in two separate tubes with a total volume of 100 ul in each tube. The two eluted fractions from the same samples were mixed and high abundant proteins were depleted from the 200 ul mixed concentrated proteins. The total proteins concentration was measured using the Bradford method.

Sample ID	OD (mean)	Concentration (ug/ml)
Sample 1	0.11	118
Sample 2	0.25	445

The quantity of protein obtained after depletion was sufficient to run on a small or a medium 1-D electrophoresis gel (minimum load 30 and 60ug respectively). 60ug of the original serum and 60ug of the depleted sample from the same participant were run on a 1-D pre-casted gel (medium gel) (Figure 5.2). The gel was stained with Coomassie blue and destained overnight. Analysis of the gel figure showed a similar protein band distribution between the raw serum and the depleted sample. Although the same amount of protein was run on both lanes, the raw serum appears to have higher quantity of protein (as observed through the band intensities) than the depleted sample on the second lane. Some bands observed in the raw serum were not seen on the depleted sample but no additional band was observed in the depleted sample. The depletion of high abundant proteins did not allow visualisation of additional bands on 1-D gel electrophoresis. The depleted lane on the electrophoresis gel was cut out and prepared for mass spectrometry analysis. The mass spectrometry positively identified a reduced number of 83 human proteins. These proteins were identified using single or multiple peptides (Table 5.4).

A second experiment was designed to quantify the detected proteins and compared their levels in active TB cases and healthy controls. To conduct this experiment, serum samples from 10 TB cases and 10 healthy controls were thawed at room temperature. The proteins were concentrated

and high abundant proteins were depleted in individual samples using ProteoSpin. After the depletion, total protein concentrations were measured in each individual sample using the Bradford method. The total protein concentrations are reported in Table 5.5. Unfortunately, most of the samples had a total protein quantity below the minimum required for 1-D agarose gel. Three of the 20 samples did not reach the minimum amount required for the small gel. Half of the participants had protein concentrations between 30ug and 60ug. The total protein obtained after depletion was too variable from sample to sample and the reproducibility of these columns was questionable and alternative methods were considered.

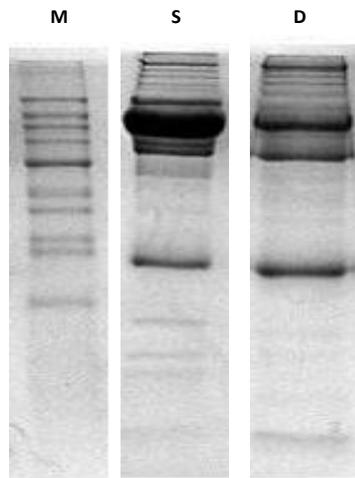


Figure 5.2: 1-D electrophoresis gel of serum proteins before and after depletion with the ProteoSpin column

M: marker; S: original serum; D: depleted sample (low abundant proteins)

Table 5.4: Data obtained after high abundant protein depletion using ProteoSpin columns

Whole blood was collected from a healthy individual in heparinized tube. Plasma was harvested and frozen at -80°C . On the day of the assay, an aliquot was thawed, proteins were concentrated and high abundant proteins were depleted. The depleted samples were denatured at 95°C for 5 minutes and run on a 1-D electrophoresis agarose gel. The gel was fragmented into 5 fractions and analysed on an Orbitrap Velos Mass Spectrometer. The Orbitrap successfully identified 83 proteins using 1 or multiple peptides.

Accession	Description	Score	Coverage	Proteins	Unique Peptides	Peptides	PSMs	AAs	MW [kDa]	pI
IPI00745872.2	Isoform 1 of Serum albumin	17436	82.27	11	48	63	1717	609	69.3	6.28
IPI00783987.2	Complement C3 (Fragment)	2841	32.41	3	35	42	166	1663	187.0	6.40
IPI00479723.5	Isoform 10 of Fibronectin	526.95	16.45	19	19	23	56	2176	239.5	5.88
IPI00022463.2	Sero transferrin	799	37.11	12	18	19	73	698	77.0	7.12
IPI00418163.3	complement component 4B preproprotein	633	17.49	11	16	19	47	1744	192.6	7.27
IPI00478003.3	Alpha-2-macroglobulin	252	16.89	3	11	16	36	1474	163.2	6.46
IPI00220327.4	Keratin, type II cytoskeletal 1	642	28.73	17	10	16	35	644	66.0	8.12
IPI00021841.1	Apolipoprotein A-I	581	39.33	2	9	9	34	267	30.8	5.76
IPI00215894.1	Isoform LMW of Kininogen-1	418	27.63	6	9	10	55	427	47.9	6.65
IPI00009865.4	Keratin, type I cytoskeletal 10	318	27.74	1	8	10	21	584	58.8	5.21
IPI00022229.2	Apolipoprotein B-100	152	3.53	1	7	12	23	4563	515.3	7.05
IPI00022431.2	cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein	151	36.03	4	7	9	19	433	46.6	6.28
IPI00478493.3	haptoglobin isoform 2 preproprotein	386	34.29	4	7	11	32	347	38.4	6.60
IPI00019359.4	Keratin, type I cytoskeletal 9	410	20.06	1	6	8	39	623	62.0	5.24
IPI00019591.2	cDNA FLJ55673, highly similar to Complement factor B	272	10.11	10	6	9	26	1266	140.9	7.18
IPI00021304.1	Keratin, type II cytoskeletal 2 epidermal	330	19.84	16	6	10	21	645	65.8	8.00
IPI00645363.2	Putative uncharacterized protein DKFZp686P15220	851	41.10	16	6	13	116	472	51.7	7.93
IPI00790784.2	Isoform 2 of Alpha-1-antitrypsin	898	32.03	4	6	9	66	359	40.2	5.47
IPI00032179.3	Antithrombin-III	311	17.89	2	5	5	10	464	52.6	6.71
IPI00017601.1	Ceruloplasmin	106	12.77	5	5	7	16	1065	122.1	5.72
IPI00022371.1	Histidine-rich glycoprotein	293	13.71	1	5	6	13	525	59.5	7.50
IPI00298971.1	Vitronectin	343	13.18	2	5	6	21	478	54.3	5.80
IPI00645038.1	Inter-alpha (Globulin) inhibitor H2	170	13.69	4	5	6	11	935	105.2	7.03
IPI00022445.1	Platelet basic protein	323	39.84	1	4	5	20	128	13.9	8.79

IPI00292530.1	Inter-alpha-trypsin inhibitor heavy chain H1	48	5.82	6	4	4	8	911	101.3	6.79
IPI00026199.2	Glutathione peroxidase 3	62	25.66	1	4	4	4	226	25.5	8.05
IPI00218732.4	Serum araoxonase/ arylesterase 1	374	16.90	1	4	4	18	355	39.7	5.22
IPI00480192.1	Retinol binding protein 4, plasma	267	25.13	3	4	4	14	199	22.9	6.09
IPI00555812.5	vitamin D-binding protein precursor	69	12.45	5	4	4	10	474	52.9	5.45
IPI00892870.1	Protein	42	15.53	5	4	6	8	470	51.5	6.46
IPI00922043.2	cDNA FLJ51742	108	7.21	7	4	5	6	888	98.3	6.60
IPI00022426.1	Protein AMBP	59	21.59	1	3	4	5	352	39.0	6.25
IPI00032291.2	Complement C5	119	3.70	2	3	5	8	1676	188.2	6.52
IPI00022488.1	Hemopexin	37	10.61	1	3	5	6	462	51.6	7.02
IPI00477597.2	Isoform 1 of Haptoglobin-related protein	457	25.00	2	3	7	20	348	39.0	7.09
IPI00022391.1	Serum amyloid P-component	103	14.80	1	3	3	5	223	25.4	6.54
IPI00930442.1	Putative uncharacterized protein DKFZp686M24218	37	16.81	1	3	5	15	476	52.4	7.77
IPI00304273.2	Apolipoprotein A-IV	104	11.36	2	2	4	5	396	45.4	5.38
IPI00019580.1	Plasminogen	51	5.93	1	2	3	3	810	90.5	7.24
IPI00009856.1	Protein Plunc	61	15.23	1	2	3	3	256	26.7	5.76
IPI00019568.1	Prothrombin (Fragment)	52	7.88	1	2	5	6	622	70.0	5.90
IPI00006114.5	Pigment epithelium-derived factor	48	5.74	2	2	2	2	418	46.3	6.38
IPI00646773.2	Isoform 2 of Gelsolin	26	7.66	5	2	3	3	731	80.6	5.85
IPI00807428.1	Putative uncharacterized protein	297	37.02	13	2	6	27	235	24.7	5.94
IPI00916734.1	Uncharacterized protein	37	17.56	2	2	2	2	131	15.1	7.84
IPI00847635.1	Isoform 1 of Alpha-1-antichymotrypsin	20	10.40	2	1	2	3	423	47.6	5.52
IPI00021854.1	Apolipoprotein A-II	47	23.00	1	1	1	1	100	11.2	6.62
IPI00006662.1	Apolipoprotein D	27	14.29	2	1	2	5	189	21.3	5.15
IPI00152007.1	Rho guanine nucleotideexchange factor 17	0	1.16	1	1	3	5	2063	221.5	6.29
IPI00479116.1	Carboxypeptidase N subunit 2	38	4.95	1	1	1	10	545	60.6	5.99
IPI00000102.1	Progonadoliberin-1	0	10.87	1	1	1	10	92	10.4	6.52
IPI00028078.1	Isoform A of Potassiumchannel subfamily K member 10	46	4.65	4	1	2	16	538	59.7	8.73
IPI00385252.1	Ig kappa chain V-III region GOL	84	31.19	13	1	2	2	109	11.8	9.25
IPI00387120.1	Ig kappa chain V-IV region Len	36	7.89	5	1	1	4	114	12.6	7.93
IPI00382440.1	Ig lambda chain V-IV region Hil	40	17.76	1	1	1	1	107	11.5	6.51
IPI00019038.1	Lysozyme C	24	6.08	1	1	1	1	148	16.5	9.16
IPI00022974.1	Prolactin-inducible	51	18.49	1	1	2	2	146	16.6	8.05

protein										
IPI00022446.1	Platelet factor 4	35	18.81	1	1	2	3	101	10.8	8.62
IPI00029717.1	Isoform 2 of Fibrinogen alpha chain	0	2.80	2	1	2	20	644	69.7	8.06
IPI00217512.4	Probable G-protein coupled receptor 115	180	7.63	2	1	2	18	695	77.7	9.01
IPI00299450.1	CD22 protein	0	1.81	3	1	1	15	662	74.3	6.79
IPI00306378.5	Isoform 2 of Mannan-binding lectin serine protease 2	31.02	8.65	3	1	1	3	185	20.6	5.96
IPI00470372.2	Isoform 2 of Leucine-rich repeats and immunoglobulin-like domains protein 1	0	2.15	2	1	2	2	1070	116.3	7.34
IPI00550731.2	Putative uncharacterized protein	721	41.00	20	1	6	55	239	26.2	8.06
IPI00552768.1	Thioredoxin, isoform CRA_b	32	15.29	2	1	1	1	85	9.4	6.04
IPI00643948.2	Complement component 1, q subcomponent, B chain	117	11.69	3	1	2	3	231	24.4	9.16
IPI00645213.1	Apolipoprotein M, isoform CRA_a	24	27.59	3	1	4	5	116	13.0	7.75
IPI00646384.1	13 kDa protein	46	11.02	4	1	1	1	118	13.1	5.47
IPI00647704.1	cDNA FLJ41552 fis, clone OLON2004478	22	12.15	16	1	3	3	494	53.3	6.52
IPI00719373.2	IGL@ protein	239	35.98	4	1	5	28	214	23.0	8.84
IPI00783024.1	Myosin-reactive immunoglobulin heavy chain variable region (Fragment)	70	14.50	1	1	1	1	131	14.1	9.63
IPI00794605.1	18 kDa protein	23	8.70	7	1	1	2	161	17.6	8.34
IPI00843764.2	Isoform C of Trypsin-3	44	8.10	5	1	1	25	247	26.7	6.10
IPI00895854.2	Isoform 3 of Protein prune homolog	28	17.34	4	1	2	4	271	30.1	6.23
IPI00903237.1	Uncharacterized protein	70	0.62	2	1	1	52	2106	232.7	5.69
IPI00909841.1	cDNA FLJ51435, moderately similar to Cofilin-1	51	17.86	2	1	1	1	112	12.5	6.60
IPI00910625.1	cDNA FLJ51265, moderately similar to Beta-2-glycoprotein 1	31	7.66	2	1	1	1	274	30.3	7.85
IPI00916916.1	Uncharacterized protein	0	6.43	4	1	1	2	140	16.2	9.03
IPI00924948.1	Uncharacterized protein	82	9.25	2	1	2	5	227	26.3	5.73
IPI00946754.1	Protease serine 1	74	20.42	8	1	2	22	142	15.4	7.27
IPI00955969.1	whirlin isoform 3	0	6.29	1	1	4	5	906	96.4	8.63
IPI00973174.1	Protein	265	21.81	4	1	7	45	431	47.5	6.87
IPI00784985.1	IGK@ protein	743	42.98	16	0	6	53	235	25.5	6.55

Table 5.5: Serum proteins of TB cases and healthy controls

Serum samples were collected from 10 TB cases and 10 healthy controls. Proteins were concentrated using the ProteoSpin kit. High abundant proteins were depleted from the concentrated samples and protein concentrations were determined using the Bradford method. Samples were diluted in 0.2 ml of elution buffer. The minimum quantities needed to load small and medium pre-casted agarose gels for mass spectrometry are 30 and 60 ug respectively. The total protein quantities for individual participants that are below the minimum required for mass spectrometric analysis from small gels are shown in bold.

Sample IDs	OD (mean)	Concentration (ug/ml)	Total quantity (ug)
N03	0.07	191	38.2
N04	0.03	94	18.8
N08	0.01	40	8
N21	0.05	152	30.4
N01	0.06	157	31.4
N05	-0.21	266	53.2
N06	-0.29	92	18.4
N07	-0.24	221	44.2
N18	-0.3	679	135.8
N19	0.39	1587	317.4
TB04	0.18	355	71
TB05	0.14	293	58.6
TB09	0.17	348	69.6
TB10	0.17	348	69.6
TB11	0.21	408	81.6
TB01	0.41	672	134
TB02	0.14	216	43.2
TB03	0.15	229	45.8
TB08	0.18	278	55.6
TB15	0.12	189	37.8

5.3.2 Heparin columns

Results obtained with the ProteoSpin led to the evaluation of a newly developed technique based on the heparin molecule. The heparin column was the second approach undertaken in this experiment to deplete high abundant proteins from serum samples. Heparin is a highly sulfated glycosaminoglycan with an affinity for a broad range of proteins and polypeptides [13]. When serum proteins from a healthy volunteer was passed through the column, high abundant proteins like albumin passed through the column and were separated from the binding proteins. These bound proteins were eluted later and analysed separately. The protein samples (high abundant proteins and low abundant protein) were denatured and run on a 1-D electrophoresis gel. The gel was stained with silver stain reagents for visualisation (Figure 5.3). The figure of the electrophoresis gel showed the efficiency of the heparin column in depleting high abundant proteins; a number of high abundant proteins like albumin (66kda) were successfully depleted allowing visualisation of low abundant proteins in the same region.

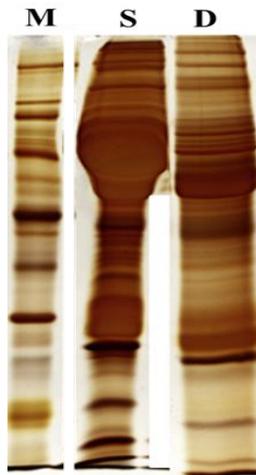


Figure 5.3: SDS-PAGE of serum samples

Whole blood was collected from a volunteer and serum was harvested and stored at -80°C . On the day of the assay, high abundant proteins were depleted from the serum sample with the heparin method. The proteins were run on the SDS-PAGE and stained with silver staining reagents. M= markers, S=raw serum, D= serum after depletion of abundant proteins.

5.3.3 ProteoPrep 20 columns

ProteoPrep 20 is an antibody based technique where antibodies are coated onto a resin and loaded into a column [16]. It was the third technique to be evaluated. The column is coated with 20 different and specific antibodies. High abundant proteins in the serum bind to these specific antibodies when low abundant proteins flow through. Through this principle, low abundant proteins are separated from the high abundant proteins which represent around 99% of the total protein quantity.

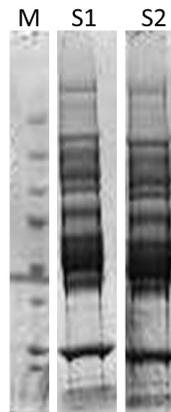
Serum aliquots from two healthy volunteers were thawed and high abundant protein was depleted using the replacement ProteoPrep 20 kits. All the through fractions (low abundant protein) were pooled together and re-concentrated using microcentrifuge filters. The total protein concentrations from the two pools were measured as shown on Table 5.6. The pooled samples were denatured and run on 1-D electrophoresis pre-casted gel. The gel analysis reveals an even repartition of proteins demonstrating an effective depletion of high abundant protein after a unique round of depletion (Figure 5.4).

To quantify and identify possible diagnostic markers, 20 samples were randomly selected (10 TB cases randomly selected and 10 healthy controls randomly selected) from our sample bank. These samples included 10 *M.tb* culture confirmed TB cases and 10 healthy controls. All participants had a HIV rapid test (Determine HIV-1/2) at recruitment and were all HIV negative (Table 5.7). Serum samples from the same clinical group were pooled together and the 20 most abundant proteins were depleted using ProteoPrep 20. All the flowthrough fractions from individual depletion cycles were pooled together for each group and re-concentrated using microcentrifuge filters. The depleted samples were denatured and run on 1-D electrophoresis gel in triplicate (Figure 5.5). The gel was fractionated into 5 fractions, digested and analysed on Orbitrap Velos mass spectrometry. The mass spectrometry positively identified 338 different proteins using single or multiple peptides. Among the identified proteins, 21 were upregulated in TB cases and 15 were down regulated (Table 5.8 and Table 5.9).

Table 5.6: Determination of depleted and non-depleted serum concentration using the Bradford method

Serum samples were obtained from 2 volunteers and high abundant proteins were depleted in these two samples using the ProteoPrep 20 kit. This kit removes the 20 high abundant proteins from human serum. These 20 abundant proteins bind to the column and are eluted with the elution buffer after the unbound proteins have been collected in the flow through. The flow through and the eluted fraction were measured with different dilution factors. The proteins concentrations were estimated using the Bradford method.

Sample IDs	Dilution factor	OD	Concentration (ug/ml)	Volume (ul)	Total quantity (mg)
Sample 1 (original serum sample)	100	0.63	749	40	3
Sample 1 eluted	01	1.31	1542	200	0.3
Sample 1 eluted	10	0.14	188	200	0.38
Sample 1 eluted	20	0.07	111	200	0.44
Sample 1 flow through	50	0.19	241	200	2.41
Sample 1 flow through	100	0.12	162	200	3.24
Sample 2(original serum sample)	100	0.86	1019	40	4.1
Sample 2 eluted	01	1.4	1676	200	0.3
Sample 2 eluted	10	0.16	205	200	0.4
Sample 2 eluted	20	0.08	120	200	0.5
Sample 2 flow through	50	0.22	278	200	2.8
Sample 2 flow through	100	0.15	193	200	3.9

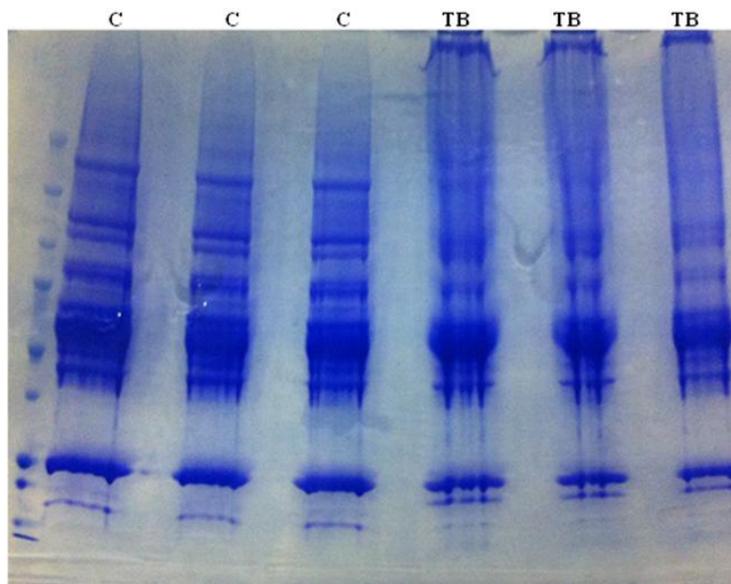
**Figure 5.4: SDS-PAGE of depleted serum samples**

The 20 high abundant proteins were depleted from serum samples using ProteoPrep 20. The flow through fractions were pooled and reconstituted using the microcentrifuge filters. The 2 samples (flow through fractions) were run on a 1-D agarose gel.

Table 5.7: Demographic and clinical characteristics of participants

A total number of 20 participants were evaluated in this study: 10 TB cases and 10 controls. They had HIV rapid test at recruitment and were all negative.

	All	TB	Controls
Number of participants(n)	20	10	10
Mean age (years)	29.4	34.3	24.6
Age range (years)	17-58	18-58	17-43
Male/female ratio	12/8	8/2	4/6

**Figure 5.5: SDS-PAGE of depleted serum samples**

Participants (n=20) were included into this study. This number included 10 controls (c) and 10 TB cases. Serum samples were pooled together according to their clinical status. The 20 high abundant proteins were depleted from pooled samples using ProteoPrep 20. The flows through fractions were pooled and re-concentrated using the microcentrifuge filters and run on a 1-D agarose gel in triplicate.

Table 5.8: Proteins down regulated during active TB

Serum samples were collected from ten TB cases and 10 controls. Serum samples from each clinical group were pooled together and treated as a single sample in each clinical group. The two pooled samples were run on mass spectrometry. A total number of 338 proteins were identified and quantified on the Orbitrap Velos. 15 proteins were found down regulated in active TB

Proteins down regulated in TB and their accession number	Median Control	median TB	Control/TB	T test
P02656 APOC3_HUMAN Apolipoprotein C-III	30.26	23.76	90.6	0.000165
P17936 IBP3_HUMAN Insulin-like growth factor-binding protein 3	27.76	25.17	6.1	0.000304
P02753 RET4_HUMAN Retinol-binding protein 4	32.08	29.60	5.6	0.003723
P02654 APOC1_HUMAN Apolipoprotein C-I	29.66	27.44	4.7	0.024765
Q96KN2 CNDP1_HUMAN Beta-Ala-His dipeptidase	29.11	27.32	3.4	0.016065
O95445 APOM_HUMAN Apolipoprotein M	27.08	25.32	3.4	0.018075
P02652 APOA2_HUMAN Apolipoprotein A-II	32.98	31.32	3.2	0.003738
P02647 APOA1_HUMAN Apolipoprotein A-I	36.84	35.21	3.1	0.00109
P06727 APOA4_HUMAN Apolipoprotein A-IV	33.38	32.01	2.6	0.003384
P27169 PON1_HUMAN Serum paraoxonase/arylesterase I	32.41	31.13	2.4	0.000326
P06276 CHLE_HUMAN Cholinesterase	30.46	29.22	2.4	0.00404
P05090 APOD_HUMAN Apolipoprotein D	30.07	28.93	2.2	0.00663
P06396 GELS_HUMAN Gelsolin	35.37	34.28	2.1	0.002957
P35858-2 ALS_HUMAN Isoform 2 of Insulin-like growth factor-binding protein complex acid labile subunit	32.67	31.67	2.0	0.010691
P29622 KAIN_HUMAN Kallistatin	32.01	31.04	2.0	0.012744

Table 5.9: Proteins up regulated during active TB

Serum samples were collected from 10 TB cases and ten controls. Serum samples from each clinical group were pooled together and treated as a single sample in each clinical group. The 2 pooled samples were run on mass spectrometry. A total number of 338 proteins were identified and quantified on the Orbitrap Velos. 21 proteins were up regulated in active TB.

Proteins up regulated in TB and their accession number	median G1	median G2	G2/G1	T test
>sp P02741 CRP_HUMAN C-reactive protein	28.78	33.06	19.4	0.000109
>sp P05109 S10A8_HUMAN Protein S100-A8	26.59	30.52	15.3	0.001219
>sp P00738 HPT_HUMAN Haptoglobin	29.82	32.63	7.0	5.09E-05
>sp P02763 A1AG1_HUMAN Alpha-1-acid glycoprotein 1	25.17	27.84	6.4	0.005326
>sp P02750 A2GL_HUMAN Leucine-rich alpha-2-glycoprotein8	31.52	34.12	6.4	0.000754
>sp P01854 IGHE_HUMAN Ig epsilon chain C region	26.61	29.08	5.6	0.000313
>sp P0DJ18 SAA1_HUMAN Serum amyloid A-1 protein	24.13	26.26	4.4	0.049286
>sp Q13103 SPP24_HUMAN Secreted phosphoprotein 24	23.75	25.86	4.3	0.001461
>sp P13796 PLSL_HUMAN Plastin-2	27.13	29.17	4.1	0.011409
>sp P68871 HBB_HUMAN Hemoglobin subunit beta	25.92	27.76	3.6	0.033379
>sp Q06033 ITIH3_HUMAN Inter-alpha-trypsin inhibitor heavy chain H3	30.86	32.64	3.4	0.00255
>sp P0C0L5 CO4B_HUMAN Complement C4-B	30.58	32.31	3.3	0.000461
>sp Q08380 LG3BP_HUMAN Galectin-3-binding protein	28.53	30.15	3.1	0.014653
>sp P01011 AACT_HUMAN Alpha-1-antichymotrypsin	36.11	37.72	3.0	0.000556
>sp P02748 CO9_HUMAN Complement component C9	32.32	33.87	2.9	0.002087
>sp P02675 FIBB_HUMAN Fibrinogen beta chain	26.89	28.44	2.9	0.004949
>sp P01876 IGHA1_HUMAN Ig alpha-1 chain C region	29.56	31.03	2.8	0.034351
>sp P02671 FIBA_HUMAN Fibrinogen alpha chain	28.40	29.80	2.6	0.001855
>sp P07359 GP1BA_HUMAN Platelet glycoprotein Ib alpha chain	26.89	28.05	2.2	0.018123
>sp P04217 A1BG_HUMAN Alpha-1B-glycoprotein	35.15	36.19	2.0	0.004704
>sp P02774-3 VTDB_HUMAN Isoform 3 of Vitamin D-binding protein	35.3	36.33	2.0	0.001857

5.4 Discussion

The three methods employed in the present study to deplete high abundant proteins in serum yielded different results. The ProteoSpin column failed to deplete high abundant proteins in serum as seen on the electrophoresis gel and the number of proteins identified on the mass spectrometry. The Heparin column method provided satisfactory results on electrophoresis gel but may require additional procedures to evaluate its reproducibility and potential usefulness in biomarker studies. The ProteoPrep20 successfully removed high abundant proteins to such a degree that the Orbitrap Velos was not able to identify albumin in the sample. The fractionation method may still be the major drawback in this experiment.

ProteoSpin column was the first method selected to deplete high abundant proteins. The method was attractive due to its cost; it is a simplified platform to deplete high abundant proteins. It was used by few authors [17][18] to identify specific proteins in specific conditions. According to the manufacturer, this kit can only deplete 70% of albumin and 50% of transferrin. The 10 most abundant proteins represent around 90% of the total serum protein content [6]. The removal of 50 to 70% of abundant proteins may not reduce significantly the shadow effect of these proteins on low abundant proteins on the electrophoresis gel or on mass spectrometry. This method is not protein specific but an isoelectric point (PI) based method. This principle could be problematic as high abundant proteins like albumin act as protein carriers in serum. By carrying other proteins, abundant proteins change to a different PI and would not be depleted. In this situation the percentage of high abundant proteins in depleted samples would remain higher, which would reduce the chance of detecting interesting proteins. Furthermore, some low abundant proteins could have the same or similar PI to a particular high abundant protein and could be depleted during the depletion process. This method may be valid to identify specific proteins in particular conditions but may not be reliable in quantitative proteomic.

Heparin chromatography is an affinity chromatography method used to separate a number of biological molecules that can bind to heparin [12]. A wide range of proteins and peptides had been shown to interact with heparin including: enzymes, serine protease inhibitors, growth factors, extracellular matrix proteins, nucleic acid-binding protein 9 initiation factors, elongation factors, restriction, endonucleases, DNA ligase, DNA and RNA polymerases, hormone receptors,

and lipoproteins[13] [8]. These proteins are present in serum in low abundance and are usually overshadowed by abundant proteins during analysis on mass spectrometry. The bound proteins, because of their different and important biological roles, could be excellent biomarker candidates. The heparin column method was developed and evaluated by Ting Lei and colleagues [8] who demonstrated the efficiency of this method in depleting high abundant proteins. The efficiency of the method was shown on 2-D as well as on 1-D electrophoresis agarose gel where an important additional number of spots or bands were observed in depleted samples compared to the raw serum. These results were confirmed in our study on 1-D electrophoresis gel. The two experiments showed the efficiency of heparin column in removing high abundant proteins in serum samples but none of the reports evaluated the reproducibility of the method. Furthermore, this method depletes all proteins lacking any ability to interact with heparin columns. The exact number of depleted proteins has not been evaluated and these depleted proteins could be biomarker candidates. This heparin method should be further validated for biomarker studies.

The difficulties encountered with the first two methods led us to employ the most complete immunodepletion kit, designed to remove the 20 most abundant serum proteins prior to proteomic analysis. This immunodepletion method yielded an important reduction in the dynamic range of protein concentration in serum and enabled us to identify a larger number of proteins by mass spectrometry. Indeed, Proteoprep20 allowed the detection of more than 300 serum proteins compared to the 80 proteins previously obtained with the ProteoSpin columns. By comparing the proteomic profile of active TB cases to healthy controls, we have identified 25 upregulated proteins including acute phase proteins (C-reactive protein and serum amyloid A) and 15 down regulated proteins during active TB. These results are in accordance with our previous findings and corroborate with the literature. Indeed, results obtained with the overnight whole blood and quantiferon supernatant using the Luminex platform showed increased levels of acute phase proteins in unstimulated samples. Agranoff and colleagues [19] also showed increased levels of these proteins using surface-enhanced laser desorption ionisation time of flight mass spectrometry. These findings may indicate the reliability and reproducibility of the present results.

The main limitation of the present study is the limited number of detected proteins. The depletion of high abundant proteins through methods like Proteoprep20 has shown to enhance the detection

of a larger panel of proteins in serum [15][6]. Although we depleted high abundant proteins, the number of proteins detected was still relatively low. This low detection could be explained by the complexity of our samples on the gels. Analysis of different results from the literature has demonstrated the relationship between the complexity of the sample and the number of proteins detected [3]. When serum or plasma samples were analysed without high abundant proteins depletion or intensive fragmentation, the number of proteins identified was low [20][21]. An example of extensive fractionation could be seen with Vitor Faca and colleagues [22]. In their experiment, they used a double fragmentation method: the first dimension was an anion-exchanged fragmentation based method and enabled the collection of 12 fractions. Each individual fraction was further fractionated based on the hydrophobicity principle, using reversed-phase chromatography. This extensive fragmentation led to the identification of more than 2500 serum proteins and importantly, they have been able to identify several proteins that are known to be of low abundance in plasma ($<1\mu\text{g/mL}$), namely ICAM2, IGFALS, L-selectin, and superoxide dismutase. In the present study, we have fractionated our gel into 5 fractions. Each individual gel fraction could still be highly complex. Table 5.10 illustrate the complexity of our samples. Three categories of proteins could be seen in this table: the low expressed proteins including IL-15 and IFN- γ (1-1000 pg/ml), medium (1001-1 000 000 pg/ml) and high expressed proteins including CRP, SAA and SAP (1 000 001- 200 000 000 pg/ml). This table excludes all the high abundant proteins. These concentrations are known from the quantiferon assay (nil value). If more than one of these categories of proteins occurs in any single fraction at similar dynamic range, only the acute phase proteins will be seen on the mass spectrometry results and IL-15 or IFN- γ will not be detected unless this fraction is further fractionated. According to the results obtained in quantiferon and presented in Table 5.10, SAP is the acute phase protein that occurs at lowest levels, below 100 000 000 pg/ml. It was, interestingly, the only undetected acute phase protein. This could lead to the extrapolation that proteins with serum concentration below $100\mu\text{g/ml}$ could not be detected in our system.

Table 5.10: Dynamic range of low abundant proteins

Proteins concentrations (pg/ml) were taken from the QFT assay in the previous chapter. Nine different analytes were selected from the QFT nil tubes into three categories of analytes based on their expression levels in serum. The division was made as follow: low expressed analytes (1 to 1000 pg/ml, in black), medium expressed analytes (1001 to 1 000 000 pg/ml, in bleu) and highly expressed analytes (1 000 001 to 200 000 000 pg/ml, in red).

Markers	Mean TB	Mean Controls	Mean All
IL-15	6	6	6
IFN- γ	23	46	35
IP10	4 077	8 980	6 613
MCP-1	8 535	13 095	10 894
MMP-2	82 007	76 385	79 807
MMP-9	680595	1 314 341	928 583
CRP	269 117 000	42 308 000	151 802 000
SAA	1 175 291 000	28 786 000	582 271 000
SAP	42 830 000	33 827 000	38 173 000

5.5 Conclusion

Proteomic analysis of serum is hampered by the presence of high abundant proteins, which obscures the detection of proteins of interest that occur at lower levels. By the use of Proteoprep20, we have successfully been able to deplete high abundant proteins to a level where we could identify additional proteins. The depletion appears to be the first important step to perform but may not be sufficient for a detailed analysis of low abundant proteins with concentration below 100pg/ml in serum or plasma. To examine these proteins, a gel fractionation protocol or alternative fractionation methods has to be developed to reduce complexity of clinically promising sample types like serum to allow analysis by mass spectrometry.

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6. Chapter VI

Bifunctional T-cell-derived cytokines for the diagnosis of tuberculosis and treatment monitoring

6.1 Introduction

Tuberculosis is an airborne infectious disease. In the year 2011 approximately 8.7 million people developed tuberculosis and 1.4 million human deaths were attributed to this disease globally [1]. In order to identify individuals with latent *Mycobacterium tuberculosis* infection (LTBI) and to prevent the development of active tuberculosis, targeted immunological testing to assess *M.tb* specific immune responses is performed in close household contacts of infective patients, persons with HIV-infection and other individuals from risk groups for the future development of tuberculosis [2]. In the absence of active tuberculosis, children and immune compromised individuals with an adaptive immune response against *M.tb*, as measured through the tuberculin skin test (TST) or an Interferon- γ Release Assay (IGRA), are offered preventive chemotherapy [3].

One of the major limitations of the currently available immunodiagnostic methods for *M.tb* infection is that neither the TST nor IGRAs are able to distinguish between individuals with LTBI and active tuberculosis [4,5].

In principle, discrimination between different states of *M.tb* infection is possible by immunodiagnostic testing. Lately it has been shown that the proportion of single-positive Tumor Necrosis Factor alpha releasing *M.tb*-specific CD4⁺ T-cells is indicative for active tuberculosis, in contrast to LTBI [6].

One of the most promising cytokine candidates to improve the immunodiagnosis of tuberculosis is the Interleukin (IL)-2 [7,8]. Millington et al[9] have reported a correlation between mycobacterial load and the frequency of IL-2⁺ and IFN- γ ⁺ secreting T-cells. Single IFN- γ ⁺ secreting cells dominate the cellular immune response in untreated active tuberculosis. This immune dominance progressively decreased during tuberculosis treatment while single IL-2⁺ secreting T-cells frequency increased.

The preferred technique to evaluate immunophenotypes on unstimulated and stimulated cells for the differentiation of active tuberculosis from LTBI is fluorescence activated cell sorting (FACS). Enzyme-Linked ImmunoSpot (EliSpot) analysis is a less expensive, highly sensitive, quantitative and easy to use, yet misses the ability of immunophenotyping by FACS. In order to overcome limitations of the EliSpot analysis, a new dual colour EliSpot technique has been developed, that allows to distinguish the expression of two cytokines at a single cell level, which is cheaper and easier to perform than FACS analysis.

This study evaluates the utility of an IFN- γ /IL-2 dual colour EliSpot to discriminate between tuberculosis and LTBI and to monitor specific cytokine profiles during tuberculosis treatment.

Declaration

Data presented in this part has been published in an international, peer reviewed journal:

Essone PN, Kalsdorf B, Chegou NN, Loxton AG, Kriel M, Preyer R, Ernst M, Walzl G, Lange C. Bifunctional T-cell-derived cytokines for the diagnosis of tuberculosis and treatment monitoring. *Respiration*. 2014;88(3)

6.2 Material and methods

6.2.1 Participants

Following written informed consent adult HIV-seronegative suspects with a first episode of tuberculosis and healthy HIV-seronegative community controls (HCCs) were recruited at local health care centres in the Ravensmead/Uitsig community in Cape Town, South Africa. Pregnancy, chronic cardiovascular or metabolic illnesses, immunosuppressive medication, and age less than 21 years constituted exclusion criteria. All participants had routinely been vaccinated at birth with *M. bovis* BCG (Danish strain 1331, Statens Serum Institute, Copenhagen, Denmark). Participants with suspected tuberculosis presented with clinical symptoms of cough, fever, night sweats and/ or weight loss. Chest X-rays, sputum smear microscopy for the presence of acid fast bacilli (AFB) and *M.tb* culture (BACTEC MGIT 960 system, Becton Dickenson Biosciences, Johannesburg, South Africa) of one sputum sample were

performed on all study participants. Tuberculosis was diagnosed by culture positivity. Individuals who were suspected to have tuberculosis with a negative *M.tb* sputum culture were excluded from further participation in the study. For all culture confirmed tuberculosis patients, clinic records attested full sensitivity against the first line drugs. All tuberculosis patients received standard treatment of two months intensive phase with isoniazid, rifampicin, ethambutol and pyrazinamid followed by 4 months isoniazid and rifampicin.

Volunteers from the same community with identical ethnic and social background who lived in a household with proven active tuberculosis patients within the past three months were recruited as HCCs. Their *M.tb* infection status was investigated either by a tuberculin skin test (TST) (2 U tuberculin RT23, Statens Serum Institute, Copenhagen, Denmark) or with a Quantiferon TB Gold in tube test (QFT, Cellestis, Victoria, Australia). A TST >10 mm diameter was regarded as positive. QFT positivity was defined according to the recommendations of the manufacturer (cut off value for a positive test was >0.35 IU/mL). The study was approved by the Ethic Committee for Human Research of the Stellenbosch University, Republic South Africa (N10/08/274), and the University of Lübeck, Germany (05-096).

6.2.2 Blood processing

Five mL of heparinized peripheral blood were collected by venipuncture and processed within two hours. Peripheral blood mononuclear cells (PBMCs) were isolated using histopaque density gradient centrifugation of blood layered on Ficoll-Paque Plus (Sigma Aldrich, Johannesburg, South Africa) [10].

6.2.3 Dual colour ELISpot for detection of IL-2⁺ and IFN- γ ⁺ secreting cells

2×10^5 PBMCs were aliquoted into all designated wells, enriched with 5% Foetal Bovine Serum (Sigma Aldrich, Johannesburg, South Africa) and co-stimulated with anti-CD28. The PBMCs were cultured overnight (16 hours) in six different conditions in duplicate: 100 μ L RPMI 1640

(Sigma Aldrich, Johannesburg, South Africa) (negative control), 100 μ l ESAT-6/CFP-10, 100 μ l MTP-65, 10 μ g/ml PPD, 5 μ g/ml Pokeweed (positive control) and anti-CD-3 (1 μ g/ml/well) (positive control). The following day, cells were washed off manually (AID wash buffer). The detection antibodies biotin-conjugated anti-human IL-2 and fluorescein isothiocyanate-conjugated anti-human IFN- γ were diluted (AID dilution buffer). 100 μ L of this secondary antibody dispersion was added per well and plates were incubated for two hours in a humid dark chamber. Unbound detection antibody was washed off. Streptavidin RED-(Cy3)-conjugate and anti-fluorescein isothiocyanate green were diluted and plates were incubated with 100 μ L/well for one hour in the humid dark chamber. Plates were washed and 100 μ L of the ready to use AID-enhancer were added. After 15 minutes of enhancer incubation the plate was blotted on a stack of towel paper and left to dry overnight in a dark place being protected from light. The IL-2/ Streptavidin RED-(Cy3) and IFN- γ / anti-fluorescein isothiocyanate green spots are invisible to the naked eye and can be read by a dual colour EliSpot Reader system (AID) as two independent signals.

6.2.4 Data analysis

The AID EliSpot software ELRIFLO5 counted all IFN- γ ⁺ and all IL-2⁺ producing cells and created an artificial image highlighting the double-stained IFN- γ ⁺IL-2⁺ spots (Figure 6.1). Cytokine producing cells were counted as spot forming cells (SFCs). Results were analysed and interpreted according to the manufacturer's guidelines. The background response of the negative control was deducted from the stimulated wells. For a correct test-interpretation, the positive controls pokeweed mitogen or anti-CD3 had to induce more than 50 SFCs/ 2×10^5 cells/ well of IL-2⁺ and more than 50 SFCs/ 2×10^5 cells/ well IFN- γ ⁺ secreting cells after subtraction of the number of spots in the negative control well and at least twice the number of spots of the negative control well, otherwise the test was indeterminate. Providing that a valid positive control result was found, the net response to ESAT-6/CFP-10, MTP-65 or PPD was considered positive when the test well had at least 10 SFCs and twice the number of SFCs in the negative control well for both cytokines. If PBMCs from a participant showed a specific (>10 SFCs/ 2×10^5 cells/ well) immune response with one cytokine, but did not secrete less than 10 SFCs/ 2×10^5

cells/ well of the other cytokine towards the same antigen, the one cytokine response below 10 SFCs was assigned 0.1 for analysis. An in vitro response of 0 to 10 SFCs/ 2×10^5 cells/ well of both cytokines to the same antigen was classified as negative response for that specific antigen. Differences between groups in SFCs for each *M.tb*-specific antigen were assessed with the Mann Whitney U test for nonparametric data analysis (GraphPad Prism version 5.00, San Diego, California, USA). Sensitivity, specificity and cut offs were ascertained by receiver operating characteristics (ROC) analysis using Statistica program (Statsoft, Ohio, USA). The study is reported according to the STARD guidelines.

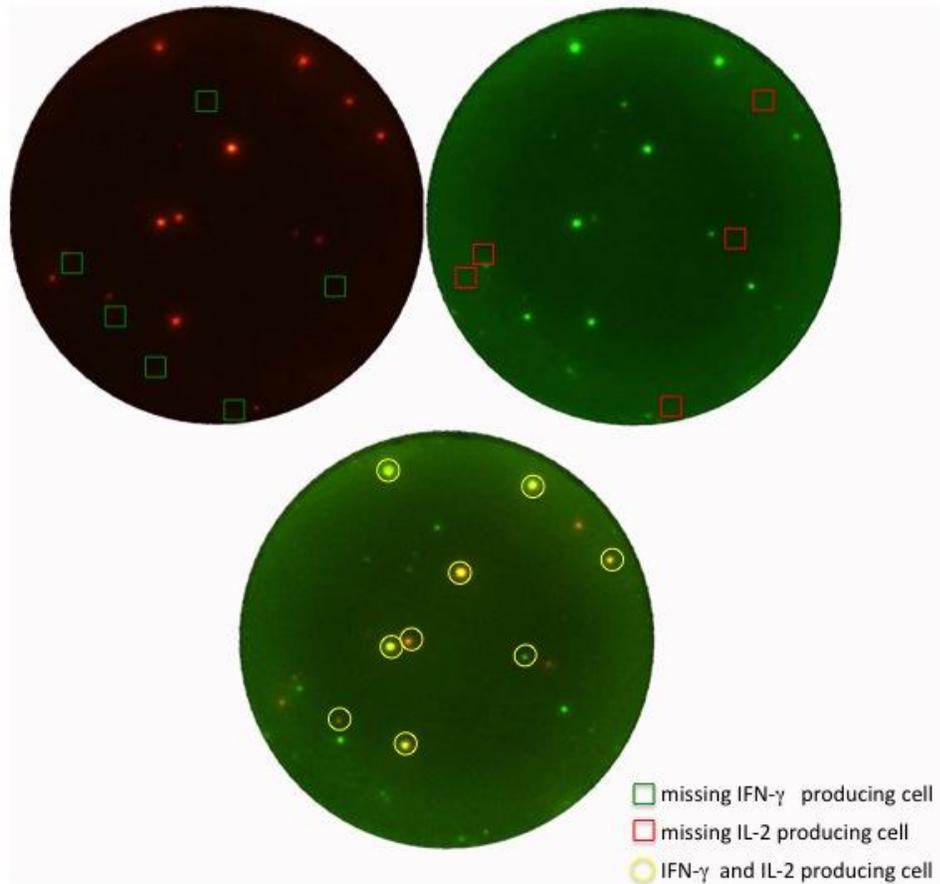


Figure 6.1: FluoroSpot analysis of three images from the bottom of the same cell culture well following ESAT6/CFP10 specific stimulation of peripheral blood mononuclear cells

On the left upper image streptavidin RED-(Cy3) bound IL-2⁺ is detected with a red narrow band fluorescence filter. In green squares, areas on the bottom of the well are highlighted, where no IL-2⁺ production can be seen, while IFN- γ ⁺ production detected by fluorescein isothiocyanate is seen in the same position on the the upper right image obtained through a green narrow band fluorescence filter. Here, red squares show areas of IFN- γ ⁺ production, where no IL-2 production is identified on the left upper image. The picture below is an automated overlay of both images. Double IFN- γ ⁺ and IL2⁺ producing cells are highlighted in yellow circles, while single cytokine producing cells are represented by red and green dots.

6.3 Results

Following written informed consent 27 individuals with suspected active tuberculosis and 26 healthy HCC were enrolled in the study between November 2010 and February 2012. One out of 27 individuals suspected to have active tuberculosis (3.7%) was excluded as he did not have a positive *M.tb* culture from sputum. In seven tuberculosis patients (25.9%) the required > 50 SFCs/ 2×10^5 cells/ well in the positive control were not obtained and their tests were classified as indeterminate. Thus 19 patients with tuberculosis were eligible for the final analysis. Among these 19 tuberculosis patients the magnitude of the immune response differed between the antigens: 16 patients showed detectable T-cell reactivity towards ESAT6/CFP10 (84.2%), 15 patients (78.9%) towards MTP65 and 13 patients (68.4%) towards PPD, respectively.

Though all 26 HCCs presented without any symptoms suggestive of tuberculosis, 2 (7.7%) had to be excluded from the study as their sputum culture was positive for *M.tb* (no further information was available to confirm the clinical status of these 2 participants). In one HCC (3.8%) the required > 50 SFCs/ 2×10^5 cells/ well in the positive control was not achieved and the test was classified as indeterminate. Thus 23 HCCs were eligible for the final analysis. Of these 23 HCCs, 5 (21.7%), 2 (8.7%) and 2 (8.7%) were low-responders towards ESAT6/CFP10, MTP65 and PPD, respectively (Figure 6.2). 9/11 (81.8%) of the HCCs had a positive TST and 10/12 (83.3%) HCCs had a positive QFT result (Table 6.1).

Table 6.1: Characteristics of participants with tuberculosis and healthy community controls

	TB	HCCs
Number of participants	19	23
Age (\pm SD)	33.74 (\pm 12.5)	33.70 (\pm 12.8)
Sex: male/female	8/11	12/11
QFT: positive / total (%)	n. a.	9/ 11 (81.82)
TST: positive / total (%)	n. a.	10/ 12 (83.33)

TB = active tuberculosis, HCC = healthy community controls, SD = Standard Deviation, QFT = QuantiFERON TB Gold in Tube, TST = tuberculin skin test, n. a. = not applicable

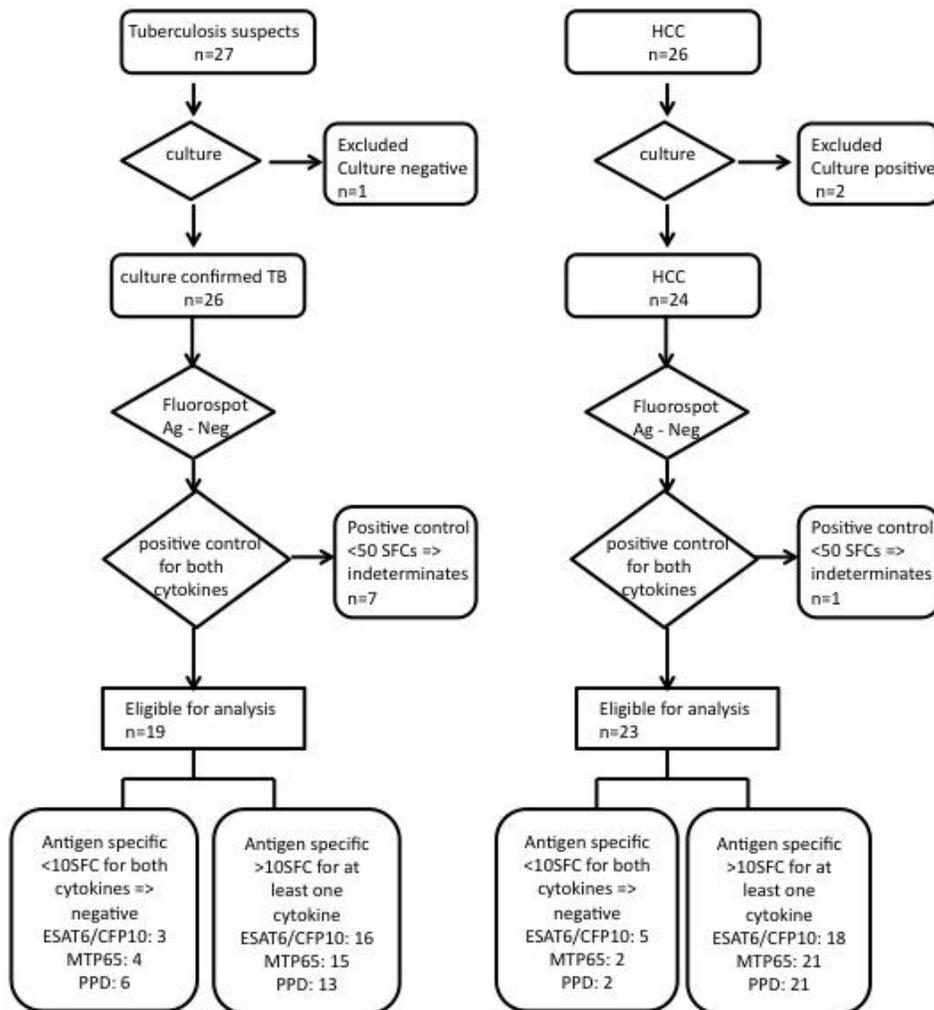


Figure 6.2: Study flow diagram

TB = active tuberculosis, HCC = healthy community controls, Ag = specific antigen, Neg = negative control, SFC = spot forming cells

6.3.1 Diagnostic utility of the number of Spot Forming Cells

After stimulation with ESAT6/CFP10, MTP65 and PPD, in each participant the numbers of IFN- γ IL-2⁺ and IFN- γ ⁺IL-2⁺ dual cytokine producing cells in tuberculosis patients and in HCCs were compared (Table 6.2). Patients with tuberculosis had a trend towards more ESAT6/CFP10-induced IFN- γ ⁺ producing cells (median 71.5 SFC) than HCC (median 31.5 SFC, p=0.062).

The frequency of MTP65-induced IL-2⁺ producing cells was higher in the HCCs (median 15.5 SFC) than in tuberculosis patients (median 0.1 SFC, p=0.032). Although statistically the median of the MTP65-reactive IL-2⁺ producing cells differed significantly between the two groups, the overlap in frequencies precluded accurate discrimination between LTBI and tuberculosis on an individual basis.

No differences in the immune response towards PPD stimulation were found between HCCs and tuberculosis patients.

Table 6.2: Diagnostic performance of stimulated spot forming cells using dual colour EliSpot

Peripheral blood mononuclear cells of active tuberculosis patients (TB) and healthy community controls (HCC) were isolated and stimulated overnight with *Mycobacterium tuberculosis* specific antigens (ESAT6/ CFP10, MTP65) and PPD. The number of Spot Forming Cells (SFC) was determined with the EliSpot Reader System ELRIFLO5. SFC of IL-2⁺ producing cells, IFN- γ ⁺ producing cells and IL-2⁺ IFN- γ ⁺ double producing cells are shown as median with interquartile ranges (IQR) in brackets. Significant p values for comparison between groups are given in bold. The area under the curve (AUC) and 95% confidence interval in square brackets [95% CI] were calculated with Receiver Operating Curve.

	host Marker	TB Median SFC (IQR)	HCC Median SFC (IQR)	P value	AUC [95% CI]
ESAT6/ CFP10	IL-2 ⁺	25.25 (0.1-123.1)	32.50(17.5-50.25)	0.704	0.54 [0.32-0.76]
ESAT6/ CFP10	IFN- γ ⁺	71.5 (241.3-234.3)	31.5 (0.1-85.38)	0.062	0.69 [0.51-0.87]
ESAT6/ CFP10	double	15.0 (4.13-55.63)	15.25 (5.13-29.63)	0.704	0.54 [0.34-0.74]
MTP65	IL-2 ⁺	0.1 (0.1–12.5)	15.5 (0.1-32.00)	0.032	0.71 [0.54-0.88]
MTP65	IFN- γ ⁺	32.5 (16-84)	40.5 (20.5-63.25)	0.748	0.53 [0.33-0.74]
MTP65	double	2 (1-5)	6.5 (2-12)	0.054	0.69 [0.52-0.87]
PPD	IL-2 ⁺	21.5 (12.25-72.25)	25.5 (10.75-55.5)	0.657	0.55 [0.34-0.75]
PPD	IFN- γ ⁺	51.0 (23.25-97.0)	23.0 (16.0-60.75)	0.103	0.67 [0.49-0.85]
PPD	double	14.5 (3.0-32.25)	9.5 (4.25-22.5)	0.915	0.51 [0.30-0.73]

6.3.2 Diagnostic utility of percentage of single and double producing cells

The lack of discrimination between tuberculosis and LTBI observed with the different antigens might be due to the high level of inter-individual variation of SFCs. To adjust for this variation, each individual cytokine response was evaluated in proportion to the overall specific immune response of the individual. Five different T-cell populations were defined: total IL-2⁺ producing cells, total IFN- γ ⁺ producing cells, IFN- γ ⁺IL-2⁻, IFN- γ ⁻IL-2⁺, and IFN- γ ⁺IL-2⁺. The total number of any cytokine producing cells in response to each stimulus was calculated by adding the SFCs of single IFN- γ and single IL-2 producing cells and SFCs of double producing cells. This sum

was used as common denominator for the calculation of the percentage of each cytokine producing phenotype, which was compared between the two study groups.

Using this algorithm, the percentage of each cell population in relation to all cytokine producing cells by ESAT6/CFP10 stimulation discriminated significantly between tuberculosis and LTBI (Table 6.3). The percentage of ESAT6/CFP10-reactive IFN- γ IL-2⁺ producing cells was higher in HCCs than in tuberculosis patients (median 34.74% vs. 15.09% respectively, $p=0.002$), as well as the percentage of ESAT6/CFP10-reactive IFN- γ ⁺IL-2⁺ double producing cells (median 30.11% vs. 18.30% respectively, $p=0.037$). The percentage of total IL-2⁺ producing cells responding towards ESAT6/CFP10 was elevated in HCCs in comparison with tuberculosis patients (median 70.38% vs. 35.88% respectively, $p<0.001$). In contrast the percentage of ESAT6/CFP10-induced IFN- γ ⁺IL-2⁻ producing cells was lower in HCCs than in tuberculosis patients (median 29.63 vs. 64.12 respectively, $p<0.001$) as well as the percentage of total IFN- γ ⁺ producing cells (median 65.27% vs. 84.91% respectively, $p=0.002$). Tuberculosis patients had increased ratios of IFN- γ ⁺ producing cells (median 84.91% IFN- γ ⁺ producing cells), whereas HCCs displayed a more balanced distribution of 34.74% IFN- γ ⁻IL-2⁺ producing, 30.11% IFN- γ ⁺IL-2⁺ double producing and 29.63% IFN- γ ⁺IL-2⁻ producing cells after ESAT6/CFP10 stimulation.

The percentage of MTP65-induced IFN- γ ⁺IL-2⁺ double producing cells was higher in HCCs than in tuberculosis patients (median 10.83% vs. 3.57%, $p=0.040$). Although the SFC of MTP65-induced IFN- γ ⁻IL-2⁺ producing cells was significantly elevated in HCCs in comparison to tuberculosis patients (Table 6.2), after adjustment for total cytokine producing cells the percentage of IFN- γ ⁻IL-2⁺ producing cells showed only a trend to be higher (median 11.57% vs. 0.62% respectively, $p=0.067$, Table 6.3).

Calculating the percentages of PPD responsive cytokine populations did not reveal any differences between tuberculosis patients and HCCs.

Using the percentages of antigen-induced cytokine populations, six host markers significantly discriminated tuberculosis from LTBI. To calculate the ability of individual markers to assign participants to their correct clinical status, receiver operator characteristics analysis were used to define the best cut off for each antigen-induced host marker. For example, the optimum cut off

for ESAT6/CFP10 stimulated IFN- γ IL-2⁺ producing cells was 28.25% (area under the curve = 0.82) and active tuberculosis was identified with a sensitivity of 72% and specificity of 81% (Table 6.3). Notably there was substantial overlap in frequencies between the two cohorts for each marker and therefore none of the antigen-induced cell population was a promising diagnostic marker on its own.

Table 6.3: Diagnostic performance of stimulated single and dual IFN- γ /IL-2 producing cells using dual colour EliSpot

Peripheral blood mononuclear cells of tuberculosis patients (TB) and healthy community controls were isolated and stimulated overnight with *Mycobacterium tuberculosis* specific antigens (ESAT6/ CFP10, MTP65) and PPD. The number of Spot Forming Cells (SFC) was used to calculate frequencies of IFN- γ IL-2⁺, IFN- γ ⁺ IL2⁻, IFN- γ ⁺ IL2⁺, total IL-2⁺, and total IFN- γ ⁺ producing cell populations. Relative percentages of each cell population are shown as median with interquartile ranges in brackets (IQR). Significant p values for comparison between groups are given in bold. Receiver Operating Curve analysis revealed the area under the curve (AUC), and the best cut off identifying the patients clinical status with the corresponding sensitivity and specificity. Sensitivity and specificity are reported with 95% confidence interval.

<i>M.tb</i> antigen	host Marker	TB Median (IQR)	HCC Median (IQR)	p-value	AUC	Cut off value	Sensitivity (%)	Specificity (%)
ESAT6/CFP10	% IFN- γ IL-2 ⁺	15.09% (0.42-27.36)	34.8% (27.4-46.9)	0.002	0.82	28.25	72	81
ESAT6/CFP10	% IFN- γ ⁺ IL2 ⁻	64.12% (46.2-83.9)	29.7% (1.0-48.0)	<0.001	0.85	42.95	67	94
ESAT6/CFP10	% IFN- γ ⁺ IL2 ⁺	18.3% (11.7-27.9)	33.1% (19.1-37.7)	0.037	0.71	20.57	78	63
ESAT6/CFP10	% total IL-2 ⁺	35.9% (16.1-53.8)	70.4% (52.1-99.0)	<0.001	0.85	57.05	67	94
ESAT6/CFP10	% total IFN- γ ⁺	84.9% (72.7-99.6)	65.3% (53.2-72.6)	0.002	0.82	71.75	72	81
MTP65	% IFN- γ IL-2 ⁺	0.6% (0.3-11.0)	11.6% (0.6-21.0)	0.067	0.68	11.25	57	80
MTP65	% IFN- γ ⁺ IL2 ⁻	88.4% (77.6-96.6)	70.3% (57.0-89.0)	0.132	0.65	76.23	62	80
MTP65	% IFN- γ ⁺ IL2 ⁺	3.6% (2.0-6.8)	11.0% (4.1-18.5)	0.04	0.71	7.1	71	80
MTP65	% total IL-2 ⁺	9.9% (2.4-22.4)	29.8% (11.1-43.1)	0.124	0.65	21.95	62	80
MTP65	% total IFN- γ ⁺	99.9% (89.0-99.7)	88.4% (79.0-99.4)	0.067	0.68	88.75	57	80

PPD	% IFN- γ IL-2 ⁺	22.1% (15.2-33.0)	27.1% (13.6-44.6)	0.620	0.55	28.81	48	77
PPD	% IFN- γ ⁺ IL2 ⁻	60.5% (51.6-65.4)	49.0% (29.7-61.3)	0.096	0.67	47.54	48	92
PPD	% IFN- γ ⁺ IL2 ⁺	19.5% (7.2-25.1)	25.5% (16.5-28.3)	0.103	0.67	24.60	52	77
PPD	% total IL-2 ⁺	39.1% (34.7-48.5)	51.0% (38.7-70.2)	0.096	0.67	52.46	48	92
PPD	% total IFN- γ ⁺	78.0% (67.0-84.8)	72.9% (55.4-86.4)	0.620	0.55	71.19	48	77

6.3.3 Diagnostic utility of combination models

In an attempt to increase specificity different combination models of three to six antigen-induced host markers were used to classify the participants. The cut off value as obtained through ROC analysis described above were used to classify individuals into the two groups. Using four, five or six markers in combination did not perform better than a three-marker model. Participants were allocated to a clinical group according to the results of the majority of the individual marker tests (2/3 or 3/3). Accordingly, the ESAT6/CFP10-induced three-host marker combination model identified 29 of the 34 participants (85.3%) to the correct clinical group with 93.8% sensitivity and 77.8% specificity. Five (14.7%) participants were misclassified, with one false negative and four false positive classifications. Grouping of individual participants, sensitivity and specificity are shown in Figure 6.3.

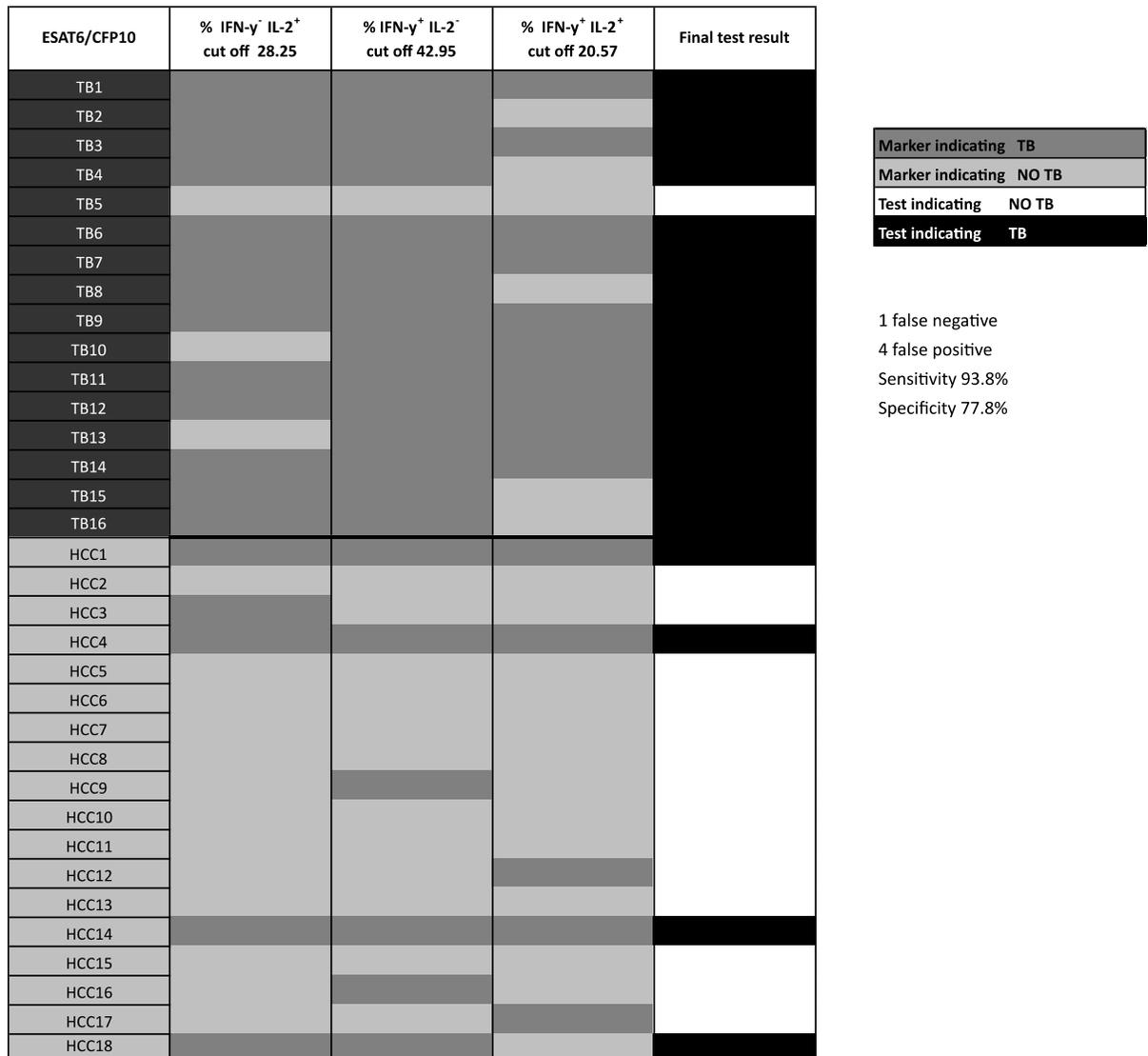


Figure 6.3: Utility of a 3 host marker combination model for diagnosis

Sixteen tuberculosis (TB) patients, defined by *M.tb* growth in culture, and 18 healthy community controls (HCC) were analysed. The relative percentage of specific cytokine producing cells and the best cut off were determined for each cell population as described in Table 6.3. The cut off was used to define whether the frequency of cell populations indicated either tuberculosis or latent infection with *M.tb* (LTBI). Each cell population was used as an independent marker. Prediction of TB is shown in dark gray, prediction of LTBI is shown in light gray for three different phenotypic markers. When ≥ 2 phenotypes supported one of the diagnoses, a final diagnosis of either TB (black) or LTBI (white) was made.

Treatment monitoring

After completion of 6 month standard tuberculosis treatment 14 tuberculosis patients and 12 HCCs were re-investigated for changes in their immune response. Again percentages of the different cell populations were calculated. Analysing the ESAT6/CFP10-induced immune response of the complete pairs during successful tuberculosis treatment the median percentage of IFN- γ IL-2⁺ producing cells increased from 9.92% at recruitment to 27.38% after 6 months of treatment, as did the median percentage of IFN- γ ⁺IL-2⁺ double producing cells (13.59% to 23.62% respectively). In contrast, the median percentage of IFN- γ ⁺IL-2⁻ producing cells decreased (71.71% to 53.19% respectively). Nevertheless, the immune response of tuberculosis patients towards the antigens ESAT6/CFP10 did not change consistently and were therefore not significant (Figure 6.4). According to the ESAT6/CFP10-induced three-host marker combination model, seven (63.63%) of the eleven tuberculosis patients were still assigned to the clinical status of active tuberculosis, despite having completed six months of successful treatment. The median percentages of HCC changed very little (IFN- γ IL-2⁺: 33.55% at recruitment to 36.73% after 6 months, IFN- γ ⁺IL-2⁺: 37.13% to 25.74% and IFN- γ ⁺IL-2⁻: 30.3% to 36.03%, respectively). One of the HCCs, who had been correctly classified at recruitment, changed his immune response to an IFN- γ ⁺ dominating profile, suggesting active disease, but in the clinical follow up of one year he did not develop any symptoms of tuberculosis. The MTP65 immune response showed no significant changes during treatment (data not shown).

Analysing the PPD-induced immune response of the nine pairs during successful tuberculosis treatment the median percentage of IFN- γ IL-2⁻ producing cells decreased from 63.24% at recruitment to 40.49% after 6 months treatment ($p = 0.02$), whereas the median percentage of IFN- γ ⁺IL-2⁺ double producing cells and all IL-2⁺ producing cells increased (19.35% to 29.25% ($p = 0.027$) and 36.76% to 59.51% ($p = 0.02$), respectively). The significant changes in the frequency of cytokine populations are shown in Figure 6.5. PPD-induced immune response in HCC did not change significantly.

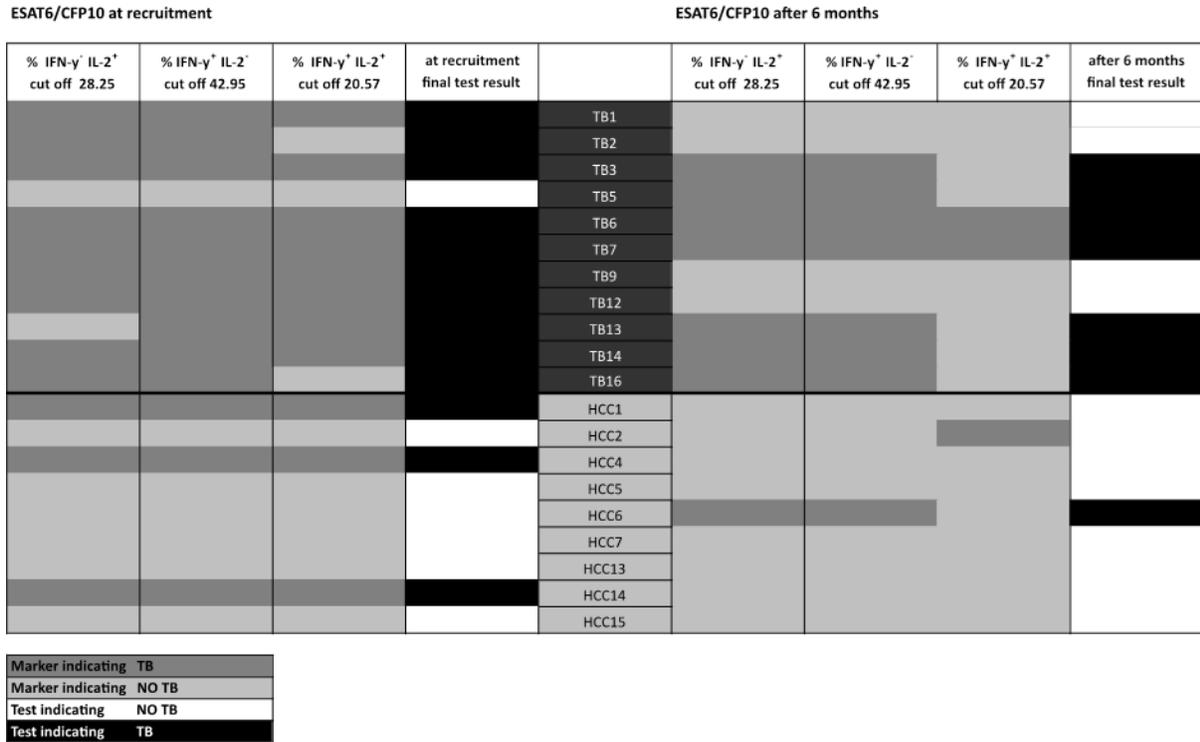


Figure 6.4: Utility of ESAT6/CFP10 in monitoring treatment

Immune responses of peripheral blood mononuclear cells towards ESAT6/ CFP10 of 11 tuberculosis (TB) patients and 9 healthy community controls (HCC) were evaluated at recruitment and after 6 months, during which the tuberculosis patients had received 6 months standard treatment. In a model the optimal AUC cut off (determined as described in Figure 6.4 and Table 6.3) was used to define TB or latent infection with *M.tb* (LTBI).). Each cell population was used as an independent marker. For each phenotype, prediction of TB is shown in dark gray and prediction of LTBI in light gray. When ≥ 2 phenotypes supported one of the diagnoses, a final diagnosis of either TB (black) or LTBI (white) was made.

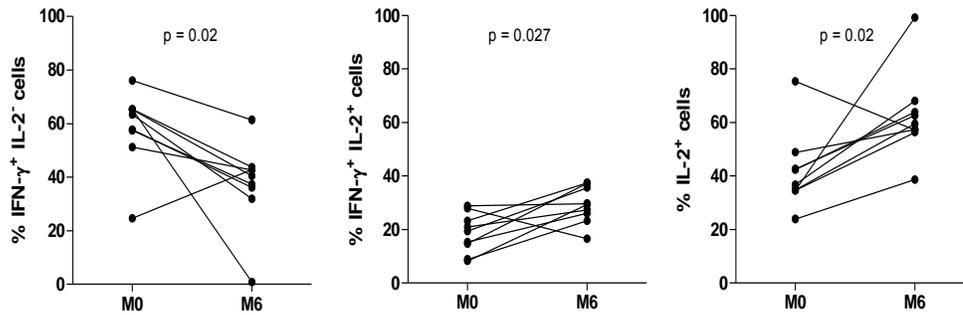


Figure 6.5: PPD-induced immune response changes during pulmonary tuberculosis treatment

6.4 Discussion

The clinical performance of the new IFN- γ /IL-2 dual colour EliSpot (FluoroSpot) to discriminate between tuberculosis and LTBI and to monitor specific cytokine profiles during tuberculosis treatment was evaluated in this study. The key findings are: 1) A three-marker combination model including ESAT6/CFP10-induced IFN- γ IL-2 $^+$, IFN- γ^+ IL-2 $^-$ and IFN- γ^+ IL-2 $^+$ double producing cells improved diagnostic sensitivity in comparison to the EliSpot. 2) ESAT6/CFP10-induced IFN- γ IL-2 $^+$, IFN- γ^+ IL-2 $^-$ and IFN- γ^+ IL-2 $^+$ double producing cells expressed as a percentage of all IL-2 and IFN- γ positive cells had a more promising diagnostic utility than analysis of absolute numbers of SFCs. 3) Analysis of PPD responsive cell populations hold promise as biomarkers for treatment monitoring.

Unlike the traditional T-SPOT.*TB* and QFT ELISA, the dual colour EliSpot allows the assessment of IFN- γ and IL-2 producing cell populations at a single cell level. The lack of diagnostic accuracy of the absolute number of SFCs of cytokine producing cells may be due to

high inter-individual variation of immune responses [10], which was decreased by calculation of percentages of cells. These results are in agreement with previous observations using FACS analysis, which showed that LTBI and tuberculosis are associated with distinct T-cell phenotypes [11,6,12]: During LTBI, the antigen specific immune response is dominated by a mixture of central memory cells (mostly IFN- γ ⁻IL-2⁺ producing cells) plus effector memory cells (IFN- γ ⁺IL-2⁺ double producing cells) whereas active tuberculosis is associated with a predominance of effector memory cells (IFN- γ ⁺IL-2⁻) [11].

Furthermore the analysis of antigen specific induction of two cytokines allows new insights into immune response capability. It has been generally accepted that a negative IFN- γ immune response in IGRAs of active tuberculosis patients are false negative test results [13,14].

However, our data show that even in the absence of antigen-specific IFN- γ production, other cytokines like IL-2 indicate sensitization to pathogen proteins. As IFN- γ ⁻IL-2⁺ phenotype occurs more commonly in LTBI, IFN- γ should therefore not be seen as the only proxy for sensitization to *M.tb*.

Although the immune signature was significantly different between LTBI and tuberculosis for five different ESAT6/CFP10-induced cytokine production patterns, the overlap between the two groups was substantial for all the markers. None of the markers could correctly classify all participants into their respective groups. However, the three-marker model allowed correct identification of active tuberculosis with a sensitivity of 93.8% and moderate specificity of 77.8%. This confirms the advantage of combination marker models instead of single markers in TB diagnostics [15,16]. A positive three-marker test may serve as screening or rule-out test to identify people with suspected tuberculosis for subsequent testing with a highly specific test such as GeneXpert MTB/RIF-or BACTEC 960 culture to optimize-diagnostic-work-up.

ESAT6/CFP10-induced immune responses as markers for treatment monitoring were not promising due to inter-individual variations. There were also fluctuations in the immune response of HCCs. These results are in line with another report [11], describing that neither ESAT6 nor CFP10 could separate untreated tuberculosis patients from another cohort of successfully treated tuberculosis patients. However, the results are in contrast with other studies on serial IGRAs, where a decline in IFN- γ production during tuberculosis treatment was observed [17–19]. We presume that kinetics of the immunological recall during tuberculosis

treatment may take longer than observed in this study.

PPD specific immune responses were superior to the ESAT6/ CFP10 specific immune responses to monitor treatment success. The frequencies of IFN- γ ⁺IL-2⁻, IFN- γ ⁺IL-2⁺ and the total IL-2⁺ producing cells changed significantly during treatment of patients with tuberculosis. Our results support previous findings by others [11] who had reported the PPD-reactive IFN- γ ⁺IL-2⁺ double producing cells may be valuable markers for treatment responses.

Our study has several limitations, including the small number of participants. Additional time points and new *M.tb* antigens and host cytokines need to be incorporated into future studies to evaluate biomarkers for successful treatment responses. The high number of indeterminate results due to inadequate cytokine responses after stimulation with the positive controls (7/26 tuberculosis patients and 1/24 HHCs) adversely affects test performance. As others observe similar reduction of immune response in the case of disease, this frequent absence of positive responses in tuberculosis may be due to immune suppression [20, 21] or the systemic predominance of TH2 cytokines over TH1 cytokines during disease [22, 23].

6.5 Conclusion

In conclusion this study has shown that ESAT6/CFP10-reactive IFN- γ ⁺ producing cells dominate in active tuberculosis, whereas in LTBI the immune response is balanced between IFN- γ ⁻IL-2⁺, IFN- γ ⁺IL-2⁺ and IFN- γ ⁺IL-2⁻ producing cell populations. A three-marker model of IL-2 and IFN- γ producing populations, expressed as percentage of all cells producing these cytokines, has improved the discrimination of LTBI from active TB patients. However, both diagnostic performance and assessment of immunological changes during TB therapy of this test require substantial improvement to warrant further clinical investigation.

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7. Chapter VII

General discussion and conclusion

7.1 Introduction

Introduction of an accurate, rapid and price effective tuberculosis (TB) test is a major priority for the control of this pandemic due to the limitations observed with the current tests in use (as discussed in chapter I). Most of the newly developed tools including GeneXpert and other nucleic acid amplification tests are not suitable in resource limited areas due to their requirements including their cost, their need for specialised laboratories and trained technicians or scientists, which are not always available in resource limited settings. The present thesis evaluated the diagnostic utility of antigen-stimulated and unstimulated host markers for the development of a rapid and simple TB tests suitable in resource limited areas.

7.2 Summary of findings and discussion

The 7-day whole blood assay was employed in the discovery phase of this thesis to select the most suitable antigen-induced host markers for TB diagnostic. This assay led to the discovery of the diagnostic utility of Rv0081. It was the most promising *Mycobacteria tuberculosis (M.tb)* antigen among the 118 antigens evaluated in our laboratory. This antigen was the only stimulus (in combination with some host markers) to correctly classify participants into their appropriate clinical groups. The role of this antigen during *M.tb* growth is not well known and the only information on its function was provided last year by Galagan and colleagues [1]. They showed that Rv0081 participates in the regulation of the initial response to hypoxia through the regulation of a number of *M.tb* genes.

The present thesis has also shown that immune responses to Rv0081 might be poorly appreciated when Rv0081-induced IFN- γ level is the only measure of sensitisation. 37% of participants responded to this antigen in our study (Table 3.8). This data stands in agreement with a recent study published by Desta Kassa and colleagues where only 33% of participants positively responded to Rv0081-induced IFN- γ [2]. When alternative Rv0081-induced host markers (other than IFN- γ) were taken into consideration, the two last mentioned studies demonstrated a considerable improvement in the evaluation of the host response to Rv0081 and a number of Rv0081-induced host markers showed their diagnostic utilities in both studies. Unfortunately, Rv0081 was not available in the laboratory to validate these results in short term assay.

The short term assay confirmed our previous observations and additionally confirmed the suitability of these antigens for use as diagnostic candidates as they elicit the production of multiple host markers. In addition to confirming our previously published observations, we show for the first time the diagnostic utility of a number of antigen-induced host markers listed in chapter IV. These stimulated markers hold promises for the development of a rapid test although they will require a second visit at the clinic due to the required culture step. A two-visit test may appear at odds with the term “rapid test” and the development of such a test could be discouraged by many researchers. It is worthwhile to notice that the proposed test mainly targets resource limited areas where sputum microscopy is the only available test for TB. The test protocol will have very limited technical, laboratory and financial requirement as the clinician will only need to draw blood into a provided tube and read the results after 24 hours on a hand-held device. Results could directly be send through SMS to the patient (as this technology is available even in resource limited areas) saving on travel. It may also be worthwhile to report that, according to the World Health Organisation, around 60% of people developing active TB live in areas where sputum microscopy could not be routinely performed for financial or technical constraint [3]. The proposed test might represent a breakthrough in these areas.

The proposed test will be more beneficial with unstimulated host markers as results might be delivered within 30 minutes after a blood draw. The present thesis has revealed the diagnostic utility of unstimulated acute phase proteins, mainly serum amyloid A (SAA). This protein could discriminate active TB from latently infected individuals in the three types of whole blood assays performed in this study (chapter IV). The discrimination utility of SAA was higher in

Quantiferon Nil supernatants. C-reactive protein (CRP) and serum amyloid P were also promising in this assay (Nil tube). The diagnostic utility of these acute phase proteins in tuberculosis was not previously assessed in our laboratory or elsewhere. The only study evaluating the diagnostic utility of acute phase proteins only concentrated on the measurement of CRP levels on HIV infected individuals [4] with moderate satisfactory results. This work has shown that SAA holds a greater diagnostic value than CRP. A combination of these two proteins in a single test might improve the accuracy of this test compared to the single marker-based test. Indeed, the inter-individual variation of immune responses toward antigens or bacteria makes a single marker-based immune test particularly challenging. Combination models of two, three or four markers may be more reliable than a single marker-based test [5][6][7]. None of the acute phase proteins or other proteins could correctly classify more than 85% of the study participants into their appropriate clinical groups. The most promising acute phase protein (SAA) with area under the receiver operator characteristics curve (AUC) = 84 had 79 and 67 sensitivity and specificity respectively. A combination model of SAA, CRP and SAP may improve the sensitivity and specificity and lead to the development of an excellent rule out test.

7.3 Future studies

Results shown in this thesis are pilot results as they were obtained in studies with limited number of participants. The whole blood assays results are further investigated in a bigger study where samples were collected in many African countries (African European-Tuberculosis Consortium [AE-TBC]). Therefore properly powered future studies should include unstimulated and *M. tb* antigen stimulated multi-marker signatures for diagnostic evaluation.

The proteomic work requires more expertise to allow successful removal of abundant proteins and to develop optimal sample preparation that will allow unbiased discovery of diagnostic markers. We are currently planning follow-on experiments with the “Centre for Proteomic and Genomic Research” (CPGR) to help with the optimisation of our sample preparation and fractionation protocol. This may lead to the simultaneous evaluation of more than one thousand

proteins using the most advanced mass spectrometry instruments which include the Orbitrap Velos and Q-Exactive.

7.4 References

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