

**Analysis of ex vivo host biomarkers in sputum samples for
diagnosis of pulmonary tuberculosis”**

By

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Declaration

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Abstract:

Background

Despite all the interventions deployed to control tuberculosis (TB), the disease still continues to be the principal cause of death from a single infectious agent in resource constrained settings. An estimated 60% of suspected TB patients do not have access to TB diagnostic tests. With the limitations of the current diagnostic tests and the importance of early diagnosis and initiation of treatment, biomarker diagnosis of TB would be an optimal option. Biomarkers are indicators of immune activity and state. Therefore, host or pathogen biomarker of TB disease would be ideal. Hence, the aim of this project was to profile a broad array of host markers for development of optimal signatures for detection of pulmonary tuberculosis from other respiratory disorders using ex vivo sputum samples

Methods

We recruited patients who were seeking medical attention at the MRCG at LSHTM outpatients department and Tuberculosis clinic with symptoms suggestive of TB, prior to clinical or microbiological diagnosis. All age groups were recruited. Sputa were collected at baseline from all participants and at 1 and 2 months from the confirmed TB cases. The sputa were digested with Sputolysin and the supernatant analysed using Luminex arrays while RNA extracted from the pellet were analysed with RT-qPCR. Statistical analyses and graphs were generated using R programming Language and GraphPad Prism, with a q value ≤ 0.05 considered significant. A receiver operating curve (ROC) was used to assess the diagnostic performance of individual and combination markers.

Results

Confirmed TB (428) and ORD (313) patients were analysed, 70 markers were assessed for diagnostic potential and treatment response. Of these, 37 were significantly different between TB and ORD. The best single marker was MMP-2 with an AUC of 0.73. An eight-marker signature (IFN- γ , IL-1 β , IL-8, IL-10, IL-12p70, MIP-1 β , RANTES and VEGF) was able to diagnose smear and culture positive TB from ORD with an AUC of 0.77, sensitivity of 78% and specificity of 70%, while a three-marker signature (IL-1 β , IL-7 and VEGF) classified smear negative but culture positive TB from ORD with an AUC of 0.74, sensitivity of 86% and specificity of 60%. Among children who had TB, a four-marker signature (FGF, IL-4, MIP-1a and RANTES) differentiated those with TB from ORD, with an AUC of 0.87, sensitivity of 82% and specificity of 87% and a five-marker signature consisting of BAFF, C3L1, IL-22, MMP-3 and sTNFR1 was able to discriminate TB and HIV co-infected from ORD with an AUC of 0.90, sensitivity of 88% and specificity of 85%. We also found a four-marker signature consisting of EGF, IL-15, MIP-1 β and TNF- β that could predict slow versus fast treatment responders at baseline with an AUC of 0.74, sensitivity of 75% and specificity of 80%.

Conclusion

We have discovered novel sputum host biomarkers and biosignatures for screening of tuberculosis and treatment response. The data is promising for potential translation into a user friendly device as a rapid screening test for pulmonary TB. However, this markers and signatures require further investigations to authenticate their usefulness.

Opsomming:

Agtergrond

Ten spyte van al die ingrypings wat toegepas word om tuberkulose (TB) te beheer, bly die siekte steeds die grootste oorsaak van die dood van 'n enkele aansteeklike middel in beperkte hulpbronne. Na raming het 60% van die vermoedelike TB-pasiënte nie toegang tot diagnostiese toetse vir TB nie. Met die beperkinge van die huidige diagnostiese toetse en die belangrikheid van vroeë diagnose en inisiëring van behandeling, sou die biomerker-diagnose van TB 'n optimale opsie wees. Vir biomerkers is dit 'n aanduiding van immuunaktiwiteit en toestand. Daarom is gasheer- of patogeenbiomerker van TB-siekte ideaal. Die doel van hierdie projek was dus om 'n wye verskeidenheid gasheermerkers te profiel vir optimale handtekeninge vir die opsporing van pulmonale tuberkulose van ander respiratoriese afwykings met behulp van ex vivo sputum monsters.

Metodes

Ons het pasiënte gewerf wat mediese behandeling by die MRCG by LSHTM-buitepasiënte-afdeling en tuberkuloseklinik opgedoen het met simptome wat dui op TB, voor die kliniese of mikrobiologiese diagnose. Alle ouderdomsgroepe is gewerf. Sputa is vanaf die beginlyn by alle deelnemers versamel en op 1 en 2 maande van die bevestigde TB-gevalle. Die sputa is met Sputolysin verteer en die supernatant is met behulp van Luminex-skikkings geanaliseer terwyl RNA wat uit die korrel onttrek is, met RT-qPCR geanaliseer is. Statistiese ontledings en grafieke is gegenereer met behulp van R-programmeringstaal en GraphPad-prisma, met 'n q-waarde $\leq 0,05$ wat as beduidend beskou is. 'N Ontvangerbedryfskurwe (ROC) is gebruik om die diagnostiese prestasie van individuele en kombinasie-merkers te beoordeel.

Resultate

Bevestigde TB (428) en ORD (313) pasiënte is geanaliseer, 70 merkers is beoordeel vir diagnostiese potensiaal en reaksie op die behandeling. Hiervan was 37 aansienlik verskillend tussen TB en ORD. Die beste enkele merker was MMP-2 met 'n AUC van 0,73. 'N Handtekening met 'n agt merker (IFN- γ , IL-1 β , IL-8, IL-10, IL-12p70, MIP-1 β , RANTES en VEGF) was in staat om die smeer- en kultuur-positiewe TB van ORD te diagnoseer met 'n AUC van 0,77, sensitiwiteit van 78% en spesifisiteit van 70%, terwyl 'n drie-merkteken (IL-1 β , IL-7 en VEGF) die negatiewe maar kultuurpositiewe TB van ORD met 'n AUC van 0,74, 'n sensitiwiteit van 86% en die spesifisiteit van 60%. Onder kinders wat TB gehad het, het 'n vierteken-handtekening (FGF, IL-4, MIP-1a en RANTES) diegene met TB van ORD onderskei, met 'n AUC van 0,87, 'n sensitiwiteit van 82% en die spesifisiteit van 87% en 'n vyf-merkerhandtekening bestaande uit BAFF, C3L1, IL-22, MMP-3 en sTNFR1 was in staat om TB en MIV wat mede-besmet was van ORD te onderskei met 'n AUC van 0,90, 'n sensitiwiteit van 88% en die spesifisiteit van 85%. Ons het ook 'n vierteken-handtekening gevind wat bestaan uit EGF, IL-

15, MIP-1 β en TNF- β wat reageer op 'n traë versus vinnige behandeling by die basislyn met 'n AUC van 0,74, 'n sensitiviteit van 75% en die spesifisiteit van 80%.

Afsluiting

Ons het nuwe sputum gasheer-biomerkers en biohandtekeninge ontdek vir die keuring van tuberkulose en reaksie op die behandeling. Die data is belowend vir moontlike vertaling in 'n gebruikersvriendelike toestel as 'n vinnige siftingstoets vir long-TB. Hierdie merkers en handtekeninge benodig egter verdere ondersoeke om die bruikbaarheid daarvan te verifieer.

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Table of Contents

Declaration	i
Abstract:	ii
Acknowledgement	vi
Table of Contents	vii
List of figures	ix
List of Tables	x
List of abbreviations	xi
Chapter 1 Introduction	1
1.1 Overview of Tuberculosis	1
1.2 Epidemiology of Tuberculosis	1
1.3 Basic immunology of tuberculosis.....	3
1.3.1 Innate immunity.....	4
1.3.2 Adaptive immunity.....	5
1.3.3 The initiation of adaptive immune response	8
1.3.4 Host - Mtb interaction	8
1.4 Spectrum of Mycobacterium tuberculosis infection	10
1.5 Distinguishing TB from Other respiratory disorders (ORDs).....	10
1.6 Tuberculosis Diagnosis.....	12
1.6.1 Clinical Investigation	12
1.6.2 Radiological Investigation.....	12
1.6.3 Sputum smear microscopy (SSM).....	13
1.6.4 Sputum Culture	13
1.6.5 Nucleic Acid Amplification Test (NAAT)	14
1.7 Tuberculosis biomarkers.....	15
1.7.1 Biomarkers	15
1.7.2 Immunological biomarkers of TB.....	15
1.7.3 Immunodiagnostic triage tests.....	18
1.8 Treatment and treatment response.....	19
1.8.1 Treatment.....	19
1.8.2 Treatment response	19
1.9 Project aims and objectives	20
1.9.1 Specific Objectives	20

Chapter 2 Materials and Methods	21
2.1 Ethics approval	21
2.2 Study participants and setting.....	21
2.3 Current microbiology test procedures	21
2.3.1 Liquid culture procedure.....	21
2.3.2 GeneXpert (XpertMTB/RIF) procedure	22
2.4 Mycobacterial load assay	23
2.4.1 Sputum digestion	23
2.4.2 RNA extraction	23
2.4.3 H37Rv Standard preparation.....	23
2.4.4 16S RNA analysis	24
2.5 Multiplex Immunoassay	24
2.5.1 Bio-Rad immunoassay procedures – 27 and 37-plex assays.....	25
2.5.2 Milliplex MAP immunoassay procedure – 38-plex assay	29
2.6 Statistical Analysis.....	32
Chapter 3 Results	33
3.1 Participant demographics	33
3.2 The Performance of individual host markers in diagnosis of TB disease	35
3.3 Usefulness of combinations of biomarkers in the diagnosis of TB	41
3.3.1 27-plex biosignature analyses.....	41
3.3.2 37-plex biosignature analysis	44
3.3.3 38-plex biosignature analyses.....	46
3.3.4 Combined biosignature analyses	49
3.4 Treatment response and monitoring	50
3.4.1 Host markers' levels during the course tuberculosis treatment.....	51
3.4.2 Biosignature of treatment responses.....	56
Chapter 4 Discussion	58
4.1 Main findings	58
4.2 Strengths and weaknesses.....	62
4.3 Implications of the study	62
4.4 Conclusion.....	63
Chapter 5 Reference.....	64

List of figures

- Figure 1.1 Estimated TB Incidence rates 2017..... 3
- Figure 1.2 CD4 T cell differentiation upon activation leading to different subsets of cells and their functions, characterised by the cytokines they produced.....6
- Figure 2.1: Plate layout for Bio-Rad Human Cytokine/Chemokine28
- Figure 2.2: Plate layout for MAP Human Cytokine/Chemokine31
- Figure 3.1 The cycle threshold (A) and quantity (B) of viable bacteria RNA in the sputum of suspected TB patients.....35
- Figure 3.2 Graphs of individual markers that performed best in classifying ORD from TB with an AUC of ≥ 0.6540
- Figure 3.3 shows overall importance of marker in a possible involvement in a biosignature formation.....48
- Figure 3.4.1A. The levels of markers been compared from corresponding samples of the same patient during course of TB treatment, sample collected 1 month interval.....52
- Figure 3.4.1B Markers showed decline in concentration levels when baseline and 2months samples from matching patients' samples were compared53
- Figure 3. 4.1C Changes in mean levels of markers during the course of treatment.....54
- Figure 3.4.1D Comparison of baseline median levels of fast (F) and slow(S) responders.....55
- Figure 3. 4.2 ROC curve of fast against slow responders to TB treatment.....57

List of Tables

- Table 1.1 WHO recommend target product profile (TPP) performance characteristics for screening and diagnosis of TB.....11
- Table 2.1. The 27-plex kit standard range.....26
- Table 2.2. 37-plex markers' standard and control ranges27
- Table 2.3. The range of controls for 38-plex markers29
- Table 3.1 Participant demographics.....34
- *Table 3.1A The quantity of viable bacteria RNA in the sputum of suspected TB patients.....34*
- Table 3.2A. Only single biomarker with significant p-values are shown (27-plex kit).....36
- Table 3.2B. Only single biomarker with significant p-values are shown (37-plex kit).....38
- Table 3.2C 1. Only single biomarker with significant p-values are shown (38-plex kit).....39
- Table 3.3.1 Biosignature classification with the 27-plex kits42
- Table 3.3.2. Biosignature classification with the 37-Plex kits.....45
- Table 3.3.3. Biosignature classification with the 38-Plex kits.....47
- Table 3.3.4 Biosignature classification of combine 27-37plex kits.....50
- Table 3.4.2. Treatment response biosignature.....56

List of abbreviations

%	percentage
<	Less than
=	equal to
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
μl	microliter
αβ	Alpha-beta
γδ	Gamma delta
°C	Degree Celsius
16SrRNA	16S Ribosomal ribonucleic acid
ABI7500	Applied Biosystems 7500 software
AEC	Airway Epithelial Cells
AFB	Acid fast bacilli
Ag-Nil	Antigen minus Nil
AIC	Akaike information criterion
AIDS	Acquired immune deficiency syndrome
AM	Alveolar Macrophage

APC	Antigen Presenting Cells
Apo A-1	Apolipoprotein-A1
APRIL/TNFSF13 superfamily member 13	A proliferation-inducing ligand/ tumour necrosis factor ligand
AUC	Area under the curve
BAFF/TNFSF13B member 13B	<i>B-cell activating factor</i> / tumour necrosis factor ligand superfamily
BCG	Bacille Calmette Guerin
BLK	Blank
BMGF	Bill and Melinda Gates Foundation
C3	Complement factor 3
C3L1	Chitinase 3-like 1
Ca ²⁺	Calcium ions
CAD	Computer-aided detection
CCR4	CC chemokine receptor 4
CD	Cluster of difference
CFP-10	10kDa culture filtrate antigen
CFU	Colony forming unit
CI	Confident interval
CO ₂	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
Ct	Cycle threshold
CT	Computed tomography

CTL	Cytotoxic lymphocytes
CXR	Chest X-ray
DAMPs	Damaged - damage-associated molecular patterns
DC	Dendritic cells
DC-SIGN	DC-specific intercellular adhesion molecule-grabbing non-integrin
ECM-1	Extracellular matrix protein 1
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme linked immunosorbent spot
EMB	Ethambutol
EpTB	Extra pulmonary Tuberculosis
ESAT-6	6kDa early secretory antigenic target
FGF- basic	Basic fibroblast growth factor
FGF-2	Fibroblast growth factor 2
FIND	Foundation for Innovative New Diagnostics
Flt-3L	FMS-like tyrosine kinase 3 ligand
FM	Fluorescence microscopy
FOXP3	Forkhead box P3
GATA-3	GATA binding protein 3
G-CSF	Granulocyte-colony stimulating factor
GLM	Generalised linear multiple
GM-CSF	Granulocyte monocyte colony stimulating factor
GRO	Growth-regulated Oncogene

HCC1	Hemofiltrate CC chemokine-1
HIV	Human immunodeficiency virus
HLA-DR	Human Leukocyte Antigen
Hr	Hour
IFN	Interferon
IFN- α 2	Interferon alpha-2
IFN- β	Interferon beta
IFN- γ	Interferon-gamma
IGRA	Interferon gamma release assay
IL	Interleukin
IL-1 α	Interleukin 1 alpha
IL-1 α	Interleukin-1 receptor antagonist
IL-1 β	Interleukin 1 beta
IL-28A/IFN λ 2	Interleukin 28A/ Interferon lambda-2
IL-29/IFN λ 1	Interleukin 29/Interferon lambda-1
IL-2R	Interleukin-2 receptor
IL-4R	Interleukin-4 receptor
ILC	Innate lymphoid cells
INH	Isoniazid
iNKT	Invariant natural killer T
IP-10	Interferon gamma inducible protein 10
IQR	Interquartile range
LAM	Lipoarabinomannan

LIGHT/TNFSF14 family protein 14	Lymphotoxin-like inducible protein/ tumour necrosis factor super
LTBI	Latent tuberculosis infection
Ltd	Limited
M. africanum	Mycobacterium africanum
M. Bovis	Mycobacterium Bovis
MAIT	Mucosal associated invariant T cells
ManLAM	Mannose-capped lipoarabinomannan
MBLA	Mycobacterial load assay
MBLs	Mannose-binding lectins
MCP	Macrophage chemotactic protein
MDC	Macrophage-derived chemokine
MDR	Multi-drug-resistant
Mg/ml	Microgram per millilitre
MGIT	Mycobacterial growth indicator tubes
MHC	Major Histocompatibility complex
MIP-1 α	Macrophage inflammatory protein-1 alpha
MIP-1 β	Macrophage inflammatory protein-1 beta
MI	Millilitre
MMP	Matrix metalloproteinase
MP 6.1	Bioplex Manager Software version 6.1
MR1	Major Histocompatibility complex class I-related
MRCG at LSHTM and Tropical Medicine	Medical Research Council The Gambia at London School of Hygiene

MRI	Magnetic resonance imaging
mRNA	Messenger Ribonucleic acid
Mtb	Mycobacterium tuberculosis
MTBC	Mycobacterium tuberculosis complex
MTBVAC	Mycobacterium tuberculosis vaccine
MVA85A	Modified vaccinia Ankara 85A
N	Number
NAAT	Nucleic Acid Amplification Test
NCAM	Neural cell adhesion molecule
NK	Natural killer
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NPV	Negative predictive value
NTM	Nontuberculous mycobacterial
OD	Optical density
ORDs	Other respiratory disorders
PAMPs	Pathogen - pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PDGF -BB	Platelet derived growth factor BB
PDIM	Phthiocerol dimycocerosate
PE	Phycoerythrin
PET-CT	Positron emission tomography and computerized tomography
POC	Point of care

PPD	Purified protein derivative
PPV	Positive predictive value,
PRRs	Pathogen recognition receptors
pTB	Pulmonary tuberculosis
PTX3	Pentraxin-3
PZA	Pyrazinamide
QC	Quality control
QFT-GIT	QuantiFERON-TB Gold In-Tube test
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RANTES	Regulated upon activation normal T cell expressed and secreted
RIF	Rifampin
RNA	Ribonucleic acid
ROC	Receiver operating curve
ROR γ t	Orphan receptor γ t
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RT	Room temperature
SAA1	Serum amyloid A 1
SAP	Serum amyloid P
sCD40L	Soluble CD40 ligand
Sec	Second
Sens	Sensitivity
SM	Streptomycin

Spec	Specificity,
SSM	Sputum smear microscopy
STAT4	Signal transducer and activator of transcription
STD	Standard
sTNFR	Soluble tumour necrosis factor receptor
TB	Tuberculosis
TBD	Tuberculosis Diagnostic
TB-LAM	Tuberculosis lipoarabinomannan
TCR	T-cell receptor
Tfh	Follicular helper T cells
TGF- α	Transforming growth factor alpha
Th	Helper T cell
TLRs	Toll Like Receptors
TNF- α	Tumour necrosis factor alpha
TNF- β	Tumour necrosis factor beta
TPP	Target product profile
Treg	Regulatory T cells
TSLP	Thymic Stromal Lymphopoietin
TST	Tuberculin skin test
UK	United Kingdom
USD	United States Dollar
VEGF	Vascular endothelial growth factor
VIF	Variance inflation factor

VPM1002	Vakzine Projekt Management 1002
WHO	World Health Organisation
XDR-TB	Extremely drug resistant tuberculosis
ZN	Ziehl-Neelsen

Chapter 1 Introduction

1.1 Overview of Tuberculosis

Tuberculosis (TB) disease continuous to be a menace to humans for centuries. More people actually died of the disease compared other infectious diseases around the world, with 1.6 million deaths in 2017 alone [1]. The disease is caused by *Mycobacterium tuberculosis* (Mtb) complex (MTBC) [2]. *M. tuberculosis sensu stricto* is the predominant sub-species while other species like *Beijing*, *M. canettii* and *M. africanum* can also cause TB in different regions [2]. The *M. africanum* strain is localised mainly in the West African sub region [2]. Robert Koch discovered the bacterium [3], [4]. However, emergence of Mtb is not well understood. Studies have postulated that the pathogen came from the soil, and the rearing of domestic animals facilitated its contact with the human population[5]. With advance in TB research, newer studies have shown Mtb not to be a descendant of, but a close relative to, *Mycobacterium bovis* (*M. bovis*), that cause similar disease in livestock, thus suggesting the two pathogens may have simultaneously evolved together [6], [7].

The Mtb pathogen requires oxygen for its survival, hence, its' preferred niche is the lungs where there is sufficient oxygen. In addition, Mtb is a nonmotile and rod-shape bacterium duplicating every 18-24 hours [7], [8]. It also has a distinct cell wall, characterised by its appearance under a Gram's stain. Even though this bacterium has been studied extensively, no consensus has been reached about its classification, due its complex cell wall [2], [9], which consists of various lipids, that provides cushion against antibiotics and dehydration [2], [10]. Moreover, the complex composition of the cell wall of the bacterium is also important for its pathogenesis and virulence [2].

1.2 Epidemiology of Tuberculosis

Mtb is transmitted through the intake of aerosolised particles containing the pathogen (bacilli) from an infectious individual. The disease mostly affects the lung (termed pulmonary TB (pTB)) but may attack other body parts of the infected individuals for instance: the spine, bones or kidneys spreading via circulatory systems (termed extra pulmonary TB (EpTB)) [4]. The World Health Organisation (WHO) annual report of 2018, projected a 1.7 billion people to be latently infected with Mtb [1], [11], thus constituting a large reservoir for potential TB reactivation. And as of 2017, 10 million people were reported to have TB, with 1.6 million TB related deaths, of which 0.3 million were HIV co-

infected individuals [12]. These figures are unacceptably high if early diagnosis and treatment were possible, since majority of active cases often have drug-sensitive TB.

Despite all the interventions deployed to control TB, the disease still continues to be the biggest killer among infectious agents, especially in low and middle income countries such as The Gambia [11], [13]. Overcrowding and poor living standards are major contributors to continued TB transmission. Although nearly 2 billion people around the globe are estimated to be infected with Mtb, less than 5-10% will go on to develop the disease in their lifetime, while most infected people would remain latently infected for life (1). The quick progression from latent infection to active TB is associated with the duration or the degree of exposure, the quantity of bacilli breathe in, the strain of infection and competence of the immune system of those infected to contain the infection. People with weakened immune systems such as those with diabetes, malnutrition or HIV, are at greater risk of progressing to disease [11]. Therefore, the availability of an effective vaccine is essential to curb TB transmission and reduce morbidity and mortality. However, Bacillus Calmette–Guérin (BCG) is the only approved vaccine against TB, developed over a century ago and mostly effective against military and Meningitis TB among children. Its effect is known to wane with age. It provides no protection against pulmonary TB or reinfection, therefore a new vaccine is needed. Due to these limitations of the BCG vaccine, an intensive search for a new vaccine against TB is ongoing. A vaccine which can protect against all forms of TB regardless of age, ethnicity or HIV status. The aim is to get a vaccine that can enhance or substitute BCG. Currently few potential vaccine candidates are on clinical trial such as: VPM1002 – this vaccine has undergone several phases of clinical trials both in new-borns and adults. Previous studies found this vaccine to be safe and immunogenic. Thus, a phase IIb trial of VPM1002 is presently ongoing in South Africa, looking at infants with and without HIV exposure [14]. A similar study of the same vaccine is also taking place in India assessing recurrent TB among cure TB patients [14]. Another vaccine under investigation is MTBVAC also at Phase 2 clinical trials in both new-borns and adults in South Africa. It was considered safe and tolerable when compared to BCG [15]. Other vaccines under investigation include DAR-901, M. vaccae, MIP, Ad5Ag85A, ChAdOx185A/MVA85A, TB/FLU-04Land M72/AS01E [16].

The failure to properly control TB have been attributed to many things such as the high operational cost, suboptimal performance of current diagnosis and the moderate performance of the current vaccine. These in conjunction with the emerging multi and extremely drug resistant forms of TB piled pressure on numerous TB control mechanisms. Nonetheless, the effort to stop TB was not in vain, for there was a significant global decline in TB related deaths by 22% from 2000 – 2016 with 53 million lives saved [17]. However, this global drop was most notable in Eastern Mediterranean and European countries with Africa lagging.

A major challenge with controlling TB is having access to an accurate, simple, cheap, and rapid TB diagnostic test. Approximately 60% of TB patients visit basic healthcare centres to seek medication, but most of these health centres have very little diagnostic infrastructure [18]. Early diagnosis is crucial in controlling TB, since each patient can transmit the bacteria to a least 10 - 15 people in a single year prior to diagnosis [19], [20]. Therefore, early diagnosis and treatment will help to block the transmission chain and ultimately help to bring TB under control.

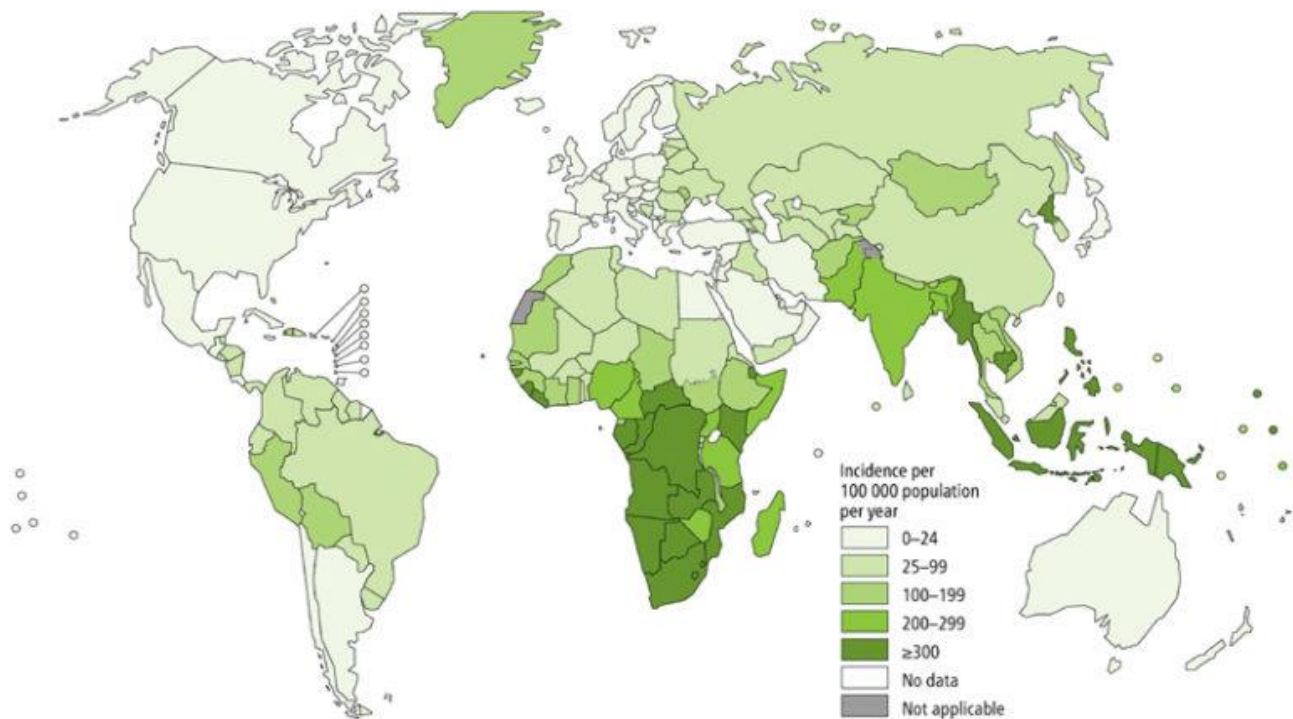


Figure 1.1 *Estimated TB Incidence rates 2018* [12]

1.3 Basic immunology of tuberculosis

The body defence against any invading microbe or pathogen is the immune system. It is a highly controlled process that is able to recognise and neutralise or eliminate any invading pathogen. The system constitutes a network of cells and molecules classified into two subclasses: the inborn, sometime refers to as the innate and the acquired or adaptive immune systems. The two arms produce various toxic materials that can kill or limit the spread of the pathogens [21]–[23].

1.3.1 Innate immunity

Innate immunity is an inborn immunity and is always the first point of call against invading pathogens. It has the ability to block, recognise and respond quickly to various pathogens attacking the body [22], [24], [25]. It constitutes the physical (skin, mucosal lining, tears, saliva, urine and cilia), chemical (fatty and lactic acids, lysozymes, chemokine and cytokines) and cellular components [22], [25]. The cellular component is only called to action when the intruding pathogen is able to penetrate physical barrier. Cells associated with the innate immune response are mostly natural killer (NK) cells, dendritic cells (DC), monocytes, macrophages, mast cells, neutrophils, eosinophil and basophils in conjunction with the complement pathway [21], [22]. Also recruited to the affected area are mucosal associated invariant T (MAIT) cells, gamma delta T cell and invariant NKT cells [26]. These innate cells are armed with pathogen recognition receptors (PRRs) which recognise similar structures present on the invading pathogen - pathogen-associated molecular patterns (PAMPs) [21], [24] or detect molecules that are released when a cell is lysed or tissue is damaged - damage-associated molecular patterns (DAMPs) [27]. In addition, it is also the function of innate immunity to distinguish what is foreign (non-self) from what is part of the body (self). Innate also instructs and directs the adaptive immune responses when overwhelm with pathogen infiltration [28].

When Mtb is inhaled, it passes via the respiratory track to the lung then picked up by its favoured host cell, the alveolar macrophages (AM) [29]. Along the surface of the respiratory track are mucus and cilia, under which lie airway epithelial cells (AEC). The AEC are considered to be the initial cells to encounter the Mtb [29]–[33]. Previous studies have also shown that AEC could be infected by Mtb on their path to lungs [33], [34] thus, inducing MAIT cells cytokine production [33] setting the stage for Mtb control. The stimulation of the MAIT induced IFN- γ production, providing resistance to Mtb prior to the arrival of the cell mediated immunity [35]. Other set of cells also present in the lung, intestine and skin are innate lymphoid cells (ILC3) [36]. The ILC3 cells are characteristic by the release of IL-22, IL-17A, GM-CSF, and/or IFN- γ in response to IL-23 and IL-1 β [36], [37]. ILCs are of three types – ILC1 known to provide protection against virus, ILC2 which resembles CD4⁺ Th2 cells protect against helminth infections and allergies while ILC3 provide immunity, promote healing and maintaining immune balance post infection [36], [37]. A previous study have shown elevated ILC3 cells among active TB, but decreased in the course of treatment period while in experimental mouse model, similar rise in ILC3 and macrophage were also seen, however, in mice that lacked ILC3, a few alveolar macrophage were noticed, as a result an inefficient control of Mtb infection was observed [38]. A similar observation was seen with IL-17, IL-22 and IL-23, ILC3 associated cytokines. Thus suggesting ILC3 resistance against Mtb infection [38]. An important step for the pathogen is to establish an infection via adhesion. During the process of establishing that contact,

the immune cells detect the PAMPs on the Mtb via Toll Like Receptors (TLRs) [30], [34], PRRs such as nucleotide oligomerization domain 2 (NOD2) [39], c-type lectin receptors, mannose receptor, mannose-binding lectins (MBLs) and DC-specific intercellular adhesion molecule-grabbing non-integrin (DC-SIGN) [19], [30], [31], [39], [40]. While complement, an essential component of innate immunity, enhances bacterial uptake by opsonisation through the complement factor 3 (C3) [40]. The absence of C3 reduces phagocytic ability of immune cells by an estimated 70% [40]. AECs or AMs release antimicrobial substances (lysozymes, lactoferrin, defensins, ROS, NO, chemokine/cytokines) [34], [41] to inhibit or kill the invading Mtb.

While the immune system is trying to get rid of the Mtb, the pathogen has developed numerous ways of escaping the immune response such as the masking from PRRs by Mtb cell surface-associated phthiocerol dimycocerosate (PDIM) lipids or the recruitment of impair macrophages that are limited in producing reactive nitrogen and oxygen species thus favouring pathogen survival [42]. In addition the presence of mannose-capped lipoarabinomannan (ManLAM) has been implicated in blocking calcium (Ca^{2+}) reaching the cytosol of macrophage, inhibiting phagosome maturation, promoting disease pathogenesis [26], [43], [44]. Furthermore, Mtb in a bid to survive obstruct cell apoptosis, a pro Mtb elimination process in favour of necrosis, a strategy to promote its spread and thus delay the adaptive immune response's arrival has also been suggested [44], [45]. In fact the increase influx of cells to the site of injury or inflammation has been attributed to promoting bacteria spread due to the infection of some the arriving cells by Mtb [44], [45]

1.3.2 Adaptive immunity

As the innate immune response becomes overwhelmed by pathogen expansion, the adaptive immune response kicks in. This is observed by arrival of specific immune responses tailored towards the disease-causing agent [28]. And the subsequent presence of memory cells to prevent reinfection by the same pathogen [25], however in TB no memory cells are shown to be associated with Mtb protection [46]. The adaptive immune responses constitutes two arms, a) humoral (B cells) and b) cell mediated (T cells). The humoral response is facilitated by B cells, which release antibodies into the blood stream to neutralise the invading pathogen. These activated B cells also aid T cell stimulation through antigen presentation hence amplifying the immune response. In turn, triggering T cells response with complex immune reactions, recruiting various cells to contain the pathogen [28]. Among the tasks of the T cells is to aid B cells and phagocytic cells activation, hence the name helper T cells. T cells are recognised by cluster of difference (CD3), which consist of two main populations: CD8 cell sometimes refer to as cytotoxic lymphocytes (CTL) and CD4 known as helper T (Th) cells. The CTL receptor bind to the major histocompatibility complex (MHC) class I of the

APC, thus killing the infected cells [28]. While the CD4 T cell when activated stimulate the arrival other immune cells to site of injury. CD4 cells can differentiate into many subsets define by the cytokine they produce: Th1, Th2, Th9, Th17, Th22, Treg (regulatory T cells)[47], [48], and Tfh (follicular helper T cells)[49], as shown in Figure 1.2 below. CD4 T cells bind to MHC class II of the APC, activating other immune cells like macrophages or promoting B cell activation.

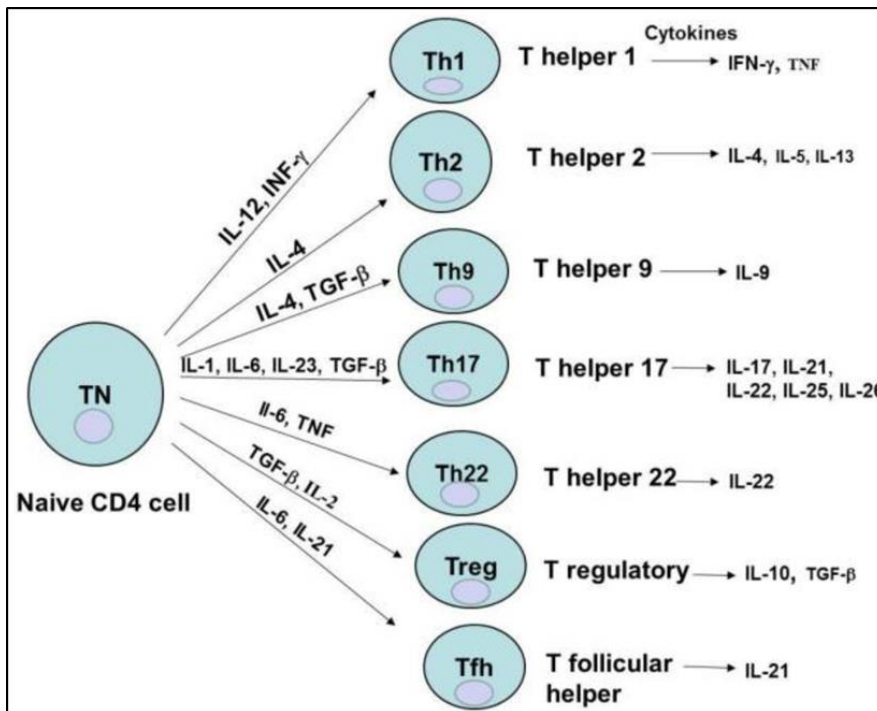


Figure 1.2 CD4 T cell differentiation upon activation leading to different subsets of cells and their functions, characterised by the cytokines they produced [48]

The best described subsets of CD4⁺ cells are Th 1, Th2, Th17 and T regulatory (Treg) cells. Th1 cells are pro-inflammatory, characterised by secretion of IFN- γ , IL-12, TNF- α and IL-2 [47], [48]. IL-12 induces STAT4 in the presence of T-bet to amplify IFN- γ release, thus aiding immune cell recruitment to fight invading pathogens. Uncontrolled Th1 responses can be detrimental, as seen in some autoimmune diseases [49]. Th2 cells are anti-inflammatory and identified by the release of IL-4 in the presence of GATA-3 transcription factor. IL-4R/STAT6 enhance the production of GATA-3, thus enhancing the differentiation of Th2 cells [49]. Other cytokines attributed to Th2 response includes IL-5, IL-9, IL-10, and IL-13 [47], [48]. In contrast to Th1 cells, Th2 cells aid B-cells in the production of antibodies against extracellular parasites such as Helminths, but excess response of Th2 cells could lead to allergic reactions and airway hypersensitivity [49]. Th2 cells can be recognised by the presence of CCR4, which is responsible for cell recruitment. Th17 cells are associated with the release of IL-17, IL-21, IL-22 and IL-26 [47], [50]. IL-6 and TGF- β enhanced CD4⁺ cells differentiation in the presence of retinoic acid-related orphan receptor γ t (ROR γ t) into

Th17 cells in mice, however, increased differentiation of these cell in human required the addition of IL-1 β [51], [52]. The task of TGF- β in human Th17 development is still not well understood; some studies found TGF- β in humans to impede Th17 development, while in cord blood it was found to aid Th17 development [51]. However, Th17 responses are mostly associated with inflammation and tissue destruction, although they are also involved in the fight against pathogens such as bacteria and fungi or other eukaryotic pathogens [47]. Finally, Treg are cells that keep in check the balance between the Th1 and Th2 responses and maintain self-tolerance [53], [54]. They can help to prevent allergy, autoimmunity, inflammation and have been shown to promote regeneration of hair follicles [55]. They are characterised by the presence of IL-2R (CD25) and Forkhead box P3 (FOXP3) gene with the release of abundant IL-10 and TGF- β [47], [54]. TGF- β enhances CD4+ differentiation to Tregs. It is noted that upregulation of Tregs might be good for treatment of autoimmune diseases and allergies while for tumour clearance, Treg downregulation is required [55]. Excessive Tregs have been implicated in reduced anti-microbial immunity [54].

Aside, the conventional T cells, other T cells for instance Gamma delta ($\gamma\delta$)T cells, Mucosal-associated invariant T cells (MAIT) cells and invariant natural killer T (iNKT) have been associated with anti-Mtb immunity [50] by connecting the innate and acquired immune systems [56]. $\gamma\delta$ T cells are a subgroup of T cells that express gamma (γ) delta (δ) on their receptor contrary to alpha-beta ($\alpha\beta$) phenotype found on conventional/classical T cells [56]. These cells constitute a tiny population in the blood, about 1-5% of the T cells [50]. They are mostly found in the epithelial and mucosal tissues [56]. They do not require MHC for antigen recognition, but instead utilise CD1d-dependent proteins for antigen presentation. Sensitised $\gamma\delta$ T cells release IFN- γ , IL-17 and TNF- α [50], [57] which are known to protect against Mtb infection and facilitate granuloma formation. iNKT and MAIT cells express TCR α chain [58] with iNKT cells accounting for about 0.01-1% [58] and MAIT cells about 5% [50] of lymphocytes in the blood. They both mature in the thymus needing no priming to function [59]. iNKT and MAIT cells recognised antigens via the MHC class I-like molecules CD1d [60] and MHC class I-related (MR1) [50], [59] respectively. iNKT cells promote DC maturation[60], [61] thus prompting pathogen destruction whereas MAIT cells are cytolytic and produce TNF- α and IFN- γ against an infection. Since the onset of adaptive immunity is slow, it was hypothesised that MAIT and iNKT cells may be the primary producers of IFN- γ required to inhibit initial pathogen evasion before the arrival of the adaptive response [56].

1.3.3 The initiation of adaptive immune response

In human Mtb infection, the arrival of the adaptive immune response can take about 5-6 weeks whereas in mice, it takes 10 -12 days post infection for Mtb specific CD4+ cell to emerge at the area of injury or inflammation [62]. The delay in priming of the Mtb specific CD4+ cells is not well understood, but it has been suggested that the increase in number of Mtb bacteria in the lymph node may be due to the late arrival of Mtb specific CD4+ or the lack of APC complexes to prime Mtb specific CD4+ cells [62], thus giving rise to the rapid increase in bacteria number and subsequently impeding the immune system's ability to completely eliminate the bacteria. Another hypothesis in regards to the failure to clear or kill the pathogen could be due the suppression of the proinflammatory response by the Treg or down regulation of bacterial antigen gene expression or resistance to the macrophage-activating effects of interferon- γ (IFN γ) [44]. Furthermore, in a bid to survive another strategy Mtb uses is necrosis, a premature cell death which is detrimental to the host as opposed to apoptosis required for proper activation of adaptive immune system [63] or the recruitment of impaired phagocytic cells which cannot controlled Mtb propagation [42].

1.3.4 Host - Mtb interaction

Macrophage - Mtb interaction induces multiple immune activations and cell recruitment, resulting in the formation of granulomas to limit bacteria propagation. Dendritic cells (DC), macrophages and neutrophils collectively known as phagocytes, are the major cells to appear at the place of injury. They pick up and kill the invading pathogen, activating more DC, which then carry the pathogen antigens to the nearest lymph node, for presentation to T cells [19], [45], [64]. The interactions amongst the DC and T cell are very important in initiating the appropriate innate-adaptive immune interaction. When the T cells are activated, they release interleukin-2 (IL-2) [65], IFN- γ , TNF- α , and IL-17 [19], [50], [66], [67]. IL-2 promotes more T Cell proliferation [65], [68], while IFN- γ and TNF- α enhance phagocyte recruitment via the secretion of IL-1, IL-6, IL-12 and IL-23 from activated DC [66]. The increased production of IL-12, IL-18 and IFN- α produces a pro-inflammatory Th1 response with IFN- γ as the major facilitator [65], [68]. Whereas secreted IL-23 by DC on the other enhance Th17 or $\gamma\delta$ T cells IL-17 release. IL-17 an inflammatory cytokine, in turn recruits neutrophil, which further promote granuloma formation thus enhancing Mtb resistance [67]. The release of IL-17 by $\gamma\delta$ T cells has been suggested to protect against early Mtb infection [67], [69]. However, a continuous secretion of IL-17 has also been associated with causing tissue damage, due to an influx of neutrophil and inflammatory proteins, for example TNF- α , IL-6 and MIP-2 [70]. In experimental mouse model, a lack of IL-17 was observed to impair granuloma form [67], whereas in humans reduced IL-17 and IFN- γ levels were observed among individuals with TB disease compared to their

counterpart the LTBI, proposing the inadequate IL-17 could lead to TB disease [69]. Conversely, type I IFN known to induce CD8⁺ cells to clear viral infections, is implicated in causing disseminated bacterial diseases [71]. In murine experimental models, an inhibition of type I IFN was seen to correspond with low bacterial load [72], whereas in human studies, a high levels of Type I IFN genes were observed in TB disease compared to their latently infected counterparts [71]–[73]. Similar studies also observed type I IFN to be highly expressed among TB patients' close contacts who later became active cases, suggesting the expression of type I IFN predisposes an individual to Mtb disease [71], [73]. Although IFN- γ is central in Mtb control, the negative feedback from type I IFN increases an individual risk of catching TB [74].

TNF- α is released by numerous cells to support granuloma formation and maintenance [66]. The importance of TNF- α in containing Mtb was observed when LTBI individuals who underwent anti-TNF- α therapy for autoimmune diseases progressed to active TB disease [75]. Hence the development of the granuloma is pivotal in the control of Mtb [19], [76]. It consists of Mtb with necrotic cells surrounded by lymphocytes and fibroblasts, thus, preventing bacterial dissemination in the host [19], [41]. TNF- α is down-regulated by Th2 cytokines (IL-10 and TGF- β) [65], thus increasing the chance of continuing active TB disease. The granuloma can be present in an individual for a life time, however, when the host immune system is weakened, dormant pathogens can reactivate [77], causing the granuloma to burst, spilling its contents into the blood stream resulting in an increase in bacterial load. This is shown best in HIV⁺ individuals, with decrease in CD4⁺ T cells resulting in lack of control of the granuloma and progression to active TB disease [78].

1.4 Spectrum of *Mycobacterium tuberculosis* infection

Exposure to Mtb has generally been oversimplified into two outcomes – latently infected and active disease. As mentioned earlier, it has been postulated that about 5-10% of infected persons with Mtb proceed to disease in their lifetime [19], while the remainder are able to control the infection. This concept of control or not defines latent and active infections as static states. Of recent, researchers have understood that there are various stages of LTBI; there are those who do not progress to active TB (non-progressors) and others who gradually progress to active TB (subclinical), even though both do not manifest signs TB. Hence the spectrum for LTBI is considered as a continuous dynamic balance between the host and Mtb pathogen. Thus, the focused has been to develop a highly sensitivity test that can detect individual who are most like to have disease. At the moment, the only available tests for Mtb infection diagnosis are tuberculin skin test (TST) and interferon gamma (IFN- γ) release assay (IGRA) [79]. The two tests measure T cell responses toward Mtb antigens. A positive test from either is an indication of infection, whereas a negative test does not necessarily mean no infection. A negative result could be interpreted in many ways such as clearance of infection by the innate immune response without engaging the adaptive immune response [79], or the activated cells homing to the place of injury without immune memory [80]. Or it could reflect cell exhaustion from persistent antigenic stimulation [81]. Importantly, neither test can distinguish latent infection from active disease.

1.5 Distinguishing TB from Other respiratory disorders (ORDs)

Respiratory disorders are diseases affecting air passages causing structural damage and narrowing of the airways, thus limiting gas exchange for the affected individual. They are a huge public health problem accounting for about 4.6 million deaths annually [32], with many of deaths occurring in developing countries. They can be classified into four main categories: obstructive, restrictive, infectious and vascular conditions and range from mild to severe forms affecting both the lower and upper respiratory tracts. The most common respiratory disorders (apart from TB) are pneumonia, chronic obstructive pulmonary disease (COPD), Asthma, Bronchitis, Emphysema, and Cystic Fibrosis. These diseases have similar aetiologies making diagnosis difficult, particularly in resource limited settings.

These common or overlapping symptoms such as fever, cough, dyspnoea, wheezing or chest pain, make the diagnosis of each particular disease challenging [4], [82], [83]. A study performed by Miravittles et al, 2013, assessing COPD patients with mixed diseases was a notable example of this challenge, where 17.4% of patients could not be clearly defined as having asthma or COPD. In

addition, some respiratory disorders such as COPD and TB share similar risk factors including diabetes, biomass pollution, smoking and nutritional status [32], [64], [84], [85]. Moreover the history of pTB is a potential risk factor to developing COPD, and vice versa [84], [85]. Early TB disease also has comparable symptoms and presentations as pneumonia [86]. Therefore, the development of a new TB test must be able to discriminate between TB and ORD (i.e. must be specific). WHO target product profile (TPP) requires >98% specificity and sensitivity for TB diagnostic tests (Table 1.1).

Table 1.1 WHO recommend target product profile (TPP) performance characteristics for screening and diagnosis of TB

Test type	Sens	Spec	Target group	Sample type	Time to Result	Price (USD)
Rapid sputum based	<ul style="list-style-type: none"> • 80% (compare to culture) • 60% (smear negatives) • 99% (smear positive) 	> 98%	pTB patients	Sputum	20mins–2hr	4 – 6
Non-sputum based	<ul style="list-style-type: none"> • $\geq 98\%$ for pTB (smear positive/culture positive) • $\geq 68\%$ (smear negative/culture positive) • Overall $\geq 80\%$ 	98%	adults and children in TB/HIV+	Non-sputum (urine, blood, oral mucosal transudates, saliva, exhaled air)	(20–60) mins	4 – 6
Community-based triaged	<ul style="list-style-type: none"> • Overall > 90% 	> 70%	Adults and children	Sputum or non-sputum	(5 – 30) mins	1 – 2

Sens= sensitivity; spec=specificity; pTB= pulmonary tuberculosis; HIV = human immunodeficiency virus and TB= tuberculosis, USD= United States Dollar

1.6 Tuberculosis Diagnosis

The diagnosis of TB includes knowing the patient clinical history, chest radiography and bacteriological confirmation [87], [88]. Though there are many new TB diagnostic tests in the pipeline, sputum smear microscopy, GeneXpert and sputum culture are still the main tools of TB diagnosis, despite various problems associated with each.

1.6.1 *Clinical Investigation*

Making a fast and correct diagnosis of tuberculosis is actually vital both for the patient and healthcare provider, to initialise early treatment and proper patient management. Thus, when a patient shows up at a health facility with symptoms suggestive of TB disease, the first thing the healthcare provider does is to subject the patient to a physical examination. This is done through the assessment of signs and symptoms of tuberculosis via interrogation about personal and family medical history. This includes assessment of any close contact to an active TB patient to establish the cause of the disease and length of exposure. Signs of TB include a continuous cough for more than two weeks, loss of appetite and weight, fever, sweat a lot in the night, coughing of blood, short breath and chest pain. This method of diagnosis is non-specific and extremely difficult in patients who may be suffering from EpTB, as symptoms may overlap with other conditions like spinal anomalies. Where there is no laboratory tests available, the patient may be put on antituberculosis treatment, with response to treatment the only confirmatory presence of the disease [89].

1.6.2 *Radiological Investigation*

Imaging plays a crucial part in the medical diagnosis of TB and subsequent patient management. Chest radiograph (CXR) is key in the diagnosis and classification of individuals suspected to have TB [90]. It is a rapid method for identifying lung abnormalities[91], assessing treatment response and detecting disease complications. Although CXR has high sensitivity for active TB and is recommended as a triage or screening test, it is not specific, so it cannot be used independently but with a confirmatory bacteriological test required [91] [92]. The impact of HIV co-infection, use of poor quality films, lack of expertise in reading the films, delay in diagnosis and sex of patients have all negatively impacted on the sensitivity and specificity performance of CXR [91] [93]. Digital radiography and portable diagnostic devices or computed tomography (CT) have been trialled too [91]. Some of these methods were noted to be more sensitive and detected more bacterial activities than standard CXR. Nevertheless, these methods are not widely supported due to infrastructure requirements and costs. There is also a gradual move to digital analysis of X-ray using computer-

aided detection (CAD) software. This system will help mitigate variability between readers, thus reducing turnaround time for a CXR result, simultaneously taking care of the scarcity of skilled personnel. Even with the advantage associated with CAD, no recommendation has currently been given by WHO for its use due to limited data to assess its accuracy [91].

1.6.3 Sputum smear microscopy (SSM)

SSM was established about one hundred years ago and still continues to be the bedrock of TB testing in least resource areas [87], [94], [95]. It is cheap, simple and requires minimal laboratory infrastructure.[96] SSM is very specific in TB-endemic settings[97] but has low sensitivity of between 20-80% [18], [86], [98]. Sensitivity is lowest in sputum smear with scarce bacilli such as childhood TB and HIV positive TB patients [18], [97]. It requires about 5000-10000 bacilli per millilitre to detect the organism in the sample specimen. The drawback in sensitivity associated with Ziehl-Neelsen (ZN) method, led to the introduction of fluorescence microscopy (FM) [95]. This test uses fluorochrome dye (e.g., auramine O or auramine-rhodamine) which is more user friendly, sensitive and faster.[86], [97] However, it is also associated with false positives, making the replacement of ZN with Auramine tricky [97]. Furthermore, the size of Mtb bacilli requires adequate training to consistently discriminate acid fast bacilli (AFB) from debris which could attribute to false positive results [87], [95]. The inability of SSM to discriminate the diverse species of MTB complex or detect resistance TB [86] has further prompted the call for a better TB diagnostic tool.

1.6.4 Sputum Culture

Sputum culture is the benchmark on which TB testing are based [18]. It is highly sensitive and specific ((80-93) % and 98 % respectively).[99] It is approximated to be 100-fold more sensitive than SSM [87]. It is mostly used as a confirmatory test to SSM and also provides further drug susceptibility testing. A culture test is either performed on solid media (Lowenstein-Jensen method) or liquid (mycobacterial growth indicator tubes (MGIT) -Becton Dickenson). The time of obtaining a positive result depends on the bacterial load. Solid culture last 6-8 weeks (56 days) to yield a positive result while the MGIT takes shorter time (42 days), with similar times to obtain a negative result [100]–[102].

Though culture is currently the gold standard, it has many limitations – it is expensive, requires adequate infrastructure, is time-consuming, laborious and prone to contamination and also requires good quality sputum [96], [103]. Moreover, culture is a luxury in resource-limited settings resulting in reliance on SSM and clinical evaluation of presumed TB cases. In laboratories where culture tests are available, the major setback is the long result turnaround time (up to 6 weeks) [87] impacting on

patient management [88]. The inclination to put clinically presumed TB cases on treatment without bacteriological confirmation carries the risk of non-TB patients being exposed to toxic TB therapy and the tendency of resistance bug to emerge. And with the changing epidemiology of TB; the increasing MDR and or HIV co-infection, and overlapping of symptoms with other respiratory diseases, a better diagnostic test which can mitigate the limitations of SSM and culture would be a great add-on in the fight against Mtb.

1.6.5 Nucleic Acid Amplification Test (NAAT)

In recent years, molecular based testing has emerged to be the main diagnostic tool for tuberculosis and drug susceptibility testing in resource-rich settings [88]. This molecular assay testing otherwise called nucleic acid amplification tests (NAAT) are now common and have the ability to detect a small bacteria genetic material as well as drug resistant Mtb directly in the specimen [104], [105]. The assays are rapid, sensitive and specific, significantly improving the time to treatment.[88] However, they are expensive and require significant infrastructure, thus limiting their use in basic healthcare facilities accessed by the majority of TB patients [105]. Furthermore, the performance of these assays largely depend on sample collection method, volume and processing [106].The need for a well-trained individual and well equipped laboratory to perform the assay is a must requirement. In addition, NAAT assays varies in accuracies across studies, with reproducibility challenge, thus its recommendation as a replacement of culture is not likely [107]. Moreover, the presence of inhibitors could affect the outcome[108]. Though NAAT assays have been shown to be very sensitive (90-100%) and specific (71-96%) on sputum smear positive respiratory samples, sensitivities dropped to (22 - 89%) while specificities are still good (97- 99%) in sputum smear-negative samples. For example, GeneXpert MTB-RIF (Cepheid) an automated method which detects both MTBC and RIF resistance bugs directly in sputum samples within 2hr,[107], [109] has sensitivity of 98.2% sputum smear positive and specificity of 99.2% while on smear negative, sensitivities ranges from 72.5-90.2% [110]. Although GeneXpert has a tremendous immediate patient and public health impacts such as earlier patient diagnosis and treatment,[111] the use of the tool in lower tier healthcare centres is still a problem. The cost of infrastructural maintenance[109] remains a challenging aspect of the assay, limiting its uptake in resource limited settings, despite being heavily subsidised by the Bill and Melinda Gates Foundation (BMGF).

NAAT's inability to differentiate dead from live bacteria also hinders identification of faster treatment responders who may benefit from a shorter treatment course [88], [96]. Due to these limitations, we still require a simple, affordable, rapid and accurate point of care (POC) test which drastically improves public health in least resource places.

1.7 Tuberculosis biomarkers

1.7.1 Biomarkers

Biological biomarkers has been defined as “biological characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention”[112]. A combination of these biomarkers is referred to as biosignature. The application of biomarkers in medical care spanned back to many decades, thus, is a common practice in medical care. With the limitations of the current TB diagnostic tests, researchers are looking into (host or pathogen specific) biomarkers as a possible solution for TB diagnosis. Biomarker diagnoses are known to be rapid, accurate and less expensive, most often requiring no or less infrastructure to perform or set up a test. An example of these are the malaria rapid test (RDT), pregnancy and HIV tests. Biomarkers can be characterised based on different parameters such as its application: 1) exposure 2) disease and 3) treatment response. Biomarkers are indicators of immune activities. Therefore, their importance as possible diagnostic markers cannot be overemphasised. They would be useful in predicting, monitoring and testing the state of a disease or effectiveness of an intervention. Of recent, studies are looking into how biomarkers can be utilised to aid personalised medicine for instance cancer treatment. Thus, finding a sputum based markers would likewise aid the diagnosis of pulmonary tuberculosis, subsequently impacting positively in the reduction of transmission and disease mortality. Such a potential biomarker should safe and easy to measure, modifiable with treatment and consistent across gender, age and ethnic groups. At the moment, the only marker of tuberculosis disease is the detection or isolation of *Mtb* bacilli or its product in the sputum or urine of the host. With culture positive conversion to culture negative sputum the only marker of treatment response. The roadblock with this procedure of disease diagnosis is suboptimal performance of the current diagnostic methods in combination with the number of bacilli in the sputum. Therefore, exploration of the host markers in sputum which is the ideal representative sample of the site of infection or disease might provide a solution. Thus is the aim of this study. We have previous shown in the pilot study, a combination three markers correctly classified TB by 96%.

1.7.2 Immunological biomarkers of TB

Previously, diagnosis of tuberculosis infection relied on a century old test called tuberculin skin test (TST), a protein – purified protein derivative (PPD) extract from *Mtb*. The protein is injected intradermally into the forearm and the monitored for 48 -72 hours, then the induration is measure. Although TST is easy, cheap and widely performed, a TST result depends on the interpreter as well

as the age, nutrition and immune competency of the patient. The estimated cut-off of a TST result ranges between 5 -15mm, which also depends on the prevalence of disease, HIV status, age or medical history of the participant [113]. Furthermore, BCG vaccination or the prevalence of NTM, can affect a TST result due to shared proteins between the TST and BCG or NTM. Moreover, TST cannot differentiate active TB from LTBI. Thus, leading to introduction interferon gamma release assay (IGRA), a blood-based assay, which relies on the IFN- γ release by the stimulation of whole blood or PBMC with Mtb specific antigens – 6kDa early secretory antigenic target (ESAT-6) and 10kDa culture filtrate antigen (CFP-10). There are two commercially available assays that utilised these antigens: Enzyme linked immunosorbent assay (ELISA) which measures IFN- γ release into plasma (QuantiFERON®-TB gold test (Cellestis Ltd., Australia)) and (Enzyme linked immunosorbent spot (ELiSpot) T-SPOT-TB assay (Oxford Immunotec, UK), which quantifies isolated peripheral blood mononuclear cells (PBMC) that releases IFN- γ . IGRA has comparable sensitivity to TST, however, it is more specific and not affected cross reactivity with either NTM or BCG. But the setbacks for IGRA are, it is an overnight stimulation assay, thus limiting its use as a POC and secondly it does not differentiate active TB from LTBI. Therefore, alternative markers are required in order to develop a test that is rapid, cheap and able to: 1) differentiate active from LTBI, 2) monitor treatment response and 3) predict relapse.

Recently, WHO has recommended a new commercial diagnostic test for TB (TB-LAM) (Alere Inc. Waltham, MA, USA), that uses lateral flow strip (non-laboratory based) to diagnose TB within 30mins [114], [115]. The AlereLAM test is an immunoassay that detects traces of the mycobacteria - Lipoarabinomannan (LAM) in urine of adult active TB [114], [116]. The test was found to be more relevant among active TB cases living with HIV, particularly advanced AIDS patients with low CD4 numbers, approximated to 100cells/ μ l than HIV negative TB patients [114]. A randomised control trial has shown reduced deaths among hospitalised TB cases by 17% [116], [117] prompting WHO to recommend for its use among TB/HIV+ and severely ill HIV patients [118]. It was also noted that the TB-LAM detects more individuals with TB in patients with lowest CD4 numbers [115], [119]. A five-study systematic review showed the sensitivity and specificity of TB-LAM among patients with CD4 cell \leq 200/ μ l to be (50 and 90) percent respectively [116], [117]. Recently, a new TB-LAM diagnostic test developed by FIND and Fuji called the Fujifilm SILVAMP TB LAM (FujiLAM; Fujifilm, Tokyo, Japan) has also been shown to diagnose TB/HIV+ patients. Studies have shown FujiLAM to be more sensitive than the WHO approved AlereLAM [115], [118]. The FujiLAM test uses an immunochromatography silver halide amplification technology to improve the test control line visibility thus improving sensitivity but taking longer to generate results compared to AlereLAM [118]. Even though TB-LAM shows promise as a point-of-care diagnostic of TB among HIV infected

individuals. not many countries currently include LAM as part of their TB national control programme [116]

While TB mainly affects the lungs, researchers are looking for alternative sample types for non-invasive and non-hazardous diagnosis. These include serum, plasma, culture supernatants and urine. A biomarker is a protein in the body that indicates a normal or abnormal condition [76] whereas, a bio-signature is a combination of biomarkers. Many researchers have reported possible biomarkers or signatures for TB. Studies that utilised stimulated blood samples have reported finding many biomarkers or biosignatures that have the potential to distinguish TB disease from either latently infected, ORD or healthy controls. For example, in a study done by Chegou et al in 2013 analysing QFT-GIT supernatants, they found IFN- α 2, IL-1Ra, IP-10, sCD40L, IFN- γ , VEGF, TGF- α and EGF to differentiate children with TB from those Mtb infection [120]. In a similar study by the same group comparing QFT-GIT supernatants of TB case and their close contacts, MIP-1 β showed potential as a single marker to differentiate TB from LTBI, whereas a combination EGF with MIP-1 β , sCD40L, IL-1 α or VEGF or a three-marker signature (EGF_{Nil}, MIP-1 β _{Ag-Nil} and IL-1 α _{Nil} (or IL-1 α _{Ag}) all showed promised to discriminate TB and LTBI as a biosignature [121]. The group did a follow up seven-day TB antigen stimulation and showed EGF and TGF- α as potential markers of TB [122] while a multisite QFT-GIT data showed a four-marker signature (IFN- γ _(NIL), TGF- α _(NIL), IL-1 α _(Agn) and MIP-1 β _(Agn) discriminated TB from ORD with 71% sensitivity and 81% specificity [123]. Since overnight stimulations are a hurdle to developing a rapid point-of-care test, researchers have now turned their focus to *ex vivo* sample types. In plasma study IL-6, IP-10, TNF- α , sCD163 and sCD14 were found to be higher in TB compared to LTBI or healthy controls but this study did not include an ORD group [124]. Another study looking at serum found a 6-marker combination of NCAM, SAP, IL-1 β , sCD40L, IL-13 and Apo A-1 could discriminate TB from ORD with a sensitivity of 100% and a specificity of 89%, increasing to 100% if HIV positive subjects were excluded [125]. In another study IL-1 β , IL-23, ECM-1, HCC1 and Fibrinogen in saliva discriminated TB from ORD with a sensitivity of 89% and specificity of 90%, and an eight-marker signature resulted in a sensitivity of 100% and specificity of 95% [126]. Though the results were very good, the author caution that this was a discovery phase and the sample size was small, thus confirmatory studies should be done. Similar studies were also carried in our laboratory in which Sutherland et al (2012) found a three-marker signature (IP-10, IL-6 and IL-10) which classified pleural TB from ORD with 96% accuracy [127]. The same laboratory showed a combination of IL-13, FGF and IFN- γ in *ex vivo* sputum resulted in 96% specificity and 85% sensitivity [128]. Furthermore, a multisite collaboration, which included MRCG at LSHTM, showed promising results with a seven-marker serum signature of CRP, transthyretin, IFN- γ , complement factor H, apolipoprotein-A1, interferon (IFN) γ inducible protein 10 (IP-10), and

serum amyloid A) discriminating TB from ORD with a sensitivity of 93.8% and 73.3% specificity [129]. A follow up study was conducted utilising a finger prick lateral flow test strip to evaluate the six of those marker (IL-6, IP-10, CRP, SAA1, Ferritin and ApoAI), with promising data on three markers (IP-10, SAA1 and CRP) which are now under further evaluation for future use in basic healthcare centres as a triage test.

1.7.3 Immunodiagnostic triage tests

New immunodiagnostic tests are currently being developed such as acute phase protein that is shown to increase and decrease rapid during and after an infection or in the course of treatment especially for children [90]. However, it has limited specificity for TB on its own. Another marker that is under investigation for use in diagnosis of TB or TB treatment response is IP-10 [90], [91]. Numerous studies in various settings showed IP-10 to be highly increased in TB patients compare to non TB controls (LTBI, ORD or Healthy) even without stimulation as reviewed by Ruhwald et al (2012) [92] and reported by other people [93]. This marker was also among the recently discovery signature (seven-marker biosignature) [94] translated to a user friendly lateral flow device to discriminate TB among other respiratory diseases in our laboratory and our collaborators' laboratories [95]. A 3-gene signature was also identified as promising signature for screening TB as well treatment response, thus a potential replacement for sputum conversion marker at 2 months. The same author has claim that the signature is able to discriminate TB from LTBI or healthy control regardless of HIV status [96]. Although many of the tests have inadequate diagnostic performance, some of them show potential to be used as screening or triage test as per the WHO recommendation for a screening or triage test, which requires sensitivity >90% and specificity >70% [97] (Table 1.1) One important requirement for a triage test is a high sensitivity in order to identify as many cases as possible [90]. A test should be affordable and obtain results within a short time (<20) mins. Moreover, for a triage test to be effective, those who are pronounced positive, should proceed to a confirmatory test on the same day, to minimise transport cost.

1.8 Treatment and treatment response

1.8.1 Treatment

The treatment for presumed susceptible TB last six months [130], however, with slow responders, treatment could be extended. Treatment is also much longer (15-20) [131] months for individuals have multiple drug resistant TB or extremely drug resistant TB. The consequence of late diagnosis and prolong initiation of treatment is death with an estimated 70% of untreated smear positive Mtb case dying [132]. Inappropriate or ineffective TB treatment could result in longer treatment duration and potential development of resistant TB or even a relapse of the disease. TB treatment regimen requires a combination of multiple anti-tuberculosis drugs [isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (SM)] and in two phases - the intensive and continuation [133], [134]. The intensive phase lasts for 2 months and includes four drugs - isoniazid, rifampicin, pyrazinamide and ethambutol. The continuation phase lasts for 4 months and consists of isoniazid and rifampicin [134], [135].

1.8.2 Treatment response

Tuberculosis treatment response monitoring poses similar challenges as TB diagnosis due to reliance on pathogen detection. Currently the only accepted marker for treatment response is a culture positive sputum conversion to negative [136] but the challenge is its predictive ability is low aside, the limitation associated with culture and smear microscopy. Of recent, a Mycobacterial load assay has been developed with a great potential to be used as treatment monitoring technique, it detects mainly the viable bacilli through detection of ¹⁶S rRNA [137]. However, it is tedious, expensive and requires skilled personnel to perform the procedure. Thus, various studies are looking into host immunological markers for potential TB diagnosis or treatment response monitoring. Many studies have also reported the essence of immunological markers in future TB diagnosis and treatment monitoring, hence more studies are focusing in this area of research. Previous Flow Cytometry based studies have shown early immune based markers such as CD38 and HLA-DR and proliferation Ki67 expression on Mtb specific CD4+ to be ideal markers of TB treatment response [138], [139]. Equally, IL-10 and MIP-1 α were also found to higher among TB cases who showed slow response to antituberculosis treatment [140], IL-10 a cytokine known to impede bacteria clearance, as a result, promoting disease progression [141], whereas MIP-1 α is known to recruitment

phagocytic cells to site of infection [142], thus suggesting these cytokine are potential indicator of disease burden. Their presence in high concentration may also suggest prolong treatment [140]–[142]. A transcriptomic study also reported a 3-gene TB score with the potential to monitor treatment outcome [143], while a positron emission tomography and computerized tomography (PET-CT) images showed the presence of lesions and MTB mRNA – Alpha-crytallin (*hspX*) to be linked to persistence MTB in the host even after completion of treatment [144]. A study done on saliva reported a combination of IL-17A, IL-23 and ECM-1 to be good indicators of treatment responses when TB cases were compared to other respiratory disorders [126]. Many studies have also found CRP, SAP, IP-10 and VEGF to be good indicators to monitor TB treatment [125], [145], [146]. Although, studies have reported identifying potential markers of TB treatment, the challenge is how to escalate this finding to a POC test. Aside, many of these used different, patient groups, sample type, technique and also in in different geographical area.

1.9 Project aims and objectives

The aim of this project is to profile a broad array of host markers for development of optimal biosignatures for detection of pulmonary tuberculosis from other respiratory disorders using ex vivo sputum samples.

1.9.1 Specific Objectives

- To determine optimal signatures in *ex vivo* sputum for diagnosis of sputum and culture positive TB patients
- To determine optimal signatures for diagnosis in difficult patient groups including:
 - Smear negative but culture positive TB patients
 - HIV co-infected TB patients
- To determine sputum host markers for monitoring of treatment response

Chapter 2 Materials and Methods

2.1 Ethics approval

Ethical approval was given by the MRCG at LSHTM/Gambian government joint ethics committee. A written informed consent was done before the initiation of the project. For participants <10 years, consent was given by their legal guardian or parents while those above 10 year personally agreed to join the study. The ethic approval code for this project was SCC1333

2.2 Study participants and setting

Study participants were recruited from patients who were seeking medical attention at the MRCG at LSHTM outpatients department and the Tuberculosis clinic with symptoms associated with TB, prior to clinical or microbiological diagnosis. Upon written informed consent, sputum samples were collected at baseline from all participants and at 1 month and 2 months from those who were subsequently found to have TB. The collected sputa were then transported in a cold box at 4-8°C to the TB Diagnostic (TBD; 5 minutes' walk from the clinic) where the samples were processed. At the TBD laboratory, the temperature and sample information were verified and logged to a sample book in the TBD laboratory. Microbiological tests (smear microscopy and/or culture and/or GeneXpert) were performed on all subjects. Participants who were smear negative without any further confirmatory tests had mycobacterial load assay (MBLA) performed to confirm the results as described in section 2.4. The participants were then classified as either having TB or other respiratory disorders (ORD). The clinical information for the participants were entered into our MRCG at LSHTM database.

2.3 Current microbiology test procedures

2.3.1 *Liquid culture procedure*

Once the samples were received in TBD laboratory and registered, a portion of each sample was aliquoted into the 50ml falcon tube. An equal volume of N-Acetyl-L-Cysteine–Sodium hydroxide (NALC-NaOH) [4% NaOH+2.9% Trisodium+0.5gm of NaCl] was then added to the falcon tube

containing the sample. The falcon tube was then tightly closed, the solution in the tube mixed by swirling on the shaker for 5sec. Then incubated for 15mins at room temperature (RT). Following the incubation, the sample was centrifuged at 3000xg for 20mins, with centrifuge set at aerosol-free and refrigerated, with sealed buckets. Following the centrifugation, the sample was allowed to settle for 5mins, then the supernatants harvested and discarded into a jar containing concentrated hycolin or phenolic disinfectant. While 0.5ml of sediment was transferred into the Mycobacteria growth indicator tube (MGIT BBL tube, 7mL) containing 2ml of 50% glycerol (sterile phosphate buffer – PH 6.8). Prior to the transfer of sediment, the MGIT PANTA Antibiotic was reconstituted with 15ml BACTEC MGIT Growth Supplement. An aliquot of 0.8ml of the reconstituted growth supplement was then transferred into the MGIT tube followed by the addition of 0.5ml of sputum sediment. The MGIT tube was then tightly closed and mixed by inverting the tube several times. Next the tube is wiped dry, scanned and inserted into BACTEC MGIT 960. The temperature is regulated close to 37°C. The tubes were left in the instrument until flagged positive but after six weeks, all samples that the instrument has not flagged up, were considered instrument negative. An acid fast bacilli (AFB) and subculture were then performed on the instrument positives as a confirmation Mtb presence. All steps were carried out in biological safety cabinet (BSC) level III in a contained facility.

2.3.2 GeneXpert (*XpertMTB/RIF*) procedure

An aliquot of one millilitre of the collected sputum was transferred into a 15ml falcon, next 2ml of sample reagent was added. The sample mixture was gently shaken by inverting the falcon tube 20 times. The sample was then incubated for 10mins at RT, followed by another gentle shake. After which an aliquot of 2ml diluted sample was transferred to the XpertMTB/RIF cartridge, then scanned to identify the module to insert the cartridge. Once the cartridge was inserted, the run would last 2hr. The results generated from XpertMTB/RIF assay was either Mtb detected as (high, medium, low or very low) or Mtb not detected. The assay also generated cycle threshold (bacterial load) as well as RIF resistant.

2.4 Mycobacterial load assay

2.4.1 Sputum digestion

An aliquot of sputum was digested with Sputolysin (1mg/ml, Merck Millipore, USA) at 1:1 dilution, then vigorously vortexed and incubated for 15 mins at room temperature (RT). After the incubation, the samples were centrifuged at 2000rpm for 10mins. The supernatants for Luminex analysis were harvested into sarstedt tubes, while pellets for MBLA analysis were re-suspended in 1ml of trizol (ThermoFisher, UK). Both tubes were stored in -80°C for later use.

2.4.2 RNA extraction

Pellets stored in trizol were thawed and 2µl of a 560 RNA extraction control (Bioline Reagents Ltd, UK) was spiked into the thawed samples. RNA extraction was performed using Qiagen RNeasy Mini Kit (250) (Qiagen, Germany). Two hundred microliters (200µl) of chloroform was added to the thawed samples, vigorously mixed and incubated for 10mins at RT. Samples were then centrifuged at 13000rpm for 15mins at 4°C. The upper aqueous phase was harvested, an equal volume of 70% ethanol was added and vortexed. The 700µl of the mixed solution was transferred to RNeasy-MinElute Spin Columns (Qiagen, Germany) and centrifuged for 15 sec at 10,000 rpm. The flow-through discarded and flow-through collection tube re-used. This step was repeated if the volume of mixed solution was more than 700µl. Next 350µl of RW1 buffer was added into the RNeasy-MinElute Spin Columns, then incubated for 5mins at RT, followed by another 15 sec at 10,000 rpm spun. The flow-through was discarded, the RNeasy-MinElute Spin Columns was then inserted into a new flow-through collection tube. 80µl of DNase master mix (10µl DNase plus 70µl RDD buffer) was added into the RNeasy-MinElute Spin Columns, incubated again for 15mins RT. Following the incubation, 350µl of RW1 buffer was added, spun for 15 sec at 10,000 rpm. The flow-through was discarded, a 500µl of diluted RPE buffer (1 part of RPE to 4 parts of 100% ethanol), spun for 2mins at 10,000rpm, this step was repeated, then flow-through discarded. The RNeasy-MinElute Spin Column was then transferred into a new collection tube and centrifuged at full speed (13,000 rpm) for 5mins. To elute the RNA, the RNeasy-MinElute Spin Column was transferred to a storage tube (eppendorf), a 30µl of elution buffer was added and spun for 2 mins at 13,000 rpm. This step was done twice to collect two 30µl aliquots of eluted RNA, which were stored at -80°C until processing.

2.4.3 H37Rv Standard preparation

800µl of growth supplement and 500µl of Mtb (H37Rv) stock were added to a mycobacteria growth indicator tube (MGIT). The tube was then placed in the BACTEC (Becton Dickinson, USA) machine for 3-5 days to confirm Mtb viability. After incubation, 20ml of 7H9 medium was used to subculture a

500µl of Mtb cell suspension for 2 weeks in a 37°C, 0.05% CO₂ incubator. The optical density (OD₆₀₀) was determined every 2 days using a spectrophotometer alongside single colony forming unit (cfu) counts to confirm growth curves. When an OD of 2.2 was reached, 1 ml of Mtb suspension was aliquoted and stored in an equal volume of trizol at -80°C for later use. To generate a standard curve, a serial dilution of one aliquot of Mtb suspension was performed, then plated on 7H9 agar plate, and 20µl of each serially diluted vial was plated on 7H11 at 3 different spots. These were then incubated for 3 weeks with a visual count of cfu performed every 2 days. RNA was then extracted and qRT-PCR was used to run the standards targeting the 16S rRNA of MTBC. The value for the top standard was estimated at 10⁸ cfu.

2.4.4 16S RNA analysis

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to determine the level of viable bacteria (16S rRNA) in the extracted sputum pellets. A SensiFAST Probe No-ROX One-Step Kit (Bioline, Reagents Ltd, UK) was used to perform the assay. A master mix containing 12.5µl 2x SensiFast Master Mix, 6.75µl of nuclease free water, 0.2µl reverse transcriptase, 0.4µl of RNase inhibitor, 0.05µl of 10uM (16^S Rox Probe, forward primer and reverse primer) was used for a single reaction. In addition, 5µl of RNA sample, standard or nuclease free water was added to their appropriate wells in a 96 well plate containing the 20µl of reaction mix for a total volume of 25µl. The plate was briefly vortexed and centrifuged at 10000rpm for 1min. A QuantStudio qPCR 7 plex system (Applied Biosystems, UK) was used to run the assay at the following thermal cycling parameters: 1 cycle at 45°C for 20mins and 95°C for 10mins followed by 40 cycles at 95°C for 10sec and 60°C for 45sec. The results were analysed with ABI7500 software version 2.3 (Applied Biosystems, UK).

2.5 Multiplex Immunoassay

Using the Luminex platform, three multiplex immunoassays were performed for each sample. 1) a 27-plex Bio-Plex Pro™ Human Th1/Th2 Cytokine Panel from Bio-Rad (Belgium) with analytes: IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL12p70, IL-13, IL-15, IL-17A, Eotaxin, FGF-basic, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF. 2) a Bio-Plex Pro™ Human inflammation panel I/II 37-plex kit (Bio-Rad, Belgium) with analytes: APRIL/TNFSF13, BAFF/TNFSF13B, sCD30/TNFRSF8, sCD163, Chitinase 3-like 1, gp130/sIL-6Rβ, IFN-α2, IFN-β, IFN-γ, IL-2, sIL6Rα, IL-8, IL-10, IL-11, IL-12(p40), IL-12(p70), IL-19, IL-20, IL-22, IL-26, IL-27(p28), IL-28A/IFN-λ2, IL-29/IFN-λ1, IL-32, IL-34, IL-35, LIGHT/TNFSF14, MMP-1, MMP-2, MMP-3, Osteocalcin, Osteopontin (OPN), Pentraxin-3 (PTX), sTNF-R1, sTNF-R2, TSLP and TWEAK/TNFSF12. 3) MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel - Premixed 38 Plex –HCYT MAG-60K-PX38 (Merck Millipore, Germany) with analytes sCD40L,

EGF, Eotaxin, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN- α 2, IFN- γ , IL-1 α , IL-1 β , IL-1 α , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, MCP-1, MCP-3, MDC, MIP-1 α , MIP-1 β , TGF- α , TNF- α , TNF- β and VEGF.

We opted for these three immunoassays because in the first place the pilot study that led to this current study was performed with 27-plex. The results from that study were encouraging, moreover, we discovered *ex vivo* host markers in sputum were higher when compared to serum or saliva. So we stucked with the 27-plex kits and added the 37-plex which contains some cytokines of interest such as the type I interferons. Finally, the inclusion of the Millipore kit was based the collaborative work performed with SUN immunology research group using culture supernatant. We found the markers in the Millipore panel to have performed well in classifying TB and ORD, hence, we opted to try the panel the current study.

The experiments were performed according to the manufacturers' specific protocols however, the beads and detection antibodies were diluted 1:1 as previously optimised in our laboratory. Following sample preparation, the specific immunoassay procedures were performed as described in the sections 2.4.1 and 2.4.2 below.

2.5.1 Bio-Rad immunoassay procedures – 27 and 37-plex assays

The lyophilised standards and controls were reconstituted with standard diluent (27-plex 500 μ l, 37-plex 781 μ l and control 250 μ l) and kept on ice for 30mins. After the incubation, the reconstituted standard was serially diluted to 8 standard points. While the reconstituted standards/control were incubating on ice, the coupled beads were sonicated for 30sec, vortexed briefly and reconstituted with assay buffer. 50 μ l per well of the reconstituted beads was added to the assay plate and then washed twice with 100 μ l per well of 1x diluted wash buffer. Followed by an addition of 50 μ l per well of samples, blank, controls and serially diluted standards to the appropriate wells (Fig. 2.1). The plates were incubated in foil for 30mins (27-plex) or 1hr (37-plex) on a rocking platform at 850rpm at RT. Following the incubation, the plates were washed, 25 μ l detection antibody added and incubated as above for 30mins. At the end of this incubation, another wash was done, then 50 μ l of Streptavidin-PE was added, incubated for 10mins, washed and 125 μ l assay buffer added. The plates were shaken briefly and read. All washes were done using a magnetic wash station (Bio-Rad, Belgium). The Magpix was used to read the plates and Bioplex Manager (MP 6.1) software was used to analyse the data. Standards from plate 1 were imported to plate 2 for analysis (Fig. 2.1). The range of the serially diluted standards (Top and lowest) and controls for 37-plex are outlined below. (Table 2.1, 27-plex and Table 2.2 37-plex)

Table 2.1. The 27-plex kit standard range

Marker	Standard Ranges (pg/ml)	
	Top standard value	Lowest standard value
IL-1 β	3992	0.24
IL-1- α	41240	2.52
IL-2	24962	1.52
IL-4	2985	0.18
IL-5	70064	4.28
IL-6	11177	0.68
IL-7	29961	1.83
IL-8	18289	1.12
IL-9	33284	2.03
IL-10	27336	1.67
IL-12(p70)	27162	1.66
IL-13	10111	0.62
IL-15	169961	10.37
IL-17A	41474	2.53
Eotaxin	5472	0.33
FGF-Basic	54963	3.35
G-CSF	150626	9.19
GM-CSF	8975	0.55
IFN- γ	18294	1.12
IP-10	47181	2.88
MCP-1	9456	0.58
MIP-1 α	2116	0.13
MIP-1 β	6935	0.42
PDGF-BB	53904	3.29
RANTES	36307	2.22
TNF- α	68937	4.21
VEGF	155565	9.49

pg= picogram, ml= microliter

Table 2.2. 37-plex markers' standard and control ranges

Marker	Standard and Control Ranges (pg/ml)		
	Top standard value	Lowest standard value,	Expected control range
APRIL/TNFSF13	995390	455	40605 - 94744
BAFF/TNFSF13B	347678	159	8458 -19736
sCD30/TNFRSF8	18377	8	389 -930
sCD163	617571	282	13860 - 32339
Chitinase 3-like 1	107240	49	2313 -5397
gp130/sIL6R β	156594	72	3613 -8430
IFN- α 2	12219	6	255 -594
IFN- β	3069	1	62 - 146
IFN- γ	15079	7	315 - 734
IL-2	10223	5	198 - 462
sIL-6R α	36429	17	1339 - 3124
IL-8	9100	4	138 - 323
IL-10	6147	3	106 - 247
IL-11	1262	1	29 - 67
IL-12(p40)	18546	8	287 - 669
IL-12(p70)	3139	1	63 - 147
IL-19	20144	9	309 - 721
IL-20	9819	4	239 - 557
IL-22	26618	12	537 - 1253
IL-26	103216	47	2019 - 4712
IL-27(p28)	19964	9	551 - 1286
IL28A/IFN- λ 2	19087	9	403 - 939
IL-29/IFN- λ 1	21154	10	380 - 886
IL-32	12104	6	409 - 953
IL-34	102537	47	5149 - 12015
IL-35	49763	23	1256 - 2930
LIGHT/TNFSF14	8293	4	195 - 455
MMP-1	226295	103	5093 - 11883
MMP-2	439914	201	10752 - 25088
MMP-3	413108	189	7809 - 18222
Osteocalcin	149899	69	3031 - 7071
Osteopontin(OPN)	301476	138	8750 - 20417
Pentraxin-3	72713	33	3525 - 8226
sTNF-R1	49154	22	941 - 2195
sTNF-R2	19939	9	400 - 933
TSLP	2085	1	36 - 83
TWEAK/TNFSF12	12292	6	198 - 463

pg= picogram, ml= microliter

Figure 2.1: Plate layout for Bio-Rad Human Cytokine/Chemokine kits Lot#.....

Plate1: **Date:**

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1	STD 1										
B	STD 2	STD 2										
C	STD 3	STD 3										
D	STD 4	STD 4										
E	STD 5	STD 5										
F	STD 6	STD 6										
G	STD 7	STD 7									QC	QC
H	STD 8	STD 8									BLK	BLK

Plate 2: import standards

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G											QC	QC
H											BLK	BLK

The most concentrated standard is STD1 and the least is STD6

STD= standard, QC= quality control and BLK= Blank

2.5.2 Milliplex MAP immunoassay procedure – 38-plex assay

The lyophilised standard and controls were reconstituted with 250µl distilled water each, then incubated for 10min at RT. The reconstituted standard was serially diluted to 6 standard points (10,000, 2000, 400, 80, 16, and 3.2) pg/ml, with 0pg/ml of assay buffer as blank. The premixed beads were sonicated, vortexed and diluted (1750µl of premixed beads into 3850µl of bead diluent). 50µl of the diluted beads was then added to the immunoassay plate and washed. 25µL of serially diluted standard, control, blank or samples were added to the appropriate wells as outlined in the plate map below (Fig. 2.2). Followed by another addition of 25µl of sample matrix into the blank, standard and control wells, while 25µl of assay buffer was added to the sample wells. The plate was then sealed and wrapped with foil. The wrapped plate was then incubated for 2hr at 850rpm at RT. After incubation, the plate was washed twice, 25µl of diluted (3200µl of concentrated detection antibody plus 2400ul of assay buffer) detection antibodies added, plate sealed rewrapped and placed on a shaker at the same incubation conditions for 1hr. Following the 1hr incubation, 12.5µl of Streptavidin-PE added, incubated again for 30mins. A final wash was done, 80µl of sheath fluid added and rocked for 5mins. All washes were done using a Magnetic wash station. The Magpix was used to read the plates while Bioplex Manager (MP 6.1) was used to analyse the data. Standards from Plate 1 were imported for Plate 2 analysis (Fig. 2.2). However, one standard curve was used to extrapolate the data for all the markers, but, each marker had its own control ranges as outline in Table 2.3.

Table 2.3. The range of controls for 38-plex markers

Marker	Control range (pg/ml)	
	QC 1	QC2
EGF	103 - 215	479 -994
Eotaxin	98 - 203	448 - 931
FGF-2	75 - 156	374 - 777
Flt-3L	101 - 210	497 - 1033
Fractalkine	112 - 232	501 - 1042
G-CSF	115 - 240	557 - 1158
GM-CSF	91 - 190	447 - 928
GRO	99 - 205	500 - 1037

IFN- α 2	88 - 184	430 - 894
IFN- γ	93 - 193	467 - 969
IL-1 α	111 - 230	492 - 1021
IL-1 β	89 - 185	425 - 883
IL-1 α	80 - 167	366 - 761
IL-2	93 - 194	461 - 957
IL-3	105 - 219	501 - 1040
IL-4	92 - 192	455 - 945
IL-5	114 - 238	513 - 1065
IL-6	104 - 216	512 - 1063
IL-7	98 - 204	489 - 1017
IL-8	101 - 209	459 - 952
IL-9	113 - 235	527 - 1095
IL-10	108 - 224	528 - 1098
IL-12(p40)	108 - 225	549 - 1141
IL-12(p70)	120 - 248	565 - 1174
IL-13	111 - 230	544 - 1131
IL-15	96 - 200	465 - 965
IL-17	101 - 211	489 - 1017
IP-10	90 - 186	462 - 960
MCP-1	105 - 217	503 - 1044
MCP-3	109 - 227	490 - 1018
MDC	135 - 281	649 - 1349
MIP-1 α	136 - 282	746 - 1549
MIP-1 β	88 - 183	450 - 935
sCD40L	83 - 172	478 - 993
TGF- α	94- 195	407 - 846
TNF- α	92 - 192	472 - 981
TNF- β	111-230	515 - 1070
VEGF	103 - 214	542 - 1166

QC= quality control, pg = picogram and ml=microliter

Figure 2.2: Plate layout for MAP Human Cytokine/Chemokine kits Lot#.....

Plate1:

Date:

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1	STD 1										
B	STD 2	STD 2										
C	STD 3	STD 3										
D	STD 4	STD 4										
E	STD 5	STD 5										
F	STD 6	STD 6										
G	QC1	QC1										
H	QC2	QC2									BLK	BLK

Plate 2: import standards

kits Lot#.....

Date:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G	QC1	QC1										
H	QC2	QC2									BLK	BLK

The most concentrated standard is STD1 and the least is STD6

STD= standard, QC= quality control and BLK= Blank

2.6 Statistical Analysis

Statistical analyses and graphs were generated using R – statistics (<https://www.R-project.org/>) and GraphPad Prism version 8.0.2 (Software Mackiev, USA) respectively. The median cytokine levels in TB and ORD groups were compared using the Mann–Whitney U-test, followed by multiple correction testing using Benjamin Hochberg test [147], [148]. Generalised linear multiple (GLM) regression analyses were performed and receiver operator characteristics curves (ROC) generated to assess the predictability and performance of the analytes in differentiating TB from ORDs. The performance characteristics and cut-offs for individual markers of the ROC analyses were generated using OptimalCutpoints-package version 1.1-4[149] in association with the Youden Index method. After performing single marker analyses, a combined marker analysis was done to evaluate the ideal biosignature for differentiating TB from ORD using multiple logistic regression. Prior to this, the data was randomly split into training and test datasets in a ratio 0.8 to 0.2. Upon performing the multiple logistic regression, a multicollinearity of the combined markers analysed was verified using variance inflation factor (VIF)[150] in the car package version 3.0-3[151]. Then a backward and forward stepwise variable selection was performed, deleting non statistical significant variables or variables that showed no contribution to the model via Akaike information criterion (AIC). The built model was then applied to the test data to determine its performance in a new data in relation to the data in which the model was trained on. Wilcoxon and Friedman tests were used to compare the individual host marker levels during the course of treatment, with a pairwise analyse done to assess the difference in the individuals who provided samples at three time points during the course of treatment. All statistical analyses with adjusted P value of ≤ 0.05 were considered significant.

Chapter 3 Results

3.1 Participant demographics

A total of seven hundred and forty-one (741) participants were included in the analysis. Of these, 428 were confirmed to have TB by microbiological tests (sputum smear, culture and/or GeneXpert), while 313 were confirmed to have other respiratory disorders (ORD). Participants who had ORD were not investigated further due to limited resources.

For the confirmed TB group, 123 were positive by culture, smear and GeneXpert (Xpert), 167 were both culture and smear positive, 13(7.22%) were culture positive but smear negative, 75 were Xpert and culture positive, and 18 were Xpert and smear positive (Table 3.1). For the ORD group, only 61 had only culture performed, of which 45 (73.78%) were negative and 16 (26.23%) were contaminated. We performed MBLA on 207 participants who were smear negative without any other evaluation. Of these, 32(15.46%) were positive by MBLA and thus classified as TB. The cycle threshold (Ct) values were obtained as an average of duplicate wells ran for each participant sample, while the quantity of the viable bacteria present was extrapolated from the standard curve. The highest standard point (top standard) had an estimated copy of 10^8 bacteria whereas the lowest was 10^2 cfu/ml. The cut-off Ct value was 36, any Ct value above the cut-off was considered negative. The median (interquartile range) of bacterial copy for the positive MBLA was 1441.79(291.2, 15091.62) with a median (interquartile range) Ct of 30.3(27.40, 33.35) whereas the negative MBLA median (interquartile range) bacterial copy was 10.82(3.72, 17.69), with a median Ct of 40 (Table 3.1A) also illustrated in figure 3.1.

Of the confirmed TB cases, 29.91% were female compared to 47.6% of the ORD group. When the median ages of TB and ORD were compared there was a significant difference between the two groups, with a p value <0.0001 . The median (interquartile range) was 31(23-43) years and 40(24-56) years for TB and ORD respectively. There were 16 HIV co-infected TB patients and 4 HIV-infected ORD patients. Of the confirmed TB/HIV co-infected, 13 were culture positive with 2 culture positive but smear negative. The 4 ORD were both smear and MBLA negative.

40 children were also analysed: 17 were confirmed to have TB while 23 had ORD. Of the confirmed TB, 7 were positive for culture, Xpert and smear, while an additional 4 were double positive for culture and smear. MBLA was also done for 8 participants who had only a smear negative result, of these, 2 were MLBA positive. The age range for the children was from 1-17 years. None of the children was HIV+.

Table 3.1 Participant demographics

Patient characteristics	TB	ORD
n= 741	428 (57.76)	313 (42.24)
Female (%)	128 (29.91)	149 (47.6)
HIV infected (%)	16(3.73)	4(1.28)
Children (<18 years)	17(42.5)	23(57.5)
Median IQR age (years)	31(23-43)	40(24-56)
Confirmed by microbiology tests:		
Smear, culture and Xpert	123 (28.74)	20 (6.39)
Smear and culture	180(42.05)	42 (13.42)
Culture and Xpert	75 (17.52)	42 (13.42)
Smear and Xpert	18 (4.21)	34 (10.86)
Smear and MBLA	32 (7.48)	175 (55.91)

n= number, TB= tuberculosis, ORD= other respiratory disorders, MBLA=Mycobacterial load assay, HIV= human immunodeficiency virus

Table 3.1A The quantity of viable bacteria RNA in the sputum of suspected TB patients

MBLA Results	Bacteria median (IQR)	Cycle threshold median(IQR)
Positive	1441.79(291.2, 15091.62)	30.3(27.40, 33.35)
Negative	10.82(3.72, 17.69)	40(40, 40)

IQR = interquartile range, Ct= cycle threshold, MBLA=Mycobacterial load assay

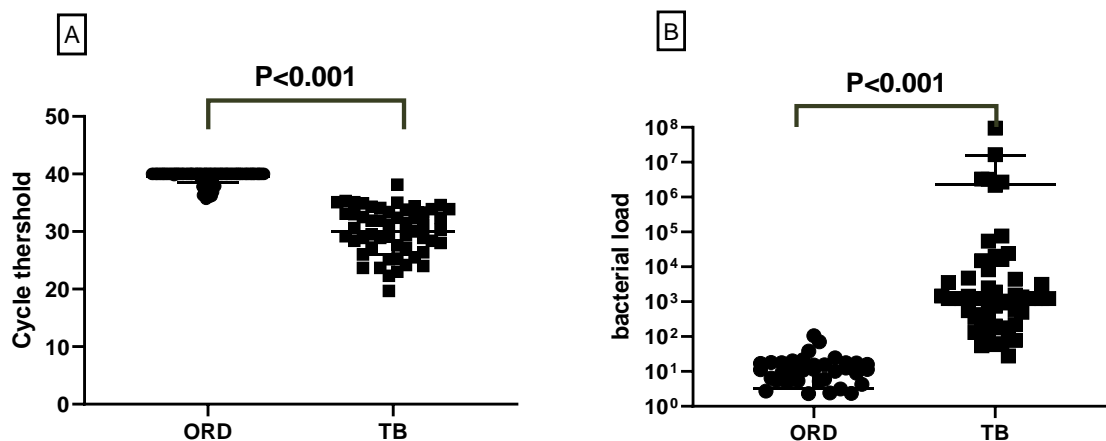


Figure 3.1. The cycle threshold (A) and quantity (B) of viable bacteria RNA in the sputum of suspected TB patients. qRT-PCR was used to quantify to perform the MBLA. ORD= other respiratory disorder, TB tuberculosis

3.2 The Performance of individual host markers in diagnosis of TB disease

Seventy (70) host markers were examined in total to assess their ability to distinguish TB from ORD. The median concentrations of host markers in sputum were compared using the Mann Whitney U test with post-test correction for multiple comparisons using Benjamini-Hochberg test[147]. We found 32 markers increased significantly between TB and ORD patients. The majority of the markers were found to be higher in the TB group compared to the ORD group except G-CSF, IL-26, IL-3 and APRIL. Of these four markers (G-CSF, IL-26, IL-3 and APRIL), only APRIL showed a significant different (p-value =7.24E-08) in their median levels with a concentration of 1103pg/ml and 118268pg/ml for TB and ORD respectively (Table 3.2B). When receiver operator characteristics (ROC) analysis was performed to determine the ability of each marker to differentiate TB from ORD. The most accurate single markers among the three kits were mostly found in the 27-plex kit. This included IL-1 β (AUC=0.69, (95% CI: 0.64, 0.73) sens=71%(95% CI: 66, 76), spec=61%(95% CI: 55, 67), FGF (AUC=0.69 (95 CI: 0.65, 0.73), sens=62% (95% CI: 56, 66), spec=70% (95% CI: 64, 75), GM-CSF (AUC=0.69 (95% CI:0.65, 0.73), sens=61% (95% CI: 55, 66), spec=73% (95% CI: 67, 78) and RANTES (AUC=0.68 (95% CI: 0.64, 0.72), sens=68% (95% CI: 63, 73), spec=65% (95% CI: 58, 70)(Table 3.2A). Nonetheless, two markers in the 37-plex also performed well. That is BAFF (AUC=0.68 (0.95 CI: 0.64, 0.72), sens=82% (95% CI: 0.78, 0.86), spec=50% (95% CI: 44, 55) and MMP-2 (AUC=0.73(95% CI: 0.70, 0.77), sens=58% (95% CI: 52, 62), spec=81 % (95% CI: 76, 85)

(Table 3.2B). MMP-2 performed the best with an AUC of 0.73 (sens=58 and spec=81%) with a high concentration cut-off of 9273 pg/ml (Table 3.2B). The least performed markers were the 38-plex with MIP-1 α been optimal performer with an AUC of 0.65, sensitivity of 53% and specificity of 70% (Table 3.2C). The single best performed markers illustrated in figure 3.2.

Table 3.2: Diagnostic performance of individual markers

Table 3.2A. Only single biomarker with significant p-values are shown (27-plex kit)

Marker	TB (median) pg/ml	ORD (median) pg/ml	Adjusted p-value	Sens (%)	Spec (%)	PPV	NPV	Youden cut-off	AUC (95% CI)
IL-12p70	1.89	1.66	4.45E-03	55.05	58.8	0.66	0.47	1.67	0.56 (0.52, 0.60)
IL-9	200.15	159.43	2.47E-04	84.6	32.21	0.65	0.59	85.92	0.58 (0.53, 0.62)
IL-8	18066.83	12048.7	3.77E-05	62.37	54.68	0.67	0.5	13634	0.59 (0.55, 0.64)
IL-15	324.87	209.79	2.55E-05	72	49.06	0.68	0.54	206.37	0.59 (0.55, 0.64)
IL-13	12.94	6.94	9.71E-06	75.25	44.57	0.67	0.55	3.82	0.60 (0.55, 0.64)
IL-6	34.315	14.41	3.39E-06	66.91	53.56	0.68	0.52	16.1	0.61 (0.56, 0.65)
MCP-1	144.25	60.45	1.39E-07	71.96	47.94	0.48	0.67	55.7	0.61 (0.57, 0.66)
PDGF-bb	157.79	108.35	1.26E-07	36.36	79.78	0.73	0.46	210.72	0.61 (0.57, 0.66)
Eotaxin	29.55	12.94	7.16E-08	67.42	54.31	0.69	0.53	15.45	0.63 (0.58, 0.67)
IFN- γ	278.71	123.32	3.90E-09	66.16	59.17	0.71	0.54	172.88	0.64 (0.59, 0.68)
IL-4	11.31	4.84	7.86E-11	64.64	61.42	0.71	0.53	7.7	0.65 (0.61, 0.69)
IL-5	20.35	9.5	1.42E-11	65.15	64.04	0.73	0.55	14.38	0.65 (0.61, 0.70)
TNF- α	671.66	106.24	7.44E-11	75.51	50.94	0.7	0.58	112.18	0.65 (0.61, 0.70)

MIP-1 α	9.28	3.15	1.14E-13	78.03	49.81	0.7	0.61	3.16	0.67 (0.62, 0.71)
MIP-1 β	1884.31	210.42	5.82E-13	78.54	49.81	0.7	0.61	211.62	0.67 (0.62, 0.71)
IL-2	25.79	6.7	5.82E-13	72.58	56.18	0.71	0.58	10	0.67 (0.63, 0.71)
IL17A	94.64	22.31	1.62E-13	71	58.8	0.72	0.58	42.87	0.67 (0.63, 0.71)
RANTES	30.26	15.06	1.49E-15	68.19	64.8	0.74	0.58	19.13	0.68 (0.64, 0.72)
IL-1 β	997.42	310.42	1.49E-15	71.21	61.04	0.73	0.59	587.88	0.69 (0.64, 0.73)
FGF	15	4.63	1.49E-15	61.61	70.41	0.76	0.55	11.18	0.69 (0.65, 0.73)
GM-CSF	5.97	3.03	1.49E-15	60.86	73.03	0.77	0.56	5.18	0.69 (0.65, 0.73)

TB = Tuberculosis, ORD= other respiratory diseases, Sens= sensitivity, Spec= Specificity, PPV= positive predictive value, NPV= negative predictive value, CI = confident interval and AUC= area

Table 3.2B. Only single biomarker with significant p-values are shown (37-plex kit)

Marker	TB (median) pg/ml	ORD (median) pg/ml	Adjusted p-value	Sens (%)	Spec (%)	PPV	NPV	Youden Cut-off	AUC (95% CI)
MMP-3	535	442	4.19E-02	81.91	27.86	0.63	0.5	219	0.55(0.50,0.59)
OPN	137.8	137.8	2.19E-02	25.88	84.73	0.72	0.43	175	0.55(0.51,0.59)
LIGHT	11	8	2.19E-02	54.02	58.02	0.66	0.45	10	0.56(0.51,0.60)
sIL-6R α	133	70	2.17E-03	38.19	76.72	0.71	0.45	256	0.58(0.53,0.62)
IL-8	18200	5299	3.12E-05	78.39	41.99	0.67	0.56	2699	0.60(0.56,0.64)
APRIL	1103	118268	7.24E-08	66.79	60.05	0.52	0.73	3843	0.63(0.59,0.68)
sTNFR1	120	22.48	1.90E-09	56.53	67.94	0.73	0.51	64	0.64(0.60,0.68)
sTNFR2	122	33	4.16E-10	64.07	62.21	0.72	0.53	66	0.65(0.61,0.69)
BAFF	9753	2612	4.07E-15	82.24	50.00	0.70	0.67	2784	0.68(0.64,0.72)
MMP-2	13683	2266	4.07E-15	56.78	80.51	0.8	0.57	9273	0.73(0.69,0.77)

TB = Tuberculosis, ORD= other respiratory diseases, Sens= sensitivity, Spec= Specificity, PPV= positive predictive value. NPV= neaative predictive value. CI = confident interval and AUC= area

Table 3.2C Only single biomarker with significant p-values are shown (38-plex kit)

Marker	TB (median) pg/ml	ORD (median) pg/ml	Adjusted p-value	Sens (%)	Spec (%)	PPV	NPV	Youden cut-off	AUC (95% CI)
EGF	835	466.72	4.63E-02	0.02	1.00	1.00	0.58	283.34	0.58 (0.52, 0.64)
TNF- α	39.87	9.5	5.73E-03	46.39	71.13	0.69	0.49	23.87	0.60 (0.54, 0.66)
MCP-1	490.8	120.8	5.73E-03	57.11	60.20	0.66	0.53	11.64	0.61 (0.54, 0.67)
MIP-1 β	80.17	59.91	8.94E-05	41.75	35.92	0.47	0.31	69.78	0.64 (0.58, 0.70)
MIP-1 α	74.68	3.2	3.03E-05	53.04	70.42	0.70	0.52	47.54	0.65 (0.60, 0.71)
IL-1 β	851.5	218.7	3.03E-05	59.02	69.03	0.72	0.55	401.49	0.66 (0.60, 0.72)

TB = Tuberculosis, ORD= other respiratory diseases, Sens= sensitivity, Spec= Specificity, PPV= positive predictive value, NPV= negative predictive value, CI = confident interval and AUC= area under the curve

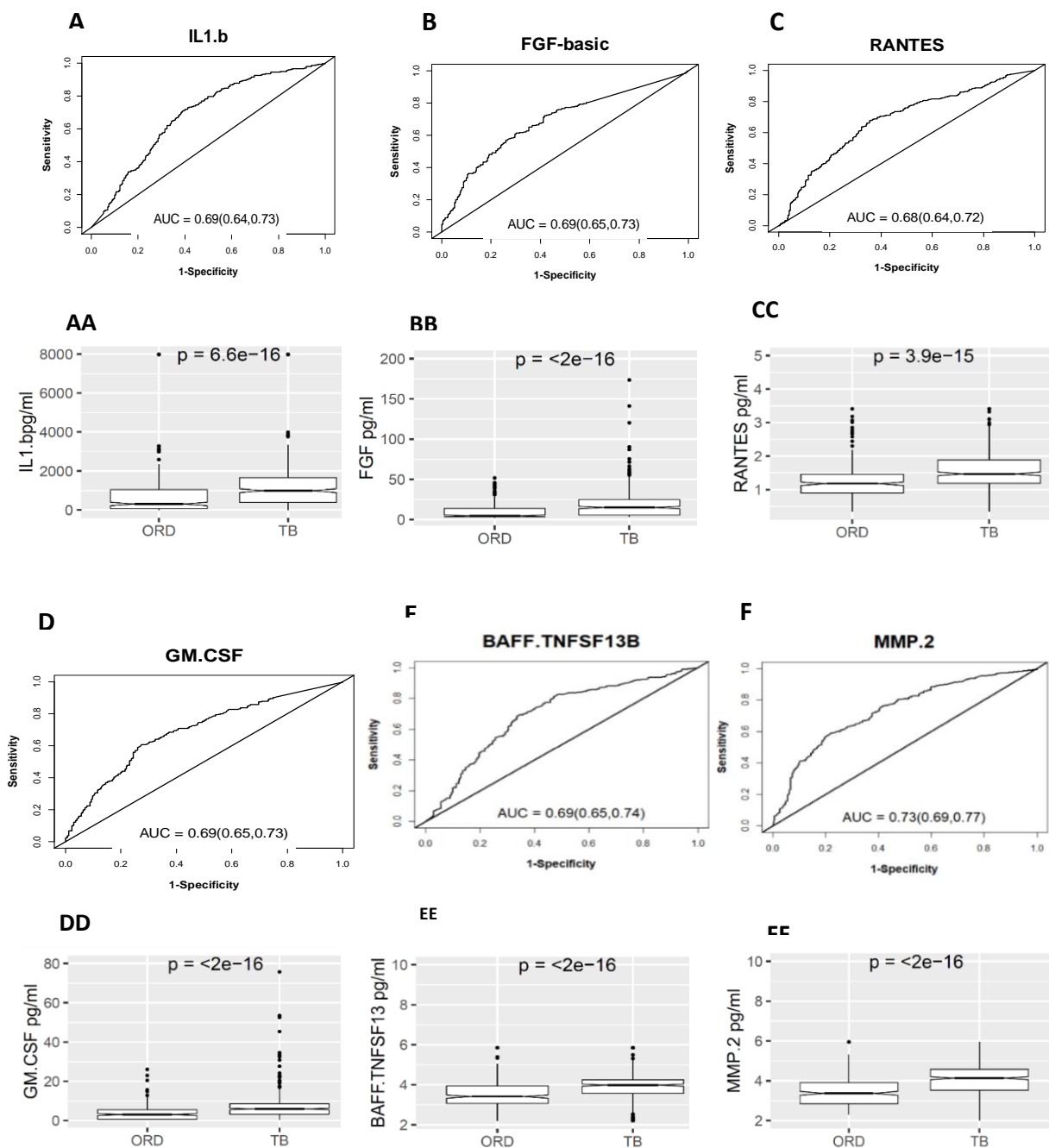


Figure 3.2 Graphs of individual markers that performed best in classifying ORD from TB with an AUC of ≥ 0.65 . The diagnostic accuracy are shown in the ROC (A-F) while the median concentration difference is represented in boxplots (AA-FF). The data were analysed using Logistic regression for ROC curves and Mann Whitney U test to compare median concentrations between ORD and TB. The notch in the boxplot indicates median.

3.3 Usefulness of combinations of biomarkers in the diagnosis of TB

After performing univariate analyses, multiple logistic regression analyses were performed to determine if better accuracies could be achieved with multiple host markers. This was determined for the 3 different kits used in the study (see section 2.4 for details) and a combined analysis. Prior to analysis, the data were divided into training – 80% and test - 20% datasets.

3.3.1 27-plex biosignature analyses

We first evaluated the performance of our previously identified biosignature (IL-13, FGF and IFN- γ), which gave 96% correct classification of TB amongst respiratory infected individual in a small sample size[152] using the same kit (27-plex Bio-Plex Pro™ Human Th1/Th2 Cytokine Panel). Analysis with our current cohort resulted in an AUC of 0.73(95% CI: 0.67,0.79) and sensitivity of 70%(95% CI 63, 77) with a low specificity of 69%(95% CI: 60, 77). The PPV and NPV were 0.75(95% CI: 0.68,0.82) and 0.63(95% CI: 0.55, 0.71) respectively on the training dataset. When the signature was applied to the test data, there was minimal change in the performance parameters (Table 3.3.1).

We next did a broad combination analyses looking at TB and ORD regardless of HIV infection among adult patients. The best signature identified was a eight-marker combination consisting of FGF, IL-1 β , IL-8, IL-10, IL-12p70, IL-13, MIP-1 β and VEGF. This combination was able to discriminate TB from ORD with an AUC of 0.78(95% CI: 0.75, 0.84) with a sensitivity of 79%(95% CI: 75, 84) and specificity of 67%(95% CI: 60, 73) for the training set. However, when the signature was applied to the test dataset, there was a drop in performance with an AUC of 0.67 (95% CI: 0.57, 0.77), with sens = 67%(95% CI: 56, 78) and spec = 67%(95% CI: 54, 80). In addition, no increased performance was seen when only HIV negative adults were analysed (data not shown).

When all age groups were analysed but excluding HIV-positive individuals, a twelve-marker signature was obtained discriminating TB and ORD with an AUC of 0.81(95% CI: 0.77, 0.84), sensitivity of 75%(95% CI: 71, 80) and specificity of 76%(95% CI: 70, 80) for training dataset. However, a drop in performance was observed when applied to test dataset with an AUC of 0.71(0.95 CI: 0.63, 0.8), sensitivity of 55%(95% CI: 44, 66) but with an increased in specificity of 90%(95% CI: 82, 97). When the analysis included the HIV-positive individuals, a small drop in sensitivity and an increase in specificity were observed (Table 3.2.1). When only individuals <18 were analysed, a four-marker signature (FGF, IL-4, MIP-1a and RANTES) differentiated TB from ORD, with an AUC of 0.87(0.95% CI: 0.75, 0.99), sens= 82%(95% CI: 64,100) and specificity of 87%(95% CI: 73, 100).

Furthermore, when individuals who were smear negative but culture positive were excluded from the TB group and only the smear positive and culutre positive were assessed, a 10-marker signature was

found (FGF, IL-1 β , IL-6, IL-8, IL-10, IL-12p70, IL-13, MIP-1 β , TNF- α and VEGF) with an AUC of 0.81 (0.95 C:I 0.77, 0.85), sens= 81(95% CI: 76, 86) and spec= 68(95% CI: 62, 75) on the training dataset, while on the test data, an AUC of 0.72 (0.95% CI: 0.63, 0.82), sensitivity of 63(95% CI: 50, 75) and specificity of 78%(95% CI: 66, 89) was observed.

Surprisingly, when HIV-positive individuals were excluded from the smear positive culture positive and ORD, there was a decrease in performance AUC=0.77(0.95% CI: 0.72,0.82) sens=78%(95% CI: 73, 84) but a gain in specificity, thus spec=70%(95% CI: 64, 77) when this signature (IFN- γ , IL-1 β , IL-8, IL-10, IL-12p70, MIP-1 β , RANTES and VEGF) was applied to test data, no change in accuracy was observed (Table 3.3.1).

When smear negative but culture positive TB patients were analysed, a three-marker signature (IL-1 β , IL-7 and VEGF) showed an optimal performance with an AUC=0.74(0.95% CI: 0.60, 0.87) , sens= 86%(95% CI: 71, 100) and spec= 60%(95% CI: 45, 75). A final analyses for the 27-Plex markers was done on coinfectd (TB/HIV+) verse ORDs, two combined markers (VEGF and RANTES) showed the potential to classify coinfectd patients from ORD with an AUC of 0.78 (95% CI: 0.65, 0.91), with low sensitivity of 56%(95% CI: 32, 81) and specificity of 90%(95% CI: 80, 99).

Table 3.3.1 Biosignature classification with the 27-plex kits

Biosignature	AUC (95% CI)	Sens (95% CI)	Spec (95% CI)	PPV (95% CI)	NPV (95% CI)
Validation					
FGF, IFN- γ and IL-13	0.73(0.67, 0.79)	70(63, 77)	69(60, 77)	0.75(0.68, 0.82)	0.63(0.55, 0.71)
	0.64(0.51, 0.78)	72(58, 86)	60(43, 78)	0.70(0.56, 0.84)	0.62(0.44, 0.80)
Only adults including HIV infected participants					
FGF, IL-1 β , IL-8, IL-10, IL-12p70, IL-13, MIP-1 β and VEGF	0.78(0.75, 0.84)	79(75, 84)	67(60, 73)	0.75(0.70, 0.80)	0.72(0.65, 0.78)
	0.67(0.57, 0.77)	67(56, 78)	67(54, 80)	0.74(0.63, 0.85)	0.6(0.47, 0.73)
All patients including HIV infected					
Eotaxin, FGF, GM-CSF, IFN- γ , IL-1 β , IL-6, IL-8,	0.80(0.77, 0.84)	70(65, 75)	78(73, 84)	0.82(0.77, 0.86)	0.66(0.6, 0.71)

IL-10, IL-12p70, IL-13, MIP-1 β and TNF- β	0.68(0.58, 0.77)	61(51, 71)	74(62,85)	0.78(0.69, 0.88)	0.55(0.43, 0.66)
All patients excluding HIV infected					
Eotaxin, FGF, GM- CSF,IFN- γ , IL1- β , IL-8, IL-10, IL-12p70, IL-13, PDGF.bb , RANTES and VEGF	0.81(0.77, 0.84)	75(71, 80)	76(70, 81)	0.81(0.77, 0.85)	0.69(0.64, 0.75)
	0.71(0.63, 0.80)	55(44, 66)	90(82, 97)	0.88(0.79, 0.97)	0.58(0.48, 0.68)
Smear positive –culture positives including HIV infected					
FGF, IL-1 β , IL-6, IL-8, IL10, IL-12p70, IL-13, MIP-1 β , TNF- α and VEGF	0.81(0.77, 0.85)	81(76, 86)	68(62, 77)	0.75(0.7, 0.81)	0.75(0.68, 0.81)
	0.72,(0.62, 0.82)	63(50, 75)	78(66, 89)	0.76(0.64, 0.88)	0.64(0.52, 0.77)
Smear positive – culture positive without HIV infected					
IFN- γ , IL-1 β , IL-8, IL-10, IL-12p70, MIP-1 β , RANTES and VEGF	0.77(0.72,0.82)	78(73, 84)	70(64, 77)	0.76(0.71, 0.81)	0.73(0.66, 0.79)
	0.77(0.68,0.87)	78(66, 89)	69(57, 82)	0.72(0.6,0. 84)	0.76(0.63, 0.88)
Smear negative – culture positive only					
IL-1 β , IL-7 and VEGF	0.74(0.60, 0.87)	86(71, 100)	60(45,75)	0.52(0.36, 0.70)	0.89(0.77, 100)
HIV infected only					
VEGF and RANTES	0.78(0.65, 0.91)	56(32, 81)	90(80, 99)	0.69(0.44, 0.94)	0.83(0.72, 0.95)
Children					
FGF, IL-4, MIP-1 α and RANTES	0.87(0.75, 0.99)	82(64,10)	87(73, 100)	0.82(0.64, 1)	0.87(0.73, 100)

Sens= sensitivity, Spec= Specificity, PPV= positive predictive value, NPV= negative predictive value, CI = confident interval and AUC= area under the curve

3.3.2 37-plex biosignature analysis

Similar analyses were also performed for the 37-Plex kit. A thirteen-marker signature was found to discriminate TB from ORD with an AUC of 0.83(95% CI: 0.80, 0.86), sensitivity of 71%(95% CI: 66, 76) and specificity of 81%(95% CI: 76, 86) in the training dataset, regardless of age bracket and HIV status of the participants. Conversely, application of this signature to the test data showed dropped in performance, AUC of 0.78(95% CI 0.70, 0.85), increased sensitivity (79(95% CI 70, 87) but decreased specificity of 70(95% CI 58, 82). When HIV+ individuals were excluded, no significant change in marker performance was observed. Furthermore, when only adults data were analysed, a fourteen-marker signature was able to classify TB from ORD with an AUC of 0.84(95% CI: 0.8, 0.88), sens=83%(95% CI: 78, 87) and spec=76%(95% CI: 70, 82) irrespective of HIV status. A similar performance was seen, when the signature was applied to the test data (Table 3.3.2.). Next, we considered only the smear positive/culture positive TB with the exclusion of HIV. For this analysis, a sixteen-marker biosignature was found to discriminate TB and ORD with an AUC=0.87(95% CI: 0.84, 0.91), with a sensitivity of 84%(95% CI: 80, 89) and specificity of 79%(95% CI: 73, 85). When the signature was applied to the test data, a drop in performance and sensitivity was observed, AUU of 0.78(95% CI 0.68, 0.87), sensitivity of 76(95% CI 64, 88) and slight change in specificity 80(95% CI 68, 91). A combination of two markers (BAFF and sIL6Ra) was able to discriminate culture positive-smear negative TB from ORD with an AUC of 0.71(95% CI: 0.58, 0.85), with a sensitivity of 64%(95% CI: 44, 84) and specificity of 74%(95% CI: 61, 88), while a five-marker signature (BAFF, C3.L1, IL-22, MMP-3 and sTNF.R1) was able to discriminate TB/HIV co-infected patients from ORD with an AUC of 0.90(95% CI: 0.82, 0.99), sens=88%(95% CI: 71, 100) and spec= 85%(95% CI: 73, 96) (Table 3.3.2).

Table 3.3.2. Biosignature classification with the 37-Plex kits

Biosignature	AUC (95% CI)	Sens (95% CI)	Spec (95% CI)	PPV (95% CI)	NPV (95% CI)
all patients' data including HIV positives					
gp130, IFN- γ , IL-10, IL-22, IL-27.p28, IL-28A, LIGHT, MMP-11, MMP-2, OPN, sTNF.R1, TSLP and TWEAK	0.83(0.80, 0.86)	71(66, 76)	81(76, 86)	0.83(0,7 9, 0.87)	0.68(0.6 3, 0.74)
	0.78(0.70, 0.85)	79(70, 87)	70(58, 82)	0,8(0.71, 0.88)	0.69(0.5 7, 0.81)
Adults patients excluding HIV co-infected					
APRIL, BAFF, gp130, IFN. α 2, IFN- γ , IL-22, IL-28A, LIGHT, MMP-1, MMP-2, Osteocalcin, P3, TSLP and TWEAK	0.84(0.80, 0.88)	83(78, 87)	76(70, 82)	0.83(0.7 8, 0.87)	0.76(0.7 0, 0.82)
	0.83(0.75, 0.91)	80(70, 91)	79(67, 90)	0.82(0.7 2, 0.93)	0.77(0.6 5, 0.89)
Smear positive – culture positive with no HIV co-infected					
APRIL, BAFF, gp130, IFN- γ , IL-2, IL-8, IL-10, IL-22, IL-28A, LIGHT, MMP-1, MMP-2, P3, sTNF.R1, TSLP and TWEAK	0.87(0.84, 0.91)	84(80, 89)	79(73, 85)	0.83(0.7 8, 0.87)	0.8(0.75, 0.86)
	0.78(0.68, 0.87)	76(64, 88)	80(68, 91)	0.79(0.6 7, 0.90)	0.77(0.6 5, 0.88)
Only smear negative – culture positive					
BAFF and sIL.6R α	0.71(0.58, 0.85)	64(44, 84)	74(61, 88)	0.58(0.3 9, 0.78)	0.78(0.6 5, 0.92)
Only HIVTB co-infected					
BAFF, C3.L1, IL-22, MMP-3 and sTNF.R1	0.90(0.82, 0.99)	88(71,10 0)	85(73, 96)	0.70(0.5 0, 0.90)	0.94(0.8 7, 1.00)

Sens= sensitivity, Spec= Specificity, PPV= positive predictive value, NPV= negative predictive value, CI = confident interval and AUC= area under the curve

3.3.3 38-plex biosignature analyses

For the 38-plex analyses, an eleven-marker biosignature identified TB from ORD with an AUC of 0.78(95% CI: 0.73, 0.84), sensitivity of 78%(95% CI: 73, 85) and specificity of 74%(95% CI: 65, 82) regardless of HIV status or age. However, when the signature was applied to test data a declined performance was observed, with an AUC of 0.71(95% CI: 0.58, 0.84) significant decrease sensitivity of 40(95% CI: 24, 56) and increased specificity of 96(95% CI: 89, 100) (Table 3.3.3). When analysis was performed on only adult patients including those with HIV infection, a biosignature of 11 markers gave an AUC of 0.82(95% CI: 0.77, 0.88), sens=83%(95% CI: 76, 90) and spec=70%(95% CI: 61, 79). When applied to the test data, its performance (AUC) and sensitivity dropped to an of 0.67(95% CI: 0.52, 0.81) and 59(95% CI:42, 76) respectively while specificity increased to 87(95% CI: 76, 97).

Likewise, when smear+culture+ TB were compared to ORD with the exclusion of the HIV infected, an eight-marker (FGF-2, GM-CSF, IFN- γ , IL-7, IL-12p40, IP-10, MIP-1 α , TNF- α) signature was able to identify TB from ORD with an AUC of 0.79(95% CI: 0.73, 0.85), sens= 71%(95% CI: 63, 80) and spec=79%(95% CI: 71, 87) as the obtained signature was applied to the test data, an AUC of 0.70(95% CI: 0.56,0.85), sensitivity equal to 73(95% CI: 58, 89) and specificity of 63(95% CI: 43, 82) were attained. No single or combination of marker(s) was able to differentiate culture positive - smear negative or TB/HIV+ from ORD.

In the 27-plex analyses, we observed that the top 7 markers (GM-CSF, FGF, IL-12p70, IL-10, TNF- α , IL-6, IL-8) were the most likely markers to frequent any potential biosignature from this dataset. With FGF, IL-1 β and IL-12p70 dominating in the number of appearance among the generated signature (Table 3.3.1). Similarly for the 38-plex analyses, FGF-2, GM-CSF, IFN- γ , MIP-1 α and IP-10 were the most common markers observed (Table 3.3.3). However, in the 37-plex, there was no particular order to determine the selection of markers, due to the number of combined markers in most signatures. The frequent of marker in various potential signatures seems to correspond with the level of importance of the marker as shown in the variable importance graph (Figure. 3.3). Thus markers with the higher levels of importance, having the greater tendency to show up in a possible ideal biosignatur

Table 3.3.3. Biosignature classification with the 38-Plex kits

Biosignature	AUC (95% CI)	Sens (95% CI)	Spec (95% CI)	PPV (95% CI)	NPV (95% CI)
All patients including HIV positive co-infected					
FGF-2, G-CSF, GM-CSF, IFN- γ , IL-12-p40, IL-13, IL- 17A, IP-10, MCP-3, MIP-1 α ,	0.78(0.73, 0.84)	78(72, 85)	74(65, 82)	0.79(0.73, 0.86)	0.72(0.64, 0.80)
TNF- α	0.71(0.58, 0.84)	40(24, 56)	96(89, 100)	0.93(0.81, 1.00)	0.55(0.41, 0.70)
Adult patients including HIV positive					
FGF-2, G-CSF, GM-CSF, IFN-y, IL-6, IL-7, IL-13, IP- 10, MIP-1 α , TNF- α and	0.82(0.77, 0.88)	83(76, 90)	70(61,79)	0.77(0.70, 0.84)	0.77(0.68, 0.85)
VEGF	0.67(0.52, 0.81)	59(42, 76)	87(76, 97)	0.86(0.72, 1.00)	0.62(0.45, 0.78)
Smear positive – culture positive TB patients only					
FGF-2, GM-CSF, IFN-y, IL- 7, IL-12p40, IP-10, MIP-1 α ,	0.79(0.73, 0.85)	71(63, 80)	79(71, 87)	0.80(0.72, 0.87)	0.7(0.62, 0.79)
TNF- α	0.70(0.56,0.85)	73(58, 89)	63(43, 82)	0.71(0.55, 0.87)	0.65(0.46, 0.85)

Sens= sensitivity, Spec= Specificity, PPV= positive predictive value, NPV= negative predictive value, CI = confident interval and AUC= area under the curve

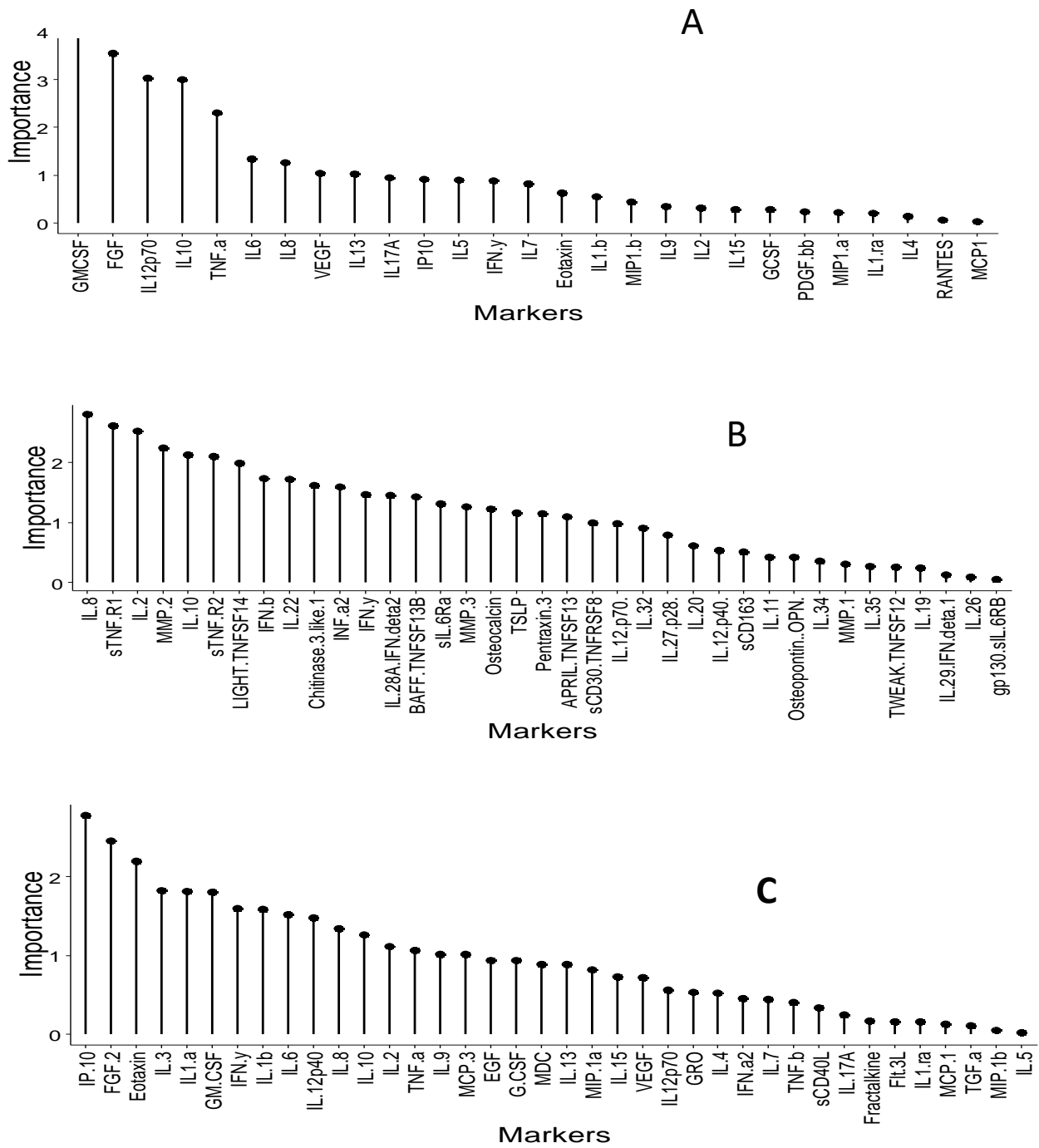


Figure 3.3 shows overall importance of marker in a possible involvement in a biosignature formation. Graph A= 27-Plex, B=37-Plex and C=38-Plex. The height of the bar indicates level of importance to building any biosignature

3.3.4 Combined biosignature analyses

Finally, when we pooled the 27- and 37-plex data together and did a combined analysis the most promising signature was generated when limited to HIV negative, smear+culture+ adult TB compared to HIV negative, adult ORD. An eighteen-marker signature (gp130, IL-1 β , IL-8, IL-10, IL-10*, IL-12p70, IL-12p70*, IL-13, IL-22, IL-28A, IFN- γ , IFN- γ *, LIGHT, MMP-1, MMP-2, OPN, RANTES, VEGF,) was able to classify TB with 83% accuracy (95% CI:82., 83.7), with an AUC of 0.89(95% CI:0.86, 0.93), sensitivity of 79(95% CI: 73, 84) and specificity of 88(95% CI: 83, 93) in the training dataset. When signature was applied to test data, there was a slight drop in accuracy of classification to 76.4 % (95% CI: 76.1, 76.7), AUC to 0.82(95% CI: 0.74, 0.89), sensitivity to 74(95%CI: 64, 84) and specificity to 80(95% CI: 70, 91). There were some markers which were duplicated because they were present in both kits such as IL-10, 12p70 and IFN- γ which are noted by an asterisk (*). The next promising biosignature was a two-marker signature (IL.29, IFN. λ 1, PTX3) which discriminated the smear-culture+ from ORD with AUC of 0.68(95% CI: 0.52, 0.85), sensitivity of 67(95% CI: 48, 86) and specificity of 95(95% CI: 88, 100) with 83.9% (95% CI: 83.4, 84.3) accuracy. The 38-plex kit was excluded from the combined analyses because we did not found discrimination with it, and most of the markers were also found to strong correlate with markers from the other two kits. Hence, we totally exclude whole data from thee kit.

Table 3.3.4 Biosignature classification of combine 27-37plex kits

Biosignature	AUC (95% CI)	Sens (95% CI)	Spec (95% CI)	PPV (95% CI)	NPV (95% CI)
Smear positive – culture positive combine data					
gp130, IL-1 β , IL-8, IL-10, IL-12p70, IL-13, IL-10*, IL-12p70*, IL-22, IL-28A, IFN- γ , IFN- γ *, LIGHT, MMP-1, MMP-2, OPN, RANTES, VEGF	0.89(95% CI:0.86, 0.93)	79(95% CI: 73, 84)	88(95% CI: 83, 93)	0.88(95% CI:0.83, 0.93)	0.78(95% CI:0.72, 0.84)
	0.82(95% CI:0.74, 0.89)	74(95%CI: 64, 84)	80(95% CI:70, 91)	0.85(95% CI: 0.76, 0.94)	0.67(95% CI:0.55, 0.79)
Smear negative – culture positive TB patients only					
IL.29, IFN. λ 1, PTX3	0.68(95% CI: 0.52, 0.85)	67(95% CI:48, 86)	95(95% CI:, 88, 100)	0.89(95% CI:0.74, 100)	0.82(95% CI:0.70, 0.93)

Sens= sensitivity, Spec= Specificity, PPV= positive predictive value, NPV= negative predictive value, CI = confident interval and AUC= area under the curve

3.4 Treatment response and monitoring

A subset of patients with confirmed TB were followed up for the duration of treatment, with sputum collected Month 1 and Month 2 for microbiological and host marker evaluation. 34 (7.94%) patients provided enough sputa at month 1 only and 39 (9.11%) at month 2 only, with 21(4.91%) patients at both time-points. At the end of the 2 months intensive TB treatment phase, 15 (3.51%) patients remained culture or smear positive while the rest were microbiologically negative. In this analyses, only markers from the 38-Plex kit were evaluated. We performed a Wilcoxon Ranked sum test to assess changes in the analyte levels during the course of treatment for those individuals who provided samples at baseline and month 1 or 2. Friedman test was used to compare differences in the mean levels of makers for patients who had samples available at both follow-up time-points, using the Pairwise Multiple Comparison of Mean Ranks Package (PMCMR)[153].

3.4.1 Host markers' levels during the course tuberculosis treatment

When the median levels of the sputum markers at 1 or 2 months were compared to the baseline, 15 markers (EGF, Eotaxin, TGF- α , G-CSF, GM-CSF, GRO, IL-1 α , IL-1 β , IL-8, IL-3, MCP-1, TNF- α , IP-10, MIP-1 β and MIP-1 α (Fig 3.4.1A) showed a significant decline in their median levels by the first month of treatment. 13 markers (EGF, Eotaxin, TGF- α , GM-CSF, IL-1 α , IL-1 β , IL-8, MDC, MIP-1 α , MIP-1 β , TNF- β , and MCP-1) were also significantly lower when Month 2 was compared to baseline. When patients with all 3 time points available were analysed, IL-1 β , IL-1 α , IL-8, Eotaxin, EGF, Fractalkine, MIP-1 α , MIP-1 β and TNF- α all showed changes with treatment (Fig 3.4.1C). IL-1 α and IL-1 β were significantly lower at Month 1 and 2 compared to baseline ($p=0.00062$ and $p=0.01514$ respectively; Figure 3.4.1C). However, there was no significant change between Months 1 and 2. IL-8, EGF, TNF- α and MIP-1 α showed a slight but significant decline by Month 1 compared to baseline ($p=0.036$, $p=0.015$, $p=0.0058$ and $p=0.044$ respectively), but remained unchanged at 2 months (Figure 3.4.1C). Eotaxin, Fractalkine and MIP-1 β were found to be significant when the three time points were compared, however, no significant difference were observed between their individual pairs (Figure 34.1C).

We also performed a Mann-Whitney U-test to compare the median levels of fast (i.e. smear negative by 2 months) and slow (i.e. smear positive at 2 months but negative by 6 months) treatment responders. EGF, FGF-2, TGF- α , GMCSF, Fractalkine, IFN- γ , IL-10, MCP-3, IL-12p40, IL-12p70, IL-15, sCD40L, IL-2, IL-7, MCP-1, TNF- α and TNF- β all showed higher levels in slow responders compared to fast responders.

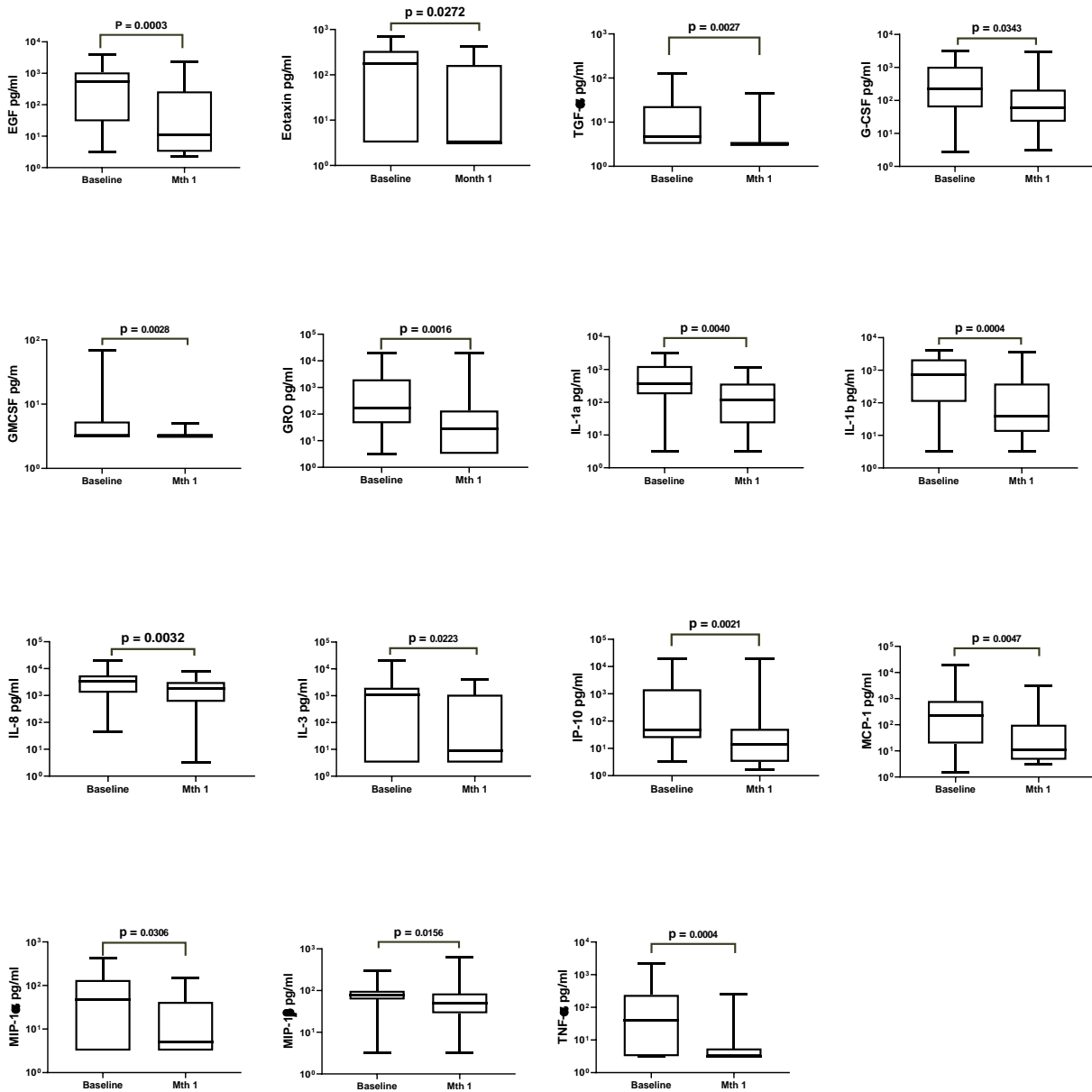


Figure 3.4.1A. The levels of markers been compared from corresponding samples of the same patient during course of TB treatment, sample collected 1 month interval

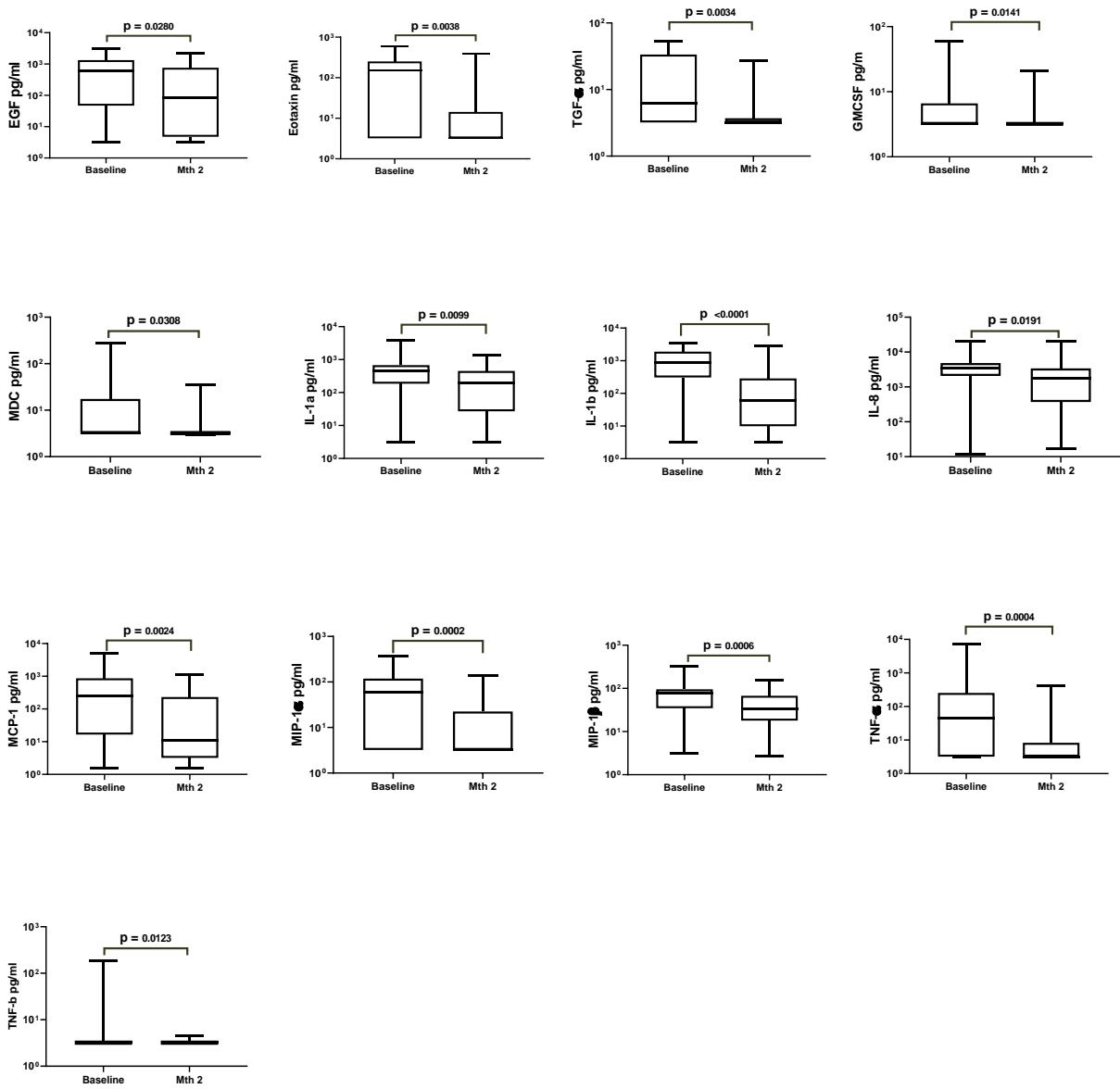


Figure 3.4.1B Markers showed decline in concentration levels when baseline and 2months samples from matching patients' samples were compared

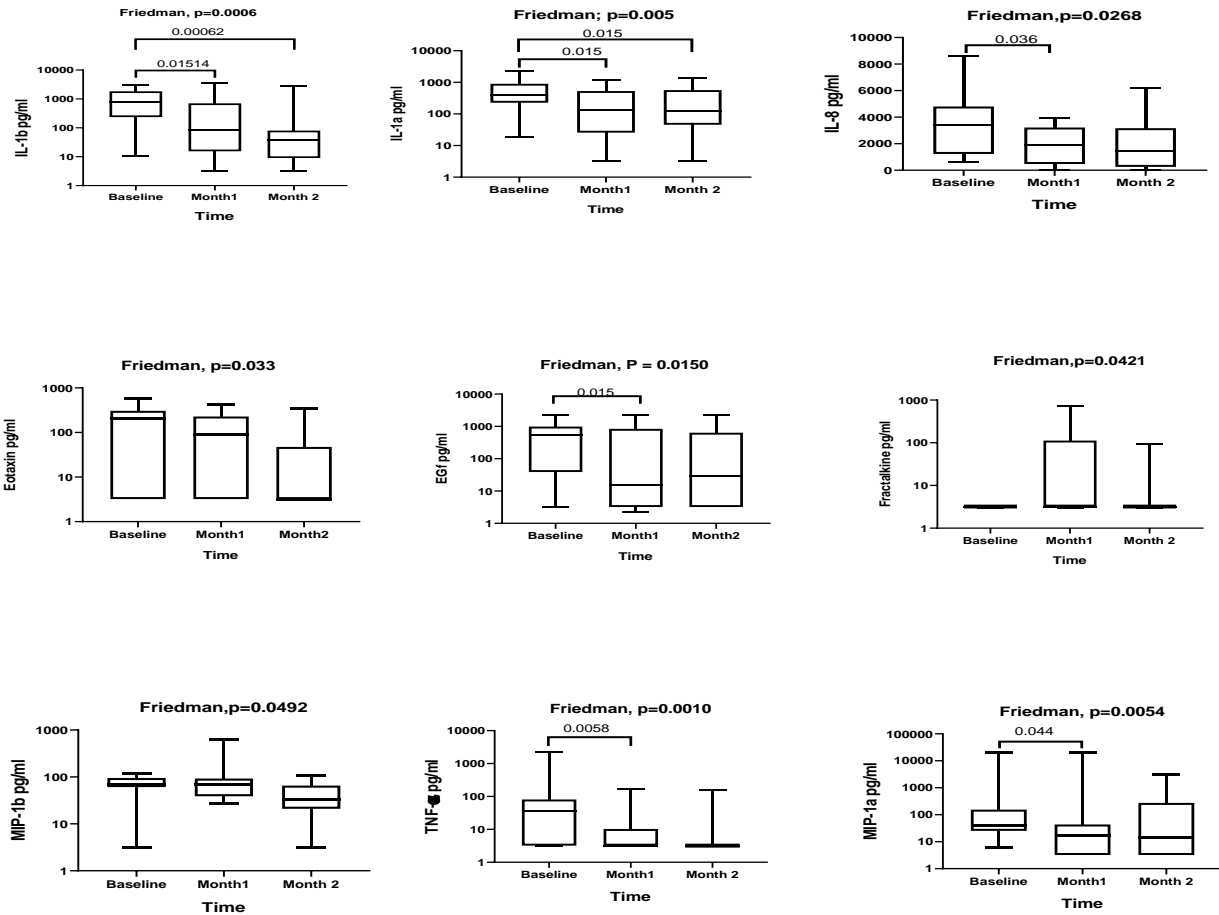


Figure 3. 4.1C Changes in mean levels of markers during the course of treatment.

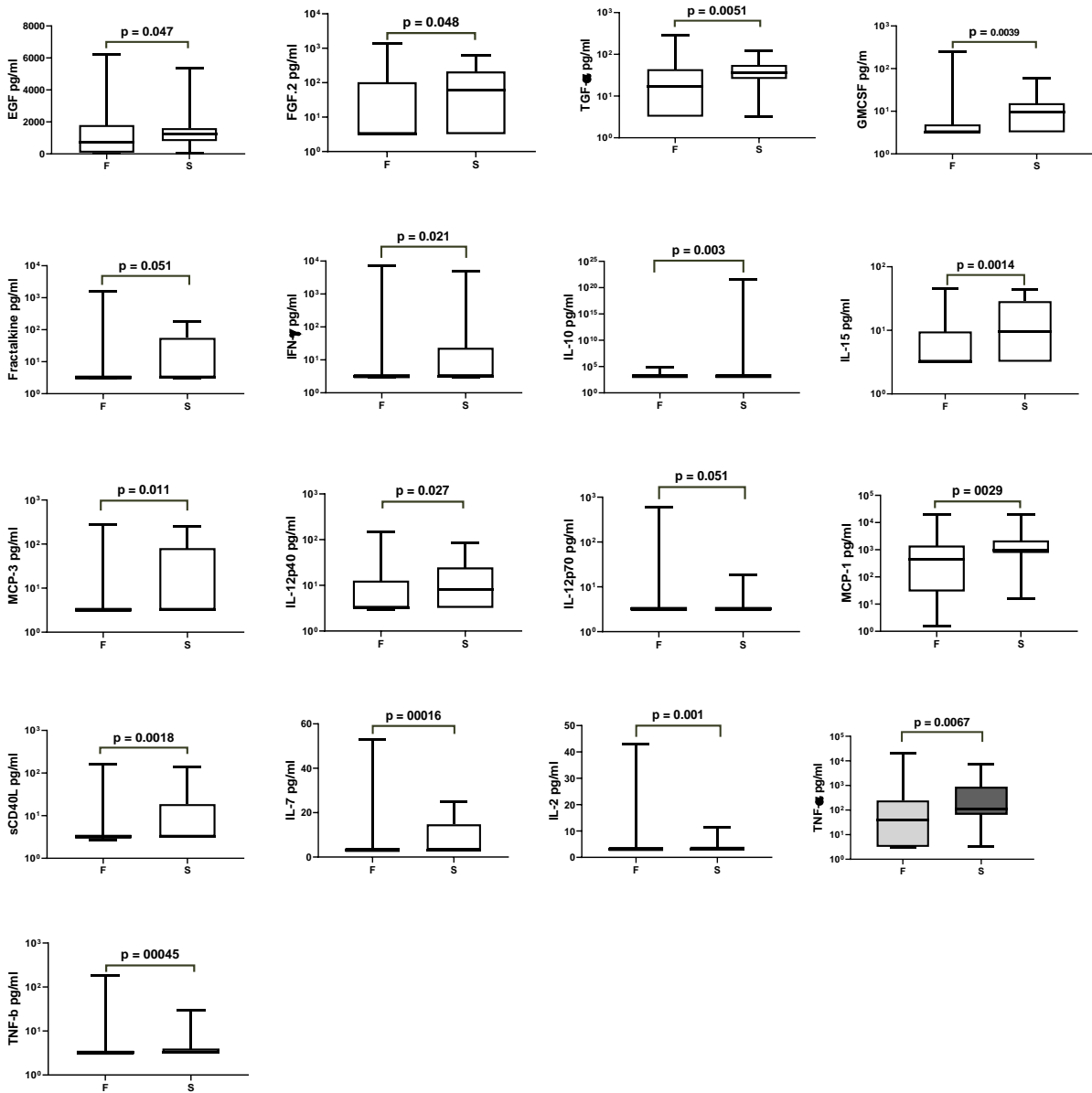


Figure 3.4.1D Comparison of baseline median levels of fast (F) and slow(S) responders

3.4.2 Biosignature of treatment responses

Following single biomarker analysis, we performed multiple logistic regression analysis to identify a potential biosignature that could predict fast and slow treatment responders. The fast responders are participants who had culture and smear converted to negative in the first 2 months of treatment, while slow responders remained culture or smear positive after 2 months of treatment. A four-marker signature (EGF, IL-15, MIP-1 β and TNF- β) was able to discriminate fast from slow responders with with an AUC of 0.74(95% CI: 0.57, 0.91), sens= 75%(95% CI: 54, 96) and spec= 80% (95% CI: 75, 86)

Table 3.4.2. Treatment response biosignature

Biosignature	AUC (95% CI)	Sens (95% CI)	Spec (95% CI)	PPV (95% CI)	NPV (95% CI)
Fast verse slow responders					
EGF, IL-15,MIP-1 β and TNF- β	0.74(0.57,0.9 1)	75(54 ,96)	80(75, 86)	0.26(0.13, 0.38)	0.97(0.95, 1.00)

Sens= sensitivity, Spec= Specificity, PPV= positive predictive value, NPV= negative predictive value, CI = confident interval and AUC= area under the curve

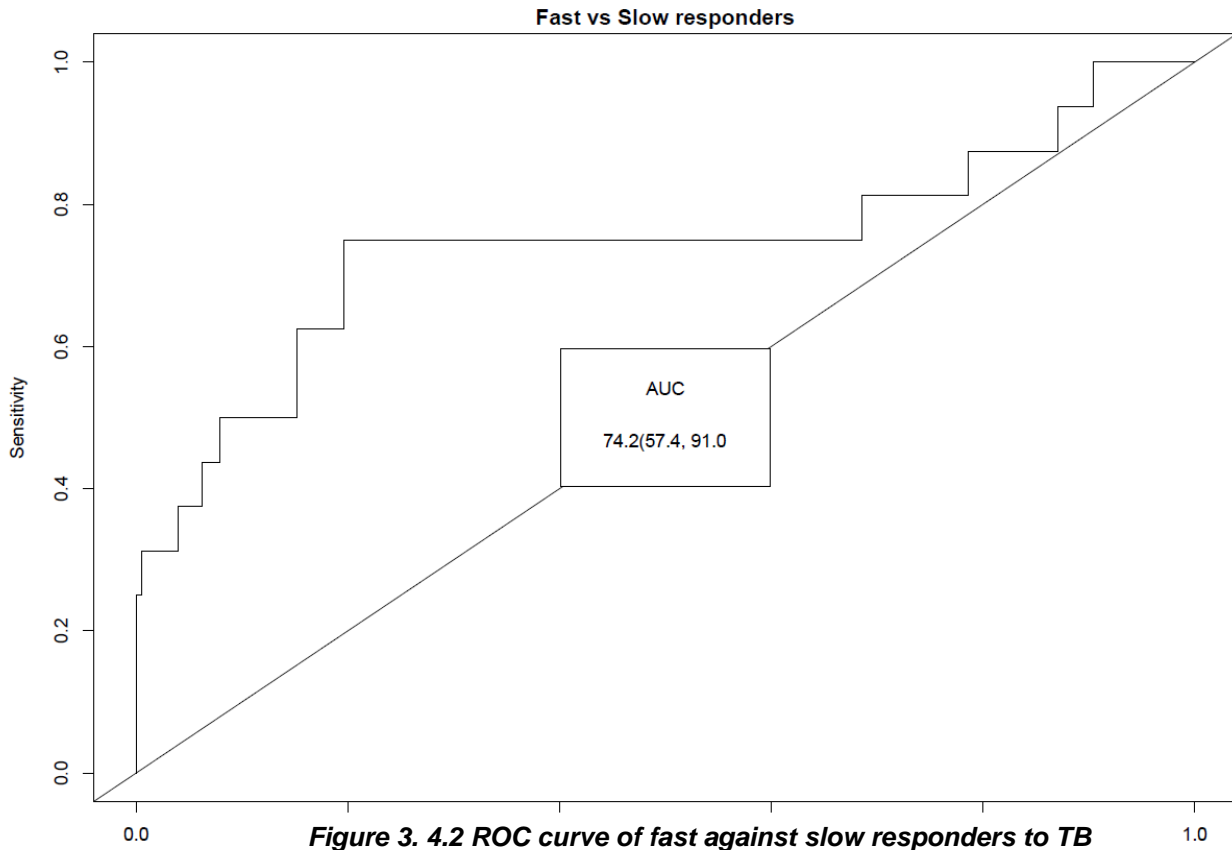


Figure 3. 4.2 ROC curve of fast against slow responders to TB treatment.

Chapter 4 Discussion

4.1 Main findings

The availability of a more efficient TB diagnostic test could revolutionise patient management, thus impacting on the transmission chain of the disease. This is particularly important since 10 million people are estimated to have TB disease every year, with a single patient prior to treatment having the potential to infect close to 15 people per annum [19]. In addition, about 70% of TB cases resided in poor resource settings [1] where they may not have access to, or be able to afford the cost of current TB tests. The worst of all, majority of these people visit primary health care facilities where there is no laboratory tests for TB, relying on clinical diagnosis despite the known limitations of clinical diagnosis, more so the overlapping symptoms of respiratory diseases [4]. Thus, the aim of this project was to determine optimal single host markers or biosignatures in ex vivo sputum samples that could diagnose pulmonary tuberculosis among individuals who present with symptoms of respiratory disease prior to microbiological confirmation. The specific objectives were a) to validate our previously identified biosignature in a larger sample size and to determine new signatures using an extensive panel of analytes; b) to assess the accuracy of the markers or biosignature in relation to different microbiological test outcomes; c) to investigate signatures that could be useful in HIV co-infected individuals or childhood and finally, to identify signatures that could predict treatment response.

In this study, we found most markers' median levels to be significantly higher in TB than ORD, with the most differential markers having a diagnostic performance (AUC) ≥ 0.60 . The best single classifiers of TB disease from other respiratory disease were: IL-1 β , FGF, GM-CSF, RANTES, BAFF and MMP-2 with an AUC ≥ 0.68 (Table 3.2A-C). In general, MMP-2 was the most outstanding single classifier in the study with an AUC of 0.73, which is still too low for a diagnostic or screening test. Though, some of the markers did not do well as single classifiers of TB, their frequent appearance in the generated signatures suggested their importance as potential markers of TB disease. The setback, however is, some of the markers such as FGF, GM-CSF and IL-12p70 which appeared in almost every generated signature has a low concentration in sputum. High concentration of the markers would be preferred. It would be easier to detect highly concentrated markers when adapted into lateral flow test or any portable diagnostic device. For instance, it is easier to detect the MMP-2 with a cut-off of 9273pg/ml than FGF with concentration cut-off 11pg/ml in sputum. With new technologies increasing the sensitivity of antibodies on lateral flow devices, this limitation may not be an issue in future.

Among the three kits analysed, the 38-plex single markers did not show good diagnostic potential in this study, with MIP-1 α and IL-1 β showing a sub optimal performance with AUCs of 0.65 and 0.66 respectively as highest performers (Table 3.2C). The poor performance could be attributed to sample type used but this is unlikely given that the other kits used were also developed for use in serum and plasma but not sputum. Another factor could be the duration of incubation after sample addition. The manufacturer recommended either a two-hour (2hr) or an overnight incubation and we opted for the 2hr incubation, which may also contribute to poor performance of the markers. However, the manufacturer must have optimised the assays for the 2hr incubation period and hence the 2hr incubation period may not completely explain our observations. Nonetheless the predictive ability of the markers were enhanced by the addition of other markers into a biosignature.

Furthermore, in this study, the data was stratified based on the microbiological test outcome (i.e. smear negative versus smear positive), HIV status and age. And the optimal signature found was an eight-marker signature (IFN- γ , IL-1 β , IL-8, IL-10, IL-12p70, MIP-1 β , RANTES and VEGF) with a sensitivity of 78% and specificity of 69% on the test data. This signature showed the potential to diagnose adult HIV negative smear positive/culture positive TB amongst total respiratory diseases but the limitation is it does not meet the WHO target product profile (TPP) criteria [sensitivity 90% and specificity 70%] for diagnosing pulmonary TB. So new studies are required to redefine the signature and some of the promising markers of in the study. We also had the previously discovered signature (IL-13, IFN- γ and FGF) in the pilot study validated in this study, however, its performance (sensitivity 70% and specificity 62%) on the test data does not meet the WHO criteria for either a TB diagnostic or triage test. Other signatures were also identified for the difficult to diagnosed form of TB (smear negative/culture positive, TB/HIV co-infected and children). In the children dataset, we had induced sputum from the under 5 years, though the numbers were small (4) but with high levels of cytokines, thus unlikely to have affected the results. Knowing the diagnostic difficulties associated with childhood, smear negative or TB/HIV+ patients due to paucibacillary disease, these signatures show promise in resolving this. The limitation with some of the identified signatures were the number of markers included were large, translating them into a single point care device would be difficult. New studies are needed to redefine the best markers in diagnosing TB as well as their applicability in a POC test.

In addition to understanding the diagnostic performance of these markers, we also investigated the usefulness of the markers in treatment monitoring amongst the TB cases. In these analyses, we only assessed the 38-plex data, at baseline, 1month and 2month. Due to limited resources the other kits were not tested. EGF, Eotaxin, Fractalkine, TGF- α , G-CSF, GM-CSF, GRO, IL-1 α , IL-1 β , IL-8, IL-3, MCP-1, TNF- α , IP-10, MIP-1 β , MDC, MIP-1 α and TNF- β were all identified as potential markers for

TB treatment response. Furthermore, we have discovered a 4-marker biosignature consisting of EGF, IL-15, MIP-1 β and TNF- β that could differentiate TB patients into slow or fast treatment responders at baseline in relation to smear or culture outcome at 2-months with a sensitivity of 75% and specificity of 80%. We also found median levels of markers in the biosignature to be elevated among slow responders compare to the fast responders, suggesting a difference in the immunological activities during TB disease between the two groups. The difference may be attributed to severity of disease, leading to prolonged inflammation, thus reflecting in the response to treatment.

Again, an ideal new TB test should be rapid (generate result within 20minutes), accurate, inexpensive and the test sample easily accessible. With the availability of lateral flow technology, the translation of immunodiagnostic markers into the lateral flow assays (LFA) would be beneficial. Lateral flow assays are known to be easy to use and provide results within a short time [114]. The employment of the technology will drastically reduce lost to follow-up occurring due long duration before results are known. More so it will impact on the number of diagnosed individuals as well prevent unnecessary treatment of non TB patients. This in itself reduces the cost associated TB patient management. Moreover, the utilisation of a laboratory free diagnostic test like sputum immunodiagnostic marker as a point of care test in the peripheral healthcare centres could mitigate the challenge of missing potential TB cases as well as fasten the time to treatment initiation. Thus, availability of the test would allow speedy testings alongside treatment acceleration or less/more referral to confirmatory testing with the standard TB tests. In addition, the test would provide information on infection/disease spectrum, identifying patients who might urgently require treatment. Of recent, exploration of ex vivo host markers have been shown to be useful and promising in diagnosing TB disease. Easily obtainable samples such as serum, plasma, saliva or urine are been investigated for a potential biomarker or signature of TB infection or disease [125], [145]. Various studies have shown promising findings in ex vivo TB biomarkers or signatures in serum, plasma and saliva [126], [129], [154]. Some of the studies observed a higher concentration of MIP-1 β , MCP-1, sCD40L, MIP-1 β , fractalkine, IFN- γ , IP-10, TNF- α , VEGF, IL-6, IL-9, IL-5, IL-17 and IL-33 among TB participant compared to ORD [154]. A similar pattern was also observed in this study, with most of the markers been higher in TB compared to ORD. In a saliva study, which was published by Jacob et al in 2016, the investigators reported a promising six-marker signature regardless of HIV status with a sensitivity of 100% and specificity of 89% [125] though the sample size were small. A similar study was by the same group using ex vivo saliva and serum discovered a five-marker signature from the saliva dataset differentiated TB from ORD with a sensitivity of 78% and specificity of 83% [145]. Urine was also assessed but the obtained urine marker (LAM) was found to be most useful for individuals with severe TB/HIV disease [118]. These findings further strengthen the belief that an

ex vivo host marker for TB disease can be attained. More so various high performed signatures were also reported by different investigators using ex vivo samples [129], [154]. Of recent a seven-marker signature obtained from serum with a sensitivity of 78% and specificity of 83% was validated in a finger prick blood test (manuscript in preparation), three markers (CRP, SAA and IP-10) from this signature showed promising results [129]. The intension is to further validate the three markers as a triage TB test in a laboratory free environment for screening of TB. These findings further stresses the important use of ex vivo samples in finding a potential immunodiagnostic marker for TB. Although a lot of biomarkers of TB infection or disease were discovered, most have not gone to the validation stage. The setback with most of these studies were; they either use small sample size, stimulated samples and/or samples (serum, plasma or pleural fluid) not obtained from the site of infection. Similarly, in our pilot study with the use of ex vivo sputum we found promising sputum biomarkers in high levels [152]. Thus this study was done to validate the performance of the previous discovered signature in the pilot study and to find potential new signatures in a large cohort. The use sputum is advantageous for many reasons; it is a routine sample for diagnosing TB, obtaining the sample is non-invasive and sample collection requires no trained personnel, but the patient's ability to produce the sputum. Moreover, the sputum sample is already been collected and used in resource limited settings, so the use of sputum signature for diagnosis of TB as a point of test would not be a challenge in these settings. More so it would help mitigate the limitation of the current diagnostic tests. In addition, an obtained sputum biomarker or signature would allow direct comparison with the current routine sputum diagnostic tests for TB thus limiting the challenge of sample variability. Again, been able to predict the treatment outcome, the sputum biomarkers or signature would provide information like disease severity in relation to smear grade or GeneXpert cycle threshold values or the chest x-ray score for personalised treatment. So a sputum marker or signature is vital in various aspect for the patient and healthcare provider in terms of time to results, treatment decision and general patient management. Above all, the available of sputum point of care test will quickly identify the most infectious patients, accelerate treatment and obviously reduce transmission. Additionally, the sputum signature would provide information for the difficult to diagnosed form of pulmonary TB. Similarly, the importance of developing an immunological marker test for treatment response cannot be overemphasised. Sputum culture conversion at 2 months is currently the only acceptable marker of treatment monitoring, and its predictive ability is lower than 40% [30]. In addition, it is based on detecting the presence of pathogen or its product, therefore its turnaround time is a limitation. It is also very difficult to obtain quality sputum during the course of treatment, therefore, the availability of markers at baseline that can predict the outcome of treatment would help to mitigate this problem. The availability of any TB diagnostic signature provide valuable information for vaccine and drug development.

4.2 Strengths and weaknesses

The study's main strength was the large sample size that allowed us to split the data into training and test datasets. It also gave us the opportunity to test our previously defined biosignature on a new dataset and to investigate accuracy in smear positive/culture positive, smear negative/culture positive, children and HIV co-infected subjects although more samples are required for validation in children and HIV-positive due to low numbers. Other advantages included the multiple microbiological tests performed on single patient samples and the number of analytes used. However, despite the large sample size, the challenges of this study were not being able to confirm the final disease classification of the ORD group (i.e. infection or chronic pathology) and obtaining sputum from TB patients during course of treatment beyond 2 months. Not collecting sputum beyond 2 months obviously affect treatment response evaluation. Moreover our samples were highly heterogynous with some samples having only smear microscopy performed as an approved diagnostic test, with MBLA as a confirmatory test. The disadvantage with MBLA was, it is not an approved TB diagnostic test even though it was sensitive in picking out TB. Finally, since Gambia has low HIV prevalence, more studies are needed to assess the effect of HIV co-infection together with CD4 counts on these markers.

4.3 Implications of the study

The result shown in this thesis is not conclusive, although it shows interesting finding which can be further investigated in various settings. In this study, we have seen promising results with ex vivo sputum markers as potential diagnostics markers of TB disease. With a combination of markers discriminating TB from ORD and also predicting the outcome of treatment. Since sputum is a readily available sample, easily collected and routinely used for TB diagnosis, obtaining a sputum based point of care test would be useful in resource poor settings with high burden of TB. Therefore, the role of these immunological markers in predicting TB or outcome of treatment should be further investigated. With this in mind, the markers discovered in thesis would enhance that understanding in future studies. The study also provide information about the performance of the markers based on the microbiological outcome, HIV status and age with data from smear positive/culture positive TB showing best performed markers or signatures. Again, these results need further investigation. Furthermore, been able to predict the outcome of treatment gives an idea of possible personalised

treatment. The growing interest in ex vivo samples analyses with intension of translating the finding into a point-of-care test which could be used in any part of the world most especially in remote setting with high burden of TB [126], [129], [152]. Therefore, this study is contributing into the pool of knowledge.

4.4 Conclusion

There is an urgent need for an accurate, rapid and inexpensive diagnostic test to curb TB, one of the single most deadly diseases to plague mankind. We have discovered novel sputum host biomarkers and biosignatures for screening of tuberculosis and predicting outcome of treatment. Since sputum is easily available, performing sputum point of care test would not be a problem in rural settings with no infrastructure. The availability of such a triage test will provide several opportunities in fight against the TB pandemic. It will mitigate most of the limitations associate with current diagnostic tests (Xpert and culture), such as cost, infrastructure, turnaround time for results and requirement for skilled personnel. With an affordable and fast triage test and an improved sensitivity, most of the possible TB would detected. At the moment, a similar triage test using fingerprick blood is under evaluation in our laboratory in collaboration with SUN. This test has been developed as a multi-plex lateral flow test in Leiden based on a finger prick blood using three markers (IP-10, SAA1 and CRP). Development of a test that is sensitive and specific for diagnosis is not enough but the cost and rolling out of the test to lower tiers of health system is key for its usefulness. Therefore, the availability of a TB triage test that could be used in remote health posts, homes or communities with less training like in the blood sugar, pregnancy or rapid Malaria tests would have great public health impact.

Chapter 5 Reference

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