Evaluation of the potential of *Mycobacterium tuberculosis* antigen-specific host biomarkers detected in QuantiFERON® TB GOLD Plus supernatants in the diagnosis of TB disease

by

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology in the Faculty of Medicine and Health Sciences at Stellenbosch University

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December 2018

Declaration

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M. P. Manngo December 2018

Abstract

BACKGROUND

The diagnosis of tuberculosis (TB) disease remains a challenge. This is mainly due to limitations with the current TB diagnostic tests including unavailability of rapid point-of-care tests. New TB diagnostic tests are therefore urgently needed. The QuantiFERON-TB® Gold (QFT) Plus test is a recently introduced test for the diagnosis of *M. tb* infection, and disease in some patient groups. As this is a relatively new test which is currently in use worldwide, it is important that its performance be evaluated, especially in high TB burden settings. Furthermore, it is not known whether measurement of host markers other than Interferon-gamma in culture supernatants of individuals with active TB or other respiratory diseases (ORD), has potential in the diagnosis of TB disease.

OBJECTIVES

1) To evaluate the usefulness of the QFT Plus test in the diagnosis of TB disease, and assess the utility of the test, when used in combination with symptoms, as a tool for diagnosis of TB disease in people suspected of having active TB in a high burden setting.
2) To evaluate alternative host biomarkers detected in QFT Plus supernatants, other than IFN-γ as biosignatures for the diagnosis of active TB

METHODS

We recruited 120 participants presenting at a primary health care clinics in Cape Town, South Africa with symptoms requiring investigation for TB disease. These participants formed part of a larger ongoing biomarker project known as the 'ScreenTB' study. Participants were later classified as TB or ORD based on the results of clinical and laboratory tests. After performing the standard QFT Plus test in study participants, the concentrations of 37 host biomarkers were evaluated in culture supernatants using a multiplex immunoassay.

RESULTS

Out of 120 individuals included in the study, 35 (29.2%) were diagnosed with active TB and were culture positive. The QFT Plus test diagnosed TB disease in all study participants with sensitivity and specificity >70%. A combination of symptoms including cough, fever and weight loss diagnosed TB disease with sensitivity and specificity >70% with an area under the receiver operator characteristics curve of 0.81. Multiple host biomarkers detected in the unstimulated and antigen-stimulated QFT Plus tubes showed potential as diagnostic markers for TB. Individual markers which diagnosed TB disease with sensitivities and specificities >60% included ITAC-1, IL-3, I-309, MIG, and EGF, P-selectin. Combinations between host biomarkers showed potential in the diagnosis of TB disease with a six-marker biosignature derived from unstimulated supernatants (APO-CII, ITAC-1, MIG, MCP-2, I-309, and NCAM-1) diagnosing TB disease with a sensitivity and specificity >78%, a four-marker TB1 and TB2 antigen-specific biosignature (TNFα, LIGHT, MIG and P-selectin) which diagnosed TB disease with sensitivity and specificity >73%, after leave-one-out cross validation.

CONCLUSION

The sensitivity of the QFT Plus test for active TB was inferior to the published >80% mentioned in the package insert by the manufacturer. Host biomarkers detected in QFT Plus supernatants showed potential in the diagnosis of active TB disease. Further validation studies are needed before such markers may be considered as candidate biomarkers for a blood-based diagnostic test for active TB.

Abstrakte

AGTERGROND

Die diagnose van tuberkulose (TB siekte) bly 'n uitdaging. Dit is hoofsaaklik te wyte aan beperkinge met die huidige TB diagnostiese toetse, insluitend die beskikbaarheid van vinnige punt van soegtoetse. Nuwe TB diagnostiese toetse is dus dringend nodig. Die QuantiFERON-TB® Goud (QFT) Plus toets is 'n onlangs ontwikkelde toets vir die diagnose van *M. tb* infeksie en tuberkulose in sommige pasiëntgroepe. Aangesien dit 'n relatief nuwe toets is wat tans wêreldwyd gebruik word, is dit belangrik dat die prestasie vd toets geëvalueer word, veral in hoë TB-lasinsweld dtrelie tellings. Verder is dit nie bekend of meting van gasheermerkers behalwe Interferon-gamma in kweek supernatante van individue met aktiewe TB of ander respiratoriese siektes (ORD), potensiaal het in die diagnose van TB-siekte.

DOELWITTE

1) Om die nut van die QFT Plus-toets in die diagnose van TB-siekte te evalueer, wanneer dit in kombinasie met simptome gebruik word, as 'n instrument vir die diagnose van TB-siekte by mense wat vermoed word dat hulle aktiewe TB heit in 'n hoë lastrehe.

2) Om alternatiewe gasheerbiomerkers wat in QFT Plus supernatante aangetref word, te evalueer, anders as IFN-γ as biomeker vir die diagnose van aktiewe TB

METODES

Ons het 120 deelnemers gewerf by 'n primêre gesondheidsorgkliniek in Kaapstad, Suid-Afrika, met simptome wat ondersoek na TB-siekte vereis. Hierdie deelnemers het deel gevorm van 'n groter voortgesette biomerkerprojek wat bekend staan as die 'ScreenTB'-studie. Deelnemers is geklassifiseer as TB of ORD gebaseer op die resultate van kliniese en laboratoriumtoetse. Nadat die standaard QFT Plus-toets in studie-deelnemers uitgevoer is, is die konsentrasies van 37 gasheerbiomerkers geëvalueer in kweek supernatante met behulp van 'n veelvuldige imuuufoetse.

RESULTATE

Uit 120 individue wat in die studie ingesluit is, is 35 (29,2%) met aktiewe TB gediagnoseer en was kultuur positief. Die QFT Plus-toets het TB-siektes in alle studie-deelnemers met 'n sensitiwiteit en spesifisiteit van> 70% gediagnoseer. 'n Kombinasie van simptome soos hoes, koors en gewigsverlies diagnoseer TB siekte met sensitiwiteit en spesifisiteit> 70% met 'n gebied onder die ontvanger operateur eienskappe kurwe van 0.81. Veelvuldige gasheerbiomerkers wat in die ongestimuleerde en antigeen-gestimuleerde QFT Plus-buise opgespoor is, het potensiaal as diagnostiese merkers vir TB vertoon. Individuele merkers wat TB-siekte gediagnoseer het met sensitiwiteit en spesifieke eienskappe> 60% sluit in ITAC-1, IL-3, I-309, MIG en EGF, P-selektien. Kombinasies tussen gasheerbiomarkers het potensiaal getoon in die diagnose van TB-siekte. Ses biomakers van ongestimuleerde supernatante (APO-CII, ITAC-1, MIG, MCP-2, I-309, en NCAM-1) het 'n sensitiwiteit en spesifisiteit getoon van >78%. Die vier-biomeker TB1 en TB2 antigenspesifieke kombinasie (TNFa, LIG, MIG en P-selektien) het TB slette gecliagnoseer met 'n sensitiwiteit en spesifisileit >73% na verlof-een-uit kruis validasie.

AFSLUITING

Die sensitiwiteit van die QFT Plus-toets vir aktiewe TB was nie soos die gepubliseerde> 80% wat in die pakketstuk deur die vervaardiger genoem word nie. Gasheer biomerkers wat in QFT Plus supernatante aangetoon is, het potensiaal getoon in die diagnose van aktiewe TB-siekte. Verdere valideringstudies is nodig voordat sulke merkers as kandidaat-biomerkers beskou kan word vir 'n bloedgebaseerde diagnostiese toets vir aktiewe TB.

Acknowledgment

First and above all, I would like to thank God for giving me the opportunity, ability, and strength to undertake my research work with perseverance and complete it sufficiently.

I would like to express my deep and sincere gratitude to my supervisor Dr Novel Chegou for his motivation, patience, enthusiasm as well as making critical suggestions and posing challenging questions. His guidance helped me throughout my research and writing this thesis. Although it was not easy, his invaluable guidance, constant encouragement and healthy criticism added considerably to my experience. I could not have imagined having a better supervisor for my master's degree.

To the head of the department of Human genetics and molecular biology and Immunology research group, Prof Walzl, thank you for giving me the opportunity to further my career under your leadership, thank you for all the contributions that you have made in my Msc research.

I would like to thank my Co-supervisor, Mrs Andrea Gutschmidt for always being there when I needed her, all the trainings, and reviewing my thesis, I wouldn't have achieved this without you. I will be forever grateful.

This journey would not have been possible without the support of my family. A special thanks to my family and friends, thank you for always being there during tough times and encouraging me to follow my dreams and for always believing in me even when I couldn't believe in myself. Thank you to my mother and grandma, Eunice and Anna, I wouldn't have done this without your constant support and unconditional love.

I would also like to thank all the experts, including clinicians, biostatisticians' research assistants, who were involved in my Msc research.

To biomarker students, thank you for always being there when I needed you, thank you for all your help with my presentations and lab work.

To the National Research Foundation (NRF) and the South African Medical Research Council for their financial assistance.

Lastly, to Hygon, Charles and Candice, thank you all your help with my lab experiment, I am grateful. To Masters Office, thanks for always making me laugh even when I was sad and being there all the time.

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List of abbreviations

%	Percentage
°C	Degree Celsius
µL	microliter
AFB	Acid fast bacilli
AIDS	Acquired immune deficiency syndrome
AUC	Area under the curve
APC	Antigen presenting cells
ADAMTS	A disintegrin and metalloproteinase with a thrombospondin type 1 motif
Ag	Antigen
Ab	Antibody
Apo	Apolipoprotein
BCG	Baccilus-Calmette Guerin
BDNF	Brain derived neurotrophic factor
Bref A	Brefeldin A
CO2	Carbon dioxide
CD	Cluster for differentiation
CDC	Centre of disease control and prevention
CF1	Complement factor 1
CI	Confidence interval
CR1	Complement receptor 1
CR2	Complement receptor 2
CT	Computed tomography

CV	Coefficient of variation
CXC	Cysteine X Cysteine
CXR	Chest X-ray
CFH	Compliment factor H
CFP10	Culture filtrate protein 1
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulphoxide
EDTA	Ethylenediaminetetraacetic acid
EGF	Endothelial growth factor
ESAT6	Early secretory antigenic target 6
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
FBS	Fetal bovine serum
FDA	Food and drug administration
GDA	General Discriminant analysis
GDNF	Glial–cell derived neurotropic factor
GM-CSF	Granulocyte monocyte colony stimulating factor
HIV	Human immune virus
HB	Haemoglobin
IU	International unit
IntCtrl	Internal control
IFN-γ	Interferon gamma
INH	Isoniazid
ITAC-1	Interferon inducible T-cell alpha chemoattractant

IP	Inducible protein
IGRAs	Interferon gamma release assay
IL	Interleukin
LTBI	Latent tuberculosis infection
LED	Light emitting diode
NAAT	Nucleic acid amplification tests
NPV	Negative predictive value
MBL	Mannose binding lectin
MIT	Mitogen
MGIT	Mycobacteria Growth Inhibitor Tube
M. tb	Mycobacterium tuberculosis
MIP	Macrophage inflammatory protein
MIG	Macrophage inflammatory protein
MCF	Monocyte chemotactic factor
MCP	Macrophage chemotactic protein
MTD	Mycobacterium direct test
ml	Millilitre
Nil	Unstimulated
NCAM-1	Neural cell adhesion molecule-1
NK	Natural killer cells
NTM	Non tuberculous mycobacteria
OD	Optical density
ORD	Other respiratory disease
PAI 1	Plasminogen activator inhibitor

PCR	Polymerase chain reaction
PE	Phycoerythrin
PET	Positron emission tomography
PET-CT	Positron emission tomography-computed tomography
PBS	Phosphate buffed saline
PDGF	Platelet derived growth factor
PGE	Prostaglandin
PMT	Phycoerythrin
PPD	Purified protein derivative
PPV	Positive predictive value
PBMC	Peripheral blood mononuclear cells
QFT	QuantiFERON
RANTES	Regulated as a normal T cell expressed and secreted
RNA	Ribonucleic acid
RIF	Rifampicin
RD1	Region of difference 1
ROCK	Receiver operator characteristic curve
rRNA	Ribosomal RNA
sICAM	Soluble intracellular adhesion molecule
sVCAM	Soluble vascular adhesion molecule
Sun-IRG	Stellenbosch University Immunology Research Group
STARD	Standards for Reporting of Diagnostic Accuracy Studies
TAM-TB	T-cell activation marker tuberculosis
TB	Tuberculosis

TCR	T-cell receptor
Th	T-helper
TIA	T-cell restricted intracellular antigen-1
TFG	Transforming growth factor
TNF	Tumour necrosis factor
TST	Tuberculin skin test
TTP	Target product profiles
VEGF	Vascular endothelial growth factor
WHB	Whole blood
WHO	World health organisation
US	Unites states

Chapter 1

Literature review

1.1 Introduction

Mycobacterium tuberculosis (M. tb) is an aerobic pathogenic bacterium that causes tuberculosis (TB) (1). *M. tb,* together with nine other mycobacterium species (*M. africanam, M. bovis, M. caprae, M. microti, M.pinnipedii, M.africanum, M. mungi, M. orygis and M. tuberculosis sensi stricto*) belongs to the family of *Mycobactericeae* (2). *M. tb* can appear as either Gram positive or Gram negative due to the presence of mycolic acids on its cell wall. Its lipids are the key virulence factors which also helps it to survive in a dry state for weeks. It can be identified with a microscope by using acid fast stains such as Zeihl-Neelsen, or fluorescent stains such as auramine (3).

M. tb is a pathogen of the mammalian respiratory system and its biology requires high levels of oxygen because it is highly aerobic (4). Lungs are the main body organs which are affected by TB (pulmonary TB), however other body parts can also be affected (extra-pulmonary TB) (1). *M. tb* is known to divide every 15 to 20 hours which is very slow when compared to other bacteria (5). TB is spread through air droplets containing bacilli which originate from a person with pulmonary active TB by either speaking, coughing, singing or sneezing. These droplets range from 0.5 to 5.0 µm in diameter and a single sneeze is capable of releasing up to 40 000 droplets. HIV amongst other TB comorbidities (diabetes and nutrition, tobacco smoking and harmful use of alcohol) is the main risk factor for developing active TB disease in people who are latently infected with *M. tb*. However, TB is also a main leading course of death in people living with HIV infection. People living with HIV infection have weak immune systems which are therefore favourable conditions for opportunistic bacteria such as *M. tb* to progress. Since TB disease depends on cell mediated immune response with CD4+ T cells being the main lymphocytes involved, people who have HIV already have low CD4 T cell counts. This therefore means that the immune system is unable to fight against two diseases at the same time, frequently resulting in death.

1.2 Tuberculosis epidemiology

The World Health Organisation (WHO) reported that TB is the ninth leading cause of death globally, and ranks above HIV/AIDS (6). The TB mortality rate has fallen globally at around 3% annually and the global incidence rate at around 2% annually. Although the mortality and incidence has decreased, WHO estimates reveal that the decline is not sufficient, and that the TB incidence rate should decrease from 4 to 5% annually by 2020, for us to meet the goals of the End TB strategy. It was estimated that 10million people fell ill with TB, and a total of 1.3 million died as a result of the disease in 2017 (Figure 1.1). Among the overall estimated 1.7 billion, 90% were adults, 64% male, and 9% (400 000) were living with HIV (figure 1.2). WHO also reported that South-East Asia and Africa regions had the most TB incidences with 45% and 25% respectively (6).

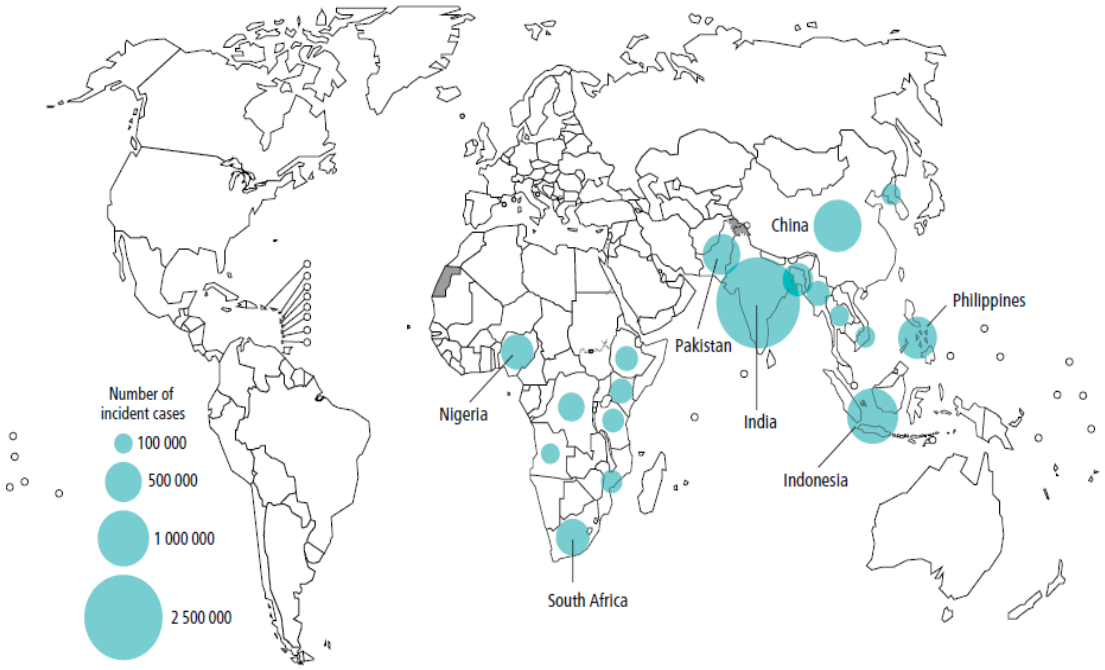


Figure 1.1: Estimated TB incidence in 2017 in countries with at least 100 000 incident cases. Source: WHO Global TB Report, 2018 (6).

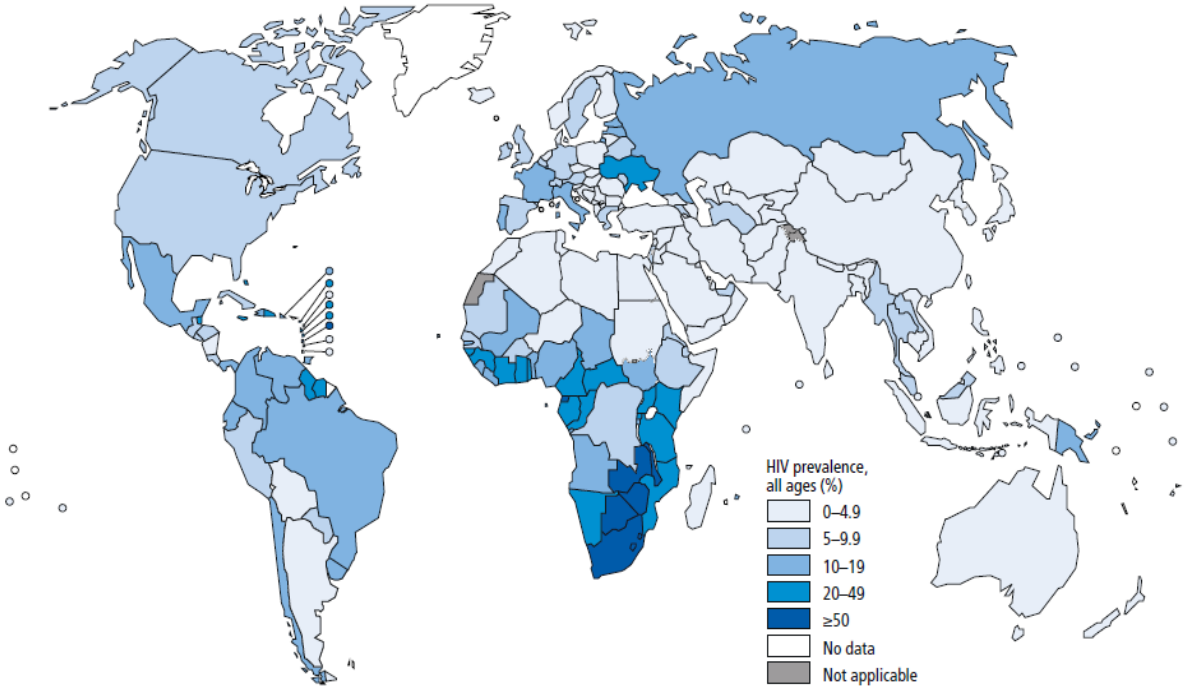


Figure 1.2: Estimated HIV prevalence in new and relapse TB cases in 2017. Source: WHO Global TB Report, 2018 (6).

1.3 Basic immunological principles relevant to the immune response against infectious diseases

Immunology refers to the body's immune defence against microorganisms capable of causing diseases as well as immune disorders. When the human body is exposed to different microorganisms, the immune system is activated in order to fight against these microorganisms, with the purpose of eliminating them. The immune system consists of a network of immune cells, tissues and organs which work together and mediate the immune response to protect the body (7). However, white blood cells (also called leukocytes) are the main players of the body's immune response against disease causing organisms. These cells are found or stored in different locations such as the bone marrow, thymus and spleen. In order for the immune system to work in a coordinated manner, these leukocytes circulate within the nodes and organs throughout the body via blood and lymphatic vessels (8). Phagocytes and lymphocytes are the two types of leukocytes which are known to digest invading organisms and enables the body to remember and recognize the past invaders and also helps the

immune system to eliminate them. The immune protection against disease is mainly mediated by natural (innate) and adaptive (9).

1.3.1 Innate immunity

The innate immunity plays an important role during infection as a non-specific defence mechanism, which happens immediately or as a first line of defence against invaders in the body (10). This type of immunity makes use of various mechanisms including external surfaces of the body such as physical barriers (the skin and mucus membranes), chemical barriers found in the blood, as well as the other immune cells which prevent invaders or organisms from entering the body. Natural immunity also recruits immune cells (neutrophils, mast cells, dendritic cells macrophages, basophiles, eosinophils and natural killer cells) to the site of infection wherein they produce chemical factors and chemical mediators such as cytokines (11). It is responsible for activating the complement system which identifies and removes bacteria or any foreign substances. The innate immune system also plays the important role of activating the adaptive immune system through presentation of antigens from innate cells to the adaptive cells (11).

1.3.2 Adaptive immunity

The adaptive immune response makes use of more specialised groups of cells in order to fight against foreign substances or bacteria and to also prevent or eliminate them from re-occurring through immunological memory (11). This type of immune response consists of cell mediated immune responses and antibody responses which are mediated by B cells and T cells. In antibody immune response, B cells are transformed to plasma cells which secrete antibodies (Abs) which circulate through the blood and lymph where they bind to specific foreign antigens (Ags) to inactivate microbial toxins and prevent them from attaching to the host cell receptors (12). During cell mediated immune response, macrophages, lymphocytes, antigen specific cytotoxic T lymphocytes are activated and release cytokines to fight against specific antigens. For

this thesis, we will focus on cell mediated immune responses which will involve the importance of T lymphocytes during *M. tb* infection (2).

1.4 Immunology of tuberculosis

Following the inhalation of *M. tb* bacilli into the lungs. The bacilli can undergo important fates were the bacilli can eliminate all bacilli such that the host never develops TB in the future, or the organism can grow and divide just after infection resulting in a clinical disease referred to as clinical infection, the bacilli can remain dormant and do not cause TB disease or the dormant/latent bacilli eventually begins to grow which will therefore result in clinical disease referred to as tuberculosis reactivation (13). Although *M. tb* bacilli remain dormant in most of the hosts, studies have shown that in all individuals with latent tuberculosis infection (LTBI) (discussed in detail below), about 3-15% develop active TB disease in their lifetime (14). Furthermore, other studies showed that reactivation of active TB can be as low as 1% over a period of 7 years (15). Additionally, individuals with compromised immune systems such as children and HIV infected individuals have around 7% chance of developing active TB disease every year post LTBI (16).

It is believed that, once *M. tb* reaches the host's lower respiratory tract, the initial host defence is mediated by alveolar macrophages which inhibit *M. tb* bacilli growth through phagocytosis. Briefly, during the process of phagocytosis, macrophages binds to *M. tb* bacilli and internalize them followed by killing of the bacteria. The complement system plays an important role during the process of phagocytosis. Experimental evidence shows that during phagocytosis process, the creation of a phagosome is followed by binding of *M. tb* to the phagocyte via complement receptors (CR1, CR2, and CR4), mannose binding receptors as well as other receptors at the cell surface (17). Prostaglandin E2 (PGE2), IL-4 and IFN-γ are some of the mediators which are activated by macrophages expressing mannose and complement receptors. PGE and cytokine receptors are known to be involved in upregulation of mannose and complement receptors and interferon gamma (IFN-γ) has been shown to have an effect on decreasing receptor function and receptor expression which therefore leads to inability of *M. tb* to adhere to the macrophage (18, 19). However mycobacterial

inhibition also involves other immune cells which help macrophages to control *M. tb* growth (20). Activated macrophages recruit and stimulate T lymphocytes during cell mediated immunity which then inhibits microbial growth (21). Although known to ingest macrophages which have engulfed *M. tb* bacilli, they can also produce small proteins such as T-cell restricted intracellular antigen-1 (TIA-1) which is a molecule found in the cytoplasm and has been demonstrated to induce apoptosis (22). Furthermore, macrophages also interact with other effector cells with cytokines and chemokines in the background. The role of these molecules is to attract and activate other inflammatory effector cells. Interleukin 8 (IL-8) is a vital chemokine from the CXC family, which is involved in mycobacterial host pathogen interaction. Its main role is to recruit neutrophils, T lymphocytes, and basophils in response to *M. tb*. IFN-γ and transforming growth factor-beta (TGF-β) are the cytokines which many researchers have been giving attention to because of their ability to activate and also deactivate the ability of macrophage to inhibit *M. tb* growth respectively. Using variety of animal and *in vitro* experiments, IFN-γ has been demonstrated to play an essential role in host defence against *M. tb*. Several studies investigated the role of IFN-γ in the control of *M. tb* including a study conducted by Holland and colleagues where they found the beneficial effect of IFN-γ, when they treated a group of patients suffering from a systemic infection caused by non-tuberculous mycobacteria (NTM) and *M. avium* using systemically administered IFN-γ (23). Furthermore, another study by Jaffe and colleagues showed that macrophages can be activated by aerosol IFN-γ which was given to normal human subjects (24). Other cytokines such as interleukin (IL)-1α/β, IL-6, have been shown to be involved in host defence against *M. tb* as well as TNF-α which has been shown to play a vital role in TB disease by controlling *M. tb* infection and is also known to play a role in maintaining granulomas (25). Inducible protein 10 (IP-10) and monocyte chemotactic factor (MCF) also fall under the CXC family of chemokines. Macrophage chemotactic protein (MCP-1) chemokine and a regulated on activation chemokine known as a normal T cell expressed and secreted (RANTES), have been shown to oppose the expression of IL-8 during TB treatment phase meaning that when MCP-1 and RANTES decreases IL-8 increases (26).

1.5 Role of T lymphocytes during host defence against mycobacteria

The adaptive immune response to *M. tb* is detectable from three to eight weeks after infection wherein, CD4+ T cells are known to play an important role as they are primarily involved in immune response against *M. tb* infection. However, CD8+ T cells have also been shown to play an important role in response to *M. tb* infection. Professional antigen presenting cells (APC) are cells whose functions are to process antigen proteins after which break them down into peptides and then present them in association with major histocompatibility complex (MHC) on their cell surface where the peptides can be recognised by T cells. While CD8+ T cells recognise *M. tb* antigens which have been presented by antigen presenting cells (APCs) on their cell surfaces from the cytosol through class I MHC molecules, through expression of α and β T cell receptors (TCRs), CD4+ T cells recognise antigens presented through MHC class II (processed in the phagosome) on the surface of APCs (27). CD4+ cells are known to be involved in host immune response amplification through recruiting more immune cells to the infection site as well as activation of effector cells. At the same time, CD8+ cells are known to have a cytotoxic effect to the targeted cell. The Th1 and Th2 cells are phenotypic classes of CD4+ T helper cells which are driven from Th0 cells. The differentiation of these cells is known to be controlled by different cytokines including IL-12 (28, 29, and 30). While Th2 cells are known to produce IL-5, IL-4 and IL-10 cytokines and recruiting eosinophils to the site of infection, Th1 cells secrete IFN-γ and IL-2, which activate inflammatory and phagocytic cells which are more likely to inhibit *M. tb* growth. However, both Th1 and Th2 may also secrete common cytokines such are granulocyte –macrophage colony stimulating factor (GM-CSF) and IL-3.

In the process of antigen driven differentiation, studies have demonstrated that both CD8+ and CD4+ T cells secrete more than one cytokine. These types of T cells are referred to as polyfunctional T cells. In order for these polyfunctional T cells to be characteristic and desirable, studies suggest that they must be tri-functionally secreting IL-2, IFN-γ and tumor necrosis factor (TNF) which therefore indicates their ability to proliferate and being effective (31). Several studies suggests that in LTBI individuals, trifunctional IL-2+ TNF+ IFN-γ+ *M. tb* specific CD2+ T cells are exhibited in higher frequencies whereas greater frequencies of *M. tb* CD4+ T cells are

associated with mono or bi functional TNF+ or TNF+IFN-γ in active TB disease (32-45).

1.6 Spectrum of tuberculosis

During *M. tb* infection, both latent and replicating bacilli are simultaneously present (46). Recent studies suggest that depending on the clinical status (57), adequate containment and progression of bacilli to replicate actively resulting in TB disease may be understood by dynamic pathology spectrum of sterile tissue, solid caseous and necrotic hypoxic lesions which contains unstable numbers of replicating bacilli can be detected during active TB (47). With the use of computer tomography and positron emission tomography imaging, the imaging results indicates that although these heterogeneous lesions coexist simultaneously, they represent various bacilli subpopulations in various microenvironments (47). However, studies have shown that the same diversity of TB lesions can also be found in LTBI cases which therefore suggests that LTBI can be described as a broad spectrum condition overlapping with conditions seen in active TB (47, 48). Animal studies have also confirmed that LTBI spectrum conditions vary wherein some subjects show a slowly progressing form of disease whereas others only show residual infection warning. In the same animal model, *M. tb* replication rate was found to be the same between LTBI and active TB which therefore suggests that instead of being in a non-replicating state, it actually replicates actively (49,50).

After primary infection followed by control of *M. tb* replication through adaptive immunity, *M. tb* may reside in various tissues in a dormant state wherein it intensifies its resistance through antimicrobial activities of host immune response (51). When the conditions are favourable, the dormant bacteria revives and then initiate active replication. However, the replicating bacilli are more likely to be killed in immunocompetent hosts which leaves the dormant bacilli predominating. This state is referred to as primary disease (52). When the host immune system is unable to control the bacilli which is metabolically active, the bacilli becomes activated and replicates which leads to secondary active TB disease. Although studies suggest that the capacity of the host immune response to clear the infection is dependent on bacterial

load, the immunological actions for this clearance is still confusing and the research is still ongoing. Transient infection is defined as the ability of the innate or adaptive immune response to prevent and clear *M. tb* infection instantly by killing of the bacilli (53). The clearance of *M. tb* infection has been shown in studies conducted for evaluation of the role of T cell adaptive immune response in clearance of *M. tb*, these studies indicate that individuals with interferon gamma release assay (IGRA) positive tests can regress to QFT negative test later and still test positive with the tuberculin skin test (TST) (53). Even though the understanding of the mechanisms involved during host immune response to *M. tb* is limited, studies suggest that enduring T cell immune responses may possibly be responsible for controlling the bacterial replication as well as prevention of disease reactivation and progression.

According to some scholars (75,76), the immune response against *M. tb* infection can be summarised under four major response spectra, based on the host immune reaction to the organism, termed the innate immune response phase, the acquired immune response phase, the quiescent infection phase, active infection phase and then clinical disease. During innate immune response phase, macrophages residing at the alveoli ingest and often destroy the *M. tb* bacilli resulting in controlled infection with some non-replicating dormant bacteria. After 2-3 weeks post-infection, acquired T-cell immune response develops wherein antigen specific T cells are recruited and proliferate within the lesions after which they activate macrophages which eventually kills the intracellular *M. tb.* In quiescent infection phase, the *M.tb* stops growing and the bacteria is controlled in a non-replicating dormant state. Lastly, the disease may progress to active infection with the immune system maintaining the bacterial replication at a subclinical level (46). The four major response spectra proposed for *M. tb* infection are illustrated in figure 1.3.

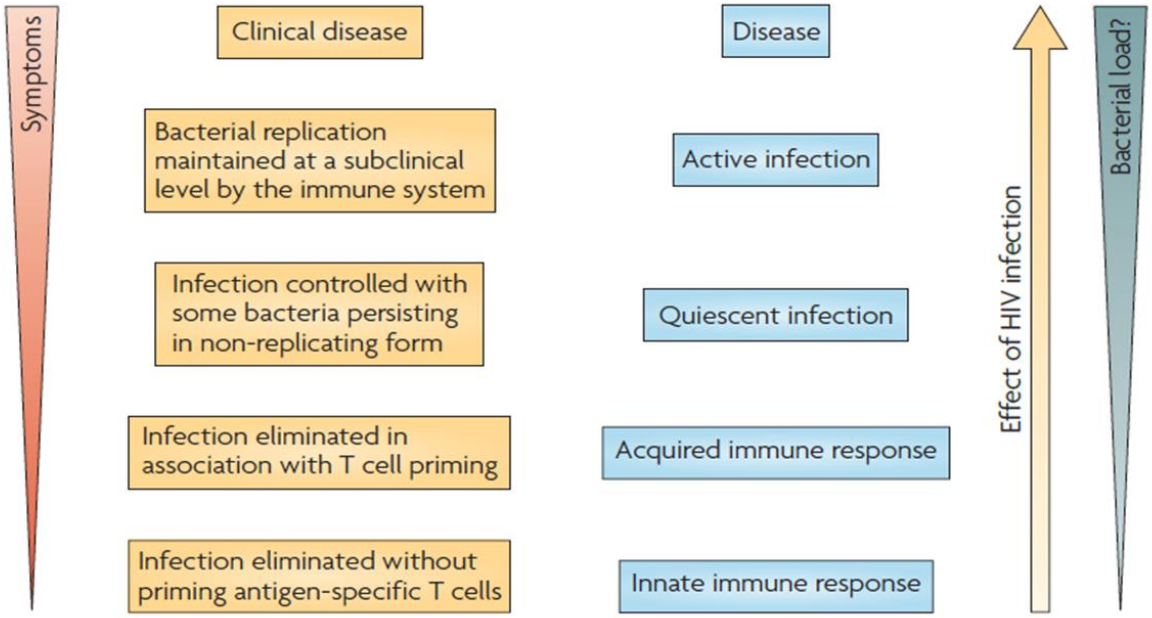


Figure 1.3*:* Spectrum of tuberculosis infection. Source: Barry et al, 2009 (47).

1.7 Active TB versus latent TB (LTBI)

Latent TB infection is the *M. tb* infection phase that is characterised by the persistence of the bacterium in the host. People with LTBI do not have any signs or symptoms of TB and they feel well and healthy. These people are not infectious and therefore cannot spread the *M. tb* to other people. About one third of the world's population (about 2 billion people) is estimated to be infected with *M. tb* (LTBI). Only about 5 to 15% of people with LTBI are believed to progress to active TB if untreated in their lifetime (75, 76, and 77). The factors that influence the progression from LTBI to active TB disease include HIV infection, smoking, alcohol and indoor air population. As there are no clinical signs or symptoms suggestive of LTBI, the term LTBI is an immunological definition, which describes the reactivity of the individual to *M. tb* antigens, thereby leading to a positive TST or IGRA test. There is no gold standard test for diagnosing LTBI as discussed further in later sections of this chapter.

1.7.1 The tuberculin skin test (TST)

The TST is the oldest still currently in use for the diagnosis of *M. tb* infection/disease. Robert Koch first defined a reaction caused by a compound known as tuberculin after preparing liquid culture with tubercle bacilli in 1990, which was followed by the development of the first tuberculin skin test in 1908 (65). This test is often used in diagnosing LTBI in countries with a low TB burden. It is however not so useful in settings with a high TB burden since almost everyone is latently infected. The TST relies on the delayed type hypersensitivity immune response which occurs when the individual taking the test is infected with *M. tb* (68, 69). It requires the injection of a purified protein derivative (PPD) intradermally in the lower part of the arm followed by reading of the amount of induration present or absent 48 to 72 hours. Although highly sensitive for *M. tb* infection, TST can produce false positive results from people who have been previously vaccinated with Baccilus-Calmette Guerin (BCG), as well as people who are also infected with other non-tuberculous mycobacteria (NTM). Furthermore, TST require two day visits to the clinic and cannot distinguish active TB from latent TB. It is still being used in high burden but resource constrained settings to guide the clinical management of *M. tb* infection/disease in special populations including children and individuals who are HIV infected. It is also used to support the diagnosis of some extra pulmonary forms of TB which are extremely challenging to diagnose including intra-ocular, spinal and tuberculous meningitis (47, 79, and 80).

1.7.2 Interferon gamma release assays (IGRAs)

IGRAs are the latest and more accurate *in vitro* T cell blood tests for diagnosing TB infection. IGRAs rely on the immune response elicited by blood cells against *M. tb* antigens including culture filtrate protein 10 (CFP10), TB7.7 and early secretary antigenic targert-6 (ESAT6) antigens (82,124). These tests, like the TST work on the principle that individuals who have previously been exposed and infected by *M. tb* harbour pre-activated T cells in circulation in their blood stream (81-83). These T cells then respond rapidly after re-challenge with *M. tb* antigens *in vitro* in the case of IGRAs, leading to the production of the cytokine IFN-γ, which is detected in culture

supernatants by an enzyme linked immunosorbent assay (ELISA) in the case of the QuantiFERON® TB Gold (QFT) (Qiagen Cellestis, Carnegie, Victoria, Australia) tests or enzyme linked immunospot assay as obtained with the T-SPOT.TB (Oxford Immunotec, Oxfordshire, Abington, UK). These tests mainly differ in the blood sample type they use and assay methods. The T-SPOT TB makes use of peripheral blood mononuclear cells (PBMCs) to detect the number of T lymphocytes producing IFN-γ using (ELISPOT) (70). Unlike T-SPOT TB, QFT uses whole blood to directly detect IFN-γ that is secreted into the culture supernatant using ELISA. Unlike TST which uses PPD antigen that is not specific for *M. tb*, the antigens used in IGRAs are coded by genes which are found at the region of difference 1 (RD1) of the *M. tb* genome. This region is deleted in most NTMs but is present in organisms belonging to the *M. tb* complex.

The first generation of the QFT test made use of PPD, just like the TST and was approved by the United States (US) food and drug administration (FDA) in 2001. Due to limitations that come with PPD and advancement in genomics, leading to the discovery of ESAT6 and CFP10, the QFT TB Gold test (done in 24 tissue culture plates) and the T SPOT TB tests were developed. Further improvements in IGRAs led to the introduction of the QFT In Tube in the mid-2000s', this version making use of a third antigen known as TB 7.7 (Rv2654) (84). The QuantiFERON® TB Gold Plus (QFT Plus) is the newest generation of the QFT test and was developed with the aim of improving sensitivity for diagnosing *M. tb* infection. Unlike the QFT In Tube which contained three tubes (nil, TB Ag and mitogen), the QFT Plus contains a second TB antigen tube (TB2) in addition to the antigen tube (TB1) which was being used in the previous QFT tests. Both the TB1 and TB2 tubes contain *M. tb*-complex associated antigen peptides from the CFP10 and ESAT 6 proteins, and not TB7.7 (71). The first antigen tube in the QFT Plus test, which originally contained peptides from three *M. tb* antigens (ESAT 6, CFP10 and TB 7.7) now only consists of peptides from CFP10 and ESAT6 (72). It is believed that the TB1 tube consists of long peptides which elicits CD4 T cell immune responses whereas the additional TB2 tube consists of both short and long peptides (also belonging to CFP10 and ESAT6) which elicit CD4 and CD8 T cell immune response (73).

IGRAs offer improved specificity over the TST and require only a day visit to the health care centre. However, despite all the advances made in the development of these

tests, they have several limitations like the TST, IGRAs cannot distinguish active TB from LTBI. Moreover, IGRAs are prone to false negative and indeterminate results in people with compromised immune systems such as HIV infected people and those with genetic immunocompromising disorders (85).

1.8 Diagnosis of active tuberculosis

TB is largely curable, but diagnosing the disease remains a major challenge worldwide. This is because of the lack of diagnostic tests which are sensitive, specific, rapid and implementable worldwide, including in resource-poor areas. (54). Many of the tests that are currently available have similar limitations including poor performance in some patient groups including people who are co-infected with HIV, people with extrapulmonary TB and young children due to either paucibacillary disease or difficulties in obtaining good quality samples, including the lack of expertise in collecting relatively invasive samples such as gastric aspirates. In this part of the current chapter, we will briefly discuss the main diagnostic tests that are routinely used in the diagnosis of active disease, followed by a brief look at relatively newer approaches (85).

1.8.1 Clinical diagnosis of TB disease

Empirically diagnosis is common in the management of TB, mostly owing to the non-availability of diagnostic tests. This is mostly done through the interrogation of symptoms and signs shown by the patient at presentation at the health care centre. TB symptoms include fever, night sweats, weight loss and cough >2 to 3 weeks, as well as lymphadenopathy. However, because TB symptoms are the same as the symptoms experience by people suffering from other conditions, empirical diagnosis often leads to over-diagnosis, which results in unnecessary chemotherapy and wastage of resources with another consequence being patients suffering with unnecessary side effects. The consequences are even grave for patients suffering from extra pulmonary TB as the symptoms will be confused with other conditions, for

example, pleural TB being mistaken for cancer or spinal TB being mistaken for other spinal anomalies.

1.8.2 Radiological diagnosis of TB disease

Chest X-rays are routinely used as a principal test for radiological assessment of suspected as well as proven TB cases. Individuals who present at the primary health clinics with symptoms such as weight loss, unexplained chronic fever and persistent cough lasting for more than 2 weeks are often evaluated for TB using a chest X-ray (54). This technique therefore allows imaging of the consolidation or infiltrates which are often found in the upper lungs with or without hilar lymphadenopathy although they can also appear at any place in the lungs (55-57). This diagnostic tool provides important information regarding follow up and management of patients which can also be useful in treatment monitoring purposes. Although useful, chest X-rays are not specific for pulmonary TB diagnosis since they may look normal when disease is actually present (58, 59). In addition to chest X-ray, computed tomography (CT) is also used to define unclear lesions as well as detecting fine lesions which may have been missed during chest X-ray visualisation (58, 60). Moreover, when plain films are inconclusive or normal, chest CT is considered as a useful diagnostic method which provides important information on how to manage the ill health. This test can therefore provide useful leads in detecting bacterial activity. The main limitation of the use of chest X-rays is that radiological facilities are not available at lower levels of the health care system. In many countries, e.g., most of Sub-Saharan Africa, patients have to travel for long distances in order to access X-ray facilities. X-rays are also not readily affordable, which is another limitation. Positron emission tomography-computed tomography (PET-CT) offers an advanced nuclear medicine imaging diagnostic technique by combining PET scanner and X-ray CT scanner in a single gantry in order to acquire subsequent images obtainable from both devices at the same time allowing the combination of two devices to produce superposed image. PET-CT may be a useful tool especially as a tool for monitoring the response to TB treatment as demonstrated in the study by Malherbe et al (74). However, implementation of PET

CT in the management of TB, even at referral hospitals is not feasible due to high costs and excessive infrastructural requirements.

1.8.3 Microscopy

Smear microscopy is recommended by the WHO as a standard TB diagnostic test and is relatively rapid, simple, specific and inexpensive. Conventional light microscopy of Ziehl-Neelsen-stained smears that are prepared directly from sputum to detect acid fast bacilli (AFB) is the most widely TB diagnostic test in resource limited settings (86). The main limitation of smear microscopy is poor sensitivity as mycobacteria are required to be present in the specimen at a concentration of 5000 to 10 000 organisms per millilitre for a positive result to be obtained (87). The main sample type that is used for smear microscopy (sputum) is difficult to obtain in some patient groups especially children, whereas, sputum may be useless if TB is extrapulmonary as briefly mentioned previously. Because of the low numbers of bacilli that are present in other biological fluids, the yield from other extrapulmonary samples is often poor. Moreover, it is not possible to distinguish between live and dead bacilli using microscopy and the technique is unable to identify drug resistant *M. tb* strains (61). However, fluorescent microscopy has been shown to be 10% more sensitive than Zielh-Nelsen test (61).

1.8.4 *Mycobacterium tuberculosis* culture

Despite ongoing research and resultant improvements in diagnostic tools for TB, *M. tb* culture remains the only WHO recommended gold standard for diagnosing active TB (88). Cultures are not only used to confirm the presence of *M. tb* but also to obtain information about important drug susceptibility testing (62, 63). Culturing of *M. tb* is done either on solid media (Lowenstein-Jensen method) or liquid media, for example, as done in the mycobacterial growth indicator (MGIT) tubes (Becton Dickenson). Solid cultures are known to be very slow. In comparison, liquid cultures yield results within 2-4 weeks. However, it still takes up to 42 days before negative culture results are confirmed, owing to the slow growing nature of *M. tb, with* the time taken for results to be positive largely depending on the bacterial load in the specimen (e.g., sputum

sample). Despite the limitation of the long turn-around time, culture remains the most sensitive method for the diagnosis of TB disease, as it requires bacilli at the concentration of 10 per ml of specimen for positive results to be obtained. Other limitations of culture include prone to contamination, costs and the requirement of biosafety level 3 environment and highly skilled staff. Because of these limitations, culture facilities are not widely available, with some countries only having a single laboratory that is capable of doing cultures, with most of these laboratories being owned by international organisations such as the Pasteur Institute, Biemerieux amongst others (64). As with smear microscopy, the reliance on a good quality sputum sample is a limitation, meaning that patient groups that cannot provide samples for microscopy as highlighted in the previous section will not also be able to provide samples for culture.

1.8.5 Nucleic acid amplification and molecular beacon-based tests

Nucleic acid amplification tests (NAATs) allow the detection of *M. tb* DNA using PCR or transcription mediated amplification. These heterogeneous tests differ in terms of accuracy and the nucleic acid sequence detected. The most commonly available tests are Amplicor *Mycobacterium tuberculosis* test (Roche diagnostics) which amplifies 19s ribosomal ribonucleic (RNA) gene region and mycobacterium direct test (MTD, Gen-Probe) which is based on reverse transcription *M. tb*-specific ribosomal ribonucleic acid (rRNA) targets. Furthermore, these tests have been studied and found to be more accurate when performed using respiratory samples instead of other specimens (65). NAATs are mostly used for diagnosis of TB on clinical specimens such as cerebrospinal fluid (CSF), sputum and lymph node aspirates, they are also useful as confirmatory TB testing by rapidly detecting *M. tb* in 50-80% of AFB smear negative and culture positive specimens. Furthermore, NAATs are also intended for diagnosis of drug resistance as a follow-up to culture positive results. Although these tests provide high sensitivity and specificity, yield results within approximately 3 hours, these tests are prone to false positive results since they do not detect viable bacteria which rule them out from monitoring TB treatment, they are also expensive and are not available in all settings.

One of the most important recent advancements in the field of TB diagnostics has been the development of the molecular beacon-based assay; the GeneXpert MTB/RIF test (Cepheid, Sunnyvale, USA). In addition to detecting *M. tb* DNA in a closed cartridge based system, the test also detects resistance to rifampicin which provide an indication for multidrug resistance, and yields results within two hours (66). Recent systematic review and meta-analysis studies showed that the sensitivity of the Xpert test is 94.4% with a pooled specificity of 98.3%. Recently, a new version of the test (the GeneXpert Ultra) was introduced. While the detection threshold of the standard Xpert tests is about 100 bacilli per millilitre of sample, Xpert Ultra reportedly has a higher sensitivity as it is reportedly capable of detecting bacilli with the same sensitivity as culture (89-90, 96). However, this test is highly expensive and its maintenance also costly (87, 91).

1.8.6 Immunological diagnostic tests for active TB

In addition to the TST and IGRAs, which are mainly *M. tb* infection diagnostic tests as discussed above, serological tests were the only alternative immunological tests that were used for the diagnosis of active TB. Although, such tests have been used for a long time (92, 93) the WHO published a negative recommendation, banning the use of all the then commercially available serological tests for the diagnosis of TB (94). Despite this ban, much research has been ongoing on the development of newer and improved versions of the tests since serological tests are rapid, relatively cheap and easily implemented as point-of-care diagnostic tests. In recent studies, making use of recently characterised *M. tb* antigens and investigating multiple classes of antibodies against these antigens, results have been very promising (95). These raise the hope for the future development of newer versions of serological tests, but although there are companies currently manufacturing such tests e. g Lionex Diagnostics and Therapeutics, Braunsweig, Germany, WHO approved commercially available serological tests do not currently exist (97). Other immunological diagnostic approaches have been investigated solely for research purposes (as discussed below) with no commercial tests currently available.

1.9 The use of host immunological biomarkers in the diagnosis of TB

In search of tools that will enable the earlier and more rapid diagnosis of TB disease, in all study participants including people living in resource-poor communities, scientists have been looking at immunological host biomarker-based approaches as possible candidate tools for the diagnosis of TB disease. According to the WHO target product profiles (TPP) published in 2014 (97), two of the four key diagnostic tests that are needed for combatting TB in the sustainable development goals era are a non-sputum biomarker based test that is capable of diagnosing TB disease including pulmonary and extrapulmonary TB, in all patient types including children, and a non-sputum triage test that is capable of being implemented for the diagnosis of TB disease at point-of-care in community health centres (97). Immunological tests are particularly very attractive and likely to fulfil these criteria because they are easily converted into point-of-care diagnostic tests, e.g., using the lateral flow technology as recently demonstrated (98, 99).

Faced with the reality that IGRAs are not useful in the diagnosis of TB in high burden settings (100) researchers including those in our research group started investigating new host biomarkers, other than IFN-γ and new antigens, other than those used in IGRAs (ESAT6 and CFP10) and which could enable the diagnosis of active TB. Studies evaluating the potential of biomarkers that are detectable in QFT supernatants revealed that multiple biomarkers detectable in QFT In Tube supernatants possessed diagnostic potential for active TB (100, 101, 102), whereas other studies showed that the use of new antigens other than ESAT6 and CFP10 (103, 104) and evaluation of biomarkers produced by these antigens (105) also showed potential in the diagnosis of TB. Given that diagnostic tests based on host biomarkers detected in overnight culture supernatants will only yield results within 24 hours, researchers have been evaluating *ex vivo* host biomarkers. Therefore studies evaluating host biomarkers detected in relatively easily collected sample types such as saliva (106, 107), urine (108) and also sputum biomarkers (109) and biomarkers detectable in other extrapulmonary fluids including pleural fluid (110,111) and cerebrospinal fluid (111) amongst others have been done. All these studies identified various biosignatures which showed potential as diagnostic tools for TB disease and investigations on some of these biosignatures are currently ongoing, in the Stellenbosch University

Immunology Research Group (SU-IRG) laboratories and other partner institutions. Concerning possible point-of-care applicability, blood-based biomarkers are currently the closest to development into diagnostic tests. Previous studies done at the SU-IRG in collaboration with partners situated in other countries identified host immunological biomarkers that are detectable in serum and plasma and which showed strong potential as a screening test for TB (negative predictive value >90% (111,112) . A point-of-care test, based on the up-converting phosphor imaging lateral flow technology (employed in (98, 99) is currently under development in a multi-institutional project involving five African countries in collaboration with European partners (www.screen-tb.eu). Host transcriptomic biosignatures have also shown potential as tools for the diagnosis of TB in both adults (115, 115) and children (116). Although, based on flow cytometry and so will probably be difficult to implement at the point of care, the T cell activation marker tuberculosis (TAM-TB) assay (118) is another recently developed test which showed potential in the diagnosis of TB disease, with further evaluations of the platform currently ongoing.

Although the biosignatures discussed in the previous paragraph have shown potential in the diagnosis of active TB, there are as yet no commercially available tests that make use of host biomarkers. Of more relevance to the work done in the current thesis is previous work that evaluated the usefulness of host biomarkers detected in QFT In Tube supernatants as diagnostic biosignatures for TB disease. One of the key such studies (102), identified 3-marker cytokine signatures in QFT supernatants which diagnosed TB disease in a case-control study with accuracy >90%. Such biosignatures were also shown to possess diagnostic potential in children (118) and adults in other studies (100, 101). Although assays based on stimulation of whole blood with TB antigens, followed by detection of host immunological biomarkers will only yield results in about 24 hours, such assays may be useful (when compared to assays making use of unprocessed, ex vivo samples) in individuals with difficult-to-diagnose TB disease such as those with extra pulmonary TB and children and they may be more specific to *M. tb*.

As discussed above, the QFT Plus is a recently introduced test for the diagnosis of *M. tb* infection. According to the manufacturer, the test has a sensitivity >95% for *M. tb* infection and is therefore more accurate than the previous generation (QFT In Tube) on which most of the studies discussed above were based. As there have been not

many independent evaluations of the test, it is imperative that the utility of the assay be evaluated in different study settings, including high burden settings such as obtained in Cape Town, South Africa. If the QFT Plus is indeed as sensitive and specific as claimed by the manufacturer, the test in combination with symptoms and signs may assist in the diagnosis of active TB. Furthermore, the host biomarkers that showed potential in QFT In Tube culture supernatants might perform better when assessed in QFT Plus supernatants. Therefore, the focus of the present thesis shall be the assessment of the accuracy of the QFT Plus test in patients that were suspected of having active TB and enrolled into a large, multi-institutional diagnostic trial (the screen-TB project), and assessment of biomarkers that previously showed potential in QFT In Tube supernatants, and recently described candidates as diagnostic tools for active TB. If promising, findings from the project will inform the planning of future larger, including studies focusing on the traditionally difficult-to-diagnose TB types such as childhood and extrapulmonary TB as mentioned in the previous paragraphs that are conducted in multiple field sites, to determine the diagnostic value of the biosignatures from the project in programmatic settings.

1.10 Study hypothesis

The QFT Plus test will be useful in the diagnosis of active TB, when used in combination with symptoms, in individuals suspected of having pulmonary TB, in comparison to a composite reference standard. Furthermore, as the test is a newer and improved version of the QFT In Tube, host biomarkers detected in supernatants from QFT Plus tubes shall be useful in the diagnosis of active TB, when compared to findings from the QFT In Tube system that are in the literature.

1.11 Study aims

1.11.1 To evaluate the usefulness of the QFT Plus test when used in combination with symptoms, as a tool for the diagnosis of TB disease in people suspected of having active TB in a high burden setting

1.11.2 To evaluate host biomarkers detected in QFT Plus supernatants as biosignatures for the diagnosis of active TB

Study specific objectives

1. To evaluate the utility for the QFT Plus test, including the use of different cut-off values in the diagnosis of active TB
2. To evaluate the usefulness of the QFT Plus test when used in combination with symptoms as a tool for the diagnosis of active TB
3. To evaluate the usefulness of host biomarkers, including analytes previously described in QFT In Tube supernatants and new host markers as diagnostic candidates for the diagnosis of active TB
4. To evaluate usefulness of combinations between host markers elicited after stimulation with QFT Plus TB1 and TB2 antigens, and unstimulated culture supernatants in the diagnosis of active TB
5. To evaluate the differential expression of host biomarkers detected in QFT Plus supernatants in patients with TB disease, individuals with LTBI and those without *M. tb* infection as these may help in understanding the biology of *M. tb* infection.

Chapter 2

Materials and methods

2.1 Study participants and setting

Participants included in the present study were recruited from primary health care clinics in urban areas of Cape Town, South Africa including Adriannse and Fisantekraal. All study participants were recruited between November 2016 and October 2017. These participants formed part of a larger ongoing biomarker project known as the 'ScreenTB' study (www.screen-tb.eu), whose main focus was the development and evaluation of a point-of-care, screening test for active TB disease in finger-prick blood samples. The main study is a collaboration between Stellenbosch University and institutions in other African institutions with the other African partners being based in Namibia, The Gambia, Uganda, Ethiopia, and the European partners being Leiden University in the Netherlands and the London School of Hygiene and Tropical Medicine in United Kingdom. Study participants enrolled in to the study were individuals presenting with signs and symptoms requiring investigation for TB disease and were recruited if they met the study's inclusion criteria. Recruitment of study participants for the main ScreenTB project is still ongoing.

2.1.1 Inclusion criteria

To be eligible for inclusion in the study, the study participants had to be willing to give written informed consent and undergo HIV testing or disclose their HIV positive status to the study nurses. Participants had to be between the ages of 18 and 70 years, and have been coughing for more than two weeks in addition to having at least one other TB suggestive symptom or sign such as fever, weight loss, haemoptysis, malaise, night sweats, chest pain or loss of appetite.

2.1.2 Exclusion criteria

Participants were excluded from the study if:

- They were on TB treatment or had received treatment in the previous 90 days.
- They were HIV positive and on isoniazid (INH) prophylaxis.
- They had no permanent address and had not been living in the community where the study was being conducted for up to 3 months.
- They were pregnant or breast feeding.
- Their HB was greater than 9 g/l
- They had been treated with quinolone or aminoglycoside antibiotics in the previous 60 days.

2.1.3 Ethics statement

As mentioned in 2.1, the study was a sub-study of the ScreenTB project. The ScreenTB study (including these sub-studies) was approved by the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences of the University of Stellenbosch (N16/05/070), and also the City of Cape Town.

2.2 Sample collection

After written informed consent blood, sputum and other samples required for the main ScreenTB project were collected from all study participants, and prior to the establishment of the diagnosis of TB or no-TB disease (Figure 2.1). About 1mL of whole blood (WHB) was collected by venepuncture directly into QuantiFERON Plus (QFT-Plus) tubes after which the tubes were transported at ambient conditions to the Stellenbosch University Immunology Research Group (SU-IRG) laboratory for further processing. Upon arrival in the laboratory, the QFT Plus tubes were incubated overnight (18 to 24 hours) at 37 °C, in a 5% CO_2 incubator after which culture supernatants and sediments were harvested and further processed as described below. Sputum samples collected from the study participants were used for

microbiological diagnosis, which formed part of the algorithm for diagnosing TB disease as discussed below.

2.2.1 Reference standard used in the classification of study participants as TB or other respiratory diseases

Sputum samples collected from study participants were processed and microbiological tests done as these tests formed part of the classification algorithm which made up the composite reference standard. Sputum samples collected from all participants were used for smear microscopy using Ziehl–Neelsen test, GeneXpert and culture using the Mycobacteria Growth Inhibitor Tube (MGIT) method. MGIT cultures which were positive were checked for contamination by the examination of Ziehl-Neelsen stained smears for acid fast bacilli. This was then followed by confirmation of organisms of the MTB complex using Capilia TB testing (TAUNS, Numazu, Japan).

As was done in previous studies (101,114), study participants were classified as either definite TB, probable TB or other respiratory diseases (ORD) using a combination of clinical, laboratory and radiological findings (Table 2.1). Similarly, participants classified as ORD had a variety of other diagnosis which included upper and lower respiratory tract infections, and acute exacerbations of chronic obstructive pulmonary diseases or asthma as described in (101,114). However, bacterial and viral cultures were not performed to identify these organisms as the main ScreenTB project was not funded to do these detailed diagnoses.

As mentioned above (section 2.1) recruitment of study participants into the ScreenTB project is currently ongoing. For the purposes of the current study, we only analysed samples from individuals that were recruited from the beginning of the study (11/10/2016) and (19/10/2017). Samples collected from study participants enrolled during this period were processed using all the techniques described in the present dissertation, for the purposes of my MSc project.

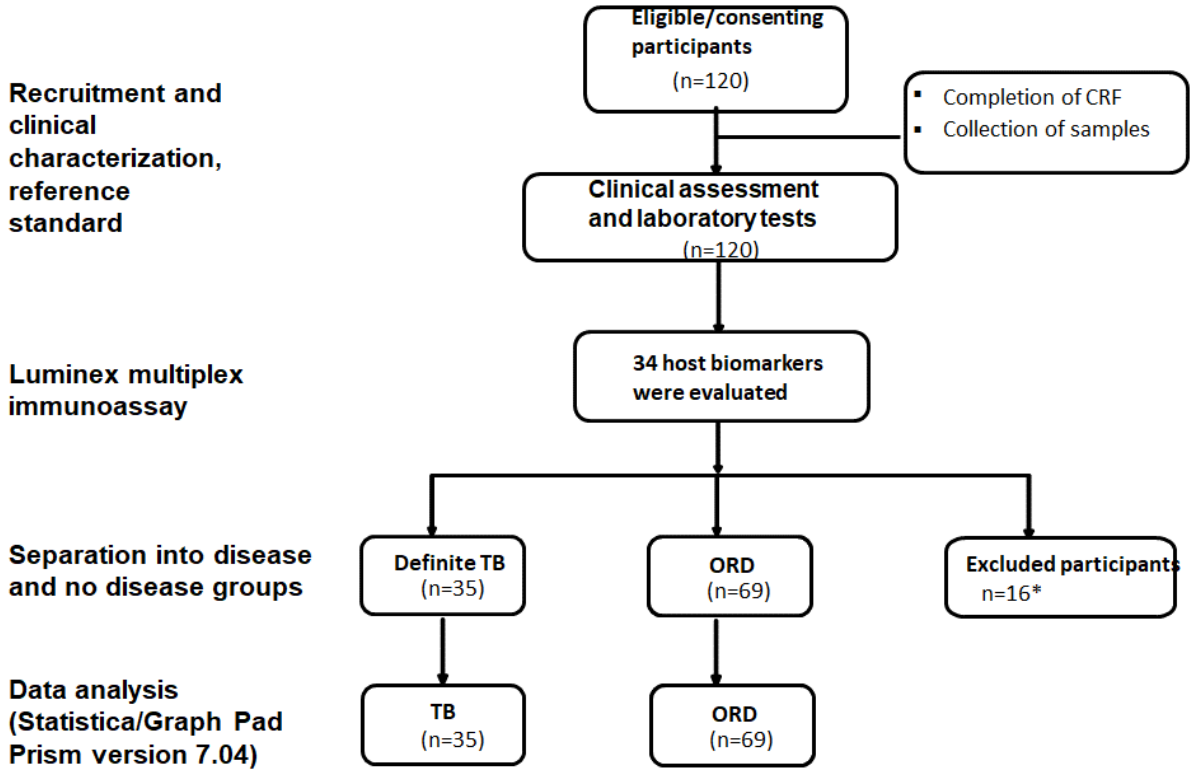


Figure 2.1: STARD flow diagram showing the study design and classification of participants. *These study participants were excluded either because they had culture contamination in addition to having negative sputum smear results (n=8) or because no final decision was arrived at as to whether they were TB patients or individuals with ORD as a result of missed week 2 and/or month 2 visits (n=8). TB= pulmonary tuberculosis, ORD= other respiratory diseases; that is, patients in whom TB disease was ruled out.

Table 2.1: Summary of algorithm used in classification of ScreenTB participants.

All participants enrolled had respiratory symptoms compatible with active TB

Classification	Definition
Definite TB	Positive sputum Culture OR Positive GeneXpert (and no previous TB episode in past year)
Probable TB	AFB smear result positive and CXR compatible with TB OR AFB smear result positive and initiated on TB treatment with good treatment response OR CXR results compatible with TB and initiated on TB treatment and shows good response OR TB treatment initiated on clinical suspicion and participant shows good response
TB Negative	Not initiated on TB treatment and symptom resolution or other diagnosis was made OR CXR signs compatible with TB but not initiated on TB treatment and resolution of symptoms or other diagnosis made
Exclude from analysis	Not able to assign category due to: crucial data missing in the form of sputa results (not done or contaminated), or CXR (not done) OR CXR signs compatible with TB and initiated on TB treatment, but participant's symptoms show doubtful response on treatment OR

	Loss to follow up and treatment response not evaluated

This diagnostic algorithm (previously published in (133)) is the same algorithm that was employed in the current study.

2.2.2 Processing of QFT Plus supernatants and sediments

2.2.2.1 QFT Plus supernatants

The QFT Plus tubes were centrifuged at 3000 x g for 15 minutes to separate the plasma from the blood. The plasma from each of the tubes was aliquoted and stored in micro centrifuge tubes as follows: 150 µL of plasma was added to the first two tubes and the remainder into the third tube. All tubes were then frozen at -80°C until used for IFN-γ measurement or the Luminex platform as described below.

2.3 QuantiFERON-TB® Gold Plus Enzyme Linked Immunosorbent Assay (ELISA)

2.3.1 Principle of the QuantiFERON-TB® Gold Plus ELISA

ELISA is a biological technique which is mainly used in immunological assays to detect and quantify antibodies or antigens of interest. QFT Plus ELISA was used to detect IFN-γ concentrations in order to diagnose *M. tb* infection in the current study participants. The QuantiFERON Plus tubes contain a negative and positive control. The mitogen tube acts as a positive control and addresses the immune competence of the participant's immune cells. More importantly, it serves as a control for proper sample transport, blood handling and incubation. A positive IFN-γ response is considered when either TB antigens response is significantly above Nil IFN-γ value (≥0.35 IU/ml). When a blood sample has a negative response to the TB antigens and a low response to Mitogen, the results is considered indeterminate. According to the manufacturer, such a result may occur due to incorrect filling or mixing of Mitogen tube, or the inability of the patient's lymphocytes to produce IFN-γ. The Nil tube serves as a negative control which adjusts for the background and therefore has to be subtracted from the IFN-γ levels of TB antigen and mitogen tubes.

2.3.2 QFT TB® Gold Plus ELISA procedure

IFN-γ concentrations in QuantiFERON supernatants were determined using QuantiFERON TB® Gold Plus ELISA kit. One hour before starting the ELISA all plasma was thawed and reagents except the 100x concentrate conjugate were brought to room temperature (22°C ± 5°C). Each plate contains a standard dilution in duplicates, a laboratory generated internal control (IntCtr) in triplicates and up to 21 plasma samples. The IFN-γ standard was reconstituted with the volume of distilled water indicated on the label of the IFN-γ Standard vial. The reconstituted standard was then mixed gently to minimize frothing and to ensure complete solubilisation. The reconstituted kit standard was the diluted 1:2 to reach a concentration of 2 IU/mL for Standard 1 followed by a 1:4 dilution series to reach concentrations of 1 IU/mL and 0.25 IU/mL for standard 2 and standard 3 respectively.

After preparing the standard dilution series, 100x concentrate conjugate was reconstituted with 300µL distilled water and mixed until dissolved completely. A working solution was prepared by pipetting 60µL of reconstituted Conjugate 100x Concentrate into 6.0mL of ‘Green Diluent’. 50 µL of the freshly prepared working solution was added to all the wells using a multichannel pipet. 50 µL of the plasma sample, internal control (IntCtrl) and lastly Standard dilution was added to the appropriate wells as indicated in figure 2.2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 N	3 N	5 N	7 N	9 N	S1	S1	13 N	15 N	17 N	19 N	21 N
B	1 TB1	3 TB1	5 TB1	7 TB1	9 TB1	S2	S2	13 TB1	15 TB1	17 TB1	19 TB1	21 TB1
C	1 TB2	3 TB2	5 TB2	7 TB2	9 TB2	S3	S3	13 TB2	15 TB2	17 TB2	19 TB2	21 TB2
D	1 MIT	3 MIT	5 MIT	7 MIT	9 MIT	S4	S4	13 MIT	15 MIT	17 MIT	19 MIT	21 MIT
E	2 N	4 N	6 N	8 N	10 N	11 N	12 N	14 N	16 N	18 N	22 N	IntCtrl
F	2 TB1	4 TB1	6 TB1	8 TB1	10 TB1	11 TB1	12 TB1	14 TB1	16 TB1	18 TB1	22 TB1	IntCtrl
G	2 TB2	4 TB2	6 TB2	8 TB2	10 TB2	11 TB2	12 TB2	14 TB2	16 TB2	18 TB2	22 TB2	IntCtrl
H	2 MIT	4 MIT	6 MIT	8 MIT	10 MIT	11 MIT	12 MIT	14 MIT	16 MIT	18 MIT	22 MIT	

Figure 2.2: QFT Plus ELISA plate layout. S1= Standard 1, S2= Standard 2, S3= Standard 3, S4= Standard 4, Nil= Control plasma, M= Mitogen plasma, TB1= *M. tb* specific antigen 1, TB2=*M. tb* specific antigen 2.

All plates were gently shaken by hand to mix, followed by incubation at room temperature (22℃ ± 5°C) for 2 hours. Wash Buffer 20x Concentrate was diluted 1:20 with distilled water. Following incubation, plate wells were washed with 1x Wash Buffer by hand, whereby each well was filled to the top of the well. The washing step was repeated 10 times. After adding 100µL of enzyme substrate solution to each well, the plate was carefully tapped on the sides and incubated for 30 minutes at room temperature (22°C ± 5°C). After 30 minutes incubation, 50µL Enzyme stopping solution was added to each well and mixed thoroughly. The addition of stop solution was done in the same order and at approximately the same speed as the Enzyme substrate addition step. Within 5 minutes, the optimal density was measured in each well using a microplate reader fitted with a 450 nm filter and 620 nm to 650nm

reference filter. The raw data were imported into the QFT-Plus Analysis Software, to generate a standard curve which has to be validated before results can be taken. The following criteria have to be met for an ELISA run to be valid (taken from QuantiFERON handbook):

- The mean OD value for Standard 1 must be ≥0.600.
- The %CV for Standard 1 and Standard 2 replicate OD values must be ≤15%.
- Replicate OD values for Standard 3 and Standard 4 must not vary by more than 0.040 optical density units from their mean.
- The correlation coefficient (r) calculated from the mean absorbance values of the standards must be ≥0.98.

QFT-Plus results are interpreted using the criteria in table 2.2

Table 2.2: QFT Plus ELISA results interpretation.

<table>
<tr><th>Nil (IU/ml)</th><th>TB1 minus Nil (IU/ml)</th><th>TB2 minus Nil (IU/ml)</th><th>Mitogen minus Nil (IU/ml)</th><th>QFT-Plus results</th><th>Report/Interpretation</th></tr>
<tr><td rowspan="3">≤8.0</td><td>≥0.35 and ≥25% of Nil</td><td>Any</td><td rowspan="2">Any</td><td rowspan="2">Positive</td><td rowspan="2">M. tuberculosis infection likely</td></tr>
<tr><td>Any</td><td>≥0,35 ≥25% of Nil</td></tr>
<tr><td><0.35 or ≥0.35 and <25%</td><td><0.35 or ≥0.35 and <25% of Nil</td><td>≥5.0</td><td>Negative</td><td>M. tuberculosis infection NOT likely</td></tr>
</table>

	< 0.35 or ≥0.35 and <25% of Nil	<0.35 or ≥0.35 and <25% of Nil	<0.50	Indeterminate	Likelihoods of *M.tuberculosis* infection cannot be determined
>8.0	Any				

2.4 Luminex multiplex immunoassay

The Luminex multiplex immunoassay platform was used for evaluation of the concentration of host immunological biomarkers in QFT Plus supernatants in the current project.

2.4.1 Principle of Luminex assay

The Luminex multiplex assay is an assay which is used to measure multiple analytes concurrently in a single well of a microtiter plate. The sample is added to a mixture of colour-coded beads which are pre-coated with analyte specific capture antibodies that binds to the analytes of interest. The biotinylated detection antibodies specific to the analytes of interest are added and form an antibody-antigen sandwich. This is followed by adding Phycoerythrin (PE)-conjugated streptavidin which binds to the biotinylated detection antibodies. The Luminex 200 platform and Flex Map 3D system, dual-laser flow based detections instrument, are used to read the polystyrene beads. The first laser is a 635nm red laser (classification laser) which segregates the beads and determines the analytes by exciting the dyes inside the beads. The second laser is 532nm green laser (reporter laser) which excites the PE. A high-sensitivity photomultiplier tube (PMT) detector detects the emission of PE, which is directly proportional to the amount of analyte bound.

The Luminex MAGPIX® is another instrument which can be used in reading the results of Luminex multiplex experiments. Unlike the Luminex 200 instruments, the Magpix only uses magnetic and not polystyrene beads. The analyser makes use of a magnet

which captures and holds the magnetic beads in a single layer. The instrument consists of two spectrally different light-emitting diodes (LEDs), the 525nm green LED and 635nm red LED, which illuminate the beads. The red LED is used to identify the beads to which analytes are bound based on their fluorescent patterns. The green LED is used to read the PE derived signal if the analyte of interest is present in the sample. The software used in the instrument then processes all images and determines the concentration of the analytes based on a standard curve. The main difference between these instruments is that the Flex Map 3D can read up to 500 analytes, the Luminex 200 up to 100, and Magpix up to 50 analytes. Figure 2.3 illustrates the principle of the luminex assay.

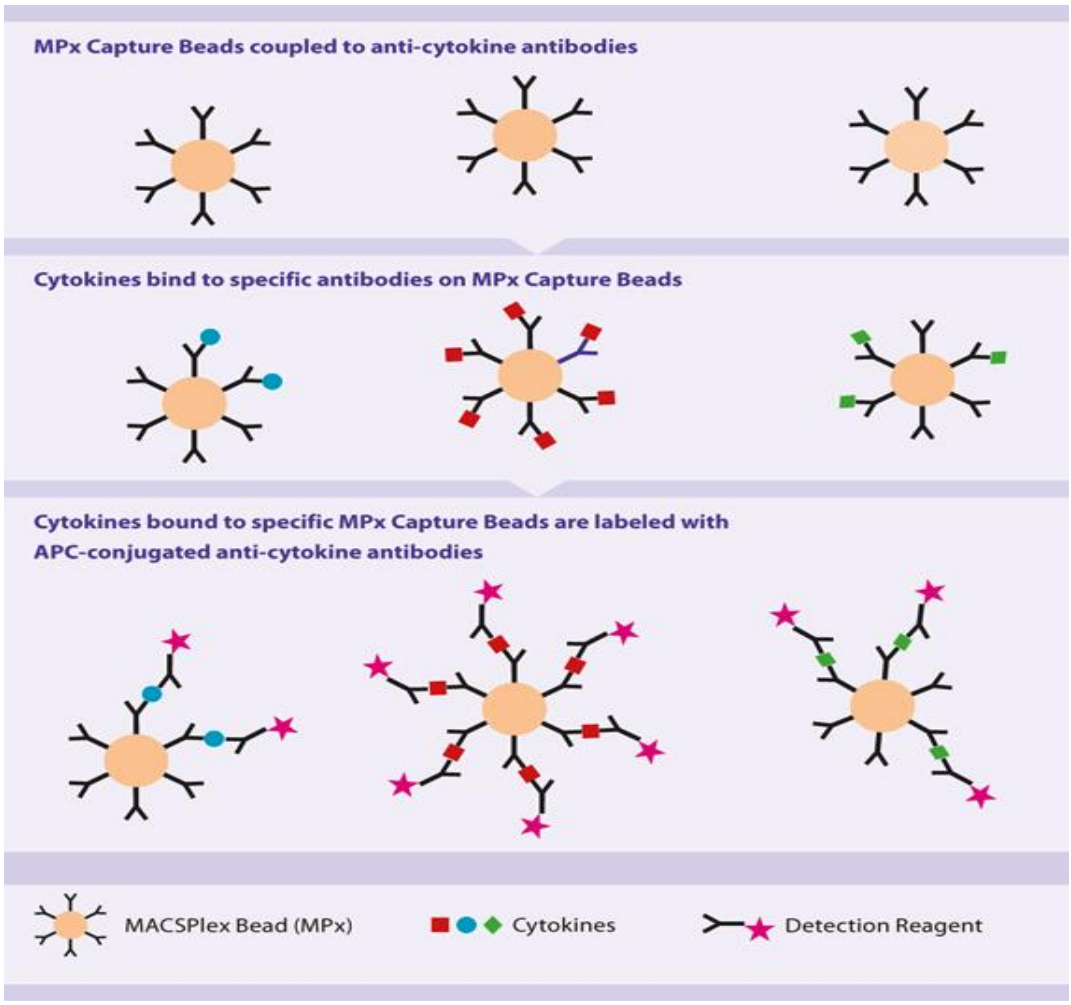


Figure 2.3: Principle of Luminex illustration. Source: miltenybiotec.com

For the reagent kits employed in the present project, all the detection antibodies were biotinylated, with the detection reagent being streptavidin-phycoerithrin.

2.4.2 Luminex assay procedure

Luminex assays were performed following the instructions of the manufacturers of the reagent kits used. All reagents used in the current project were purchased from Merck Millipore (Billerica, MA, USA) and R&D systems (Biotechne, Minneapolis, MN, USA).

We evaluated the concentrations of 37 analytes in QFT Plus nil, TB1 and TB2 antigen-stimulated supernatants from all study participants. All samples and reagents were brought to room temperature prior to the start of experiments. After thawing, samples were vortexed and centrifuged for 3 min at 3000xg followed by preparations of all reagents, standards and quality controls as directed by the manufacturers. The Luminex plates were pre-wetted with appropriate wash buffer and incubated on a shaker for 10 minutes (if recommended by the manufacturer) followed by the addition of standards and the controls from the manufacturer if available with the kit being evaluated. For the Milliplex assays, a serum matrix solution was added to the standards and controls wells, followed by addition of samples to the appropriate wells. Mixed beads were added to each well and the plate was incubated for 2 hours at room temperature or overnight at 4 degrees Celsius on a plate shaker, following the speed recommended by the kit manufacturers. Following the incubation, all plates were washed using the respective wash buffers as recommended by the manufacturers in the kit inserts. Detection antibodies were then added to each well followed by 1 hour incubation. The plates were then washed again, followed by addition of Streptavidin-Phycoerythrin and incubated for 30 minutes. This was then followed by a further wash step, resuspension in either Sheath or Drive fluid and then reading on a Bio-Plex 200 instrument (Bio-Rad laboratories, Hercules, CA, USA) and or MAGPIX (Bio-Rad laboratories). Bead acquisition and analysis of median fluorescence intensity were done using the Bio-Plex Manager™ version 6.1 software (for plates read on the Bio-Plex 200 instrument) or the Bio-Plex MP software, followed by Bio-Plex Manager 6.1, if the Magpix instrument was used. All samples were analysed in a blinded manner. Host biomarkers evaluated in the study are listed in table 2.3. Prior to analysis, samples were pre-diluted as shown in table 2.4.

Table 2.3: List of Luminex kits and analytes employed in the study

Luminex kits	Analytes
Reagent kit catalogue numbers and analytes purchased from Merck Millipore	
Human Neurodegenerative disease magnetic bead panel 1 HNDG1MAG-36 K	Interleukin 4 (L-4), IL-6, Macrophage inflammatory protein 1-apha (MIP)1-α , -interferon alpha 2 (IFN)-α2
Human Neurodegenerative disease magnetic bead panel 1 HNDG1MAG-36 K	Complement C3
Human Neurodegenerative disease magnetic bead panel 2 HNDG2MAG-36 K	Complement C4
Human Cytokine/Chemokine Magnetic Bead Panel HCYTOMAG-60K	Apolipoprotein A1, Apolipoprotein CIII, Complement Factor H
Human Cardiovascular Disease HCVD2MAG-67K	D-dimer, GDF-15,Myoglobin, MPO, NGAL, sVCAM-1,
Human Neurodegenerative disease magnetic bead panel 3 HNDG3MAG-36 K	BDNF, Cathedpsin -D, ICAM-1, MPO, PDGF-AA, RANTES,PDGF-AA/BB, VCAM-1, PAI 1
Human complement HCMP1MAG-19K	Complement C2, Complement C5, Complement C4b, Complement 5a, Complement C9, Complement factor 1, MBL
Reagent kit catalogue numbers and analytes purchased from R&D systems	
R&D LXSAHM-02	NCAM-1/CD56, TGF-α
R&D LXSAHM-02	Ferritin, MMP-9
R&D LXSAHM-08	CXCL9/MIG,EGF, P-Selectin, LIGHT, IL-3, CCL/MCP-2, IL-15, VEGF
R&D LXSAHM-20	ADAMTS13, CCL2/MCP-1, CD40 Ligand, CXCL11/ITAC-1, Fas, IFN-γ, IL-beta, IL-13, IL-2, IL-33, CCL1/I-309, CCL4/MIP-1,

	CXCL10/IP, CXCL8/IL-8, GM-CSF, IL-1 alpha, IL-10, IL-1 rα, IL-22, TNF-alpha

Table 2.4: Dilution of samples prior to use in the Luminex experiments. All dilution factors used were as prescribed by the reagent kit manufacturers**.**

Kits	Dilution factors
HCVD2MAG-67K	1:100 dilution
HNDG1MAG-60K	Neat sample
HNDG1MAG-36K	1:40,000 Dilution
HCMP1MAG-19K	1:2 Dilution
LXSAHM-02	1:2 Dilution
LXSAHM-08	1:2 Dilution
LXSAHM-20	1:2 Dilution

2.3 Statistical analysis

All statistical analysis was performed using Graph Pad Prism version 7.04 (San Diego, CA, USA) and Statistica (TIBCO Software Inc, CA, USA). The Mann-Whitney U-test was used for the evaluation of differences in the concentrations of individual host biomarkers between any two groups e.g. TB vs. ORD. The diagnostic accuracy of individual biomarkers was evaluated by receiver operator characteristics (ROC) curve analysis. Cut-off values and associated sensitivity and specificity were determined using Youden's Index. The General discriminant analysis (GDA) procedure was used to assess the predictive abilities of combinations between multiple host biomarkers. Prediction accuracy was ascertained by leave-one-out cross validation. P-values ≤ 0.05 were considered significant.

2.4 Role of the candidate in the project

After joining the research group in February 2017, processing of all QFT Plus samples for the main ScreenTB project and the extra steps done as part of the Candidate's project were done by the candidate. All the QFT Plus ELISA experiments were performed by the candidate and results quality controlled by experienced laboratory staff and imported into the main ScreenTB data base for use by the consortium. The candidate also performed all the Luminex experiments (with assistance of research assistants), cleaned all data in preparation for analysis, analysed the data with help from the Centre for Statistical Consultation of Stellenbosch University (Professor Martin Kidd) especially on the diagnostic biomarker models, and wrote the thesis. I have also been responsible for writing a draft manuscript on the results as well as presenting the work obtained from this project at conferences and departmental seminars.

Chapter 3

Evaluation of the potential usefulness of the QuantiFERON® TB Gold Plus test as a tool for adjunctive diagnosis of TB disease

3.1 Introduction

TB continues to be one of the leading causes of death worldwide and resulted in the deaths of an estimated 1.3 million adults, with 10 million reportedly falling ill with the disease in 2016 (6). As also discussed in chapter 1, Interferon gamma release assays (IGRAs) and the tuberculin skin test (TST) remain the only approved immunological diagnostic tests for TB. These tests are generally not recommended for the diagnosis of active TB disease, especially in high burden settings, such as obtained in South Africa, because of the high prevalence of latent *M. tb* infection (119). However, positive IGRA or TST results do lead at least, to preventative treatment with isoniazid in some patient groups, including children under the age of 5 years, HIV infected individuals and those undergoing immunosuppressive therapy (120, 121). In the case of the diagnosis of some particularly difficult-to-diagnose extra pulmonary TB types such as ocular TB and TB meningitis, these tests form part of the diagnostic algorithms that are employed in ruling-in or ruling-out TB, in the course of differential diagnosis.

As discussed in chapter 1, the QuantiFERON® TB Gold (QFT) Plus test is a new generation of IGRA that was introduced for the management of TB recently (122) and replaced the QFT In Tube. After the introduction of the QFT In Tube to the market in the mid-2000's, several investigations were done, aimed at determining whether the test could contribute to the diagnosis of active TB, leading to the recommendations against the use of the test in high burden settings (123). As the QFT Plus is a new test, without much independently published data about its use currently available, and with the manufacturer claiming that it is more sensitive than the QFT In Tube (>94% sensitivity for *M. tb* in individuals with active TB disease depending on the extant of TB disease and the TB settings), we sought to evaluate the accuracy of the test in the diagnosis of active TB disease in patients with suspected TB and to investigate whether the use of the test, in conjunction with symptoms has the potential to make any contribution to the diagnosis of active TB in a high burden setting. Knowledge

gained may contribute to further research on the potential use of the test in the management of TB in the difficult-to-diagnose TB types (paediatric and extrapulmonary TB) or inform the design of research studies in these patient groups.

3.2 Materials and methods

3.2.1 Study participants

As described chapter 2 (section 2.1), we recruited individuals that were suspected of having pulmonary TB disease from primary health care clinics in Cape Town, South Africa, prior to the diagnosis of TB or other respiratory diseases (ORD) in them. This was a sub-study of a larger biomarker study that is currently ongoing in multiple African countries and known as the “ScreenTB” project (www.screen-tb.eu) and the inclusion and exclusion criteria were described in chapter 2 (section 2.1.1-2.1.2).

3.2.2 QuantiFERON® TB Gold Plus ELISA

As also described in chapter 2 (section 2.5) , whole blood was collected from all study participants by venepuncture, directly into tubes that were supplied by the manufacturers of the QFT Plus test (1 ml per tube, 4 tubes corresponding to the nil, TB1, TB2 and mitogen) as recommended by the manufacture (Qiagen, Germany). The tubes were then transported at ambient conditions to the laboratory after which they were incubated overnight (18 to 24 hours) at 37 °C, in a 5% CO_2 incubator, followed by centrifugation (3000 x g for 15 minutes) as recommended by the manufacturer. Culture supernatants were then harvested, aliquoted and frozen until used for ELISA. IFN-γ concentrations in the supernatants were then determined using QFT Plus ELISA kit and the results interpreted as positive, negative or indeterminate using the analysis software provided by the manufacture (Qiagen, Germany).

3.2.3 Statistical analysis

The sensitivity and specificity of the QFT Plus for TB disease was evaluated against the composite reference standard described in Chapter 2 (section 2.7). The

concentrations of IFN-γ detected in the nil, TB1, TB2 and Mitogen stimulated supernatants were compared between patients with TB disease or ORD using the Mann Whitney U test. Alternative cut-off values for discriminating between TB and ORD using any of these stimulation conditions were assessed using receiver operator characteristics curve analysis. The General discriminant analysis (GDA) procedure was used in evaluating combinations between the nil, TB1, TB2 and mitogen–specific IFN-γ values to ascertain whether the use of alternative cut-off values for each of these conditions could potentially provide an algorithm for the diagnosis of active TB. Finally, we evaluated whether the use of symptoms in combination with the test result obtained using the manufacture's cut-off value had potential in the diagnosis of active TB disease. Prediction accuracy was evaluated using leave-one-out cross validation. As mentioned in chapter 2, all statistical analysis was performed using Graph Pad Prism version 7.04 and Statistica (Dell Software), with p-values ≤ 0.05 considered significant. This report especially the discussion section was written in the method proposed by Sacket and Haynes (124).

3.3 Results

3.3.1 Patient characteristics

As previously described in chapter 2 a total of 104 study participants were prospectively recruited in to the study between (11/10/2016) and (19/10/2017). The mean age of all the study participants was 40 ± 12.6 years, with 14 (11%) being HIV infected. Using the manufacturer's recommended cut-off value (0.3 IU/ml), 87 (72.5%) the study participants were QFT Plus positive. Using the reference standard described in chapter 2 (section 2.2), which made use of clinical information, laboratory (smear, GeneXpert and culture) and imaging results, 35 (33.6%) of the study participants were definite TB patients. In fact, all the TB patients were culture positive (table 3.1). The prevalence of different symptoms that led to the suspicion of TB disease, hence the inclusion of the study participants in the current study is shown in chapter 2 (section 2.1.1).

Table 3.1: Clinical and demographic characteristics of study participants.

No of participants	All	TB disease	ORD
No. of patients	104	35 (33.6%)	69 (66.3%)
Female's n (%)	64 (53.3%)	10 (28.5%)	46 (66.6%)
Age mean ± SD	40.06 ±1 2.58	34.8 ± 12.14	41.92 ± 12.6
HIV positive n (%)	14 (13.4 %)	7 (20.0 %)	7 (10.1%)
QFT plus Positive n (%)	87 (72.5%)	27 (77.1%)	49 (71%)
QFT plus Negative n (%)	32 (26.7%)	8 (22.8)	20 (28.9)
Indeterminate n (%)	1 (0.8)	0 (0)	0 (0)
Symptoms at enrolment			
Coughing	115 (95.8%)	30 (85.7%)	69 (100%)
Night sweats	99 (82.5%)	28 (80.0%)	56 (81.2%)
Weight loss	79 (65.8%)	28 (35.4%)	38 (55.1%)
Chest pain	91 (75.8%)	29 (31.8%)	51 (73.9%)
Feverish	57 (21.1%)	25 (71.4%)	24 (34.8%)
Weak and tired	75 (62.5%)	26 (74.3%)	41 (59.4%)
Not feel like eating	73 (60.8%)	21 (60%)	42 (60.9%)

35 of the study participants were definite TB and 69 were Non-TB (ORD). Participants were classified as Definite TB if they were smear positive, culture positive and had a chest X-ray suggestive of TB. Participants were classified as ORD if they were culture negative, smear negative and negative chest X-rays, and have never been under TB treatment initiated by health providers. QuantiFERON results was obtained and defined as positive, negative, and indeterminate by using manufacture's software. Abbreviations: TB= Tuberculosis, ORD= other respiratory disease, SD=standard deviation, HIV= Human immunodeficiency virus, QFT= QuantiFERON TB Gold plus**.**

3.3.2 Analysis of IFN-γ responses obtained in respective QFT Plus tubes

We evaluated the magnitude of IFN-γ produced in the respective QFT Plus tubes from all study participants and also assessed differences between TB patients and those with ORD. The mean IFN-γ levels obtained in the nil tube was 0.34 ± 0.45 IU/ml. After

subtraction of the nil values, the mean TB1, TB2 and mitogen-specific IFN-γ responses were1.93 ± 1.11 IU/ml, 2.36 ± 3.11IU/ml and 10.97 ± 2.90IU/ml respectively. When the mean IFN-γ levels were compared between TB and ORD, the mean IFN-γ levels in TB was higher than that of ORD. When IFN-γ levels were compared between TB and ORD groups, nil and mitogen-nil were the only tubes which showed significant difference between TB and ORD. the mean, standard deviation and p values from individual tubes are shown in table 3.2.

Table 3.2: IFN-γ (IU/mL) concentration detected in unstimulated QFT Plus supernatants from individuals with TB disease (n=35) and other respiratory diseases (n=69). The concentrations were measured in each QFT Plus tube.

Stimulant	All (mean ± SD), (IU/ML)	TB (mean ± SD), (IU/ML)	ORD (mean ± SD), (IU/ML)	P-value
Nil	0.34 ± 0.45	0.34 ±0 .26	0.29 ± 0.27	0.0129
TB1	2.21 ± 2.54	1.70 ± 1.84	2.35 ± 2.83	0.8017
TB2	2.25 ± 2.81	2.458 ± 2.84	2.580 ± 2.84	0.6350
Mitogen	1.86 ± 4.46	8.89 ± 4.46	10.20 ± 1.67	0.9808
TB1-nil	1.93 ± 1.11	1. 36 ± 1.75	2.13 ± 2.81	0.5024
TB2-nil	2.36 ± 3.11	2.45 ± 3.16	2.17 ±2 .82	0.8608
Mitogen-nil	10.97 ± 2.90	9.98 ± 3.73	11.64 ± 1.76	0.0472

3.3.3 Performance of QFT Plus in the diagnosis of TB disease

As shown in table 3.1 (above), 72.5% of all study participants (patients with TB plus those with ORD) were positive with the QFT Plus test. Out of all the 35 TB patients, the test, using the cut-off values recommended by the manufacturer yielded positive results in 27, corresponding to a sensitivity of 77.1% (95% CI: 59.9-89.9%), specificity of 71.0% (95% CI: 58.98.3%), positive and negative predictive values of 57.5 (95% CI: 47.32-67.1%) and 86.0 (95% CI: 76.59-92.0%) respectively. The area under the ROC

curve for the QFT Plus test in the diagnosis of active TB in individuals in whom TB disease is suspected was 53.0% (95% CI: 44.3-61.8%).

Given the relatively poor sensitivity and specificity of the test (that is, sensitivity falling below the expected threshold of 95% that is claimed by the manufacturer), we sought to determine whether there were alternative cut-off values derived using ROC curve analysis, which would yield better accuracy. When ROC curve analysis of IFN-γ values was done, values detected in the nil tube had the highest area under the ROC curve (AUC) as shown in table 3.3. The cut-off values, sensitivity, specificity, positive and negative predictive values as well as area under the ROC curves are shown in table 3.3.

Table 3.3: Diagnostic accuracies for IFN-γ detected in different QFT Plus tubes from individuals with TB disease (n=35) and other respiratory diseases (n=69).

Stimulant	Cut-off	AUC (95%CI)	Sensitivity	Specificity	P value
Nil	<0.2650	0.65 (0.55-0.75)	68 (0.56-0.79)	51 (0.34-0.69)	0.0129
TB1	<0.7300	0.51 (0.41-0.63)	61 (0.48-0.72)	40 (0.24-0.58)	0.8017
TB2	<0.83	0.52 (0.42-0.64)	0.63 (0.45-0.79)	0.40 (0.29-0.53)	0.6350
Mitogen	<11.00	0.50 (0.36-0.65)	77 (0.65-0.86)	0.51 0.34-0.67)	0.9808
TB1-nil	<1.335	0.54 (0.43-0.65)	66 (48-81)	42 (30-55)	0.5024
TB2-nil	<1.830	0.51 (0.40-0.62)	61 (0.48-0.74)	40 (0.23-0.58)	0.8608
Mitogen-nil	>11	0.62 (0.50-0.74)	96 (0.87-0.100)	28 (0.15-0.46)	0.0472

AUC= Area under the ROC curve, CI= Confidence interval. p<0.005 is considered significant

After performing GDA analysis to evaluate combinations between the QFT Plus tubes, the sensitivity of the QFT Plus test with the GDA derived cut-off value (0.44) was inferior to that obtained using the manufacturer's recommended cut-off value, even though there was an improvement in the specificity. The sensitivity of the GDA combinations of antigen-specific IFN-γ values was 42.9% (95% CI: 23.3-60.7%) and the specificity was 88.4% (95% CI: 78.43-94.9%). After leave–one-out cross validation, the sensitivity and specificity were 44.9% (95% CI: 26.3-60.7%) and 87.0% (95% CI: 16.7-93.9%) respectively. The positive and negative predictive values were 62.5% (95% CI: 44.8-77.4%) and 72.12% (95% CI: 62.5-80.5%) respectively (Figure 3.2).

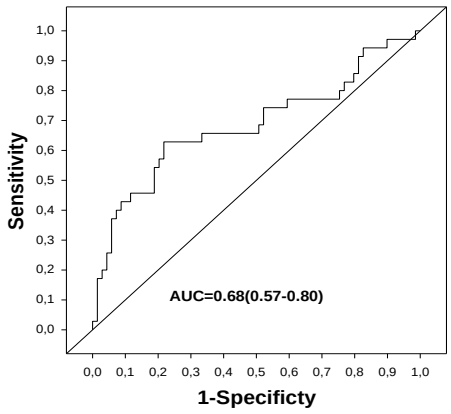


Figure 3.1: ROC curve for diagnosis of TB disease using combination of IFN-γ values detected in QFT Plus antigen tubes (nil, TB1-nil, TB2-nil and Mitogen-nil) using alternative cut-off values (A). The scatterplot (B) represents the classification of each study participant by the GDA derived model.

3.3.4 Performance of QFT-Plus test in the diagnosis of TB disease when used in combination with symptoms.

We investigated whether the use of symptoms in combination with the QFT Plus test result obtained using the mufacturere's cut-off value had any potential in the diagnosis of active TB. A combination of three symptoms diagnosed TB disease with sensitivity and specific of 77.0% (95%CI: 56.4-91.0 %) and 70.0% (95% CI: 56.0-81.2 %)

respectively, with an AUC of 0.81 (95%CI: 0.71-.91%). After leave-one-out cross validation, there was no change in the sensitivity and specificity (Table 3.4 A). When the QFT Plus was used in combination with symptoms, there was an added benefit of combing both results as a combination of QFT Plus results and four symptoms including not feel like eating, weight loss, feverish and sharp chest pains which resulted in an increased sensitivity of 80.0% 95% (CI: 91.4-92.3%) and specificity of 77.0% (95%CI: 54.9-81.3%) and after leave-one-out cross validation, the sensitivity and specificity were 76.7% (95%CI: 57.2-90.1%) and 69% (95%CI: 54.9-81.3%) respectively (Table 3.4 B).

Table 3.4: Diagnostic biosignatures identified in the current study. The GDA modelling procedure was performed in all TB study participants

	Classification matrix		**Leave-one-out cross validation**			
	Sensitivity (%)	**Specificity (%)**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
Biosignature (i): symptoms only						
Coughing + feverish+ weight loss	77.2 (6/26)	70.4 (39/56)	77.2 6/26)	70.4 (39/56)	52.6 (41.80-63.2)	86.7 (75.1-93)
Biosignature (ii): symptoms and QFT Plus positive results						
Not feel like eating + weight loss + feverish + sharp chest pains + QFT Plus results	80.0 (24/30)	69.2 (36/52)	77.0 (23/300	69.2 (16/52)	59.1 (47.6-69.3)	83.7 (72.4-91.1)

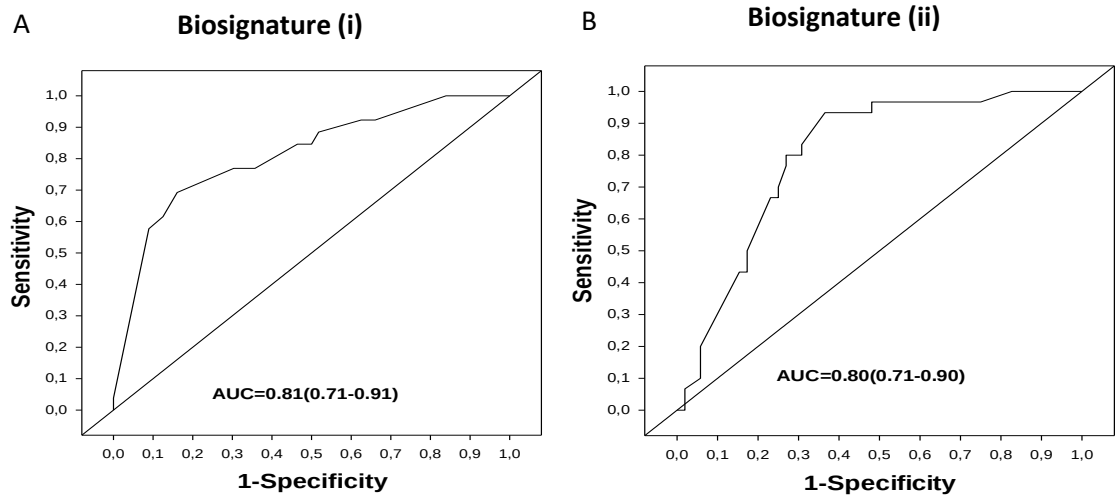


Figure 3.2: ROC curve for diagnosis of TB disease using combination of symptoms only and symptoms plus QFT Plus positive results. A, represents combination of symptoms only and B, represents combination of symptoms and QFT Plus positive results.

3.4 Discussion

Main findings

We evaluated the accuracy of QFT Plus test in the diagnosis of active TB amongst patients with suspected TB disease. Additionally, we investigated whether this test had potential in diagnosing TB when alternative cut-off values were used and also when used in conjunction with TB symptoms. Our main findings was the ability of the QFT Plus test, using the manufacturer's recommended cut-off value to diagnose active TB with sensitivity and specificity of 77.1% and 71.0% respectively as shown in table 3.1. When used alone, a combination of three symptoms coughing, being feverish plus weight loss diagnosed TB disease with sensitivity and specificity of 77.2% and 70.4% respectively, and an AUC of 0.81.We hypothesized that the use of the two modalities (symptoms and QFT Plus results) in combination would lead to an incremental sensitivity and specificity, compared to the values obtained with these tests (symptoms and QFT Plus result) alone. When the QFT Plus positive results were used in combination with symptoms, the addition of QFT Plus did not have any additional value over the symptoms alone. On the contrary, QFT Plus positive results + symptoms resulted in inferior accuracy to the QFT Plus alone or combination of symptoms alone.

Study strength and weaknesses of the study

There are several studies in the literature in which the QFT (especially previous versions as the current is still relatively new) were assessed for their potential in the diagnosis of TB disease (125-126) in both high and low TB burden settings. It is known that the QFT tests are primarily designed for the diagnosis of infection with *M. tb.* However, as there is no gold standard test for the diagnosis of *M. tb* infection, data obtained from TB studies provide evidence of the true sensitivity of the test, since people with newly diagnosed, untreated active TB (such as those employed in the current study) are definitely infected. A limitation to this approach though is the fact that TB patients may have anergic T cells, thereby compromising on the sensitivity evaluation, however, as there is no gold standard for *M. tb* infection, TB patients represent the best group in which the sensitivity of tests for *M. tb* infection can be assessed.

According to a systemic review and meta-analysis by Sester et al (123), reported that the pooled sensitivity of IGRAs do not show any supplemental value in diagnosing active TB even when combined with TB diagnostic standard methods (123). However, none of these studies were conducted in individuals with symptoms suggestive of TB disease. As QFT Pus is a newly introduced *in vitro* test which has been reported to offer improved sensitivity, mostly because of the additional TB2 antigen tube, this study had an opportunity to evaluate its performance in a TB endemic region in people with TB symptoms. Furthermore, given the reportedly high sensitivity and specificity of this test, according to the manufacturer, the current study was able to able to assess the ability of this test to be used in combination with TB symptoms as this might be helpful in diagnosing TB, with higher predictive values.

While the sample size of this study was reasonable, especially as it was a study in which study participants were recruited prospectively, it might have been better if we had more study participants so as to increase the proportion of people for example, who are HIV co-infected. The small number of HIV infected individuals led to the inability of this study to assess the effect of HIV infection on the QFT Plus test since evidence from previous studies showed that the previous generation of the test was affected by HIV infection but current evidence suggests that the QFT Plus test is not affected by HIV infection although the viral and CD4 count was not assessed, in fact

it has been shown to work better in HIV and other immunocompromised individuals (128). This limitation is however not major as the strengths of the current study outweigh the limitations. That is, participants were recruited prospectively, therefore the proportion of HIV individuals is concurrent with what one would normally expect at a community health care centre, such as where the study participants were recruited. Power calculations done for the main ScreenTB project showed that the number of study participants enrolled into the project were sufficient. However, more independent studies in which the manufacturer of the kits is not involved are required to confirm the findings for this study in participants from both high and low TB endemic countries.

The relation of the current study with other studies

The data for the performance of QFT Plus test in the diagnosis of TB of either *M. tb* or active TB disease is currently limited. However, studies which were conducted in previous QFT (QFT-TB Gold In tube) tests have demonstrated that this test may be useful in diagnosing TB disease in high TB settings (129), with a pooled sensitivity and specificity of 80% and 79% in case control study performed in all patients with TB (non-confirmed and confirmed by culture) (123). The manufacturer of the QFT Pus test has reported that this test offers increased sensitivity as high as 87% against culture confirmed TB (122) with sustained high uninterrupted specificity more especially to immunocompromised individuals as well as individuals living with HIV infection. QFT Plus results from culture confirmed TB varies. A study by Barcellini et al (74) reported a sensitivity of 87.9% (85), Hoffman et al, (132) 95.8% in culture confirmed TB. Our study found a sensitivity of 77.1% which is similar to the findings from these studies. The low specificity (71.0%) reported in the present study maybe because unlike other studies which included selected study participants in case-control or cross-sectional studies, our study included participants who were suspected of having active TB disease, prior to the confirmation or rule-out of TB disease, which is a stronger (phase III) design for a diagnostic study (131).

Implications of the current study

Although some studies reported against the use of QFT test in diagnosing TB in TB endemic regions. A positive QFT Plus test together with a combination of symptoms may still be useful in the diagnosis of TB in certain patient groups and such a diagnostic modality may act as a screening tools for active TB, for later confirmation

with gold standard TB diagnostic tests. This may be of particular relevance to extrapulmonary TB or childhood TB diagnosis, where current diagnostic approaches have limited applicability.

In the current study, the levels of IFN-γ obtained in individual antigen tubes did not show any significant difference between the ORD and TB group although the median TB2 IFN- γ levels were higher than in TB1 antigen tube (Table 3.2). This might have resulted due to our small sample size. HIV-confected active TB patients may have also affected the concentration levels of IFN-γ although no conclusions can be made about the effect of HIV infection, hence the CD4 count and vial load was not assed. However, the similar results were obtained in a study conducted by YI et al (72). They reported that no significant difference was found between the two antigen tubes. The difference shown in these TB antigen tubes may suggests that QFT Plus was sensitive in diagnosing active TB disease which may have been influenced by the additional TB2 antigen tube (72). Recent studies suggests that the TB2 antigen tube is highly sensitive in people with active TB, children, HIV infected patients and recent exposure which may therefore supports our findings. Additionally, the current study evaluated the relative cut-off values for accuracy of QFT Plus and individual tubes. The best cut-offs selected for individual tubes in this study showed promising optimal sensitivity and specificity, however no conclusion can be made from these findings until more studies are done. Furthermore, our statistically derived cut-off value of 0.4 UI/mL did not show any significant difference at all when compared to the manufacturer cut-off (0.35 UI/mL).

Unanswered questions and future research

Considering the relatively small number of HIV infected individuals enrolled in the current study, future studies for evaluating the performance of this test in individuals with TB symptoms and HIV infection may be beneficial and will add to the few studies that have been reported in the literature about the QFT Plus test. As this study was conducted in a high TB endemic region, similar studies conducted in low TB settings may be beneficial in order for conclusions about the performance of the test in the diagnosis of active TB to be made. Additionally, as diagnosing TB in children is problematic, it may be important that future studies should look at the performance of this test in children with and without HIV infection in both high and low TB settings.

HIV infected individuals included in these further studies should be appropriately staged with CD4 counts and viral loads to determine the effect of extensive HIV infection on the test results. Furthermore, confirmed other respiratory diseases will help in identifying specific groups e.g. patients with pneumonia, which may influence the specificity of the TST.

Conclusion

To conclude, this study was one of the first to evaluate the accuracy of the QFT Plus in people in whom TB is suspected, independently and in a high burden setting. Although 71.0% of individuals without active TB and who presented with other respiratory diseases yielded positive results with the test, thereby indicating the high LTBI in the population, the sensitivity and specificity of the test was still 77.1% and 71.0% respectively. When combination of symptoms was used to diagnose TB and was 80.0% and 77.0% respectively when positive QFT Plus results were used in combination with symptoms. The test may therefore have a role in the diagnosis of active TB, especially in difficult to diagnose TB groups such as HIV infected individuals, children and those with extrapulmonary TB. However, as that was not the focus of the current study, such investigations were not done. Therefore further studies evaluating the accuracy of the QFT Plus are needed before any conclusions can be made about current findings.

Chapter 4

Evaluation of the potential of host biomarkers detected in QuantiFERON® TB GOLD Plus supernatants in the diagnosis of TB disease

Declaration:

The work presented in this chapter:

1) Has been presented at "The Union Student late-breaker session related to lung health" the forth-coming 49th Union World Congress on Lung Health, which was held in The Hague, Netherlands.

   Abstract No: **A-0996-0057-03295**

   Abstract title: **Host biomarkers detected in QuantiFERON Plus supernatants show promise as diagnostic candidates for active TB disease**

   Authors: MP. Manngo, G. Walzl, N. Chegou

2) Is being written up as a research article. Formatting of the manuscript shall be completed and the work submitted for possible peer-review and publication after submission of my thesis.

***Proposed Target Journal**: The Journal of Infection*

4.1 Introduction

An estimated 2 billion people were reported to be infected with *Mycobacterium tuberculosis* (*M. tb*), the causative agent of tuberculosis (TB), with about 10 million reported to have fallen ill with active TB in 2017 (6). About 1.3 million people were reported to have died of the disease in the same year (6). The widely publicised limitations of the currently existing diagnostic procedures (132, 133) is one of the reasons why TB continues to be a problem. The most widely used TB diagnostic test (smear microscopy) (86) has poor sensitivity, whereas culture; the gold standard has long turnaround time, is prone to contamination, expensive and it is not available in many resource poor settings (111). The Gene Xpert MTB/RIF test (Cepheid, Sunnyvale, CA, USA), the most important recent development in the TB diagnostic field yields results within 2 hours, and detects resistance to rifampicin, one of the first-line TB drugs, but is expensive and cannot distinguish between DNA from dead and live bacteria (6). These tests rely on the availability of a good quality sputum specimen, which is problematic for the diagnosis of TB in children and individuals with other paucibacillary forms of the disease such as those with extrapulmonary TB (133). Immunological diagnostic approaches have been shown to be potentially useful and may be easily converted into point-of-care screening tests (95, 98, and 100).

Despite ongoing research into the development of immunological diagnostic tests, the tuberculin skin test (TST) and interferon gamma (IFN-γ) release assays (IGRAs) remain the only approved immunodiagnostic tests for the management of TB. These tests are useful in identifying people who are infected with *M.tb* but do distinguish active TB from latent infection (LTBI) [100, 102,136). They are therefore not recommended for use in high burden settings, due to the high prevalence of LTBI (6). Given the probable easier application of blood-based tests in the diagnosis of TB, multiple investigators have evaluated other antigens other than those used in IGRAs (ESAT-6, CFP10 and TB 7.7) or alternative host biomarkers other than IFN-γ; the read-out biomarker in IGRAs (100, 102) as active TB diagnostic tools. Most of these investigations, which were done in QuantiFERON® TB Gold (QFT) In Tube culture supernatants (100, 103) identified promising biosignatures for the diagnosis of active pulmonary TB disease in both adults (100, 102, 134) and children (118. 135). The QFT

In Tube has since been replaced by the QFT Plus. This new version of the test (QFT Plus), unlike the QFT In Tube which had three tubes namely; nil control, TB antigen and mitogen, contains a second TB antigen tube. Furthermore, the TB antigen tube in the QFT In Tube system contained ESAT-6, CFP10 and TB 7.7 peptides, but the QFT Plus only contains CFP-10 and ESAT-6 peptides (123) with the TB antigen (TB1) tube containing long peptides that elicit CD4 cell immune responses and the TB2 tube containing short and long peptides which elicit CD4 and CD8 T cell immune response (139).

Given that host immunological biomarkers detected in QFT In Tube supernatants were shown to have potential as diagnostic biomarkers for TB disease in several studies, reviewed in (136), it is not certain whether the findings from these studies can be replicated in supernatants from the QFT Plus system. Furthermore, as the QFT Plus is reportedly more accurate in the diagnosis of *M. tb* infection than the QFT In Tube, owing to the inclusion of the second antigen tube (122, 130), it is not known whether the use of alternative biomarkers detectable in QFT Plus supernatants will perform better in the diagnosis of TB disease than what was obtained with the QFT In Tube system.

The aim of the present study was therefore to evaluate the utility of host immunological biomarkers that have previously been shown to possess potential as biomarkers for active TB or discrimination between LTBI and active TB using the QFT In Tube system, in QFT Plus supernatants, in individuals suspected of having TB disease, recruited from a high TB endemic setting. We therefore evaluated the utility of biomarkers that have previously been shown to possess potential in the diagnosis of *M. tb* infection or disease in previous studies including studies done in serum, plasma and QFT In Tube supernatant samples (section 4A) and 19 relatively newer host biomarkers, mainly complement proteins in a subset of study participants (section 4B). The relatively newer host biomarkers (reported in section 4B) were only evaluated in a limited number of study participants because our aim was to generate preliminary data on the potential utility of these biomarkers, with the aim of evaluating promising candidates in future larger studies. The reasons for not evaluating these host biomarkers in all study participants were primarily cost related, as Luminex immunoassay kits containing these relatively newer markers are expensive.

4.2 Materials and Methods

As described in chapter 2.1 (figure 2.2), QFT Plus supernatant from a total of 120 study participants were evaluated. Sample collection and processing, diagnostic tests including QFT Plus ELISA and the reference standard were described in chapter 2 (sections 2.4 and 2.5 and 2.7.2 respectively).

4.2.1 Luminex multiplex immunoassay

As described in chapter 2, section 4.2.5, we evaluated the concentrations of 37 host biomarkers which showed potential as TB diagnostic candidates in previous studies (103, 113, 114) in the nil, TB1 and TB2 stimulated supernatants from all study participants. These host markers included apolipoprotein (Apo) A-1, Apo-CIII, complement factor H (CFH), IFN-α-2, macrophage inflammatory protein 1-alpha (CCL4/MIP-1 α), interleukin 4 (IL-4), IL-6 which were purchased form Merck Millipore (Merck Millipore, Billerica, MA, USA), and neural cell adhesion molecule-1 (NCAM-1/CD56), transforming growth factor (TGF)-α, IFN-γ, monokine induced by IFN-γ (CXCL9/MIG), p-selectin, epidermal growth factor (EGF), tumour necrosis factor superfamily member 14 (TNFSF14/LIGHT), monocyte chemotactic protein (MCP)-1 (CCL2), MCP-2 (CCL8), macrophage inflammatory protein-1 beta (MIP)-1β, vascular endothelial growth factor (VEGF), a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), CD40 ligand (CD40L) , interferon inducible T-cell alpha chemoattractant (CXCL11/ITAC-1), IFN-γ inducible protein (IP) -10 (CXCL10), tumour necrosis factor (TNF)-α, Fas , interleukin (IL)-1β, granulocyte monocyte colony stimulating factor (GM-CSF), IL-8 (CXCL8), IL-3, IL-15, IL-13, IL-2, IL-33, IL-1α, IL-10, interleukin- 1 receptor agonist (IL)-1ra, and IL-22 which were purchased from R&D Systems Inc. (Biotechne, Minneapolis, MN, USA). All experiments were run on the Bio-Plex 200 or Magpix platform (Bio-Rad Laboratories, Hercules, USA) with the Bio-Plex Manager Software (version 6.1) used for bead acquisition and analysis of median fluorescent intensity (Bio-Plex 200) or Bio-Plex MP,

followed by Bio-Plex manager 6.1 (for assays run on the Magpix). The values of the proteins in the respective quality control reagents were within the expected ranges.

4.2.2 Statistical analysis

As described in chapter 2 (section 2.9), Graph Pad Prism version 7.04 (San Diego, CA, USA) and Statistica (TIBCO Software Inc., CA, USA) were used for all statistical analysis done in this chapter. The differences in the concentrations of individual host biomarkers between TB vs. ORD groups were assessed using Mann-Whitney U-test. The diagnostic accuracy of individual biomarkers were evaluated by receiver operator characteristics (ROC) curve analysis. Youden's Index was used to determine the Cut-off values and associated sensitivity and specificity. The predictive abilities of combinations between multiple host biomarkers was assed using general discriminant analysis (GDA), with prediction accuracy ascertained by leave-one-out cross validation. $p \leq 0.05$ were considered significant.

4.3 Results

Section 4 A: Evaluation of the potential of previously identified host biomarkers as tools for the diagnosis of *M. tb* disease or infection, in QFT Plus supernatants

In this section, we evaluated the utility of the 37 host biomarkers which previously showed potential in serum, plasma or QFT In Tube supernatants as potential biomarkers, in the QFT Plus supernatants. Our primary aim was to determine whether biomarkers that previously showed potential in QFT In Tube supernatants would perform better in QFT Plus supernatants, given that the kit manufacturer claims that the QFT Plus has superior performance, compared to the QFT In Tube assay. We therefore also included the recently identified biomarkers (from serum and plasma based studies), to ascertain the potential of antigen-specific levels of these biomarkers in the diagnosis of TB disease or *M. tb* infection.

4.3.1 Patient characteristics

A total of 104 participants; mean age, 40 ± 12.6 years, 56 (46.7%) of whom were males were included the study (Figure 2.1) with 14 (13.5%) of the participants being HIV infected. Using the manufacturer's recommended cut-off value (≥0.3 IU/ml), 87 (72.5%) of the study participants were QFT Plus positive. We planned to use a pre-established composite reference standard that includes clinical information, laboratory (smear, GeneXpert and culture) and imaging results to classify study participants as definite TB, probable TB or ORD as done in previous studies (101,114) but surprisingly, all the 35 (29.1%) individuals that were finally diagnosed with TB were culture positive (Table 4.1).

Table 4.1. Clinical and demographic characteristics of study participants. All study participants were culture positive.

		TB disease	ORD
No. of patients	104	35 (29.1%)	69 (70.8%)
Female's n (%)	64 (53.3)	10 (28.5)	46 (66.6%)
Age mean ± SD	40.06 ± 12.58	34.8 ± 12.14	41.92 ± 12.6
HIV positive n (%)	14 (13.5%)	7 (20.0 %)	7 (10.1%)
QFT plus Positive n (%)	87 (72.5%)	27 (77.1)	49 (71%)
QFT plus Negative n (%)	32 (26.7%)	8 (22.8)	20 (28.9)
Indeterminate n (%)	1 (0.8)	0 (0)	0 (0)

QuantiFERON results were obtained and defined as positive, negative, and indeterminate by using the manufacture's software. Abbreviations: TB= Tuberculosis, ORD= other respiratory disease, SD= Standard deviation, HIV= Human immunodeficiency virus, QFT= QuantiFERON TB® Gold plus.

4.3.2 Utility of individual host markers in the diagnosis of TB

We assessed the usefulness of all 37 host markers that showed potential in previous studies (99-107) in QFT Plus supernatants using the Mann Whitney U test. The

concentrations of the host markers detected in the nil (unstimulated) supernatants and the TB1 and TB2 antigen-specific values (TB1 minus nil and TB2 minus nil) respectively, were evaluated separately so as to assess the potential usefulness of markers detected in the nil supernatants as done in previous studies (100, 113).

4.3.3 Host markers detected in unstimulated (nil) supernatants

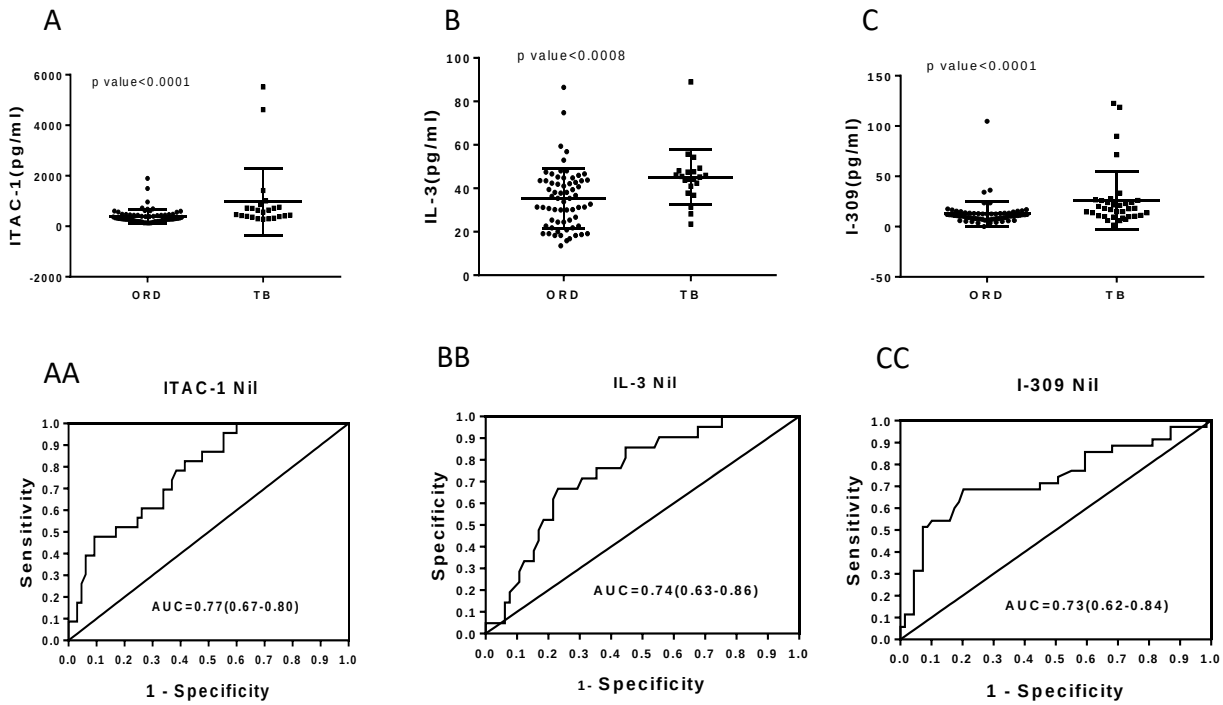
When the values obtained in nil supernatants from TB patients were compared to responses obtained in the individuals with other respiratory diseases (ORD), regardless of HIV infection status, significant differences (P≤0.05) were obtained in the concentrations of 14 of the 37 markers. The median concentrations of ITAC-1, IL-3, I-309, MIG, Apo-A1, ADAMTS13, GM-CSF, IL-22, and TGF-α were significantly higher in the TB patients whereas, the concentrations of IL-1α, IL-33, Apo-A1, and IL-2 were significantly higher in the ORD group (Table 4.2). When the diagnostic accuracy of the markers detected in the nil supernatants were assessed using ROC curve analysis, the areas under the ROC curves (AUCs) were all ≥0.65 for the same 14 analytes, with ITAC-1, IL-3, I-309, and MIG being the most accurate individual markers as determined by AUCs > 0.70 (Figure 4.1). When only the HIV uninfected individuals were considered, only the same 14 host markers showed significant differences (p≤0.05) between the two groups.

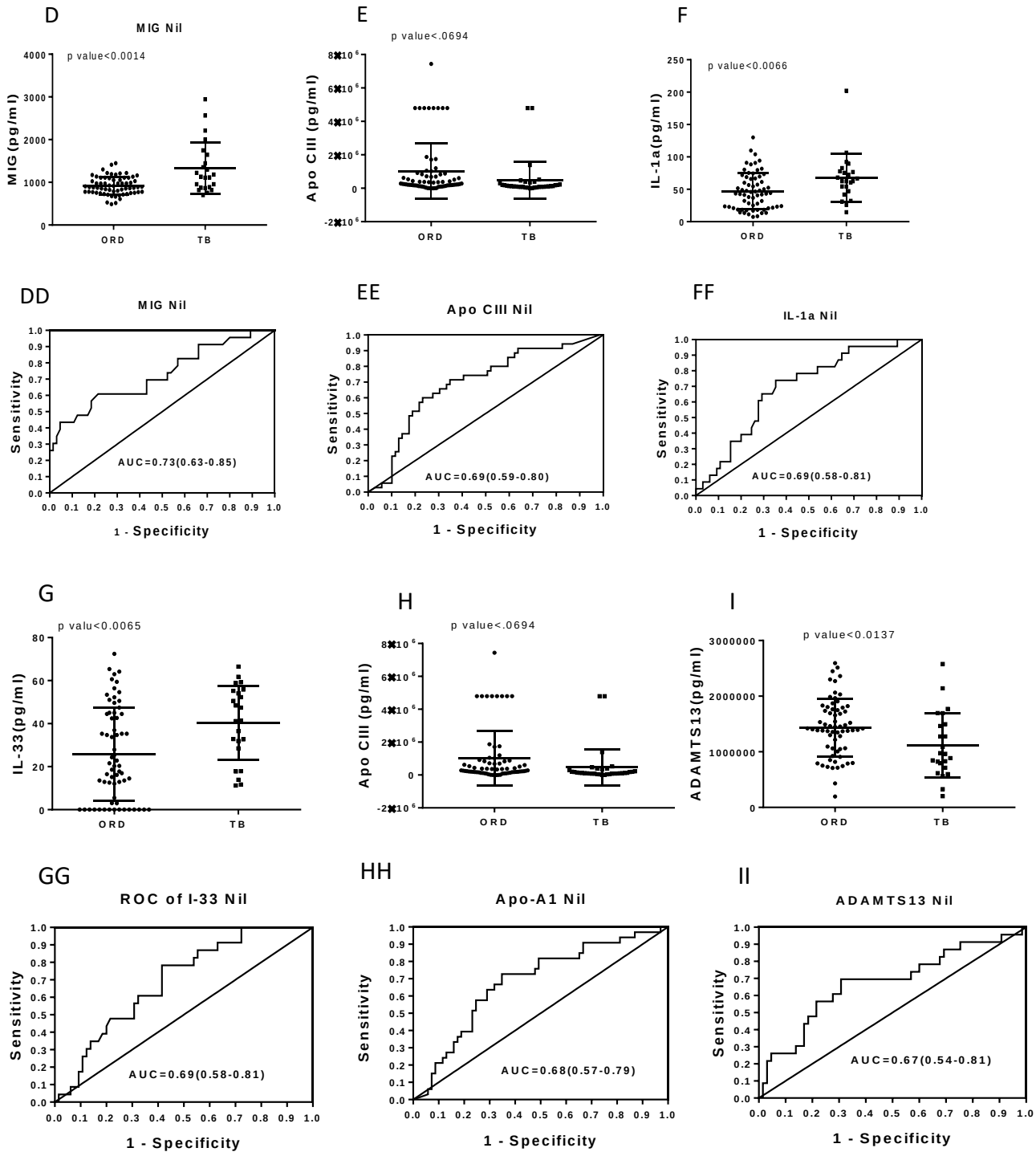
Table 4.2: Median (Inter-quartile ranges in parenthesis) levels and diagnostic accuracies of host biomarkers detected in unstimulated QFT Plus supernatants from individuals with TB disease (n=35) and other respiratory diseases (n=69).

Host marker (pg/ml, ng/ml)	ORD (IQR)	TB (IQR)	P value	AUC (95 %CI)	Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)
ITAC-1	310 (238-450)	561 (381-753)	0,0001	0.77 (0.67-0.80)	>381.70	78 (56-92)	62 (48-73)
IL-3	35 (24-43)	44 (38-48)	0,0008	0.75 (0.63-0.86)	>40.85	76 (53-92)	65 (52-76)
I-309	11 (8-13)	18 (10-26)	0,0001	0.73 (0.62-0.84)	>11.09	71 (54-85)	55(43-67)
MIG	890 (772-1095)	1133 (873-1645)	0,0014	0.73 (0.60-0.85)	>940.30	70 (47-87)	57 (44-69)
Apo CIII	281738 (169471-838212)	122386 (80337-295193)	0,0013	0.69 (0.59-80)	<203602	71 (54-85)	65 (53-76)
IL-1α	43 (23-67)	62 (47-78)	0,0066	0.69 (0.57-0.81)	>50.87	74 (52-90)	62 (49-73)
IL-33	20 (3-45)	41 (29-55)	0,0065	0.69 (0.58-0.81)	>28.20	78 (59-92)	58 (45-71)
Apo-A1	710225 (394468-1525800	346446 (190034-653295	0,0032	0.68 (0.57-0.59)	<536691	73 (54-87)	65 (53-76)
ADAMTS 13	1418300 (1061200-1762700)	71289 (71289-1496500)	0,0137	0.67 (0.54-0.81)	<203602	67 (47-86)	64 (57-80)
IL-2	1180 (563-1552)	1555	0,0145	0.67 (0.55-0.76)	>1363	70 (47-87)	65 (50-75)

		(1275-1707)					
NCAM-1	126811 (19041-154833)	100864 (75770-132990)	0,0047	0.67 (0.56-0.78)	<124132	72 (53-86)	54 (41-66)
GM-CSF	37 (23-56)	62 (25-94)	0,0134	0.65 (0.53-0.77)	>40.81	71 (54-85)	55 (43-65)
IL-22	28 (15-41)	36 (31-44)	0,0389	0.65 (0.53-0.77)	>29.12	90 (69-99)	57 (44-96)
TGF- α	23 (18-34)	31 (19-51)	0,0213	0.64 (0.53-0.76)	>28.32	64 (45-80)	65 (53-76)

Only biomarkers which showed significant differences or trends in the Mann Witney U test are shown in the table. AUC= Area under the ROC curve, CI= Confidence interval.





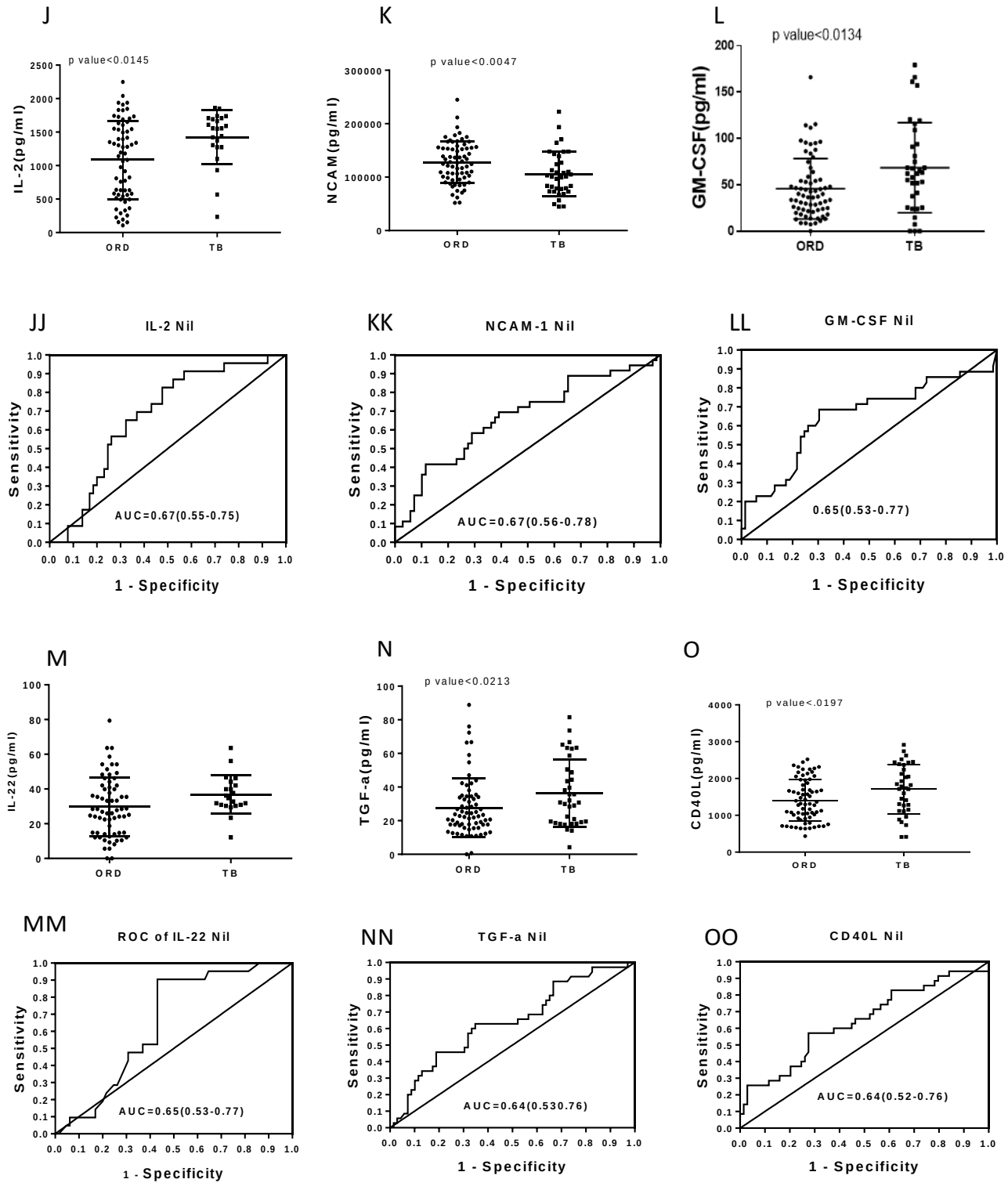


Figure 4.1: Concentrations of host biomarkers detected in unstimulated (Nil) QFT plus supernatants from individuals with TB disease and individuals with other respiratory diseases (graphs A-O). Receiver operator characteristic curves showing the accuracies of with these host biomarkers are shown (graphs AA-OO),

where AA is the ROC curve for the marker shown in figure A, BB, that for the marker in figure B.

4.3.4 Host markers detected in TB1 antigen stimulated supernatants

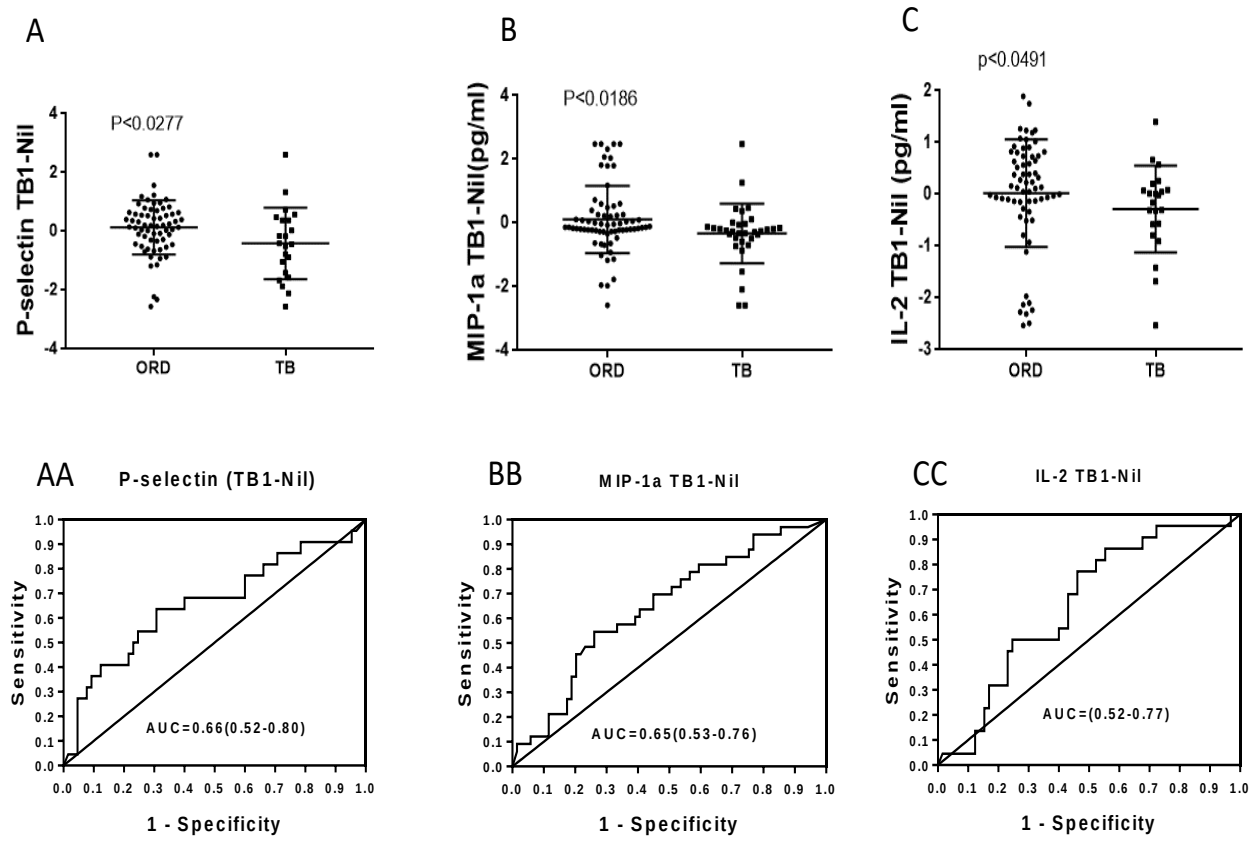
When the TB1 antigen-specific levels of the host markers were compared between the TB and ORD groups, median levels of MIP-1α, LIGHT, and IL-13 were significantly higher in the TB patients whereas those of P-selectin, IL-2, IL-10 and GM-CSF were higher in ORD group (Table 4.3). When ROC curve analysis was done, seven of the 37 markers discriminated between the two groups with AUC ≥0.60, with MIP-1α, p-selectin, and IL-2 being the most accurate TB1-speficic host markers (Figure 4.2).

Table 4.3: Median levels (inter-quartile ranges in parenthesis) of QFT Plus TB1-antigen-specific host markers as detected in individuals with TB or other respiratory diseases and accuracies in the diagnosis of TB.

Host marker (pg/ml)	ORD (IQR)	TB Disease (IQR)	P value	AUC (95% CI)	Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)
P-selectin	0.197 (-0.3806-0.6405)	-0.4173 (-1.447-0.4049)	0.0277	0.66 (0.52-0.80)	0.04329	68 (45-86)	60 (47-72)
MIP-1α	-0.1339 (-0.2646-0.3281)	0.2562 (-0.537-0.04445)	0.0816	0.6445 (0.53-0.76)	-0.1554	70 (51-84)	55 (43-67)
IL-2	-0.137 (-0.2132-0.7377)	-0.088795 (-0.0341-(-0.1149)	0.0491	0.6409 (0.52-0.74)	0.4564	68 (45-86)	60 (44-69)
LIGHT	-0.0934 (-0.7485-0.7281)	-0.4049 (-0.792-0.1191)	0.0794	0.63 (0.50-0.73)	-0.2076	71 (48-89)	52 (40-65)

IL-13	-0.0892 (0.2827.518)	-0.2463 (-0.518-0.0051)	0.0484	0.6212 (0.51-0.57)	-0.128	70 (0.51-0.84)	54 (41-66)
GM-CSF	0.2213 (-0,3758-0.2015)	-0.2611 (-0.7483-0402)	0.05834	0,6228 (0.51-0,73)	-0.02804	66 (48-81)	62 (50-74)
IL-10	0.2727 (-0.2287-0.7325)	0.00814 (-0.4544-0.2281)	0.0548	0.6179 (0.51-0.73)	0.2201	73 (55-88)	52 (40-64)

Only biomarkers which showed significant differences or trends between the two groups using the Mann Witney U test are shown. Cut-off values were selected based on the Youden's index. The negative values shown indicate that the concentrations of the host biomarkers detected in the QFT Plus TB1 tube were lower than values obtained in the unstimulated tube. AUC= Area under the ROC curve, CI= Confidence interval.



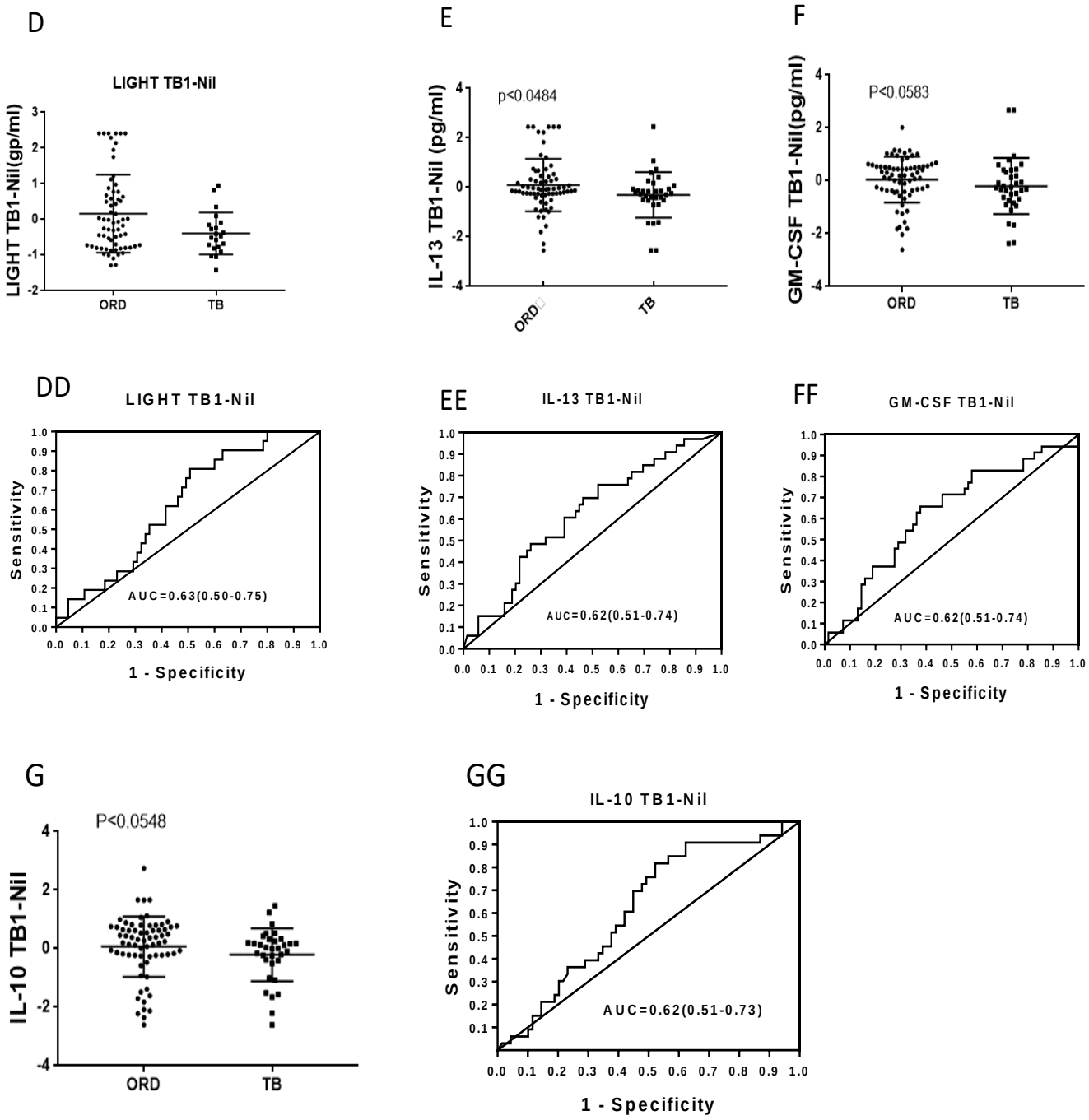


Figure 4.2: Concentrations of host biomarkers detected in QFT Plus TB1 supernatants. Representative plots for the antigen-specific levels of the host markers (TB1-nil) that showed significant differences with the Mann Whitney U test ($p \leq 0.05$) are shown in graphs A to G and receiver operator characteristic curves showing accuracies of these markers in diagnosing TB disease are shown in figures AA-GG). The negative values in figures A to G indicate that the unstimulated levels of the specific analyte were higher than the values obtained in the TB1 antigen-stimulated samples.

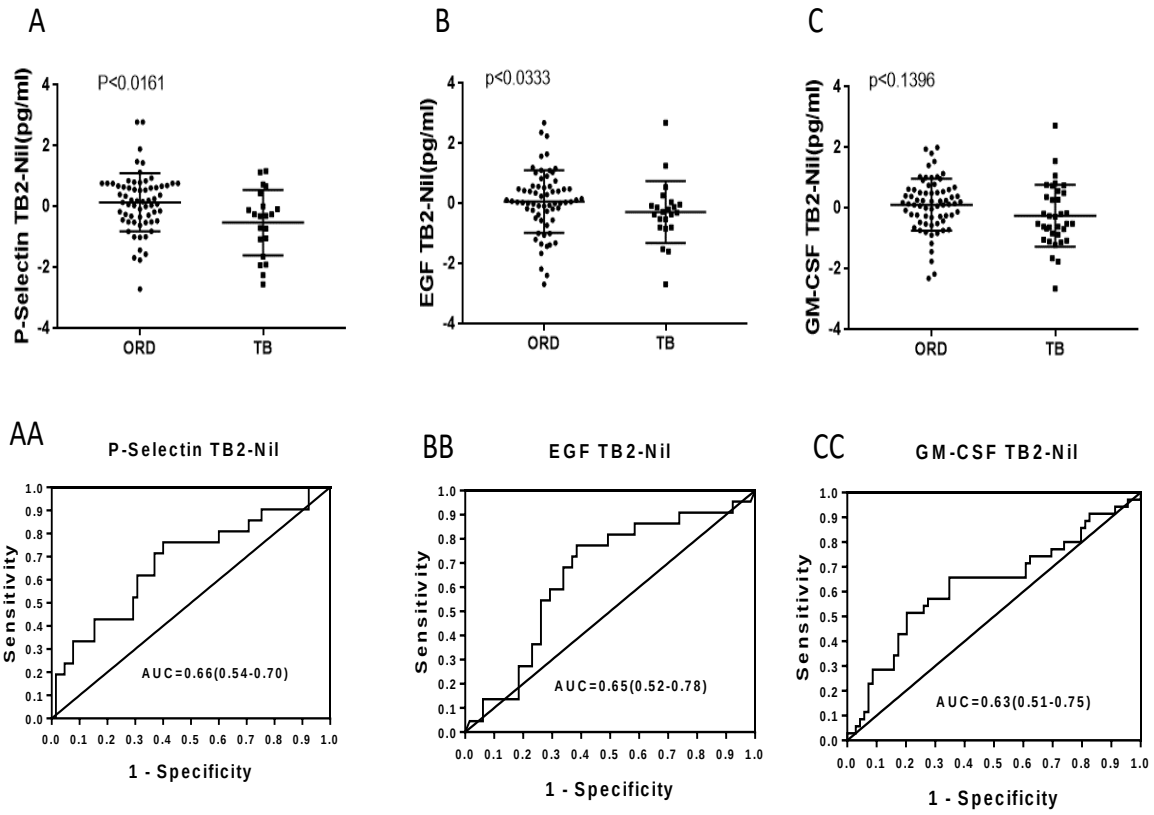
4.3.5 Host markers detected in TB2 antigen stimulated supernatants

When the TB2-antigen-specific concentrations of the host markers were compared between patients with TB Vs. ORD, the median levels of IL-13, TGF-α, and MIP-1α were higher in the TB group whereas p-selectin, EGF, IL-10, and GM-CSF levels were higher in the ORD group (Table 4.4). EGF and p-selectin were the most accurate individual TB2-antigen-specific markers with AUCs of 0.66 (95%CI, 0.54-0.81) and 0.65 (95% CI, 0.52-0.78) respectively (Figure 4.3).

Table 4.4. Median levels (interquartile ranges in parenthesis) and diagnostic accuracies for host biomarkers detected in TB2 stimulated QFT Plus sups from individuals with TB disease or other respiratory diseases.

Marker (pg/ml)	ORD (IQR)	TB (IQR)	P value	ACU (95% CI)	Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)
P-selectin	0.1928 (-0.402-0.7307)	-0.2961 (-1.35-0.2293)	0,0161	0.66 (0,54-0.81)	0.0309	76 (53-92)	60 (47-72)
EGF	0,6551 (-0.4189-0.5737)	0.2845 (0.7855-0.0088)	0.0333	0.65 (0.52-0.78)	-0.0172	77 (55-92)	62 (49-73)
GM-CSF	0.1559 (-0.3593-0.6323)	-0.1459 (-0.6546-0.1884)	0.0327	0.63 (0.51-0.75)	-0.1396	66 (48-81)	65 (53-75)
IL-10	0.1892 (-0.5080-0.6323)	-0.5043 (0.8695-0.5049)	0.0384	0.63 (0.51-0.74)	0.0988	73 (54-88)	61 (48-72)
TGF-α	0.086 (0,8910-0.3405)	0.152 (-0.5266-0.9270)	0,02068	0.56 (0.45-0.70)	-0.0823	60 (38-63)	51 (38-63)

Values shown are TB2 antigen-specific levels, obtained by subtraction of the nil from the TB2 antigen stimulated values. Only biomarkers which showed significant differences between the two groups with $p<0.05$ in the Mann Witney U test are shown. AUC= Area under the ROC curve, CI= Confidence interval.



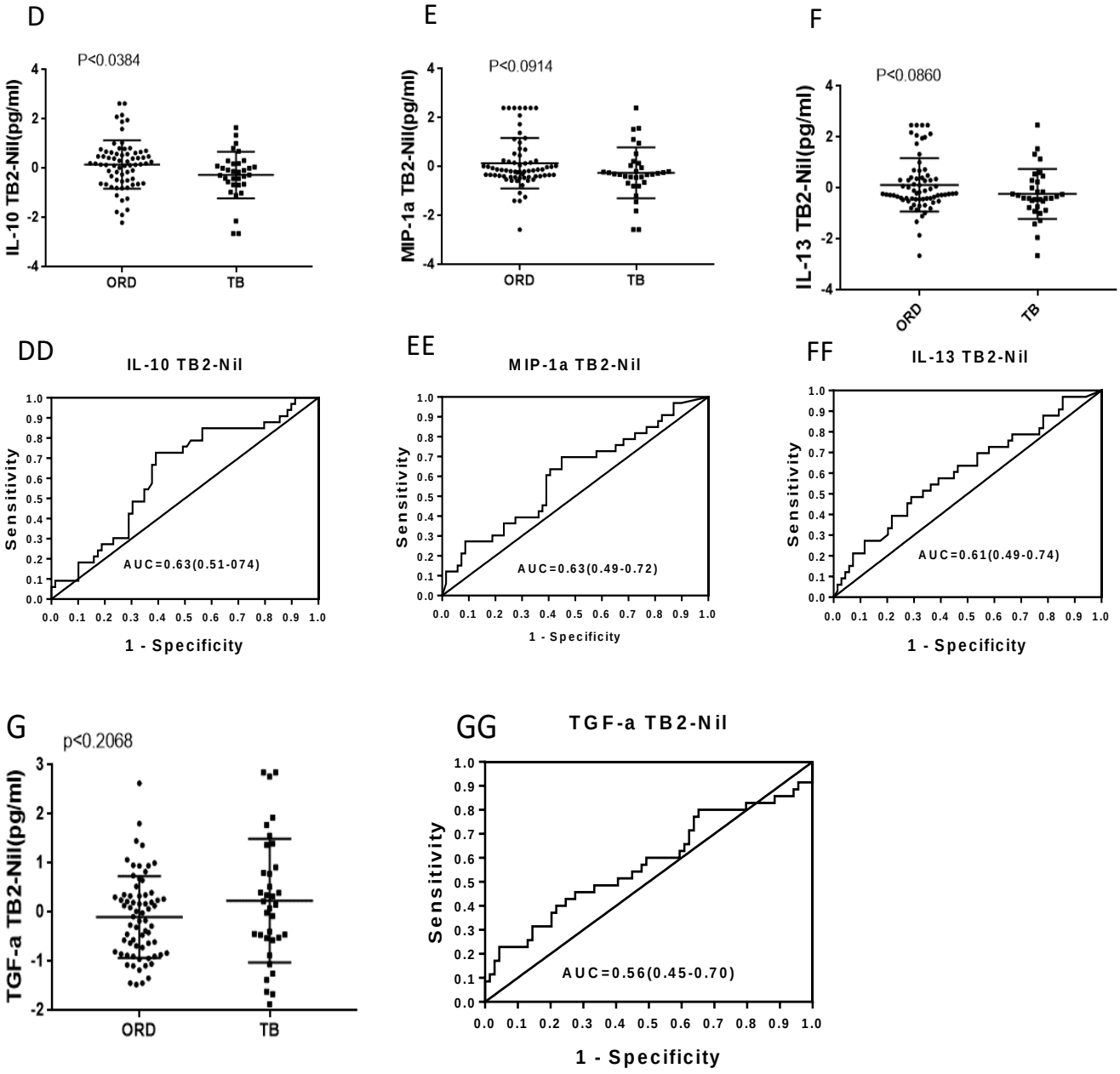


Figure 4.3: Concentrations of host biomarkers detected in TB2 stimulated QFT Plus supernatants from individuals with TB disease and individuals with other respiratory diseases (graphs A-G) and receiver operator characteristic curves showing accuracies of these markers in diagnosing TB disease. Representative plots for markers with AUC >0.50 are shown (graphs AA-GG).

4.3.5 Utility of combinations between different biomarkers in the diagnosis of TB

We evaluated the utility of combinations between host biomarkers detected in culture supernatants from the different stimulation conditions alone by general discriminant analysis (GDA), followed by evaluation of biosignatures comprising of combinations between biomarkers derived from the antigen stimulation conditions (TB1-nil and TB2-nil together).

When we evaluated the usefulness of combinations between biomarkers detected in **unstimulated supernatants**, regardless of HIV infection status of study participants, a six-maker biosignature comprising of Apo-A1, ITAC-1, I-309, MIG, MCP-2 and NCAM diagnosed TB with an AUC of 0.91 (0.88-0.97) , corresponding to a sensitivity and specificity of 73.9% (95% CI, 51.6-89.8%) and 87.61% (95% CI, 77.2-94.5%) respectively (Table 4.5). After leave-one-out cross-validation, the sensitivity of the biosignature was 73.91% (95% CI, 51.6-89.8%) and specificity was 86.2% (95% CI, 75.3-93.5%). The positive and negative predictive values (PPV and NPV) of the biosignature after leave-one-out cross validation were 68.0% (95% CI, 51.5-81.0%) and 90.5% (95% CI, 82.6-91.0%) respectively. When only the HIV uninfected participants were considered, a four-marker (unstimulated) biosignature comprising of Apo-CIII, I-309, MIG and NCAM similarly diagnosed TB disease with an AUC of 0.91 (0.85-0.98) , corresponding to a sensitivity of 82.4% (95% CI, 56.6-96.2%) and specificity of 87.3% (95% CI, 76.5-94.4%) and with sensitivity and specificity of 76.5% (95% CI, 50.1-93.2%) and 85.7% (95% CI, 74.6-93.3%) respectively after leave-one-out cross validation. The PPV and NPV after leave-one-out cross validation were 59.1% (95% CI, 42.8-73.4%) and 93.1% (95% CI, 85.0-96.8%) respectively.

When the **TB1 antigen-specific marker values** were similarly fitted into GDA models, regardless of HIV infection status, a four-marker signature comprising of Apo-CIII, I-309, MIP-1α and TNF-α diagnosed TB disease with an AUC of 0.72 (95% CI, 0.61-0.88), with sensitivity and specificity of 62.9% (95% CI, 44.9-78.2%) and 73.3% (95% CI, 66.7-87.3%) respectively . After leave-one-out cross validation, the sensitivity and specificity of the biosignature were 54.3% (95% CI, 36.7-71.2%) and 72.5% (95% CI,

60.4-82.5%) respectively (Figure 4.4). The predictive values after leave-one-out cross validation are shown in table 4.5.

When **TB-2 antigen-specific markers** were fitted into GDA models, again regardless of HIV infection status of participants, a four-maker biosignature comprising of IL-6, MIP-1a, GM-CSF, TGF-α diagnosed TB disease with an AUC of 0.72 (95% CI, 0.60-0.84), corresponding to a sensitivity 60.0% (95% CI, 42.0-76.1%) and specificity of 79.1% (95% CI, 68.3-88.4%) (Table 4.5). After leave-one-out cross validation, the sensitivity of the biosignature was 57.1% (95% CI, 65.1-86.1%) and the specificity was 71.6% (95% CI, 39.4-73.7%). The PPV and NPV of the signature after cross validation were 60.0% (95% CI, 46.9-72.0%) and 79.7% (95% CI, 63.5-81.3%) respectively (Figure 4.4).

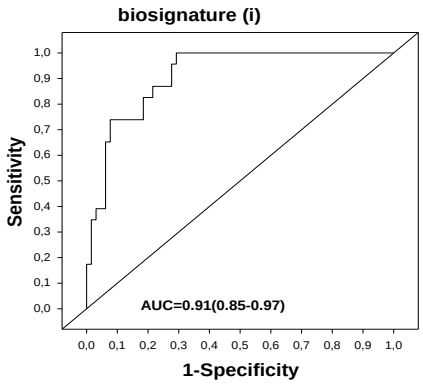
When we evaluated **combinations between TB1 and TB2 antigen-specific markers**, again, regardless of HIV infection status, a four-marker biosignature comprising of TNF-α, LIGHT, MIG, and p-selectin diagnosed TB disease with sensitivity and specificity of 77.3% (95% CI, 54.6-92.2%) and 72.3% (95% CI, 59.8-82.7%) respectively (Table 4.5). After leave-one-out cross validation, the sensitivity and specificity of the biosignature were 77.3% (95% CI, 54.5-92.2%) and 69.2% (95% CI, 56.6-80.1%) respectively (Figure 4.4).

Table 4.5: Summary of biosignatures [] in unstimulated, TB1, TB2 and TB1&TB2 stimulated supernatants) identified in the current study. AUC = Area under the ROC curve, Spec= Specificity, Sens= Sensitivity, PPV= Positive predictive values, NPV= Negative predictive value.

Biosignature	**AUC (95% CI)**	**Classification matrix**		**Leave-one-out cross validation**			
		Sens (%)	**Spec (%)**	**Sens (%)**	**Spec (%)**	**PPV (%)**	**NPV (%)**
***Biosignature (i)**: Markers detected in unstimulated (nil) supernatants, regardless of HIV infection*							
APO-CII+CXCL1/ITAC-1+CXCL9/MIG+CCL	0.91	73.9 (17/23)	87.3 (57/65)	73.9 (17/23)	86.2 (56/65)	68.0 (51.2-80.9)	90.5 (82.6-95.0)

8/MCP-2+I-309+NCAM-1/CD56	(0.88-0.97)						
***Biosignature (ii)**: Markers detected in unstimulated (nil) supernatants, only in HIV uninfected participants*							
CXCL9/MIG+CCL1/I-309+CCL8/MCP-2+NCAM-1/CD56	0.91 (0.85-0.98)	82 (14/17)	87 (55/63)	76.5 (13/17)	85.7 (54/63)	59.1 (42.1-73.6)	93.1 (85.1-97.0)
***Biosignature (iii)**: TB1 antigen-specific biomarkers (TB1 Ag-minus nil), regardless of HIV infection status*							
CFH+CCL1/I-309+CCL4/MIP-1α+TNF-α	0.72 (0.61-0.88)	62.9 (22/35)	78.3 (54/69)	54.3 (19/35)	72.5 (50/69)	60.0 (46.7-71.1)	80.6 (72.6-86.7)
***Biosignature (iv)**: TB2 antigen-specific biomarkers (TB2 Ag-minus nil), regardless of HIV infection status*							
IL-6+CCL4/MIP-1α+GM-CSF+TGF-α	0.72 90.60-0.84)	60.0 (21/35)	79.7 (55/69)	57.1 (15/35)	76.7 (53/69)	60.0 (46.6-72.0)	79.7 (72.0-85.7)
***Biosignature (v)**: Combinations between TB1 and TB2 antigen-specific biomarkers, regardless of HIV infection status*							
TNFα+LIGHT+CXCL9/MIG+P-Selectin	0.84 (0.73-0.95)	77.3 (17/22)	72.3 (47/65)	77.3 (17/22)	69.2 (45/65)	48.6 (37.5-59.8)	81.1-95.4)
***Biosignature (vi)**: Combinations between TB1 and TB2 antigen-specific biomarkers, only in HIV uninfected patients*							
ADAMTS13+CCL1/I-309+CCL4/MIP-1α+p-selectin	0.84 (0.73-0.95)	62.5 (10/16)	82.5 (52/63)	56 (9/16)	76 (48/63)	37.5 (24.4-52.7)	87.3 (79.5-92.4)

A



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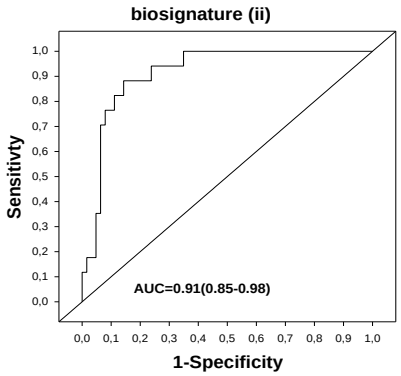

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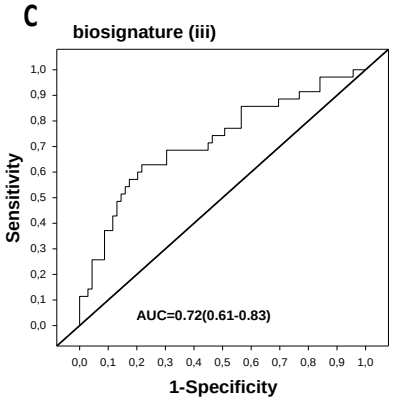

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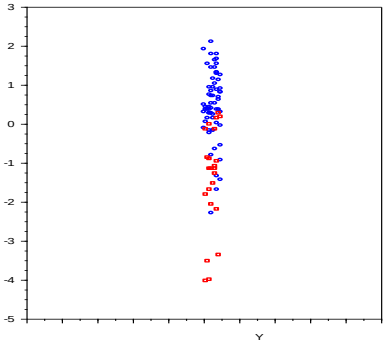

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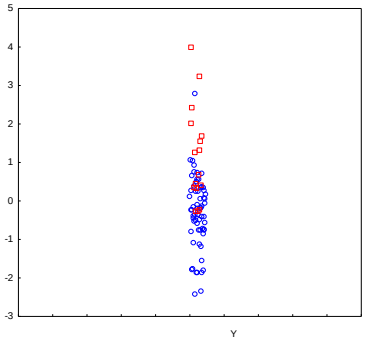

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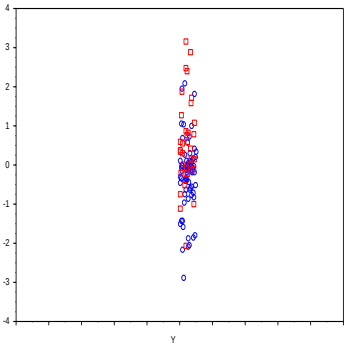

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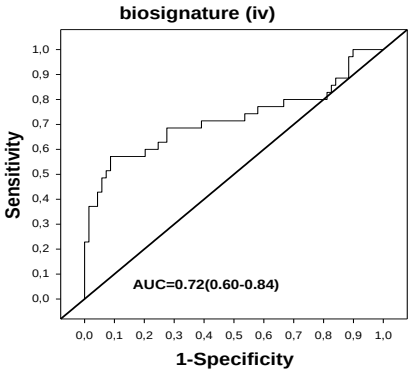

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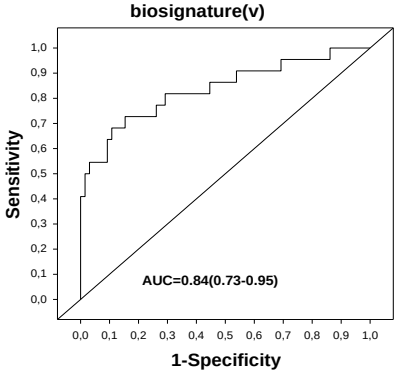

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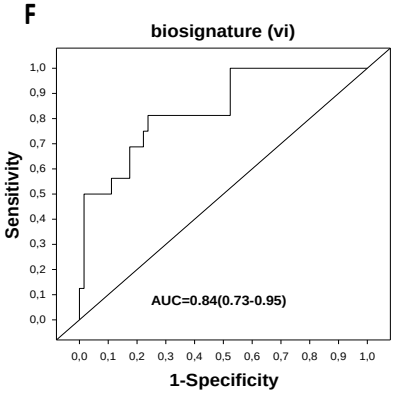

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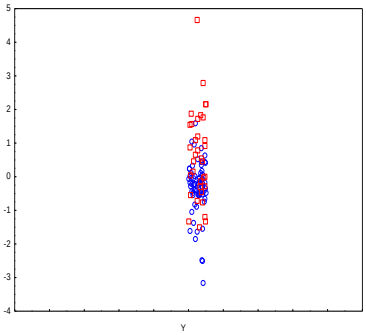

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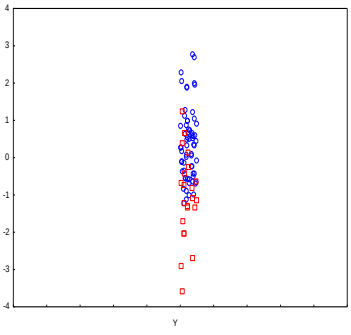

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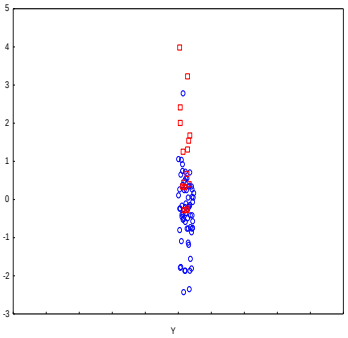

Figure 4.4. Usefulness of combinations of host markers detected in QFT Plus supernatants in diagnosis of TB disease. ROC curves and scatter plots showing the accuracies of the different biosignatures described in the text and table 4.5. The red squares in the scatter plots represent TB group and the blue squares represent ORD group from the scatter plots. A, ROC curve showing the accuracy of biosignature (i): combinations between unstimulated host markers regardless of HIV infection, and AA, scatter plot showing the distribution of TB patients and those with ORD as classified by biosignature (i). B, ROC curve showing the accuracy of biosignature (ii): combinations between unstimulated markers in HIV uninfected individuals and BB, scatter plot showing the distribution of TB patients and those with ORD as classified by biosignature (ii). C, ROC curve showing the accuracy of biosignature (iii): combinations between TB1 antigen-specific host markers regardless of HIV infection, and CC, scatter plot showing the distribution of TB patients and those with ORD as classified by biosignature (iii). D, ROC curve showing the accuracy of biosignature (iv): combinations between TB2 antigen-specific host markers regardless of HIV infection, and DD, scatter plot showing the distribution of patients and those with ORD as classified by biosignature (v). E, ROC curve showing the accuracy of biosignature (v): combinations between TB1&TB2 antigen-specific host markers regardless of HIV infection, and EE, scatter plot showing the distribution of TB patients and those with ORD as classified by biosignature (v). F, ROC curve showing the accuracy of biosignature (vi): combinations between TB1&TB2 antigen-specific host markers in HIV uninfected individuals, and F, scatter plot showing the distribution of TB patients and those with ORD as classified by biosignature (vi).

4.4 Differential expression of host biomarkers detected in QFT Plus supernatants in patients with TB disease, individuals with LTBI and those without *M.tb* infection

We evaluated the ability of host markers detected in unstimulated, TB1 and TB2 antigen-specific QFT Plus supernatants to discriminate LTBI from active TB as well as *M. tb* uninfected individuals. Patients classified as active TB included all the TB patients described in previous sections of this thesis whereas those defined as LTBI

were patients in the ORD group who had positive QFT Plus results, as defined by the manufacturer. The patients with ORD, who were QFT Plus negative were classified as *M. tb* uninfected individuals for the purposes of this analysis.

Out of 37 host markers that showed potential in previous studies, 16 markers that were detected in the unstimulated supernatants were significantly different between individuals with TB and those with LTB1. Among these markers, I-309 (p=0.00006), ITAC-1 (p=0.00006), IL-3 (0.00100), IL-33 (p=0.00143), MIG (p=0.003800), and APO-CIII (p=0.0039) were the top discriminatory between the two groups, with the median concentrations of ITAC-1, I-309, IL-3 and IL-33 being higher in TB patients (Table 4.6). For the comparison between patients with active TB and the *M. tb* uninfected individuals, eight unstimulated host markers discriminated between the *M. tb* uninfected and TB disease groups including APO-A1 (p=0.0051), MIG (p=0.0061), APO-CIII (p=0.0089), ITAC-1 (p=0.0188), and IL-1α. None of the 37 analytes unstimulated analytes discriminated between LTBI and *M. tb* uninfected individuals (Table 4.6).

Table 4.6: Differential expression of host biomarkers in unstimulated (nil) culture supernatants from patients with TB disease, individuals presenting with symptoms suggestive of TB, but who had LTBI and individuals with symptoms suggestive of TB, but who were M. tb uninfected. The data shown is for all study participants, regardless of HIV infection status. Only markers that showed significant differences between any two of the three groups being compared (TB Vs LBI, TB Vs Uninfected or LTBI Vs Uninfected) are shown. p-value 1 = active TB Vs, LTBI, p-value 2 = Active TB Vs *M. tb* uninfected, p-value 3 = LTBI Vs. *M.tb* Uninfected

Variable	All groups Median (n=120)	Median in TB (n=35)	Median (LTBI, n=48)	Median (*M. tb* uninfected, n=20)	p-value (ATB Vs. LTBI)	p-value (ATB Vs uninfected)	p-value (LTBI vs *M. tb* Uninfected)
Apo-AI	522829,835 (239527,1-1010055)	340003,3 (190034,4--653295)	718436 (305090-1547050)	667845 (485276,2-1373600)	0,0071	0,0050	0,9480

Apo-CIII	199290 (102457,5-490713)	122386,4 (80336,6-295193	279161 (131266-857590)	335950 (176600,3-832119)	0,0039	0,0089	0,9479
ADAMTS13	1385550 (923210,4-1696100)	963729,3 (712891,4 - 1496500)	1448300 (1061200-1736400)	1389450 (957148,0-1908100)	0,0159	0,0787	0,9490
CD40L	1440 (1009,8-2043)	1738,3 (1153,3-2371)	1411 (834-1786)	1274 (1068,6-1955)	0,0266	0.0989	0,8296
ITAC-1	362 (258,7-561)	560,6 (382,9-753)	300 (204-438)	390 (258,2-560)	<0.0001	0,018776	0,2109
IL-2	1278 (611,3-1610)	1554,8 (1274,8-1707)	1180 (562-1546)	1084 (597,4-1619)	0,0210	0,0559	0,8256
IL-33	27 (12,9-47)	41,4 (28,5-55)	19 (3-35)	34 (15,7-54)	0,0014	0,3298	0,1157
I-309	11 (8,1-16)	17,8 (10,4-26)	10 (7-13)	12 (9,0-15)	<0,0000	0,0329	0,1423
GM-CSF	46 (24,7-75)	62,2 (25,5-94)	35 (22-55)	40 (23,3-68)	0,0144	0,1154	0,6294
IL-1a	47 (26,0-72)	62,5 (47,2-78)	43 (21-67)	42 (24,6-66)	0,0153	0,0170	0,8926
IL-10	14 (5,8-33)	16,5 (7,6-36)	12 (3-23)	14 (6,2-38)	0,0406	0,4463	0,2876
IL-22	31 (22,4-42)	36,2 (30,8-44)	26 (15-40)	29 (19,0-43)	0,0167	0,0859	0,5552
MIG	919 (787,6-1133)	1133,0 (873,1-1645)	925 (777-1108)	885 (762,9-1000)	0,0038	0,0062	0,4384
IL-3	39 (30,0-45)	44,3 (38,1-48)	31 (22-43)	35 (30,9-43)	0,0010	0,0250	0,3976

NCAM-1	113070 (88636,8-151756)	101134,9 (78004,8-139029)	120588 (99924-153382)	138978 (85579,6-166486)	0,0279	0,0129	0,2593
TGF-α	24 (17,8-39)	30,8 (19,1-51)	22 (16-32)	25 (17,9-34)	0,0227	0,1194	0,4188

When the host markers detected in supernatants after stimulation with the TB1 antigens (TB1-nil) were compared between the three groups, fourteen markers discriminated between active TB and LTBI with significant p-values including IL-13 (p=0.0061), MIP-1α (0.0058), IL-3 (p=0.0216), GM-CSF (p=0.0258), IL-1α (p=0.0333) and IL-10 (p=0.0447, with the levels of IL-10 and GM-CSF being higher in patient with active TB/LTBI. For the comparison between patients with active TB and *M. tb* uninfected individuals, IP-10 (p=0.0332) and p-selectin (p=0.03665) were surprisingly the only two markers which significantly differentiated between the two groups. Furthermore, for comparison between individuals with LTBI and the *M. tb* uninfected individuals, IFN-γ (p=0.02734), IL-13 (p=0.02284), IL-22 (p=0.0265), MCP-2 (p=0.0113), and ITAC-1 (p=0.0114) were markers which showed significant difference with levels of IL-13, IL-2 and ITAC-1 being high in LTBI/*M. tb* (Table 4.7).

Table 4.7: Differential expression of TB1 antigen-specific host biomarkers in culture supernatants from patients with TB disease, individuals presenting with symptoms suggestive of TB, but who had LTBI and individuals with symptoms suggestive of TB, but who were M. tb uninfected. The data shown is for all study participants, regardless of HIV infection status. Only markers that showed significant differences between any two of the three groups being compared (TB Vs LBI, TB Vs

Uninfected or LTBI Vs Uninfected) are shown. p-value 1 = active TB Vs, LTBI, p-value 2 = Active TB Vs. *M. tb* uninfected, p-value 3 = LTBI Vs. *M.tb* Uninfected

Variable	All groups Median (n=120)	Median in TB (n=35)	Median (LTBI, n=48)	Median (uninfected, n=20)	p-value (ATB Vs. LTBI)	p-value (ATB Vs uninfected)	p-value (LTBI vs *M. tb* Uninfected)
MCP-1	-0,072868 (-0,282325-0,459761)	-0,067396 (-0,29333-0,258874)	0,000797 (-0,230440-0,758232)	-0,228515 (-0,47440-0,255531)	0.2307	0.3269	0.0449
CD40L	0,098992 (-0,372639-0,607542)	0,004483 (-0,68304-0,385254)	0,250728 (-0,173490-0,817956)	-0,203741 (-1,03025)-0,352699	0.0592	0.5590	0.0423
IFN-γ	-0,120690 (-0,345800-0,470378)	-0,076108 (-0,38603-0,612980)	-0,031872 (-0,255192-0,397685)	-0,225043[(-0,62042-(-0,156615)]	0.5096	0.1248	0.02734
IL-13	-0,158508 (-0,419695-0,267908)	0,005612 (-0,34379-0,348592)	-0,057102 (-0,289931-0,661018)	-0,296560-(-0,56801) (-0,080530)	0.0061	0.8819	0.2284
IL-2	0,046608 (-0,336224-0,630012)	-0,280739 (-0,52764--0,066524)	-0,013665 (-0,250622-0,602939)	-0,245551 (-0,90206-(-0,075036)	0.0216	0.9243	0.0301
MIP-1α	-0,161030 (-0,290859-0,206659)	-0,112931 (-0.29086-0.562437)	-0,191948 (-0,310707-0,246760)	-0,210221 (-0.27332-(-0.079519)	0.0058	0.4944	0.0861

GM-CSF	0,089219(-0,444020-0.526170)	-0,219415 (-0,74830-0,401807)	0,330827 (-0,335770-0,626016)	-0,253817(-0.062542-0.484057_	0.0258	0.8897	0.0996
IL-1α	0,051399 (-0,496773-0,516707)	-0,156397 (-0,99778-0,263018)	0,127250 (-0,255514-0,657574)	-0,115602 (-1,30341-0,294889)	0.0332	0.8244	0.0630
IL-10	0,143093 (-0,247530-0,616650)	0,018938 (-0,40113-0,256452)	0,373737 (-0,223484-0,731481)	-0,063260 (-0,52430-0,526355)	0.0447	0.9763	0.0655
IL-22	0,021480 (-0,358551-0,721595)	-0,016114 (-0,79408-0,085347)	0,231700 (-0,095873-0,807096)	-0,149744 (-1,11030-(0,275707)	0.3575	0.2746	0.0265
MCP2	-0,451233 (-0,609290-0,132487)	0,005531 (-0,47405-0,727645)	0,257143 (-0,238657-0,776592)	-0.0233158(-1,18681-0,201871)	0.0899	0.3922	0.0113
ITAC-1	-0,154998 (-0,357620-0,212799)	-0,417857 (-0,61839--0,213829)	-0,316357 (-0,563309-1,097509)	-0,576163 (-0,62093-(-0,281495)	0.8984	0.1282	0.0114
IP-10	-0.225249 (-0.243271-(-0.148163)	-0.23852-(-0.180209)	-0.221627 (-0.242848-(-0.133254)	-0.240422 (-0.24708-(-0.225705)	0.5699	0.0332	0.2179
P-selectin	0.115645 (-0.534426-0.581989)	0.099251 (-0.449230-0.581989)	-0.43127 (-0.40690-0.484360)	0.400482 (0.11565-0.704964)	0.0638	0.0365	0.0655

We then evaluated the ability of TB2 antigen-specific host markers (TB2-nil) to discriminate TB form LTBI. Nine host markers discriminated TB from LTBI including IL-6 (0.0489), IL-13 (0.0041), GM-CSF (0.00300), IL-10 (0.0138), IL-2 (0.0112), and MIP-1α (0.0139). For the comparison between patients with active TB and the *M. tb* uninfected individuals, three TB2-stimulated host markers discriminated between the *M. tb* uninfected and TB disease groups including IFN-γ (p=0.0366) and P-selectin (p=0.0385) (Table 4.8). Furthermore, for comparison between individuals with LTBI and the *M. tb* uninfected individuals, IFN-γ (p=0.0031), MIP-1α (p=0.0200), IL-22 (0.0049), IL-13 (p=0.0016), and GM-CSF (p=0.0114) were markers which showed most significant between the two groups. The most useful host markers in discriminating between groups, regardless of whether they were detected in unstimulated, TB1 or TB2 antigen-stimulated samples included IL-10, GM-CSF and IL-2.

Table 4.8: Differential expression of TB2 antigen-specific host biomarkers in culture supernatants from patients with TB disease, individuals presenting with symptoms suggestive of TB, but who had LTBI and individuals with symptoms suggestive of TB, but who were M. tb uninfected. The data shown is for all study participants, regardless of HIV infection status. Only markers that showed significant differences between any two of the three groups being compared (TB Vs. LBI, TB Vs. Uninfected or LTBI Vs Uninfected) are shown. p-value 1 = active TB Vs, LTBI, p-value 2 = Active TB Vs. *M. tb* uninfected, p-value 3 = LTBI Vs. *M.tb* Uninfected

Variable	All groups Median (n=120)	Median in TB (n=33)	Median (LTBI, n=51)	Median (uninfected, n=20)	p-value (ATB Vs. LTBI)	p-value (ATB Vs *M. tb* uninfected)	p-value (LTBI vs *M. tb* Uninfected)
IL-6	-0.12443(-0.41034-0.09644)	-0.06644 (-0.17730-0.76335)	-0.14133 (-0.20570-(-0.02184)	-0.12577 (-0.14793-0.785911)	0.0489	0.3716	0.3251

IL-13	-0,25125 (-0,49942-0,36351)	-0,39921 (-0,76622-0,01781)	-0,02905 (-0,38411-0,70593)	-0,43650 (-0,78285--0,22408)	0.0041	0.0679	0.0016
IL-2	-0,07970(-0,55695-0,64166)	-0,34614 (-0,59848-(-0,06261)	0,24070 (-0,32108-0,73350)	-0,27379 (-1,03420-0,20622)	0.0112	0.6567	0.0278
CD40 L	0,098992 (-0,372639-0,607542)	0,004483 (-0,68304-0,385254)	0,250728 (-0,173490-0,817956)	-0,203741 (-1,03025)-0,352699	0.0604	0.3718	0.0112
ITAC-1	-0,154998 (-0,357620-0,212799)	-0,417857 (-0,61839--0,213829)	-0,316357 (-0,563309-1,097509)	-0,576163 (-0,62093-(-0,281495)	0.5092	0.5096	0.0075
CCL4/ MIP-1a	-0,20486 (-0,42811-0,21296)	-0,30407 (-0,67177--0,03698)	-0,05459 (-0,34047-0,73869)	-0,38316 (-0,57313--0,11101)	0.0139	08344	0.0200
GM-CSF	0,07182 (-0,60698-0,56209)	-0,27921 (-0,86950-0,50489)	0,24752 (0,22045-0078888)	-0,41737 (-0,72919-0,15589)	0.00310 .0124	0.8344	0.0031
IL-10	0,07535 (-0,53770-0,50659	-0,20582 (-0,64358-0,18449)	0,27644 (-0,42411-0.66378)	0,00654 (-0,66790-0,39388)	0.0138	0.5947	0.0049
P-selectin	0.05079 (-0.51284-0.67969)	-0.29609 (-1.06398-0.16163)	0.14514 (-0.49938-0.29609	0.20139 (-0.16630-0.63207)	0.0356	0.0385	0.8371

IFN-γ	-0,120690 (-0,345800-0,470378)	-0,076108 (-0,38603-0,612980)	-0,031872 (-0,255192-0,397685)	-0,225043[(-0,62042-(-0,156615)	0.4921	0.0366	0.0012
IL-1α	0.02143 (-0.50292-0.54729)	-0.31379 (-0.68781-0.41121)	0.1327 (-0.29773-0.60549_	-0.28833 (-0.83299-0.16161)	0.2045	0.6567	0.0452
IL-22	0,021480 (-0,358551-0,721595)	-0,016114 (-0,79408-0,085347)	0,231700 (-0,095873-0,807096)	-0,149744 (-1,11030-(0,275707)	0.0759	0.4343	0.0049

Section 4 B

4.4 Utility of the relatively new host markers evaluated in the current project as potential TB diagnostic candidates

We evaluated the concentrations of 20 additional host biomarkers, which have not frequently been evaluated in serum, plasma or culture supernatants in the TB field in this part of the study. Although some commonly evaluated host markers were part of the reagent kit panels, most of these biomarkers were mainly complement proteins and other proteins that are produced by various cell types during the immune response against infectious conditions. These biomarkers included: complement C2 (CC2), CC4b, CC5, CC5a, complement factor D (CFD/adipsin), mannose binding lectin (MBL), complement factor 1 (CF1), soluble intracellular adhesion molecule (sICAM), soluble vascular adhesion molecule (sVCAM), D-DIMER, GDF-15, Myeloperoxidase (MPO), (Lipocalin2-NGAL), Glial–cell derived neurotropic factor (GDNF), cathepsin, platelet derived growth factor (PDGDF-AA), PDGF-AB-BB, plasminogen activator inhibitor (PAI1), BDNF (brain derived neurotrophic factor and CCL3. As with section 4A, we evaluated the utility of individual analytes using the Mann Whitney U test, followed by ROC curve analysis. We did not evaluate the usefulness of combinations between these biomarkers because of the limited numbers of participants in one of the study groups as highlighted below.

4.4.1 Study participants

The individuals in whom the biomarkers evaluated in this section of the study were assessed (n= 17) had a mean age of 40.31±11.76. As study participants were selected from the biorepository and assessed in a blinded manner, 15 (88.2%) of the participants included in this pilot part of the project were finally diagnosed with TB disease, with only 3 (17%) being ORD. All the 17 study participants evaluated (Table 4.9) were a subset of the study participants described in section 4A.

Table 4.9 Clinical and demographic characteristics of study participants evaluated in section 4B of this dissertation.

	All	TB disease	ORD
No. of patients	18	15 (88.2%)	3 (17.6%)
Female's n (%)	7 (41.2%)	4 (57.1%)	2 (28.6%)
Age mean ± SD	40.31± 11.76	38.95 ±12.26	42.09 ± 13.23
HIV positive n (%)	1 (5.9%)	0 (0)	0 (0)
QFT plus Positive n (%)	15 (88.2%)	12 (80%)	3 (17.6)
QFT plus Negative n (%)	1 (5.9%)	0 (0)	1 (1%)
Indeterminate n (%)	0 (0)	0 (0)	0 (0)

4.4.2 Utility of individual host markers in the diagnosis of active TB disease

As done for the host markers described in section 4A, we assessed the potential of the analytes detected in the nil (unstimulated) culture supernatants and the TB1 and TB2 antigen-specific levels separately, using the Mann Whitney U test.

When the concentrations of the 19 host markers were compared between patients with TB disease and those with ORD, four markers namely, complement factor D (CFD/adipsin), adipsin and sVCAM1 showed significant differences between the two groups and their levels were higher in TB patients. None of the other 15 host markers showed differences between the two groups (Table 4.10).

When the TB1 antigen-specific levels of these markers were compared between the TB patients and those with ORD using the Mann Whitney test, the levels of sVCAM1 was significantly elevated in individuals with ORD with none of the other 18 markers showing any differences (Table 4.10). When the TB2-antigen specific levels of the markers were assessed, only BDNF concentrations were significantly different between the two groups, with the levels of this marker significantly higher in individuals with ORD (Table 4.6). Taken together, these results indicate that TB1 and TB2-antigen specific levels of most of the 19 relatively new biomarkers evaluated in this section of the study may not be useful in the diagnosis of active TB, when evaluated

in QFT Plus supernatants. However, the limited number of study participants, especially the disproportionately lower number of patients in the ORD group does not allow for conclusions to be made about the potential usefulness of these host markers. It is for this reason that differences between LTBI, active TB and no-*M.tb* infection were not accessed, to avoid over- or under-interpretation of the results.

Table 4.10: Median levels (interquartile ranges in parenthesis) and diagnostic accuracies for host biomarkers detected in unstimulated, TB1 and TB2 stimulated QFT Plus sups from individuals with TB disease or other respiratory diseases. Only host markers that showed differences in either the unstimulated, TB1 or TB2 antigen stimulated culture supernatants are shown.

Marker (pg/ml)	ORD (IQR), n=15	TB (IQR), n=3	P value	ACU (95% CI)	Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)
Markers detected in unstimulated (nil) supernatants							
MPO	455700 (4106000-5740000)	5000000 (4759000-8837000)	0.0892	0.78 (0.49-1.09)	<4.73E+006	0.75 (0.19-0.99)	0.85 (0.565-0.98)
Adipsin	411.9 (341.1-786.0)	547.0 (493.5-411.9	0.1742	0.73 (0.31-1.15)	<427	0.75 (0.19-0.99)	0.92 (0.64-100)
TB1 antigen-specific marker levels							
sVCAM 1	908290 (55881-2140000)	1004000(672958-1094000)	0.8208	0.54 (0.19-0.89)	>1.024E+006	50 (0.06-0.93)	0.61(0.32-0.86_
TB2 antigen-specific marker levels							
BDNF	25705 (5417-64711)	23387 (15346-37319_	0.9099	0.5192 (0.13-0.91)	>17123	0.77 (0.46-0.94)	0.50 (0.07-0.93)

Discussion

Main findings

The main aim of the current study was to evaluate the usefulness of host biomarkers detected in QFT Plus supernatants as diagnostic candidates for active TB disease among individuals suspected of having pulmonary TB. Selected host biomarkers that showed potential in previous studies including studies done in serum, plasma or culture supernatants were evaluated in the QFT Plus supernatants. We furthermore, assessed the potential of these biomarkers in discriminating between active TB, LTBI and *M. tb* uninfected individuals as data obtained from such comparison may help in enhancing our knowledge of the immune response against TB, especially as relevant to the peptide pools used in the recently introduced QFT Plus test.

The main findings of the work presented in this chapter, included the identification of multiple host biomarkers that are detectable in QFT Plus supernatants including markers detected in the nil, TB1 and TB2 stimulated tubes, which showed potential individually in the diagnosis of TB disease, as well as combinations between different biomarkers which showed strong potential as presented in table 4.5. The potentially most useful individual host biomarkers detected in either unstimulated, TB1 or TB2 antigen-stimulated supernatants for the diagnosis of active TB as determined by AUC included ITAC-1, IL-3, I-309, MIG, Apo-A1, P-selectin, MIP-1α, EGF, GM-CSF, IL-2 and IL-10. Biomarkers that might not necessarily have shown potential individually, but that formed parts of various combinations of analytes, representing the biosignatures from nil, TB1 and TB2 antigen-specific, or TB1+TB2 antigen-specific biosignature as shown in table 4.5 included NCAM-1, MCP-2, CFH, TGF-α LIGHT, Apo-CIII and ITAC-1. Furthermore, host markers which discriminated active TB from LTBI and *M. tb* uninfected included MIP-1 α, IL-3, CD40L, IP-10, MCP-2, IL-1 α, IFN-γ and ITAC-1. These host markers may therefore represent the most important candidate biomarkers which may be taken forward for further investigations in future larger studies. However, as the main purposes of the present study was to evaluate the usefulness of biomarkers detectable in QFT Plus supernatants in the diagnosis of active TB amongst individuals with suspected TB, the most accurate biosignature for the diagnosis of TB disease in all study participants, regardless of HIV infection or QFT Plus results was a combination between APO-CIII, ITAC-1, MIG, MCP-2, I-309,

and NCAM-1. This biosignature diagnosed TB disease in all study participants with a sensitivity of 73.9% and specificity of 83.7%.

Study strength and weaknesses of the present study

Several studies have demonstrated the potential of host markers detected in supernatants obtained after stimulation of whole blood cells in previous generations of QuantiFERON in the diagnosis of TB disease. The current study was about host markers detected in QFT Plus supernatants, a new generation of QuantiFERON test which consist of an additional antigen stimulation tube (TB2). Based on literature searches, there are as yet no studies that reported on the potential utility of host markers detected in especially QFT Plus supernatants in the diagnosis of TB disease in individuals in whom TB is suspected (phase III diagnostic studies as done in the present study. We have shown in the current study that some of the host markers that were described in previous studies (125) may also be useful, when measured in QFT Plus supernatants in the diagnosis of TB disease.

The study participants included in the current study were the same participants included in the study reported in chapter 3. As already discussed in that chapter, a phase III study, such as described in the present study is the best study design for evaluating the accuracy of diagnostic biomarkers or tests, especially when done blindly as was the case in the current project. The fact that this study was conducted in an area with one of the highest burdens of TB disease in the world, with a high prevalence of LTBI (6), strengthens the findings, especially regarding possible use of any biomarkers identified in the project in the diagnosis of active TB. However, the use of only a single study site in the project may be seen as a limitation. That notwithstanding, the findings from a phase III study such as this will be very relevant and will be informative in the design of future studies, including studies done at multiple sites, e.g. in other African countries or other continents. Although, this study had reasonable sample size, the small number of HIV infected individuals led to the inability of this study to assess the effect of HIV infection on the performance of the current individual host markers which showed potential in diagnosing and differentiating TB between active TB and other respiratory diseases as well as *M tb* uninfected individuals. Although the current study discovered new host markers which showed potential in diagnosing TB disease, the disproportionately lower number of

ORD group did not allow us to make any conclusions about the usefulness of these new markers. Furthermore, we were unable to evaluate all markers from previous studies which showed potential in diagnosing TB disease due to the fact that the luminex kits used in this study had already been purchased before the beginning of this study. Although most of the markers in unstimulated supernatants seemed to not be affected by HIV infection, small number of active TB individuals with HIV infection made it impossible for us to compare the effect of HIV infection in the performance of individual host markers between the two groups.

Relation of the current study with other studies

In previous work done at the Stellenbosch University Immunology Research Group laboratory (SU-IRG), host biomarkers other than IFN-γ which were detected in culture supernatants left over after the QuantiFERON TB Gold In Tube test showed potential as diagnostic biomarkers for active TB disease and for discriminating between active and latent *M. tb* infection in both adults and children (100, 102, 118, 134, 135). The biomarkers identified in these studies were put together and evaluated in a larger Africa wide project that included seven institutions situated in six different countries, with this larger study validating some of the analytes described in the single site study (100). Other recent studies from the group indicated that biomarkers detected in unstimulated samples including serum, plasma and saliva showed potential in the diagnosis of TB disease (112, 113). As no previous studies have evaluated the usefulness of these biomarkers in the supernatants obtained after stimulation of blood cells with the antigens used in the recently introduced QFT Plus test, it was imperative that the potential value of these biomarkers in this test system be established. Out of the 15 host markers that showed potential in diagnosing TB disease in unstimulated QFT Plus supernatants in this study, four individual host makers (ITAC-1, IL-3, I-309 and MIG) which were amongst the best of these analytes were described as showing potential in a previous study by Jacobs et al (112). Furthermore, other host biomarkers which showed potential including EGF, TGF-α, IL-2, IL-33, APO-A1, ADAMTS13, GM-CSF and IP-10 also showed potential in previous studies (100, 101, 118, 120). The findings from the present study are therefore in agreement with these published studies which were based on QFT In Tube supernatants.

One of the markers that showed potential in the present study (IL-3) is a cytokine that is secreted by basophils and activated T cells and helps in improving natural host immune response against diseases (137). It was the second best individual host marker which diagnosed TB disease with promising sensitivity and specificity. IL-3 concentrations were significantly higher in TB patients in comparison to patients with ORD and this was only observed in the unstimulated and not the TB1 or TB2 stimulated samples. This may suggest that IL-3 may be a useful baseline biomarker, which may be detectable in simple serum, plasma or finger-prick based tests for differentiating TB from Non TB patients. ITAC-1 is a chemokine which is known to attract activated T cells to the site of infection. This chemokine showed promise in diagnosing TB disease either as a single marker or in combination with other cytokines. A previous study by Jacobs et al (112) showed that ITAC-1 has potential in diagnosing TB disease in plasma which is consistent with findings from our current study. Other chemokines such as I-309, MCP-1, MIP-1α showed promise in diagnosing TB disease. The most promising TB1 and TB2 antigen-specific host markers identified in this study including P-selectin, MIP-1, GM-CSF, IL-10, IL-2 and LIGHT are amongst the mostly investigated biomarkers for diagnosis of TB disease.

It is widely known that CD4 T cells play an important role in the immune response against *M. tb* infection through production of IFN-γ and other host markers. However, in addition to CD4 T-cells, evidence from previous studies have shown that CD8 T cells also play an important role during *M.tb* infection through production of cytokines (138). Cytokines produced by CD8 T cells either activate macrophages which suppressed the growth of *M. tb* or directly lyse *M. tb* intracellularly (138). The addition of CD8+ peptides (TB2) in the QFT Plus test is hypothesised to have increased sensitivity for diagnosing *M. tb* infection as well as active TB (139, 140, 73). In this study, the most promising antigen-specific host markers for the diagnosis of active TB were mostly anti-inflammatory cytokines and growth factors which have previously been associated with the disease. Two growth factors (EGF and TGF- α) showed significant differences between TB patients and ORD groups and were detectable in the TB2 antigen stimulated and not the TB1 culture supernatants. EGF stimulates cell growth and differentiation amongst other functions, a study by Bermudez and colleagues demonstrated that EGF enhances the growth rate of extracellular and intracellular *M. tb* in the site of infection (141). Another study by Chegou et al also

reported the upregulation of these growths in factor of active TB individuals (100). Transforming growth factor (TGF- α) plays an important role as an EGF receptor ligand, which activates the signalling pathway for proliferation, activation and development of the cell. Since TB2 antigen tube contains peptides which stimulates both CD4 and CD8 T cell immune response, the presence of these growth factors only in TB2 antigen tube may indicate that CD8 T cells were the main cells which stimulated EGF and TGF-α not CD4 T cells as these responses were not observed in TB1 culture supernatants. Although EGF levels were not significantly different between ORD and TB groups, the presence of these markers only in TB2 confirms the role for CD8 T cell immune function during TB disease as CD8+ T cells have been reported to be associated with active TB or *M. tb* bacterial loads (73). However, this does not fully explain previous observations about EGF in previous studies. Therefore this finding requires further investigation.

Although there are no biomarkers approved for differentiating LTBI from active TB yet, hence there is no gold standard for LTBI, many studies have shown various markers which are differentially expressed between people with TB, without TB and with other respiratory diseases (101,114,) and also markers discriminating between LTBI, healthy controls and active TB (101). Host markers which showed great potential in discriminating active TB from LTBI either in stimulated or unstimulated supernatants were mostly pro/anti-inflammatory cytokines/chemokines, growth factors and few other proteins. During adaptive immune response to *M. tb* infections, it is known that CD4+ and CD8+ T cells are the main cells involved during host immune defence against *M. tb*. During these response, cytokines such as IL-2 and IFN-γ plays different roles including signalling, activation, development, proliferation and control of the infection. This may explain the why different host markers were found in unstimulated supernatants. A study by Nonghanphithak et al (142), showed that IL-2 and MCP-1 may have potential in discriminating between active TB and LTBI. In our study IL-2 and ITAC-1 were the most informative host markers for discrimination between active TB, LTBI and *M. tb* uninfected , and they were also common markers between antigen stimulated and unstimulated QFT Plus supernatants. In a meta-analysis study for host markers in discrimination of LTBI from TB, IL-2 was found to be a valid marker to discriminate TB from LTBI with a high pooled sensitivity and specificity (143). Worth noting, IP-10 and P-selectin were the only antigen stimulated host markers which

significantly discriminated TB from *M. tb* uninfected individuals. This was surprising as it was expected that biomarkers differentiating between LTBI and active TB would also differentiate between active TB and uninfected controls if these are the groups and after re-analysis of the data. In the previous study by Chegou et al (102), the same phenomenon was observed, but not for all biomarkers. Therefore, the reason for this is unknown but requires further evaluation in other studies. Since QFT Plus relies on T cell mediated immune response. The difference and changes of the discriminative host markers could be due to the *M. tb* burden or antigen load. The ability of IP-10 to discriminate these two groups in the present study is in agreement with previous studies which have shown its importance during active TB (99,144-146).

Although individual host biomarkers showed potential, combinations between host markers were more promising as diagnostic signatures as has been observed in other studies. In a study by Eun-Jung Won et al (147), a combination of stimulated and unstimulated levels of GM-CSF, IL-2, and IL-3 in QFT-GIT supernatants correctly discriminated between 100% of *M. tb* uninfected and 92.06% *M. tb* infected groups. In a more recent study by Chegou et al, combinations of various host markers including TGF-α and MIP-1β diagnosed TB disease regardless of HIV infection with sensitivities above 80 % (100). In the current study, the most accurate combination of host markers was a combination of six markers in unstimulated QFT Plus supernatants (Apo-ACIII, MIG, ITAC-1, MCP-2, I-309 and NCAM-1) which diagnosed TB disease regardless of HIV status with sensitivity and specificity >70%. However, when only HIV uninfected individuals were considered, another combination of markers MIG, I-309, MCP-2 and NCAM-1 diagnosed TB disease with an increased sensitivity and specificity >80%. The increased sensitivity and specificity after excluding HIV infected individuals therefore indicated that HIV has an influence in performance of host biosignatures in unstimulated QFT Plus supernatants. Furthermore, combinations between host markers detected in both TB1 and TB2 antigen stimulated tubes resulted in two biosignatures which diagnosed TB disease with accuracies >70%. When both antigen tubes were combined, the combination of host markers resulted in an increased accuracy for diagnosis of TB disease. These data indicate *M. tb* antigen-specific and also unspecific responses detectable in QFT Plus supernatants may contribute to adjunctive diagnosis of TB disease. However, given recent showing that biomarkers detectable in serum, plasma, urine and saliva samples may be useful and even

performed better than the accuracy obtained for QFT Plus supernatants in the current study as per literature reports (103,104,106,107, 112), the place of QFT Plus based host biomarkers might be limited and such biomarkers may be relevant to specific niche groups, but more research is needed to ascertain the place of such biomarkers in the TB diagnostic landscape.

Implications of the study

Although tests based on ex vivo samples may be more rapid and so be more useful in the diagnosis of TB disease as discussed in the previous paragraph, there are no diagnostic tests based on these samples currently in the market. Potential tools based on these sample types are still under development. During the evaluation of the performance of such tools in routine clinical settings in the TB programs, it may be realised that antigen-specific biomarkers might still play a role, in terms of specificity of the tests for TB disease, since biomarkers from ex vivo samples will be unspecific to TB. Given the roles of cytokines/chemokines/growth factors in immune response against *M. tb* antigen stimulated markers can be used in diagnosing TB disease in low TB endemic settings or in people with difficult to diagnose TB such as extrapulmonary TB (150). Furthermore, elevation of unstimulated host markers provides a good basics for the development of a more rapid test to detect *M. tb* or active TB (149).

Furthermore, a blood-based point-of-care screening test for active TB which is based on the detection of host biomarkers in blood samples is currently under development and clinical evaluation in our research group (www.screen-tb.eu). Therefore, host markers detected in the current study especially, markers that were detected in unstimulated culture supernatants are amongst the biomarkers that are on the ScreenTB test that is currently under development. These host markers can be used for optimisation of point-of-care diagnostic tests for TB disease in resource-constrained settings. More diagnostic tools at a point of care will be easily accessible to anyone for early diagnosis and then treatment will be initiated early. This prevents transmission and risk of drug resistance.

Unanswered questions and future suggestions

In the current study, we identified multiple candidate biomarkers and biosignatures which showed potential in the diagnosis of TB disease amongst people who were suspected of having TB disease. The biosignatures shown in table 4.5 represent the

best signatures from unstimulated, TB1 antigen-stimulated, TB2 antigen stimulated and TB1& TB2 antigen stimulated tubes, with performances in only HIV uninfected individuals shown for some of the biosignatures. Due to the design of the project, analysis of study participant samples blindly and assaying on plates in the order in which they were recruited, there was a disproportionately low number of people with ORD in the samples on which panels containing the relatively new biomarkers (same panels contained some well-known markers) were investigated. Results from that specific analysis (Section 4B) are therefore not conclusive. Therefore the true potential of these biomarkers as possible diagnostic biomarkers in unstimulated or QFT Plus antigen-stimulated samples remains unknown, even though significant differences and promising AUCs were obtained for some. Taking the findings as shown in the applicable tables in section 4B may be considered as over- or under-statement of the study's findings. Future studies should evaluate all the promising analytes identified in the present study with more study participants. Participants included in such studies should include children and adults, both with suspected pulmonary and extrapulmonary TB. HIV infected individuals included in such studies should be properly staged e.g. with viral loads or CD4 counts so that the influence of severe HIV infection on the host makers may be determined. Data obtained from the current study may be used in power calculations to ensure that these future studies are well-powered. These future studies will need to be done in other African countries and other continents to determine the global relevance of the findings.

Conclusion

In conclusion, this was the first study to demonstrate that host biomarkers detected in QFT Plus supernatants may be useful as diagnostic candidates for TB disease, in high burden settings. Although findings from this study hold promise in improving TB diagnosis, more studies are needed to confirm the findings from the study. In light of the recent finding showing that ex vivo host biomarkers obtained in serum, plasma, saliva and urine samples may be useful in the diagnosis of TB, it will be necessary that future studies determine the best scenarios where biomarkers detected in *M .tb* stimulated samples may be useful. Such information may then be useful in the development of novel diagnostic tools that may be useful in the diagnosis of TB disease in specific clinical circumstances where the tools currently being developed may have limitations.

Chapter 5

Summary and conclusion

5.1 Thesis overview

As discussed in previous chapters within this thesis, proper control of TB is dependent on early diagnosis of the disease and early initiation of treatment. Several highly publicised limitations of the currently existing diagnostic tests include poor sensitivity, long turn-around times and, high costs amongst others. TB therefore remains a global problem because of these reasons. However, recent studies have shown that measurement of the concentrations of host biomarkers which are detectable in plasma, serum, *M. tb* antigen-stimulated supernatants and other sample types may be useful in diagnosing TB disease. The importance of especially diagnostic approaches based on host immunological biomarkers is that they can be easily incorporated into simple point-of-care tests. Therefore, the main aim of this thesis was to evaluate the potential of host biomarkers detected in culture supernatants obtained from stimulation of whole blood samples in the recently developed QuantiFERON® TB GOLD Plus (QFT Plus) supernatants as biomarkers for the diagnosis of TB disease. This was done in study participants who presented with symptoms requiring investigation for TB disease at a primary health care centre in Cape Town, an area with a high burden of TB. As the QFT Plus is a relatively new test, we also evaluated the usefulness of the test in this study population, especially as the manufacture claims a sensitivity >95% and also whether when used in combination with symptoms the test had potential in the diagnosis of TB disease in people suspected of having active TB.

5.2 Summary of findings

In chapter 3 we evaluated the usefulness of QFT Plus in the diagnosis of TB disease in people suspected of having active TB in a high burden setting. The sensitivity and specificity of the QFT Plus test in our setting was 77% and 71% respectively. When QFT Plus was used in combination with TB symptoms (8 symptoms including coughing, night sweats, feverish, weight loss, chest pains, weak and tiredness as well as not feel like eating), three symptoms including coughing, weight loss and feverish

diagnosed TB disease with the same accuracy as the QFT Plus test (sensitivity of 77% and specificity of 70%) and when the QFT Plus positive results were used in combination with symptoms, the addition of QFT Plus did not have any additional value over the symptoms alone. On the contrary, QFT Plus positive results + symptoms resulted in inferior accuracy to the QFT Plus alone or combination of symptoms alone. In chapter 4, we evaluated the potential of 37 biomarkers in unstimulated and TB antigen-stimulated QFT Plus supernatants as candidates for diagnosis of TB disease. In unstimulated supernatants, 14 host markers showed potential for diagnosing TB disease as determined by the area under the ROC curves (AUC). From these host markers, ITAC-1, IL-3, I-309, MIG, and APO-CIII were the top individual markers. In TB1 antigen stimulated supernatants, seven host markers including p-selectin, MIP-1α, and TGF-α, IL-13, GM-CSF and IL-10 showed potential. In TB2 antigen stimulated supernatants, six host markers including p-selectin, MIP-1α, GM-CSF, LIGHT, IL-13, GM-CSF and IL-10 showed potential as individual host markers.

Despite the potential shown by individual host markers in diagnosing TB disease, combinations between different host markers were more promising in all antigen stimulation conditions. In unstimulated culture supernatants, the most promising biosignature that diagnosed TB disease regardless of HIV status or QFT Plus results included a six-maker biosignature comprising of Apo-A1, ITAC-1, I-309, MIG, MCP-2 and NCAM; diagnosing TB with an AUC >0.91, sensitivity of 73.91% (95% CI, 51.6-89.8%) and specificity of 86.2% (95% CI, 75.3-93.5%) after leave-one-out cross validation. However, it was shown that unstimulated host markers were affected by HIV infection, given that in only HIV uninfected individuals, a four-marker biosignature comprising of Apo-CIII, I-309, MIG and NCAM diagnosed TB disease with better accuracy (an AUC >0.91 (0.85-0.98), with sensitivity and specificity of 76.5% (95% CI, 50.1-93.2%) and 85.7% (95% CI, 74.6-93.3% respectively after leave-one-out cross validation). In TB1 antigen stimulated supernatants, the most useful biosignature in all study participants was a four marker combination between I-309, TNF-α, MIP-1α and CFH , which diagnosed TB disease with an AUC of 0.72, sensitivity of 60.0% and specificity of 78.8% after leave-one-out cross validation. In TB2 antigen stimulated supernatants, the most useful biosignature in all study participants was a four marker combination between IL-6, MIP-1α, GM-CSF and TGF-α, which diagnosed TB disease with an AUC of 0.72, sensitivity of 60.0% and specificity of 78.7% after leave-one-out

cross validation,. When TB1 and TB2 antigen-specific host biomarkers were combined, the most useful biosignature was a four combination of TNF-α. LIGHT, MIP and P-Selectin which diagnosed TB disease with an AUC of 0.84 corresponding to sensitivity of 48.6% specificity of 87.3% after leave-one-out cross validation. A second biosignature showed promised in diagnosing TB disease was when TB1 was combined with TB2. The biosignature comprised of TNF-α, LIGHT, MIG, and p-selectin which diagnose TB disease with sensitivity and specificity of 77.3% (95% CI, 54.6-92.2%) and 72.3% (95% CI, 59.8-82.7%) respectively. After leave-one-out cross validation, the sensitivity and specificity of the biosignature were 77.3% (95% CI, 54.5-92.2%) and 69.2% (95% CI, 56.6-80.1%) respectively.

Given that 71.1% of the patients with ORD were infected with *M. tb* according to the QFT Plus test, we evaluated whether these host protein biomarkers were differentially expressed between patients with active TB, individuals with LTBI and *M. tb* uninfected individuals. As shown in table 4.6 of chapter 4, the most important unstimulated host biomarkers that discriminated between LTBI and active TB included I-309, ITAC-1, IL-3, those that discriminated between TB and *M. tb* uninfected individuals included APO-A1, MIG and APO-CIII, and surprisingly, there were no significant differences between LTBI and *M. tb* uninfected individuals for any of the 37 biomarkers evaluated in all study participants. The most important TB1-stimulated host biomarkers that discriminated between LTBI and active TB included MIL-1α, GM-CSF, IL-3, surprisingly those that discriminated between TB and *M. tb* uninfected individuals only included IP-10 and P-Selectin and , for comparison between individuals with LTBI and the *M. tb* uninfected individuals, IFN-γ, lL-13 , IL-22 , MCP-2 , and ITAC-1. Furthermore, the most important TB2-stimulated host biomarkers that discriminated between LTBI and active TB included IL-6, GM-CSF, IL-2 and IL-10, those that discriminated between LTBI and *M. tb* uninfected individuals included IFN-γ,MIP-1α, IL-3, IL-22 and GM-CSF, for comparison between individuals with LTBI and the *M. tb* uninfected individuals, IFN-γ, lL-13 , IL-22 , MCP-2 , and ITAC-1. Surprisingly those that discriminated between TB and *M. tb* uninfected individuals only included IFN-γ and P-selectin. The most useful host markers in discriminating between groups, regardless of whether they were detected in unstimulated, TB1 or TB2 antigen-stimulated samples included IL-10, GM-CSF and IL-2.

5.3 Significance of the finding from this thesis

This was the first study to evaluate the performance of host markers in QFT Plus supernatants, at least in a setting with one of the highest burdens of TB in the world. Findings from this work are being written up for submission for peer review and publication and will contribute to knowledge about the potential utility of these host markers especially in high burden settings. The findings on the accuracy of the QFT Plus test in patients presenting with symptoms in this study are also very relevant, given the high sensitivity and specificity promised by the test manufacturer in the package insert. The strong study design (phase III) of the parent study adds to the high relevance of findings from the project, opposed to several QFT In tube-based previous studies which were based on a case-control design. It is known that case-control studies tend to over-estimate the accuracy of diagnostic studies (132). It is now known whether the high accuracy values reported by the manufacturer in the package insert were based on such studies or in studies conducted in extreme groups. Our study's findings are therefore highly relevant regarding the performance of the QFT Plus test and also host biomarkers detected in QFT Plus culture supernatants in the real-world setting, in high burden countries. In light of the recent finding showing that ex vivo host biomarkers obtained in serum, plasma, saliva and urine samples may be useful in the diagnosis of TB, it will be necessary that future studies determine the best scenarios where biomarkers detected in *M .tb* stimulated samples may be useful. Furthermore, a blood-based point-of-care screening test for active TB which is based on the detection of host biomarkers in blood samples is currently under development and clinical evaluation in our research group (www.screen-tb.eu). Although the main aim of this chapter was to evaluate antigen specific host markers, unstimulated host markers performed better when compared to the stimulated host markers and are amongst the biomarkers that are on the ScreenTB test that is currently under development. These host markers can be used for optimisation of point-of-care diagnostic tests for TB disease in resource-constrained settings. Furthermore, tests based on ex vivo samples may be more rapid and useful in the diagnosis of TB disease, however, there are no diagnostic tests based on these samples currently in the market. During the evaluation of the performance of such tools in routine clinical

settings in the TB programs, antigen-specific biomarkers might still play a role, in terms of specificity of the tests for TB disease, since biomarkers from ex vivo samples will be unspecific to TB. Given the roles of cytokines/chemokines/growth factors in immune response against *M. tb* antigens, stimulated markers can be used in diagnosing TB disease in low TB endemic settings or in people with difficult to diagnose TB such as extrapulmonary TB.

5.4 Direction for future studies

Although the main aim of this study was to evaluate the antigen stimulated host biomarkers/biosignature, host biosignature in the unstimulated QFT Plus supernatants showed better accuracy for diagnosing TB disease and the combination of these markers has never been shown in any study. Future studies should therefore evaluate this biosignature in ex vivo samples as elevation of unstimulated host markers found in this study may provide a good basis for the development of more rapid tests to detect *M. tb* or active TB. As HIV co-infection may have influence on the performance of individual host markers as indicated by findings from the current study, it may be important to include more properly staged HIV co-infected (low/high CD4 count, viral loads) individuals in future studies as discussed in chapter 4. Cytokine profiles of *M. tb*-specific T cells have been studied with the aim of finding a correlation with *M. tb* infection. However, existing data are currently inconclusive due to contrasting findings on the distribution of the various cytokine-producing CD4+ and CD8+ T cell subsets. Furthermore, few studies have been conducted on cell types producing the biomarkers that are detectable in serum and plasma samples during *M. tb* infection. Human immune responses to *M. tb* may also lead to transcriptional patterns in blood that are not present in other conditions. RNA transcripts have shown ability to distinguish tuberculosis from other diseases, but most of the studies to date were conducted in unstimulated blood samples as obtained with RNA obtained from Paxgene tubes and not RNA obtained from *M. tb* specific antigen stimulated blood cells. In the course of my MSc studies, I collected sediments that were left over after performance of the QFT test. These sediments were either cryopreserved for flow cytometry or stored in RNA later. Therefore future studies may use these sediments for the investigation of novel, *M. tb* specific transcriptomic biosignatures for active TB disease. As flow Cytometry,

is a potential tool to improve TB diagnosis by phenotypical and functional characterization of antigen-specific T cells, information obtained about the expression of biomarkers intracellularly or at the surface of cells may help improve our understanding of T cell immunology- especially as related to *M. tb* antigen-specific stimulation of blood cells as obtained with the sediments stored after stimulation with the antigens employed in QFT Plus supernatants in my MSc project. Therefore there are many opportunities for future follow-on studies from this project, given that little is known about the immunology of *M. tb* infection especially regarding what constitutes protective immunity, correlates or protection, correlates of risk or biomarkers for TB treatment monitoring. The findings from the work conducted in the course of my project including findings from the stored, unused samples mentioned above may help shed light on these important questions about the immune response against TB.

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