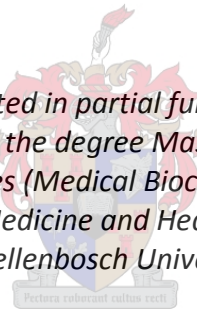


**Utilization of antigen-specific host
responses in the evaluation of
Mycobacterium tuberculosis
infection, development of disease
and treatment effect**

By
Angela Maria Menezes

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Requirement for the degree Master of Science in
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Promoter: Professor Gerhard Walzl
Department of Biomedical Sciences

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Declaration

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Abstract

Setting

This study was conducted in the Tygerberg district, Cape Town, in the Western Cape, South Africa

Background

The evaluation of early tuberculosis (TB) treatment response is based on month 2 sputum culture status. This method of evaluation has a number of limitations: the test requires relatively advanced laboratory infrastructure and procedures, it takes several weeks to obtain results and is a relatively a poor marker at predicting treatment response. The discovery of potential host markers which reflect the efficacy of early treatment would be of great importance for clinical management of individual patients. The treatment failure would be detectable earlier than at week 8 of treatment. The duration of clinical trials of new anti-tuberculosis drugs may also be substantially reduced by such markers if these would be measurable earlier than at week 8 of therapy.

Objectives

- 1) To evaluate diluted, 7-day whole blood cultures stimulated with live *Mycobacterium tuberculosis* (*M.tb*) for the presence of host markers of early TB treatment response
- 2) To evaluate an overnight, undiluted, *M.tb* antigen stimulated whole blood culture Quantiferon Gold In Tube (QFT-GIT) supernatants for host markers of early TB treatment response

The study designs were as follows:

In study one, baseline samples and samples from week 1, week 2 and week 4 of treatment from 30 cured TB patients were selected from a larger biomarker study, in which whole blood was stimulated with live *M.tb* or left unstimulated. Fifty seven host markers were measured in supernatants by multiplex cytokine arrays.

In study two, baseline samples and samples from week 2 and week 8 of treatment from 19 cured TB patients were randomly selected from the placebo group in a micronutrient supplement study. QFT-GIT supernatants from these participants were assessed through multiplex cytokine arrays for levels of fifty seven host markers.

All of the participants in both studies were Human Immunodeficiency Virus (HIV) negative.

Changes in marker expression over time and between fast and slow responders to treatment were evaluated. Comparability between the two culture methods was assessed for markers that were evaluated in both studies.

Results

In study one, the majority of host markers showed significant changes over time in the unstimulated supernatants. Only GRO and IL-1beta changed significantly in an antigen-specific manner (background levels subtracted). No significant changes were observed between fast and slow responders.

In study two, the majority of host markers showed significant changes over time in the unstimulated supernatants whereas only MDC and IL-4 changed during the observation period in antigen stimulated levels. Significant differences were observed between fast and slow responders at pre-treatment for IL-13_{Ag-Nil} and IL-1beta_{Ag-Nil}.

Conclusion

This study revealed, antigen-specific responses showed only limited potential for early TB treatment response monitoring, but may have potential in differentiating between treatment outcomes. Future investigations may have to include later time points during treatment as these were not included in the present assessment. The QFT-GIT samples do not appear to be equivalent to live *M.tb* stimulated 7-day whole blood assays.

Opsomming

Instelling

Die studie is uitgevoer in die Tygerbergdistrik, Kaapstad, Wes-Kaap, Suid-Afrika.

Agtergrond

Die evaluering van die respons op vroeë tuberkulose (TB) behandeling word gebaseer op die status van maand 2 sputum kulture. Hierdie evalueringmetode het 'n paar beperkinge: die toets benodig relatief gevorderde laboratorium infrastruktuur en prosedures, die toetsuitslae is eers na 'n paar weke beskikbaar en dit is 'n relatiewe swak merker om respons op behandeling te voorspel. Die ontdekking van potensiële selfmerkers wat die doeltreffendheid van vroeë behandeling weerspieël sal van groot belang wees vir die kliniese bestuur van individuele pasiënte. Mislukking van die behandeling sal sodoende voor week 8 van behandeling waargeneem kan word. Die tydsduur van kliniese proewe van nuwe anti-tuberkulose medikasie mag ook baie verkort word met sulke merkers as dit voor week 8 van behandeling gemeet kan word.

Doelwitte

- 1) Om verdunde, 7-dae oue volbloedkulture, met lewende *Mikobakterium tuberculosis (M.tb)* gestimuleer, te evalueer vir die teenwoordigheid van vroeë TB behandeling respons selfmerkers.
- 2) Om die supernatant van oornag, onverdunde, *M.tb* antigeen gestimuleerde volbloedkulture Quantiferon Gold In Tube (QFT-GIT) vir vroeë behandeling respons selfmerkers te evalueer.

Die studie-ontwerpe was soos volg:

Met studie een is basislynmonsters en monsters verkry na week 1, week 2 en week 4 van behandeling van 30 geneesde TB-pasiënte geselekteer uit 'n groter biomerkerstudie waarin die volbloed met lewende *M.tb* gestimuleer is of ongestimuleer gelaat is. Sewe-en-vyftig selfmerkers is in die supernatante gemeet deur middel van multipleks sitokine *arrays*.

Met studie twee is basislynmonsters en monsters verkry na week 2 en week 8 van behandeling van 19 geneesde TB-pasiënte lukraak uit die plasebo-groep in 'n

mikrovoedingstowwe-aanvullingstudie geselekteer. Vlakke van 57 selfmerkers is in die QFT-GIT supernatante van hierdie deelnemers, deur middel van die multipleks sitokine *arrays*, bepaal. Al die deelnemers van beide studies was HIV negatief.

Veranderinge in merker-uitdrukking oor tyd, asook tussen vinnige en stadige respons tot behandeling, is ge-evalueer. Die vergelykbaarheid van die twee kultuurmetodes is geassesseer ten opsigte van die ge-evalueerde merkers in albei studies.

Resultate

Met studie een het die meerderheid van die selfmerkers in die ongestimuleerde supernatante kenmerkende verandering oor tyd gewys. Slegs GRO en IL-1beta het aansienlik verander in die antigeenspesifieke wyse (agtergrond vlakke afgetrek). Geen kenmerkende veranderinge was waargeneem tussen die vinnige en stadige respons pasiënte nie.

Met studie twee het die meerderheid van die selfmerkers aansienlike veranderinge oor tyd in die ongestimuleerde supernatante gewys, in vergelyking waar net die MDC en IL-4 veranderinge gedurende die observasie periode in antigeen gestimuleerde vlakke getoon het. Kenmerkende verskille is tussen die vinnige en stadige respons pasiënte in voorbehandeling vir IL-13_{Ag-Nil} en IL-1beta_{Ag-Nil} waargeneem.

Gevolgtrekking

Die studie bewys dat antigeenspesifieke response slegs beperkte potensiaal vir vroeë TB behandeling respons monitoring het, maar mag die potensiaal vir onderskeidende behandeling uitkomstê hê. Toekomstige ondersoekes sal dalk latere tydpunte gedurende die behandeling moet insluit aangesien dit nie in hierdie evaluasie ingesluit is nie. Die QFT-IT monsters verskyn nie as gelykwaardig met die lewendig *M.tb* gestimuleerde 7-dae volbloed toetse nie.

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List of Abbreviations

AFB	Acid fast bacteria
Ag	Antigen
ANOVA	Analysis of variance
BCG	Bacillus Calmette–Guerin
°C	Degrees Celsius
CI	Confidence Interval
CMI	Cell-mediated immunity
CO₂	Carbon dioxide
CFP-10	Culture filtrate protein 10
CRP	C-reactive protein
CTL	Cytotoxic T cells
CXR	Chest x ray
DTH	Delayed type hypersensitivity
DOTS	Directly Observed Treatment Short
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMB	Ethambutol
ESAT-6	Early secreted antigen target-6
FGF-2	Fibroblast growth factor-2
FLT-3	Fms-like tyrosine kinase 3
G-CSF	Granulocyte colony-stimulating factor
GDA	Genera discriminant analysis
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRO	Gene regulated oncogene
HIV	Human immunodeficiency virus
IFN-γ	Interferon-gamma
IGRAs	Interferon-Gamma Release Assays
IL	Interleukin
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin- 1 beta
IL-1ra	interleukin-1 receptor antagonist
INH	Isoniazid
IP-10	Interferon-inducible protein
IUATLD	International Union Against Tuberculosis and Lung Disease
LSD	Least significant difference
LTBI	Latent TB infection
MCP-1	Monocyte chemotactic protein-1
MCP-3	Monocyte chemotactic protein-3
MDC	Macrophage-derived chemokine
MDR-TB	Multi-drug-resistant Tuberculosis
MIP-1α	Macrophage inflammatory protein-1 alpha
MIP-1β	Macrophage inflammatory protein-1 beta
MGIT	Mycobacteria growth indicator tube
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
NAATs	Nucleic Acid Amplification Tests
NHLS	National health laboratory service
NTM	Non Tuberculoses Mycobacterium
OD	Optical density
PAS	Para-sailicyclic acid
PCR	Polymerase chain reaction

PHA	Phytohaemagglutinin
PPD	Purified protein derivative
PZA	Pyrazinamide
QFT	Quantiferon
QFT-GIT	Quantiferon Gold-In Tube
RPMI	Roswell Park Memorial Institute medium,
RMP	Rifampicin
RNA	Ribonucleic acid
SAA	Serum amyloid A
SAP	Serum amyloid P
SD	Standard deviation
sEGFR	Soluble epidermal growth factor receptor
sgp130	Soluble gp130
sIL-1R1	Soluble interleukin-1 receptor 1
sIL-1R2	Soluble interleukin-1 receptor 2
sIL-2Ralpha	Soluble interleukin-2 receptor alpha
sIL-4R	Soluble interleukin-4 receptor
sIL-6R	Soluble interleukin-6 receptor
sRAGE	Soluble Form of Receptor for Advanced Glycation End Products
sTNFR1	Soluble tumour necrosis factor receptor 1
sTNFR2	Soluble tumour necrosis factor receptor 2
Streptavidin-(PE)	Streptavidin-Phycoerythrin
sVEGFR 1	Soluble vascular endothelial growth factor receptor-1
sVEGFR 2	Soluble vascular endothelial growth factor receptor-2
SVEGFR 3	Soluble vascular endothelial growth factor receptor-3
SEM	Standard error of the mean
TB	Tuberculosis
TGF-α	Transforming growth factor alpha
TNF-α	Tumour necrosis factor-alpha
TNF-β	Tumour necrosis factor-beta
TST	Tuberculin skin test
TTP	Time to positivity
VEGF	Vascular endothelial growth factor
WHO	World health organisation
XDR-TB	Extremely drug-resistant Tuberculosis
ZN	Ziehl-Neelsen

CHAPTER 1:

Introduction

1.1 Global Tuberculosis Burden

Tuberculosis (TB) is one of the most common infectious diseases in humans, has been described as the “white plague”¹ and is caused by the pathogen *Mycobacterium tuberculosis* (*M.tb*).

The World Health Organisation (WHO) has estimated that one third of the world’s population is infected asymptotically with *M.tb*, of whom 5 to 10% have a life time risk of developing clinical disease.² In 2010 the WHO, reported 8.8 million incident TB cases and 1.4 million TB deaths. Currently, South East Asia and Africa rank the highest in the absolute numbers of cases per year with South Africa in third position following India and China.³

Despite the fact that TB itself is a threat to the world population, co-infection with HIV has further increased the risk of TB disease progression and deaths. It was estimated that 1.2 million incident cases of TB were infected with HIV and 350 000 deaths were reported due to HIV/TB co-infection. In the African region it was estimated that 82% of the TB cases were co-infected with HIV in 2010.⁴ HIV infected individuals have a 10% annual risk of developing TB whereas HIV negative individuals have a 10% life time risk.⁵

Despite the availability of anti-tuberculosis chemotherapy, some regions battle with an increase in multidrug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB) strains of *M.tb*. Incorrect treatment regimens and non-adherence to anti-tuberculosis therapy have facilitated the spread of disease. The XDR-TB epidemic in Kwazulu–Natal was an example of how dangerous and rapid a disease can spread without the correct monitoring and treatment.⁶

The use of optimally working vaccines is the most cost efficient measure for the control of any disease. The only recommended vaccine for TB is Bacilli Calmette Guerin (BCG), it was developed in 1921 and found to be useful and cost effective against miliary TB in neonates.⁷ In adult pulmonary TB, the BCG vaccine has proven to be very inconsistent in their protection mechanisms.⁷⁻⁸

The spread of drug resistant strains, HIV co-infection, the absence of short and effective treatment regimens and the absence of effective new vaccines pose serious challenges for TB control.

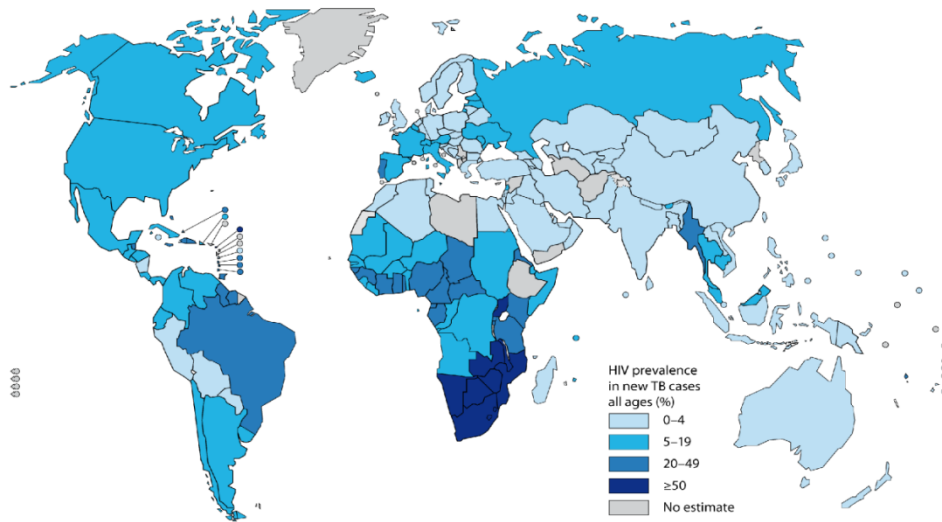


Figure 1.1 Estimates of the prevalence of HIV in new TB cases by country in 2010. Dark coloured areas represent regions with a high rate of HIV/TB co-infection, while grey and lighter blue areas reflect areas with lower HIV/TB co-infection. Source: WHO REPORT 2011: Global tuberculosis control

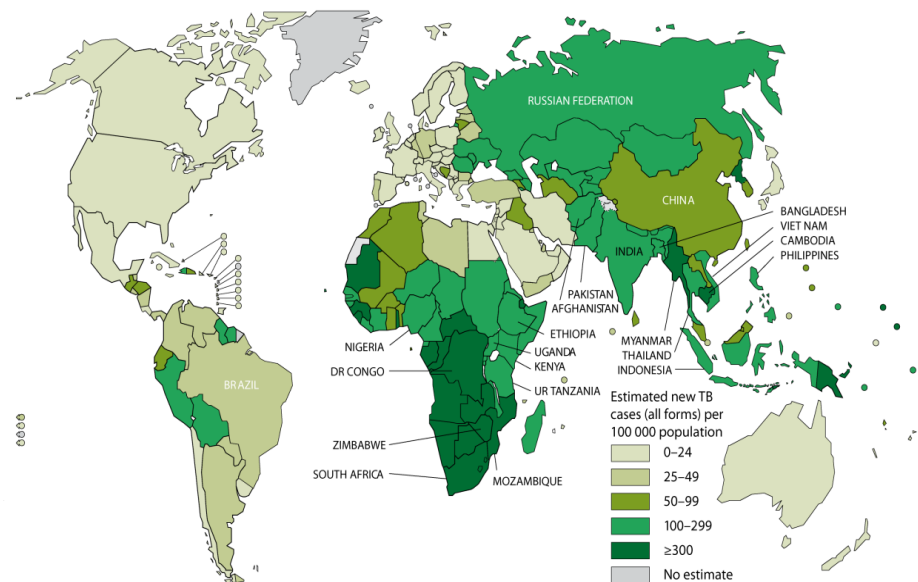


Figure 1.2 Estimates of rates of TB incidence, by country in 2010. Dark green areas represent regions with high TB incident cases, while the grey and lighter green reflects areas with lower TB rates. Source: WHO REPORT 2011: Global tuberculosis control

1.2 *M. tb*: Causative agent of human TB

M. tuberculosis, which causes TB is a rod shaped, slow-growing, acid-fast, aerobic, non-motile bacterium with a replication time of 15-20 hours.⁹ The cell wall structure of mycobacteria is unique compared to fast-growing bacteria. The complex mycobacterial cell wall is composed of unusual amounts of lipid moieties i.e. peptidoglycolipids (mycosides), cord factors and sulpholipids which provide hydrophobic characteristics, acid fast properties and enable intracellular survival of the bacterium within the macrophage.^{10,11}

1.3 Tuberculosis – Clinical Forms

M.tb has the ability to cause infection anywhere in the body but it mainly resides in the oxygen rich lungs.¹² The route of infection occurs via inhalation of aerolised droplets which are either coughed up or sneezed out by a person with active TB. The droplet nuclei are particles of 1-5µm in diameter and can remain suspended in the air for a number of minutes or hours. The likelihood of *M.tb* infection is increased by the number of bacilli inhaled, frequency of exposure and the general immune status of the potential host.^{13,14}

The host response plays a major role in determining the clinical manifestations and outcomes of persons who are exposed to the *M.tb* pathogen.

There are a number of possible outcomes that can occur when a person first encounters *M.tb*. First, some hosts have the ability to destroy the bacillus through the activity of innate responses, in particular through the antimicrobial activity of alveolar macrophages, which results in the host remaining uninfected. These hosts do not develop immunological sensitization to the bacterium (T or B cell responses). Second, the bacilli may escape eradication and a population of bacilli may remain in a state of dormancy within the host. This is termed as latent infection, which is manifested by a positive tuberculin skin test (TST) or positive interferon gamma release assays (IGRAs). Finally, if this state of latency is compromised active disease occurs, accompanied by common clinical symptoms, i.e. chronic cough, fever, night sweats and weight loss.¹⁵

Only 10% of people who are exposed to the bacillus will progress to active disease within 2-5 years of becoming infected.¹⁶ The remainder (90%) of people who are infected but not diseased are not able to spread the bacilli. Immunosuppressive factors enhance the progression of TB from infection to disease. These triggers can

involve HIV¹⁷, therapies such as tumour necrosis factor (TNF) neutralization therapy¹⁸, diabetes and immune senescence (old age).¹⁹

1.4 Immune responses to *M.tb* Infections

A foreign molecule that enters into the host has the ability to initiate a series of defence mechanisms in the body.¹² The immune system has two branches namely, the innate and the adaptive immune system. *M.tb* has the ability to stimulate both of these branches. As *M.tb* has adapted well to survive inside some immune cells, like macrophages, a major component of the protective response against this pathogen is the cell mediated immune response. For the destruction of *M.tb*, lymphocytes need to be activated, which in turn activate macrophages to improve their ability to kill intracellular pathogens. Ninety percent of exposed individuals do not develop active TB disease and this demonstrates that the interplay between innate and adaptive immune responses is generally effective in preventing progression to active TB disease.

1.4.1 Innate Immunity

Following inhalation, the bacillus travels down the trachea of the new host and reaches the small airways and lung parenchyma. The bacillus encounters alveolar macrophages (AMOs) and dendritic cells (DCs), which form the first line of defence within the lung.¹² Many receptors such as the complement and mannose receptors aid the entry of the bacillus into the host phagocytic cells.¹² These receptors can directly or indirectly attach to the surfaces of the sugar residues within the pathogen cell wall²⁰ and result in the expression of pro-inflammatory signals.²¹ Toll-like receptors (TLRs) are pattern recognition receptors that are able to identify pathogen molecules, including mycobacterium components. TLRs activate the intracellular signalling cascades that promote inflammation but can also activate signals that reduce the innate immune response.²² TLRs also play a role in the differentiation of monocytes into macrophages.²³

After bacillary uptake, the bacteria enter a phagosome which traffics within the AMO and fuses with lysosomes to form a phago-lysosome which results in a reduction of the intra-phagosomal pH and the production of nitrogen and oxygen radicals.²⁴ This may lead to the destruction of the bacteria with no persistence of infection. When the host fails to kill the bacteria but manages to inhibit growth, a state of 'dormancy' or quiescent infection may be established. The infected macrophage release inflammatory mediators, which induce the extravasations of phagocytic cells (granulocytes and monocytes), natural killer (NK) cells and T cells.²⁵ Two to three

weeks after infection, a cell mediated T cell response becomes detectable which results in a further release of inflammatory molecules. This releases cytokines and chemokines that regulate the migration of leukocytes to the site of infection and further activate other macrophages to initiate the formation of a granuloma.²⁵

1.4.2 Granuloma Composition and Function

The granuloma is the hallmark of the host immune response against mycobacteria and it is believed that these cellular structures play an important role to contain the bacillus. The production of TNF and Interferon (IFN)- γ from the infected macrophage stimulate the involvement of neutrophils; NK cells, CD4, CD8 T lymphocytes, DCs and B lymphocytes. Each of these cells produces their own cytokines that increase cellular recruitment and remodelling of the infection site.^{26,27}

The function of the granuloma is to contain and prevent the spread of the infectious bacilli at the primary site of the infection by starving the bacilli from oxygen and nutrients which are essential for their survival. A microenvironment for immune cross-talk between host cells leading to macrophage and lymphocyte activation, cytokine production and the killing of the bacteria by CD8 T lymphocytes is also a function of the granuloma.²⁵ This limits inflammation to the infection site and decreases multiplication of the bacilli.

The granuloma structure comprises of a necrotic centre, which results in the death of the majority of bacteria. This area is surrounded by a layer of macrophages, epitheloid cells and multinucleated Langerhans cells and lymphocytes.²⁸

The surviving bacilli remain in a quiescent or latent state and can become reactivated due to immunosuppression, either due to HIV infection or due to a poor health status of the infected person. These are the main causes for the unchecked replication of bacilli enclosed in the granuloma, ultimately resulting in active tuberculosis.^{29,27}

The innate immune system therefore has important functions in initiating the immune response against mycobacteria but also provides some of the final effector functions of the adaptive immune response.

1.4.3 Adaptive Immunity

(i) Cell mediated immunity to M.tb

CD4 T lymphocytes play an essential role in the protective immune response to *M.tb*.^{30,31} The primary residence of the bacteria is within the phagosome of the macrophage resulting in *M.tb* antigens being presented to CD4 T cells via the Major

Histocompatibility Complex (MHC) class II pathway. The importance of CD4 T cells in the protective immunity to *M.tb* has been demonstrated in several studies using mice models and humans. Studies using mice that were deficient in CD4 T cells were characterised by an impaired ability to control the infection due to the steady increase of mycobacterial growth in all organs which resulted in death.³² In humans, the protective role of CD4 T cells in TB is striking because HIV-mediated decrease of CD4 T cell numbers results in progressive primary infection, reactivation of latent infection and exacerbate TB disease.³³⁻³⁶

CD4 T cells comprise of subclasses, among which T helper (TH1) and TH2 cells produce cytokines which are important in the outcome of the immune response. Due to the release of IL-12 from the activated macrophages, CD4 T cells can develop into TH1 cells and release cytokines such as IFN- γ , TNF- α and Interleukin 2 (IL-2). These cytokines have the ability to activate macrophages to kill or inhibit the growth of mycobacteria.¹⁷ The release of IL-4 leads to the development of TH2 cells, which are able to produce cytokines such as IL-13, IL-5 and IL-10 and support B cell growth and differentiation³⁷ but suppress the TH1 immune response.³⁸

CD4 T cells can also develop into TH17 cells due to the release of IL-23, IL-6, and IL-21 to produce IL-17 and IL-22 and stimulate defensin production and recruit monocytes to the inflammation site.³⁹

(ii) CD8 T Cells

M.tb antigens are processed and presented to CD8 T cells, also called cytotoxic T lymphocytes (CTL), via MHC class I molecules. It is believed that CD8 T cells are less important than CD4 T cells in the protection against TB, but a murine study showed that a defective function of CD8 T cells due to a loss of function of β 2 microglobulin, resulted in increased susceptibility to *M.tb*.⁴⁰ CD8 T cells are activated due the mediation of *M.tb* which enhances perforin, granulysin, IFN- γ and Fas-L synthesis, which kill infected cells, and hence intracellular bacteria through different mechanisms.⁴¹ Granulysin alters the integrity of the bacterial cell and together with perforin, reduces the viability of *M.tb*.⁴² The ability for CD8 T cells to release IFN- γ and the ability to use different mechanisms to kill bacilli makes this cell type an effective killer during *M.tb* infection.⁴³ A subset of CD8T cells expressed the chemokine RANTES together with perforin and granulysin. RANTES attracted the *M.tb* infected cells and thereby enhanced their clearance.⁴⁴

1.4.4 Cytokines and *M.tb* Infection

Cytokine profiles may serve as indicators of the immune activation status. Different cytokines possess biological overlapping functions which give them the ability to control and regulate the production of other cytokines. The analysis of the function of a complete set of cytokines expressed within a micro-environment (ie. the site of inflammation) is often of more value when looking at a single cytokine at a time.¹²

(i) *IL-12*

IL-12 is produced by activated macrophages or dendritic cells upon phagocytosis of *M.tb*, triggering a TH1 response and IFN- γ production by CD4 and CD8 T cells.^{45,46} IL-12 is a critical cytokine in controlling *M.tb* infection. In one study it was shown that mice that had a deficiency in their IL-12p40 gene were highly susceptible to intravenous infection with *M.tb* and were unable to control bacterial growth within the lung⁴⁷ and IL-12 that was administered exogenous to BALB/c mice showed to prolong their survival.⁴⁸ Humans with mutations in IL-12p40 or the IL-12 receptor genes showed a reduction in IFN- γ and were more susceptible to mycobacterium infections.⁴⁹ In a previous study it was shown that the administration of IL-12 DNA could significantly reduce bacterial numbers in mice suffering from chronic *M.tb*.⁵⁰ This suggests that the induction of this cytokine could play a major role in the design of TB vaccines.

(ii) *IFN- γ*

IFN- γ plays a central role in controlling *M.tb* infection. It is produced by CD4, CD8 T cells and NK cells during *M.tb* infection. Individuals who have defective genes for IFN- γ or IFN- γ receptors are prone to serious mycobacterial infections.⁵¹ In a previous study, patients presented with disseminated infection with *Mycobacterium bovis* BCG or environmental mycobacterium, due to a IFN- γ receptor deficiency which resulted in some cases of death and survivors were required to continue anti-mycobacterial treatment.⁵²

Mechanisms have been developed by *M.tb* to limit the activation of macrophages by IFN- γ .^{53,54,55,56} This gives an indication that the amount of IFN- γ produced by T cells may be less predictive of outcome than the ability of the cells to respond to this cytokine. In one murine study it was shown that the IFN- γ levels produced in response to a potential vaccine do not always correlate with how effective the vaccine is during *M.tb* infection.⁵⁷ Therefore IFN- γ is important in the development of an immune response that lengthens the life span of an infected animal, but this response is not sufficient to eliminate an *M.tb* infection.

(iii) TNF

TNF is a cytokine which is produced by macrophages in response to a pathogen. TNF plays a role in recruiting inflammatory cells to the site of an infection which results in the containment of bacilli within a macrophage but it is not required for the initiation of an antigen-specific T cell response.^{25,58,59} In previous studies involving mice and zebra fish embryos, the absence of TNF caused granuloma formation to be halted and variable increases in bacterial growth. These observations indicate that TNF plays a direct role in controlling mycobacteria in granuloma macrophages and this may indicate that TNF functions indirectly in decreasing mycobacterial growth by modulating the maintenance of the granuloma.⁶⁰⁻⁶² The over production of TNF is associated with autoimmune diseases such as Crohn disease and psoriatic arthritis. Neutralizing TNF is used for the treatment of these diseases but promotes the possible reactivation of latent tuberculosis in individuals that are *M.tb* infected.⁶³

(iv) IL-4

IL-4 is one of the cytokines that promotes the immune system to induce the TH2 response. This cytokine is considered to be detrimental to the host protective mechanisms against TB. High levels of IL-4 have been shown to impair BCG vaccine-induced protection in the developing world where IL-4 elevation in people with helminth infection exists.⁶⁴ Increased levels of IL-4 have been noted to correlate with serum IgE concentration and with the extent of cavitations in CXR in patients with pulmonary TB.^{65,66} In a study using IL-4 delta 2 (IL-4 δ 2), a splice variant and an inhibitor of IL-4, it was found that both were increased in active TB and only IL-4 δ 2 was elevated in individuals with latent infection.^{67,68}

1.6 Diagnosis of Tuberculosis

There is no technique available for the diagnosis of TB that is sensitive, specific, cost effective, rapid and freely available at high incidence areas. Therefore the development of a secure and quick test is important in order to interrupt the transmission of TB. Microbiological tests such as microscopy and culture are used to diagnosis TB. Currently, immunological techniques are available which show an improvement in the detection process. *M.tb* infection without disease relies on immunological tests.

(i) TST

In 1891 Robert Koch isolated a compound called tuberculin which was prepared from a liquid culture of tubercle bacilli in order to use as a therapeutic vaccine against TB.⁶⁹ Today Koch's method for the preparation of tuberculin is used in the production

of purified protein derivative (PPD) which is used to diagnosis *M.tb* infection in the TST.⁷⁰ The TST is an *in vivo* test in which PPD is injected intra-dermally in the forearm and read within 48-72 hours. A positive reaction shows indurations and swelling, which is caused by delayed type hypersensitivity (DTH) reaction.⁷¹ Even though the TST has a high sensitivity for infection with *M.tb* its specificity is poor as many antigens present in the PPD are also present in the BCG and in other Non-tuberculous mycobacteria (NTM).⁷² Further the TST cannot differentiate latent TB from active TB, which is a problem in high incidence areas.⁷³

(ii) Radiology

Radiographic screening or Chest X ray (CXR) allows for the visualisation of cavities and infiltrates in the upper lungs. This method is used to screen and monitor the progression of pulmonary TB (PTB).⁷³ CXR have been discouraged for the diagnosis of PTB as it is restricted in diagnosing smear negative TB among TB suspect patients.²⁹ The sensitivity and specificity of the CXR to detect culture-positive TB cases depends on the presentation of the disease and its intensity⁷⁶. This is influenced by a number of factors which include; HIV status, delay in diagnosis, sex of the patient and misdiagnosis is common due to the lack of specialist radiology support.⁷³ Radiology remains only a supportive diagnostic tool and is subject to confirmation by microbiologic culture.

(iii) Microscopy and Culture

Microscopic visualization of *M.tb* using the Ziehl-Neelsen (ZN) staining method to detect acid-fast bacilli (AFB) is the most widely used method, inexpensive and results can be delivered within hours.⁷⁴ AFB testing by microscopy requires 5×10^4 bacilli/ml concentrations in sputum samples due to this, many immuno-suppressed individuals are missed as they present with low bacterial counts.⁷⁵ Diagnosis in children is difficult as they do not always produce collectable sputum.⁷⁶ Some modifications prior to staining, including cyto-centrifugation or overnight sedimentation, have been found to increase the concentration of AFB in the smear and results in higher sensitivity.⁷⁷

In order to confirm the presence of *M.tb*, culture is regarded as the gold standard to confirm a case of TB. The sputum has to be cultured in solid or liquid media, a process which also involves decontamination and speciation (confirmatory biochemical analysis or Polymerase chain reaction (PCR)).⁷⁸ The Mycobacterial growth indicator tube (MGIT) is the most commonly used culture system, as it is run in an automated system and positive results can be obtained within two weeks compared with 4-8 weeks when using the Lowenstein-Jenson and Middlebrook

culture systems.⁷⁹ The advantage of using sputum culture is that its more sensitive than AFB testing but culture does have many disadvantages such as it takes up to two weeks or more to confirm results, with delays in the confirmation process, it's a costly process, requires a complex laboratory system with a bio safety level 3 laboratory and the possibly of contamination, which would produce false positive results⁷⁹.

(iv) Nucleic-Acid Amplification Tests (NAATs)

NAATs involve a two-step PCR method which involves the amplification of a specific sequence of *M.tb* DNA.⁷⁸ Tests include “in-house” which are developed in non-commercial laboratories and commercial kits. These commercial kits use different methods to amplify the specific nucleic regions in *M.tb*.⁷⁶ The Roche Amplicor *M.tb* PCR which amplifies the region of 16s ribosomal RNA gene of *M.tb*.⁷⁸ The advantages of using these tests is that results can be obtained within 3-6 hours and can distinguish *M.tb* from NTM which is useful in populations with a high incidence of NTMs.⁷⁴ Although the NAATs have shown in several studies to have a high specificity and low sensitivity, these tests cannot replace culture and microscopy but should be used in accordance with these tests together with the clinical data to diagnose TB.⁷⁸

NAATs are not useful in monitoring the progress of TB treatment as these tests detect non-viable bacteria and produce false positive results.⁷⁴

(v) Gene Xpert MTB/ RIF

The Gene Xpert MTB/RIF is an automated *ex vivo* gene amplification molecular test which has the ability to diagnose TB and detect any resistance to the drug RIF which is one of main drugs used in the treatment of TB and it also serves as an indicator for multidrug resistance.⁹⁸ Test results can be obtained within two hours with high levels of sensitivity and specificity. However its use is limited in detecting latent *M.tb*.¹⁰⁸

(vi) Interferon gamma release assays (IGRAs)

Other assays rely on proliferation of lymphocytes after exposure to *M.tb* antigen. To be able to detect *M.tb* infection without detecting NTM's or *M.bovis* BCG, antigens that are specific for *M.tb* have to be used. The region of difference (RD1) codes for antigenic proteins which are restricted to the *M.tb* complex, including the 6-kDa early secreted antigen target-6 (ESAT-6) and culture filtrate protein 10 (CFP-10).⁸⁰ Those antigens have been used in two commercial interferon gamma release assays (IGRA), the Quantiferon-TB Gold (QFT) (together with TB 7.7) and the T-SPOT.TB test. These tests depend on IFN- γ production in response to these antigens.⁸¹ Only T

cells of sensitized individuals who have re-encountered *M.tb* antigens will produce IFN- γ .

The T-SPOT.TB test is an *in vitro* test in which isolated mononuclear cells are stimulated with ESAT-6 and CFP-10. Effector cells, T cells which have been recently exposed to the TB antigen, will be able to produce IFN- γ ⁸⁴ while the memory T cells are less likely to produce IFN- γ due to the short incubation time.

The Quantiferon-TB GOLD test is an Enzyme Linked Immunosorbent Assay (ELISA) based test whereby IFN- γ in culture supernatant is measured by ELISA.^{82, 83}

The WHO has issued a set of guidelines for the use of IGRAs in low and middle income countries, it stated that IGRAs and TST could not truly predict the likelihood of an infected individual to progress to active TB and due to this, IGRAs and TST should not be used to diagnose active TB. Due to the cost and technical implications, IGRAs cannot replace the TST test.¹

IGRAs and TST tests are used to detect latent TB, they are “indirect tests” which do not detect the viable *M.tb* bacilli but an immune response will indicate a past or present exposure to *M.tb*.⁸³

The IGRA is a short time assay and only effector T cells are detected, the T cells in the TST test have more time to expand to memory T cells.^{85,86} Overall it is believed that that the IGRAs were not more sensitive when compared to the TST for the diagnosis of TB.⁸⁴

1.7 TB treatment

In the 19th century, TB symptoms were not recognized to belonging to a single diseases entity, let alone being caused by an infectious disease.⁸⁷ In 1854 Herman Brehemar, who suffered from TB, went to the Himalayas and when he was subsequently cured this was ascribed to the climate. He opened the first TB treatment sanatorium where TB patients were treated by enforcing good nutrition, bed rest and fresh air.⁸⁸ These sanatoria were introduced throughout Europe. After 1919 more drastic methods were applied such as induction of artificial pneumothorax and surgery but these methods remained controversial and had potentially serious side-effects.⁸⁷ In 1946 streptomycin was the first antibiotic discovered to be effective against TB.⁸⁹ Other drugs such as para-salicylic acid (PAS) and isoniazid (INH) were also introduced, but the bacteria developed resistance within a short period of time⁹⁰ The combination of these three drugs proved to be more efficient and were not

associated with the development of drug resistance. Today INH is still used as a first line drug together with Rifampicin (RMP), Pyrazinamide (PZA) and Ethambutol (EMB) in the two month intensive phase of treatment, followed by four months of INH and RMP.⁹¹

1.8 Directly Observed Treatment (DOT) and Directly Observed Treatment- Short Course (DOTS)

A study directed by Wallace Fox of the British Medical Research Council in Madra, India, was one of the first studies to implement directly observed treatment (DOT). Home and sanatorium based drug therapy were compared and it was concluded that the sanatorium based patients, despite good nursing, rest and constant monitoring, did not fare better than those that were treated at home. This meant that the great expense of hospitalisation could be avoided and treatment would be more cost effective and more appropriate for resource poor settings.⁹² This study resulted in the implementation of DOTS strategy by the World Health Organisation (WHO), which was launched in the mid 1990's which consists of a five-point policy, which the third policy states that standard short course chemotherapy should be administered to all individuals that are smear-positive under the correct and controlled case management conditions.⁹³ DOT seeks to improve the adherence of people to TB treatment through health workers, family or community members who directly observe patients taking their anti-tuberculosis treatment.⁹⁴ The advantage with this system is that people are closely monitored, but many patients stop taking their medication as soon as they start to feel better. The DOT strategy prevented prolonged illness and decreased the continued transmission of TB in many communities.⁹⁵

Despite the implementation of DOTS, the relapse and failure rates are still too high. Patients receiving the standard six month regime who have drug susceptible organisms still have an estimated failure rate of 1-4% and a relapse rate of up to 7%.⁹⁶

1.9 The potential of Biomarkers to evaluate anti-TB Therapies

The tools used to evaluate TB treatment, especially in clinical trials, are expensive and time-consuming. The clinical indicator of treatment success is the sum of treatment failures which is defined as the presence of continued or a recurrence of positive cultures during the course of anti-tuberculosis treatment and a relapse is defined as an individual who has become culture negative at the end of anti-tuberculosis treatment but becomes culture positive again or exhibits radiographic or

clinical evidence that is consistent with active TB¹¹² at the end of a 2 year period after treatment. A phase III trial of new TB treatment with relapse as an end-point would require the participation of over 2500 participants and a follow up period of four years.⁹⁷ In a study done by Visser, *et al* it identified a number of factors which could delay the sputum culture conversion at month 2 in patients with positive smear tuberculosis; these included a shorter Time to detection (TTD) at baseline, tobacco smoking, the appearance of cavities in the upper lungs and the W-Beijing genotype.¹¹³

It has been suggested that if predictive biomarkers existed for TB treatment response it would help in clinical practice and shorten clinical trials.⁹⁸

Several biomarker discovery approaches are being developed for TB. For instance, specific mRNA molecules are being used to monitor mycobacterial viability. Antigen 85B RNA has shown to clear more rapidly than viable sputum colony counts during therapy and may be a good marker for early treatment effect.^{99,100} There has been some interest in looking at pathogen markers in urine as such samples can be easily collected and relatively safe and easy to process. The presence of small fragments of *M.tb* IS6110 DNA in the urine of 79% (34/43) of TB patients but not in healthy controls and may have treatment response applications but this needs further investigation.¹⁰¹

Other approaches have looked at non-specific immune activation markers such as CRP¹⁰², neopterin¹⁰³ and pro-calcitonin¹⁰⁴ to follow TB and other bacterial diseases in humans. In principle, following any of these markers would be useful for assessing responses to anti-TB therapy as host immunity is essential for controlling TB and for suppressing reactivation.¹⁰⁵

More prospective studies need to be conducted in order to validate the potential candidate biomarkers. Candidate biomarkers may have to be discovered in smaller study groups due to cost-implications of the discovery techniques like next generation sequencing, metabolomics or proteomics.¹⁰⁶ It is essential that these studies are well designed with clear clinical phenotypes. Clinical characteristics that are important include extent of disease at baseline, time of culture conversion and time to positivity (TTP), sputum culture status after two months of therapy, and final treatment outcome, including cured, treatment failure and relapse.¹⁰⁷ Studies should also be conducted in different settings, including different geographical locations to ensure universal applicability of biomarkers. Genetic backgrounds of host or

pathogens or environmental factors like concurrent parasitic infections or other regional conditions may otherwise affect the performance of these markers.¹⁰⁸

1.10 Study Hypotheses and Objectives

1.10.1 Hypotheses

Stimulation of blood samples from people with active *M.tb* infection with *M.tb* antigens will result in the expression of biomarkers, which will correlate with early TB treatment response.

1.10.2 Objectives

- To evaluate diluted, 7-day whole blood cultures stimulated with live *M.tb* for the presence of host markers of early treatment response
- To evaluate Quantiferon supernatants for host markers of early treatment response

CHAPTER 2:

Material and Methods

2.1 Study Setting

These studies were conducted at the Immunology Research Laboratory, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences of the University of Stellenbosch. The participants all enrolled from the Western Cape Province, South Africa. One study (Micronutrient Study) recruited from the Delft clinic and the second study (Surrogate Marker Study) from five primary health care TB clinics near the Tygerberg Academic Hospital (Ravensmead, Uitsig, Adriaanse, Elsiesriver and Leonsdale). The clinics in these communities apply the DOTS strategy for treating tuberculosis. The Ravensmead/Uitsig community has an extremely high prevalence of TB infection as the prevalence has been reported as in this area as 1000 per 100 000.¹⁰⁹

2.2 Ethical Issues

For the Surrogate Marker studies (Chapter 3) ethics was obtained from the Committee for Human Research of the University of Stellenbosch (ref number 99-039). Ethical approval for the Micronutrient study (Chapter 4) was obtained from the University of Cape Town Research and Ethics Committee (Project ethics number 137/2003). Written informed consent was obtained from all participants. All participants were counselled before and after their HIV test was performed.

2.3 Study participants

2.3.1 A prospective evaluation of surrogate markers for treatment response and outcome in adult patients with pulmonary tuberculosis (Surrogate Marker Study) - Chapter 3

The participants for this study were recruited from the five primary health care TB clinics mentioned in 2.1 between 15 May 1999 and 15 July 2002. Details of this study have been published by Hesselning *et al.*, 2010.¹¹⁰ This study's main focus was to assess immunological and micro-bacterial markers for TB recurrence after initial successful TB treatment amongst South African participants. Principal investigators for this study included Prof. N. Beyers, P. Van Helden and G. Walzl from Stellenbosch University. The study was part of the GlaxoSmith Kline sponsored Action TB program.

The inclusion and exclusion criteria for participants were:

Inclusion criteria:

- Two positive sputum smears using the ZN staining
- Age between 20-65 years
- Willingness to undergo HIV testing
- Willingness to give informed consent for participation in the study

Exclusion criteria:

- Refusal to participate, including for HIV testing
- History of previous TB
- HIV infection
- Presence of resistance to both RIF and INH (Multi-drug resistant TB)
- Diabetes, malignancy, lung cancer, chronic bronchitis, sarcoidosis
- Use of systemic steroids.

The study included 336 patients of which 42 participants with pulmonary TB were selected as described below. Twelve patients relapsed within 24 months after initial cure and the remaining were matched to 30 cured patients. Each relapse patient was matched to two cured patients without relapse. The matching was done according to gender, extent of disease on chest X-ray (CXR) and where possible, duration of sample storage. Classification of CXR extent of disease was based on the criteria described by Simon, G 1971.¹¹¹ Briefly, the presence and number of cavities as well as the overall extent of alveolar involvement (using the size of the right upper lobe or a whole lung as reference volumes) were used. The overall extent classified as either moderate or extensive in an adaption of the Simon method.

- **Relapse** was defined as a second episode of TB with the same MTB strain as in the initial episode as determined by restriction fragment length polymorphism (RFLP) fingerprinting. The treatment outcome for episode one had to be 'cure' or 'treatment complete', as defined by WHO criteria¹¹²
- A **cured** patient for the purposes of the present study did not develop a repeat episode of active TB during the two year follow-up period and had a favourable outcome of their TB treatment episode as defined above ('cured' or 'treatment complete').

The participants used for this study (Chapter 3) included only the 30 cured tuberculosis cases. These participants were longitudinally recruited from the study area and followed up for over 24 months. Blood samples were collected from every patient at each of the seven time points (from pre-treatment week 1, 2, 4, 6, 8 and 24 months post-treatment) and stored at -80°C. Host markers using the Bio-plex platform were only evaluated on the whole blood culture samples that were only collected at diagnosis, week 1, week 2 and week 4.

2.3.2 The evaluation of new host markers in Quantiferon supernatants (Micronutrient study) – Chapter 4

This is a sub-study of a project entitled “The Effect of Vitamin A and Zinc supplementation on the Bacteriologic Response of persons with Pulmonary Tuberculosis in the Western Cape”. This was collaboration with Dr Marianne Visser from the University of the Western Cape (UWC) as the principle investigator. The aim of the main project was to evaluate the effect of vitamin A and zinc supplementation on the early (week eight) treatment response in adult, sputum smear positive TB patients. The findings of the main study were published by Visser et al., 2011¹¹³

This study followed a community-based randomized double blind, placebo-controlled study design, where prospective patients were recruited and followed up at three separate time points, namely baseline, week 2 and 8. The recruitment of these participants was done between March 2005 and August 2008.

All eligible patients presenting at Delft Clinic with newly diagnosed pulmonary tuberculosis were recruited in the study. The 154 patients that were recruited were randomly assigned to the micronutrient or placebo groups by the use of a random-number generator using a block design, without stratification for HIV infection status. One study group of 77 participants received a single dose of 200 000 IU Vitamin A (retinyl palmitate) at study entry plus daily (five days per week) supplementation of 15mg of zinc. The other group of 77 participants received the placebo (sunflower oil) supplement for two months. Both groups received standard anti-tuberculosis treatment in addition to the supplement or placebo.

For the purpose of this study subjects were recruited based on the following criteria:

Inclusion criteria

- Aged 18-65 years
- Two sputum samples testing positive for acid-fast bacilli by smear microscopy or one positive sputum specimen plus CXR findings suggestive of active TB as assessed by a pulmonologist experienced in the use of the Chest Radiograph Reading and Recording system.¹¹⁴
- Willingness to undergo counselling and testing for HIV

Exclusion criteria:

- Previous treatment of TB disease
- Known or suspected multi-drug resistance tuberculosis
- Extra-pulmonary tuberculosis
- Pregnancy
- Birth six months prior to study entry
- Liver disease, renal failure, congestive heart failure or neoplasm
- Use of corticosteroids
- The use of supplements containing Vitamin A, zinc or iron during the month preceding recruitment into the study.

For the present study 19 pulmonary TB cases were randomly selected from the 77 participants receiving the placebo supplement by using online randomization software (www.random.org). All of the participants' samples were evaluated using the QFT IT. Host markers were evaluated on the additional aliquots of the QFT supernatants from the selected participants, from all time points (Diagnosis, week 2 and week 8), using the Bio-plex platform as described below.

2.4 Treatment Protocol

All participants received the six-month DOTS anti-tuberculosis therapy which is recommended by the South Africa National Tuberculosis Programme. Briefly, the drug regimen consisted of a weight-related combination of INH, RIF, PZA and EMB during the first two months of therapy, followed by RIF and INH for the remaining four months. The intensive phase of treatment was prolonged to 3 months if sputum smear conversion had not occurred at 2 months.

2.5 Laboratory Procedures

2.5.1 Sputum and Culture Examination

Sputum samples were cultured in liquid media using the BACTEC MGIT 960 system (Becton Dickinson, Sparks, MD). Direct sputum smear microscopy was performed using the ZN method with quantitative smear grading using the International Union against Tuberculosis and Lung Disease guidelines. Sputum samples and smears were processed at the National Health Laboratory Services (NHLS).

2.5.2 QFT-IT

QFT-IT is an assay that measures the cell mediated immune (CMI) responses against *M.tb* proteins. It is used as an *in vitro* diagnostic test for prior exposure to *M.tb* (primarily latent TB infection) and detects the release of IFN- γ by effector memory T lymphocytes when stimulated with *M.tb* specific antigens.

2.5.2.1 Incubation and harvesting of blood

Each sample of venous blood was aseptically collected into three vacutainer 1ml QFT-IT tubes (Nil, TB Antigen and Mitogen) that were supplied by the manufacturer (Cellestis, Victoria, Australia). The Nil tube contains saline and serves as a negative (background or unstimulated) control tube. The antigen tube contains the overlapping peptides representing the entire sequence of ESAT-6, CFP-10 and TB7.7 (Rv2584) and the mitogen tube contains phytohaemagglutinin (PHA) and is used as a positive control. After blood collection, each tube was shaken vigorously to ensure that the entire surface was coated with blood as recommended by the manufacturer.¹¹⁵ The tubes were then incubated at 37°C for 16-24 hours. After incubation, the tubes were centrifuged at 3000 x g for 15 minutes. Plasma (120 μ l) was harvested from all three tubes and aliquoted into 500 μ l tubes. The samples were then stored at -80°C until further analysis or until ELISAs were performed.

2.5.2.2 QFT-IT ELISA procedure

The QFT-IT ELISA was performed according to the manufacturer's instructions.¹¹⁵ In short, the QFT-IT kit and samples were taken out one hour prior to the assay to ensure all samples and reagents reached room temperature. The ELISA plate was coated with the conjugate, samples and standards and left to incubate for two hours in the dark. This was followed by a washing step and enzyme substrate solution was added to all wells and mixed on a plate shaker for two minutes. The plate was then incubated at room temperature in darkness for thirty minutes. Enzyme stopping solution was dispensed into all wells and mixed well. The optical density (OD) of

each well was measured using a micro plate reader and read at 450nm and 650nm. These values were then analysed using QFT-IT analysis software from Cellestis version 2.50 to obtain results.

- A **positive** result was determined if the TB specific antigen IFN- γ response (TB antigen – Nil) were ≥ 0.35 IU/ml regardless of the mitogen values.
- A **negative** result was defined if the TB specific antigen IFN- γ response was ≤ 0.35 IU/ml provided that the mitogen value was ≥ 0.5 IU/ml.
- An **indeterminate** result was defined if the TB specific antigen IFN- γ response was < 0.35 IU/ml and the mitogen – nil response was < 0.5 IU/ml.

2.5.3 Seven-Day whole blood assay

Blood was collected into sodium heparin tubes (Becton Dickenson). Aliquots of the blood sample (1ml each) were transferred into two 50ml tissue culture flasks and diluted with 9ml of Roswell Park Memorial Institute medium (RPMI)-1640 (Gibco). After thawing at 37°C for at least two hours, a previously frozen aliquot of an isolate of *M.tb* (thawed from a frozen aliquot of H37Rv) was resuspended by gently shaking, and mixed by passing through a 1ml syringe. Both control and test cultures were prepared. The test whole blood cultures were inoculated with *M.tb* at 1×10^5 cfu's/ml in tissue culture flasks. One hundred and fifty micro litres 1×10^5 CFUs/ml were added to the test culture and 100 μ l of RPMI were added to the control culture. The cultures were incubated at 37°C and 5% CO₂ for seven days after which both the supernatant and cell pellet were harvested. The supernatant (removed without disturbing the cell pellet), was filtered with a 5ml syringe, aliquoted and frozen at -80°C until further analysis. The cell pellet was preserved for future analysis by adding 10ml of Ribonucleic acid (RNA) stabilisation reagent (Roche) and frozen at -80°C. All steps in the whole blood assay were done in a bio safety level 3 laboratory.

2.5.4 Principle of the Luminex Assay

The technology makes use of 5.6 μ m polystyrene or magnetic microspheres which are internally colour-coded by two fluorescent dyes (a red and an infra-red dye) such that each microsphere set is unique and especially distinct from other sets. This allows for simultaneous quantitation of up to 100 analytes in a single assay well.

Antibody specifically directed against the cytokine of interest is coupled to the colour-coded polystyrene beads. The antibody coupled beads react with the sample (supernatant) containing the unknown concentration of the cytokine. After a series of washes to remove the unbound protein, a biotinylated detection antibody, specific for

a different epitope on the cytokine is added to the beads. This results in the formation of a sandwich of antibodies around the cytokine. The reaction mixture is detected by streptavidin-phycoerythrin (streptavidin-PE), which binds to the biotinylated detection antibodies. The well contents are drawn up into the Luminex suspension array system, which identifies and quantitates each specific reaction based on the bead colour and fluorescence.

Biomarker levels were measured using the above mentioned kits according to the manufacturer's instructions. All samples (QFT and 7-day WBA supernatants) were brought to room temperature, vortexed and spun down at 14000 rpm for two minutes to pellet any precipitate cellular debris.

For the 3-plex cardiovascular disease/acute phase protein panel, a 1:8000 dilution of all the supernatants was performed in a two-step process: 5µl of the sample was added to 495µl of assay buffer and then mixed thoroughly (1:100 dilution) after which the sample was further diluted to 1:8000 by addition of 10µl of the 1/100 diluted sample to 790µl of assay buffer, followed by thorough mixing.

For the 14, 13, and 27 plex assays, QFT supernatants were diluted 1 in 3 with the serum matrix diluent provided in the kits, while the 7-day WBA samples were tested neat. The dilution factors were decided after passed optimization experiments.¹¹⁶

All the samples, standards and quality controls were assayed in duplicate. The levels of the host markers in the quality control reagents were all within the manufacturer's recommended expected ranges. The range of the standard curve for all the markers in the 27-plex assay was 3.2-10000 pg/ml. The standard curve of the high sensitivity assay was 0.13-2000 pg/ml and for the acute phase proteins SAA and SAP 0.08-250 ng/ml and for CRP between 0.016-50.0 ng/ml.

The soluble receptor assay had different standard curves for the various receptors as seen in Table 2.1

Table 2.1 The standard curves for the soluble receptors

Soluble Receptors			
Standards	sIL-4R, sIL-6R, sRAGE, TNFRI, sTNFRII	sCD30, sgp130, sIL-1RI, sIL-2R α	sEGFR, sIL-1RII, sVEGFR1, VEGFR2, sVEGFR3
1-7	12.2 – 50.000 pg/ml	24.4 – 100.000 pg/ml	122.1 – 500.000 pg/ml

2.5.4.1 Evaluation of Supernatants by the Luminex multiplex immunoassay

A total of 57 analytes were evaluated in the samples supernatants from the participants using Milliplex multiplex immunoassay kits (Millipore, St. Charles, Missouri, USA). The levels of all the analytes were evaluated in the unstimulated (Nil) and TB antigen QFT-GIT and live *M.tb* seven day whole blood stimulated and unstimulated supernatants. The detection of all the analytes were determined using four separate kits (pre-designated by the manufacturer), based on assay compatibility. These included a 14-plex soluble receptor panel kit, a 13-plex high sensitivity kit, a 3-plex acute phase proteins kit, and a 27-plex 'normal sensitivity' kit as seen in Table 2.2.

Table 2.2 The analytes present in the soluble receptor, 13-plex, 27-plex and the 3-plex kits.

Luminex Kits	Soluble receptor kit	13-plex high sensitivity kit	27-plex kit	3-plex acute phase proteins kit
	• soluble CD30 (sCD30)	• Interleukin (IL)- 1B	• Epidermal Growth Factor (EGF)	• Serum Amyloid protein A (SAP A)
	• soluble Epidermal Growth Factor Receptor (sEGFR)	• IL-2	• Eotaxin	• Serum Amyloid protein P (SAP P)
	• soluble Interleukin-4 Receptor (sIL-4R)	• IL-4	• Fibroblast Growth Factor 2 (FGF-2)	• C-reactive protein (CRP)
	• soluble Tumour Necrosis Factor Receptor 1 (sTNFR1)	• IL-5	• FMS-like tyrosine kinase 3 (FLT-3)	
	• sgp130	• IL-6	• Fractalkine	
	• soluble Interleukin 1 Receptor-1 (sIL-1R1)	• Granulocyte Monocyte Stimulating Factor (GM-CSF)	• Granulocyte Colony Stimulating Factor (G-CSF)	
	• sIL-6R	• IFN- γ	• GM-CSF	
	• sTNFR2	• TNF- α	• Growth Regulated Oncogene (GRO)	
	• sIL-1R2	• IL-7	• Granulocyte Macrophage Stimulating Factor (GM-CSF)	
	• sIL-2R α	• IL-8	• Tumour Necrosis Factor-beta (TNF-beta)	
	• soluble Receptor for Advanced Glycation End products (sRAGE)	• IL-10	• IFN- α	
	• soluble Vascular Endothelial Growth Factor Receptor 1 (sVEGFR1)	• IL-12p40	• Interleukin 1 Receptor Antagonist (IL-1ra), IL-1 α , IL-3, IL-9, IL-12p40, IL-15, IL-17	
	• VEGR2	• IL-13	• Vascular Endothelial Growth Factor (VEGF)	
			• Interferon inducible protein 10 (IP-10)	
			• Monocyte Chemotactic protein 1 (MCP-1)	
			• Macrophage Derived Chemokine (MDC)	
			• Macrophage Inflammatory protein (MIP-1 α)	
			• Transforming Growth Factor- α (TGF- α).	
			• TNF-beta	
			• MIP-1beta	
			• sCD40L	

2.5.4.2 The Luminex assay procedure

As recommended by the manufacturer, plates were pre-wetted with assay buffer and left at room temperature on a shaker (at 700 rpm) for 10 minutes. The assay buffer was then removed by vacuum aspiration. The standard and control reagents were then added to their respective wells, followed by the addition of assay diluent to the sample wells. The standards, controls, internal control and the supernatant solution were added to the appropriate wells, followed by the addition of antibody-coated fluorescent beads.

After one hour incubation on a plate shaker at room temperature, the fluid was aspirated by the vacuum. The biotinylated secondary antibody was added, followed by streptavidin-labelled phycoerythrin antibody, with alternate washing and incubation steps between the additions of each antibody. Finally, the sheath fluid was added to each well and the beads were re-suspended by mixing the plate on the plate shaker, after which the plate was read on the Bio-rad Luminex reader. A standard curve was automatically generated by using the Bio-Plex Manager software version 4.1.1 (Bio-rad Laboratories) to determine the medium fluorescent intensities of the different analyte bead sets and respective concentrations. An illustration of this technique can be seen in Figure 2.1.

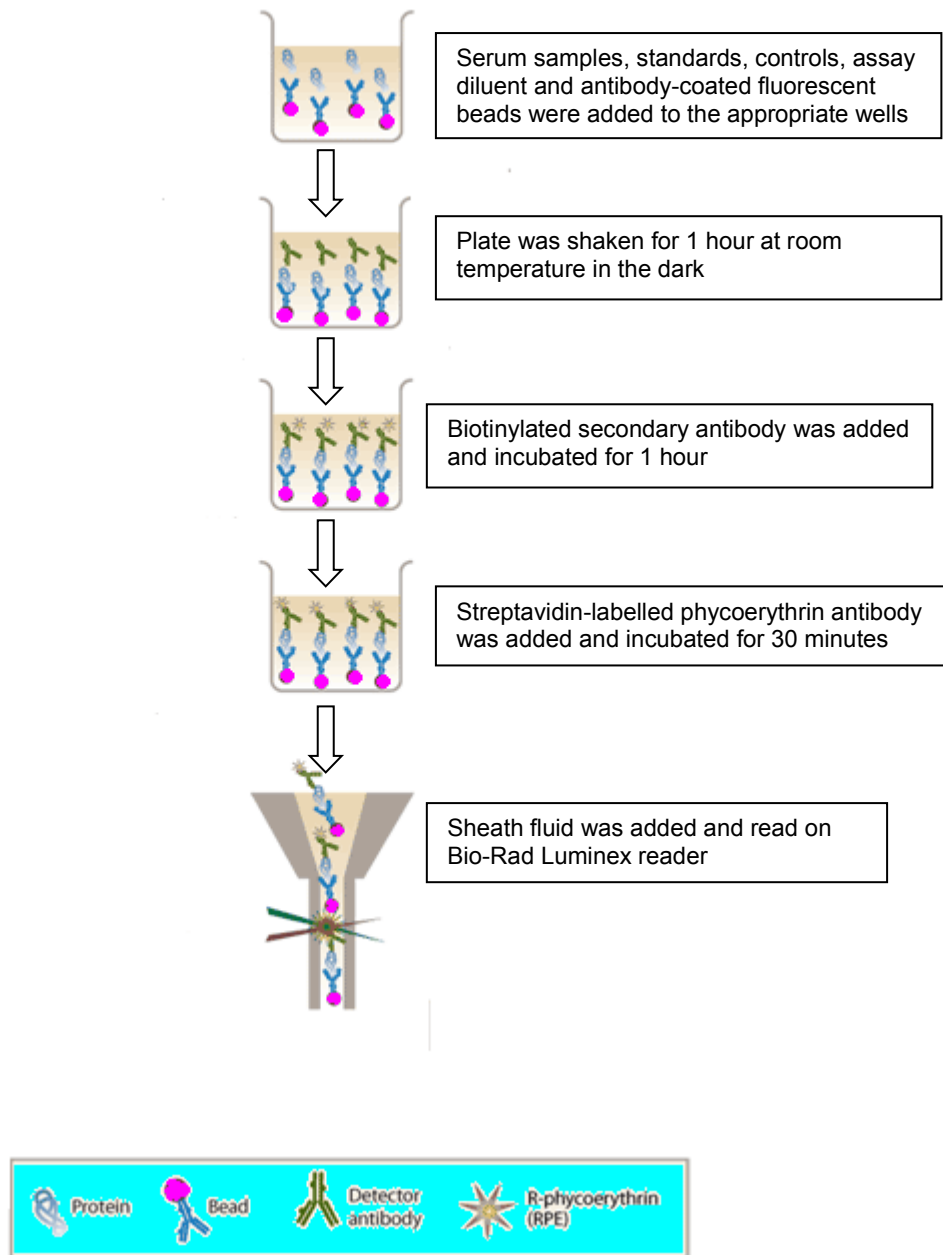


Figure 2.1 Principle of the Luminex technique

2.6 Data and Statistical analysis

All data was statistically analysed using Statistica version 9. All QFT analyte levels obtained from the Luminex assay were multiplied by 3 to correct for the dilution. All results were transformed using the logarithm for the base 10 to reduce variance in distributions. The unstimulated (nil) values have been subtracted from the antigen (Ag) stimulated values. A one-way repeated measure Analysis of Variance (ANOVAs) with least significant difference (LSD) post-hoc was conducted to compare time points. Values with a $p \leq 0.05$ were considered significant.

In an unbiased analysis approach, Qlucore Omics Explorer, Version 2.0 Beta (Qlucore AB, Lund Sweden) was used to construct heat maps. The clustering of the study participants was based on the host markers expression across the course of TB treatment. The colour assigned to a point on the heat map indicates how much of a particular host marker was expressed in a given participant sample. The expression level is generally indicated by red for high expression and green for low expression.¹³¹

The Ingenuity Pathway Core analysis (IPA) (Redwood City California, USA) was used to interpret the host marker data according to biological processes, pathways and networks. Qlucore analysis identified significant host markers which expressed distinct patterns. The host markers in each pattern was defined as a value parameters for the IPA analysis. After the analysis, networks were generated by a score meaning significance. The Fisher exact test p value was used to determine the significance of the bio functions and pathways. Bio functions were grouped in disease and molecular functions. The pathway was generated by the ratio value which is the number of molecules in a given pathway that meet cut criteria, divided by total number of molecules that make up that pathway.¹³¹

CHAPTER 3:

Feasibility assessment of biomarker discovery for early TB treatment response in live *M.tb* stimulated whole blood culture assays.

3.1 Introduction

The current treatment of TB consists of a six month regime divided into a four-drug intensive phase for two months, followed by a continuation phase of two drugs for four months.⁹¹ Since the discovery of rifampin in the 1960's, no new drugs against TB have been introduced into clinical practice.³⁸ The onset of MDR and XDR TB has created a demand for novel and effective treatments to reduce the burden of this disease. The WHO initiative DOTS is the most effective means of monitoring the treatment of tuberculosis.⁹³

The International Union against Tuberculosis and Lung Disease (IUATLD) has recommended that smear or culture status after two months of treatment, be used as a marker to evaluate early treatment response.¹¹⁷ However, the short coming of this procedure is that a diagnosis for unresponsive patients to therapy can only be made after two months. This enables the continual tissue damage¹⁰⁷ and the possible development of drug resistance.¹¹⁸ In addition; this leads to lengthy trial periods in the evaluation of new drugs in clinical trials.^{105,107} Culture status at month two is a poor reference standard for measuring relapse or treatment failure.¹⁰⁸

It has been suggested that if predicative biomarkers existed which reflected the efficacy of early treatment response it would allow patients to be stratified into relative risk groups according to treatment needs.⁹⁸ This would allow the shortening of treatment in a majority of patients, who do not require the six month treatment regime. The strain on health care systems would also be alleviated as TB programs could focus on patients who do require the intensified drug regimen since these patients are at increased risk for poor treatment responses or relapse.^{97,107} The cohort sizes and costs in clinical trials would be reduced, due to the appropriate stratification of patients with similar risk profiles into treatment arms. These advancements would allow for the acceleration of new drugs to be produced for tuberculosis.¹⁰⁷

A previous study conducted in our laboratory has identified several serum host markers including EGF, IL-10, IL-12p40, IL-13, MCP-1, MIP-1 α , MIP-1 β , IL-6, IP-10, TNF- α and sCD40 ligand which were shown to change during TB chemotherapy. Some of these markers could discriminate between patients that responded quickly to treatment and those with a slower response time as measured by week 8 sputum culture results.⁹⁷

The markers detectable in serum and plasma of patients may be non-specific for *M.tb* infection. This may be a result of the immune system being restricted in its array of responses against infectious diseases or other inflammatory disorders. This is evident in diseases such as diabetes, cardiac disease and several forms of cancer, where levels of acute phase proteins, chemokines and pro-inflammatory cytokines were shown to increase or decrease.^{119,120–123}

The identification of TB antigen specific markers might be more useful than the serum/plasma markers as antigen-specificity is a hallmark of the adaptive immune system and T cells, one of the central role players in this arm of the immune system, play such a crucial role in protection against TB.¹²

The aim of this section of work was to evaluate if the stimulation of whole blood samples with live *M.tb* from TB patients undergoing treatment, results in the production of host markers which correlate with early treatment response as assessed by sputum culture conversion at week 8 of therapy.

3.2 Study Design and Methods

In this study, the sample population consisted of 30 TB participants, who were successfully treated without relapse (they were matched according to age, gender and CXR extent of disease to 12 participants who did develop relapse as part of another study). These participants were selected from a sample bank containing 336 participants who were recruited as part of the Surrogate Marker study as mentioned in Chapter 2 (Figure 3.1). All these participants started anti-TB treatment at study entry, after the first sample collection. For this study a healthy control group was not available to compare cytokine levels against.

Whole blood samples were collected from all participants after which samples were cultured in the presence of live *M.tb* for 7 days as described in Chapter 2, (section 2.5.3). Supernatants were harvested, aliquoted and frozen at -80°C after which the levels of 57 host markers were measured in the unstimulated and antigen-stimulated culture supernatants using the Luminex technology.

Samples from four time points were available for analysis; at the onset of anti-TB treatment and 1, 2, 4 weeks thereafter (refer to chapter 2.3.1). No samples were collected after week 4 of treatment in this study. A one-way repeated measures ANOVA with LSD post-hoc was used in the analysis of the data. Statistical analysis of the immune response between groups (fast and slow responders) was performed using variance estimation, precision and comparative analysis including the LSD-test. The predicative power of the measured analytes for sputum culture status at week 8 was assessed by general discriminant analysis (GDA). GDA were used to evaluate the predictive abilities of combinations of biomarkers for differentiating between fast and slow responders. Prediction accuracy were estimated using leave-one-out cross validation. This method was used due to the small sample size.

The Qlucore software (Qlucore AB, Lund Sweden) was used to construct heat maps to generate patterns of changes in marker expression during treatment.¹¹³

Ingenuity Pathway Core Analysis (IPA) (Redwood City California, USA) was used to identify signalling and metabolic pathways, molecular networks and biological processes that were affected by the host marker patterns observed.¹¹³

For all analyses, the unstimulated, antigen-stimulated and *M.tb* specific marker levels (background subtracted from antigen stimulated levels) were used as separate variables during the data analysis.

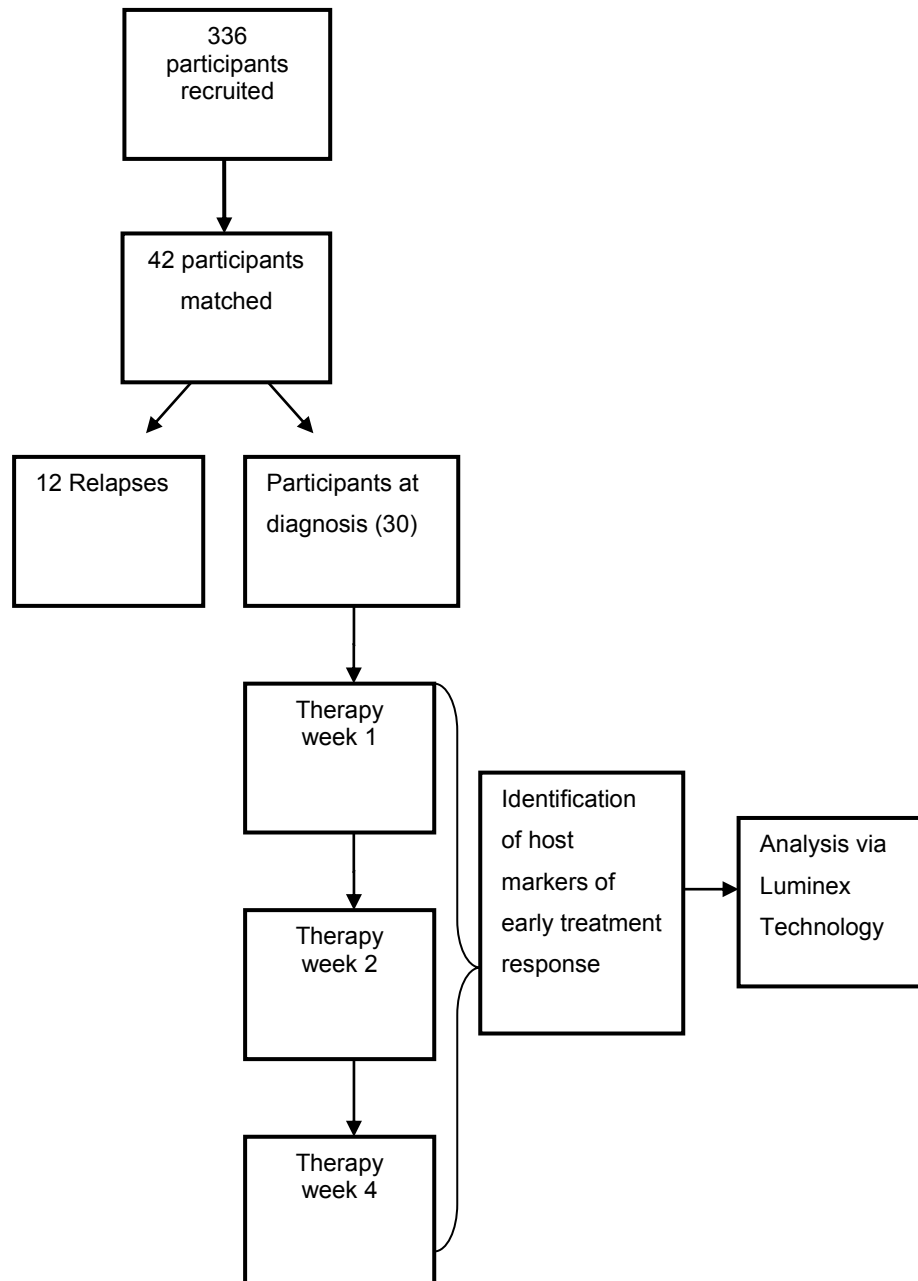


Figure 3.1 Flow diagram and summary of study design. 336 participants were initially recruited for the Surrogate Marker study. Forty two participants were selected for biomarker analysis: 12 TB relapses and 30 matched cured TB participants. The 30 participant's culture supernatants were used in the present study for the identification of host markers of early treatment using the Luminex technology

3.3 Results

3.3.1 Clinical and microbiological markers of treatment response

Of the 30 participants who were cured after 26 weeks of standard DOTS, 23 (76.7%) were male and 7 (23.3%) were female. They ranged between the ages of 19 to 64 years.

The clinical information collected on the participants is shown in Table 3.1

Participants were classified into fast and slow responders to chemotherapy based on their culture status at week 8. The sputum *M.tb* culture and smear for acid fast bacilli results revealed that after 8 weeks of treatment 16 (53.3%) of participants were culture positive and 7(23.3%) were smear positive. In 7 instances, sputum cultures were contaminated precluding their interpretation.

Smear results were not available for one participant at week 2 and for two participants at week 4. All of these participants were cured at month 6 (end of treatment).

The whole blood cultures from the 16 slow responders (sputum culture-positive) and 9 fast responders (sputum culture-negative) with available week 8 culture results were used for this section of work.

The mean total time to positivity (TTP) increased over the eight weeks of treatment. No significant differences were observed over the eight weeks of treatment.

Table 3.1 Clinical characteristic of TB participants in this study

	DX	Week 1	Week 2	Week 4	Week8
Culture					
Positive	30 (100)	30 (100)	27 (90) [#]	26 (86.7) [#]	16 (53.3) [#]
TTP	3.5 ±1.81 (1-7)	8.63 ± 3.47 (2-19)	10.67 ± 4.94 (3-27)	12.83 ± 4.86 (3-29)	18 ± 4.22 (13-29)
Smear					
Positive, n (%)	29 (96.7)	26 (86.7)	22 (73.3) [†]	14(46.7) [†]	7(23.3)

Definition of abbreviations: SD = Standard deviations, TTP = Total Time to Positivity

Values are expressed as mean ± SD, range (min-max)

[#] Week 2 and 4 had one contaminated culture and week 8 had five contaminated cultures

[†]Week 2 has one missing smear result and week 4 has two missing smear results

3.3.2 Response patterns observed in levels of host markers in participants with active tuberculosis at the onset of anti-TB treatment and 1, 2, 4 weeks thereafter

Out of the 57 host markers used in this study, G-CSF, IL-3, IL-9, IL-15, IL-17, MIP-1 α , IL-2, IL-13, sCD30, sIL-1R1, sRAGE, and sVEGFR3 were present at very low to undetectable concentrations in culture supernatants.

Differences in levels of FLT-3 ligand, IL-1 α , GM-CSF, FGF-2, MCP-1, IL-7, IL-5, IL-10, IL-2, TGF- α , MDC, MCP-3, IL-12p40, IL-12p70, MIP-1 β , IFN- γ , sVEGFR1 and sVEGFR2 were detected between time points but not at significant levels ($p \leq 0.05$).

The levels of 27 markers showed significant changes during treatment and these followed 5 distinct patterns. The mean values of host markers and p values between the different time points are in Table 3.2

(i) Host markers which showed a gradual decrease across all time points

The unstimulated levels of chemokines and inflammatory cytokines i.e. TNF- β ($p < 0.05$), MCP-3 ($p < 0.01$), Fractalkine ($p < 0.001$) and sCD40 ligand ($p < 0.001$) were higher at pre-treatment levels and dropped significantly during the four weeks of therapy (Figure 3.2). Ingenuity Pathways Analysis suggests that these markers play a pivotal role in the IL-17A signalling pathway (Figure 3.3). The primary function of these cytokines in this pathway is to support an inflammatory response to infectious disease by participating in antigen presentation, cell to cell signalling and interaction (Table 3.3).

(ii) Host markers which showed a significant decrease by week 1

The unstimulated levels of chemokines, acute phase proteins and inflammatory cytokines i.e. Eotaxin ($p < 0.01$), GRO ($p < 0.05$), IL-6 ($p < 0.001$), IL-1 α ($p < 0.01$), IP-10 ($p < 0.001$), IL-8 ($p < 0.001$), TNF- α ($p < 0.05$), SAP P ($p < 0.05$), sTNFR2 ($p < 0.001$), SAP A ($p < 0.001$), CRP ($p < 0.001$), IL-1 β ($p < 0.05$), sIL-2R α ($p < 0.05$), EGF ($p < 0.01$) and the corrected stimulated levels (background corrected antigen stimulated levels) of IL- β ($p < 0.05$) and GRO ($p < 0.05$) expressed high levels at pre-treatment, dropped significantly by week 1 and remained at lower levels throughout the four weeks of treatment (Figure 3.4) These host markers play a role in the IL-17 and acute phase response signalling. (Figure 3.5) They are involved in the process of

inflammation and function both in cellular movement and antigen presentation (Table 3.4).

(iii) Host markers which showed a significant increase at week 4

The unstimulated levels of soluble receptors, pro and anti-inflammatory cytokines i.e. IL-4 ($p < 0.05$), sEGFR ($p < 0.05$), sIL-1R2 ($p < 0.001$), sTNFR1 ($p < 0.05$), and IFN- α 2 ($p < 0.01$) showed an increase between week 2 and week 4 of treatment (Figure 3.6). These markers are mainly involved in the inflammatory response to invading infectious pathogens by mediating communication between immune cells (Figure 3.7). Their molecular and cellular functions include cellular development, maintenance and death (Table 3.5).

(iv) Host markers which showed a transient increase at week 1

Soluble cytokine receptors regulate inflammatory and immune events by functioning as agonists or antagonists of cytokine signalling.¹²⁴ The unstimulated levels of the soluble receptors i.e. ssIL-6R ($p < 0.01$), sIL-4R ($p < 0.01$) and gp130 ($p < 0.05$) (Figure 3.8) had transiently elevated levels at week 1. Ingenuity pathway core analysis was only done for groups of four or more markers expressing the same pattern.

(v) Markers which increased during treatment

VEGF ($p < 0.05$) was the only marker which showed a low response at pre-treatment followed by a continual increase throughout the four weeks of treatment. (Figure 3.9)

Table 3.2: Mean log transformed levels of cytokines in TB participants at the onset of anti-TB treatment and 1, 2, 4 weeks thereafter

Cytokine	Diagnosis	Week 1	Week 2	Week 4
TNF-beta _{Nil}	1.59 ± 0.14	1.50 ± 0.10*	1.28 ± 0.26***	1.28 ± 0.14***
MCP-3 _{Nil}	1.82 ± 0.62	1.68 ± 0.48	1.50 ± 0.56**	1.46 ± 0.53**
Fractalkine _{Nil}	1.43 ± 0.41	1.40 ± 0.44	1.24 ± 0.47	1.17 ± 0.57*
sCD40L _{Nil}	1.17 ± 0.48	1.10 ± 0.40	0.97 ± 0.48	0.73 ± 0.37**
Eotaxin _{Nil}	1.02 ± 0.32	0.91 ± 0.36*	0.88 ± 0.32**	0.92 ± 0.35*
GRO _{Nil}	2.70 ± 0.47	2.48 ± 0.44**	2.36 ± 0.34**	2.41 ± 0.42**
IL-6 _{Nil}	2.22 ± 0.46	1.91 ± 0.54**	1.91 ± 0.53**	1.97 ± 0.33 **
Il-1ra _{Nil}	2.37 ± 0.39	2.08 ± 0.43**	1.93 ± 0.51**	2.12 ± 0.35 *
IP-10 _{Nil}	2.54 ± 0.39	2.33 ± 0.52*	2.25 ± 0.47**	2.29 ± 0.46*
IL-8 _{Nil}	2.40 ± 0.29	2.09 ± 0.34***	2.15 ± 0.30 ***	2.08 ± 0.21 ***
GRO _{Ag-Nil}	2.43 ± 0.80	1.82 ± 1.04**	1.91 ± 0.99 **	1.83 ± 1.05**
TNF-alpha _{Nil}	1.28 ± 0.42	1.02 ± 0.46**	1.07 ± 0.43**	1.06 ± 0.36**
SAP P _{Nil}	3.74 ± 0.19	3.55 ± 0.19***	3.73 ± 0.14	3.64 ± 0.20*
sTNFR2 _{Nil}	3.05 ± 0.15	2.87 ± 0.17***	2.86 ± 0.18 ***	2.85 ± 0.18***
SAP A _{Nil}	4.20 ± 0.65	3.46 ± 0.66 ***	3.35 ± 0.73 ***	3.30 ± 0.61 ***
CRP _{Nil}	4.71 ± 0.30	4.19 ± 0.48 ***	4.21 ± 0.43***	4.09 ± 0.52***
IL-beta _{Ag-Nil}	0.88 ± 0.49	0.55 ± 0.40**	0.57 ± 0.49**	0.73 ± 0.49
IL-beta _{Nil}	0.89 ± 0.46	0.62 ± 0.53**	0.64 ± 0.41 **	0.73 ± 0.45
sIL-2Ralpha _{Nil}	1.98 ± 0.30	1.87 ± 0.27 **	1.83 ± 0.34 **	1.92 ± 0.36
EGF _{Nil}	1.19 ± 0.57	1.16 ± 0.41	0.92 ± 0.38**	0.97 ± 0.41**
IL-4 _{Nil}	0.40 ± 0.10	0.42 ± 0.13	0.38 ± 0.14	0.45 ± 0.15*
sEGFR _{Nil}	3.32 ± 0.27	3.37 ± 0.19	3.31 ± 0.19	3.41 ± 0.17**
sIL-1R2 _{Nil}	2.22 ± 0.30	2.27 ± 0.28	2.22 ± 0.26	2.46 ± 0.35***
sTNFR1 _{Nil}	1.85 ± 0.40	1.82 ± 0.31	1.73 ± 0.30*	1.87 ± 0.35
IFN-alpha 2 _{Nil}	1.20 ± 0.45	1.17 ± 0.37	1.11 ± 0.40	1.36 ± 0.30*
sIL-6R _{Nil}	2.79 ± 0.19	2.87 ± 0.14 **	2.81 ± 0.15	2.83 ± 0.14
sIL-4R _{Nil}	1.42 ± 0.23	1.64 ± 0.19 ***	1.45 ± 0.19	1.46 ± 0.28
sgp130 _{Nil}	3.89 ± 0.18	3.95 ± 0.20**	3.92 ± 0.20	3.88 ± 0.21
VEGF _{Nil}	1.08 ± 0.61	1.18 ± 0.70	1.35 ± 0.66	1.49 ± 0.52*

P values are for differences between time points on treatment versus baseline levels.

*p<0.05, **p<0.01, ***p<0.001

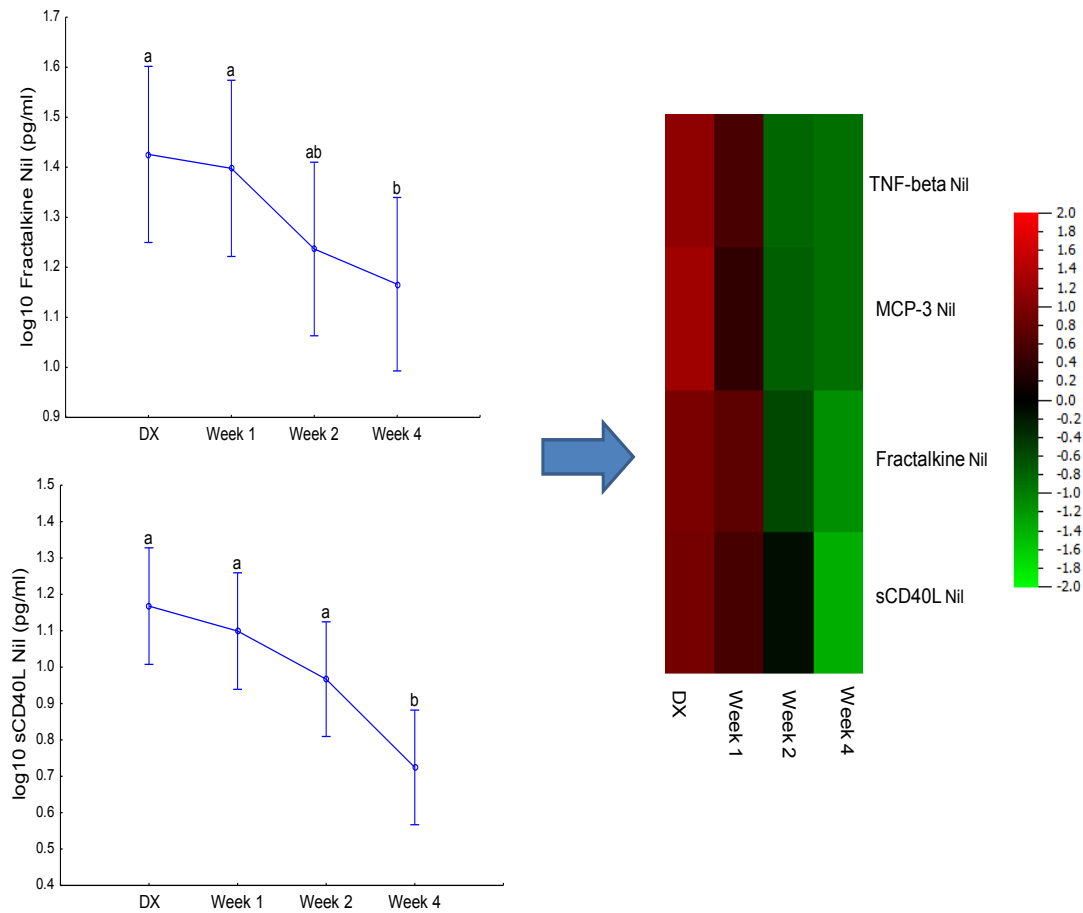


Figure 3.2 Host markers showing a gradual decrease during the first 4 weeks of treatment: Markers were grouped according to response patterns (only markers with significant changes are shown). The heatmap was created using mean log values for each time point using the QluCore Explorer Software where red indicated high expression and green indicates low expression. The line graphs are representatives for the response pattern that are shown. The data was analysed by one-way, repeated measures ANOVA with LSD post-hoc test. Different letters on the line graphs indicate that they were significantly different from each other with a p value of ≤ 0.05 . Values with the same letters are not statistically different from each other.

Table 3.3 The Ingenuity Pathway Analysis software illustrates the disease, molecular function and the pathway for the set of host markers which expressed a gradual decrease across the time points

Response Pattern	Disease Function	Molecular Function	Pathways
A gradual decrease during the first 4 week of treatment	<ul style="list-style-type: none"> Inflammatory response and disease 	<ul style="list-style-type: none"> Cell to cell signalling and interaction Antigen presentation 	<ul style="list-style-type: none"> IL-17 A signalling Pathway

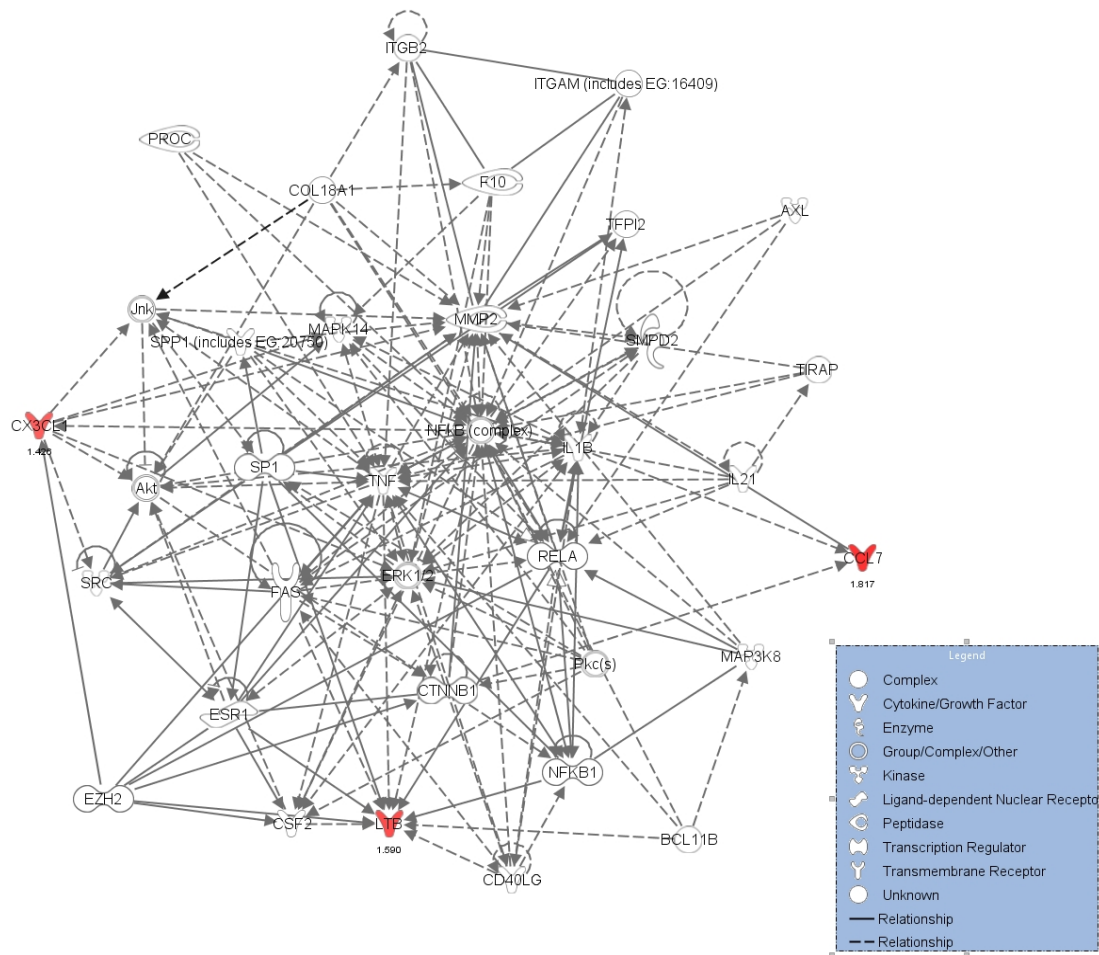


Figure 3.3 Ingenuity network analyses for specific host markers with a gradual decrease during the 4 weeks of treatment: The main pathway that was identified in this network was the IL-17A pathway. The diagram shows interactions between measured and unmeasured, putative molecules in this pathway. Red indicates measured markers with decreasing levels during treatment.

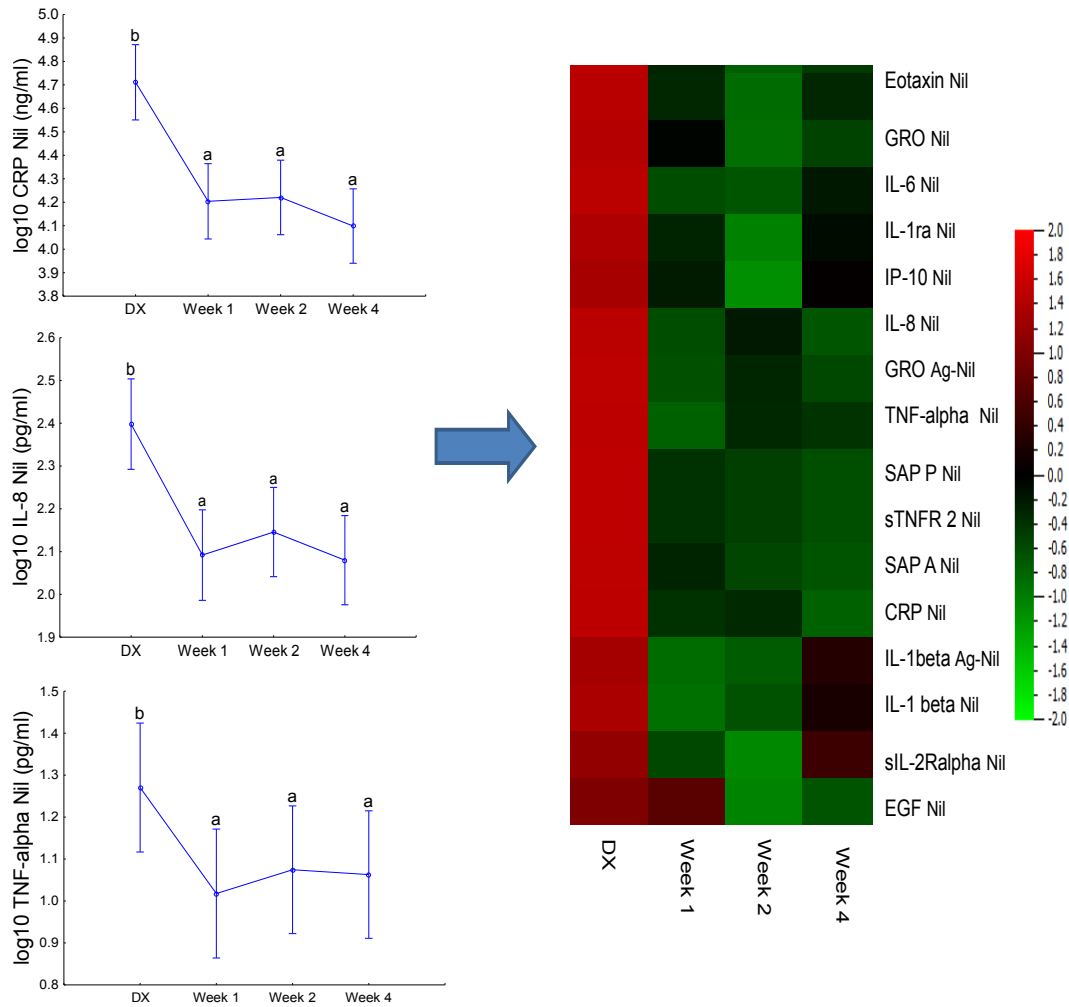


Figure 3.4 Host Markers showing a significant decrease by week 1: Markers were grouped according to response patterns (only markers with significant changes are shown). The heatmap was created using mean log values for each time point using the Qlucore Explorer Software where red indicates high expression and green indicates low expression. The line graphs are representatives for the response pattern that are shown. The data was analysed one-way, repeated measures ANOVA with LSD post-hoc test. Different letters on the line graphs indicate that they were significantly different from each other with a p value of ≤ 0.05 . Values with the same letters are not statistically different from each other.

Table 3.4 The Ingenuity Pathway analysis software illustrates the disease, molecular function and the pathway for the set of host markers which expressed a significant decrease by week 1

Response Pattern	Disease Function	Molecular Function	Pathways
A significant rapid decrease by week 1	<ul style="list-style-type: none"> Inflammatory response and disease 	<ul style="list-style-type: none"> Antigen presentation Cellular Movement 	<ul style="list-style-type: none"> IL-17 A signalling Pathway Acute Phase Response Signalling

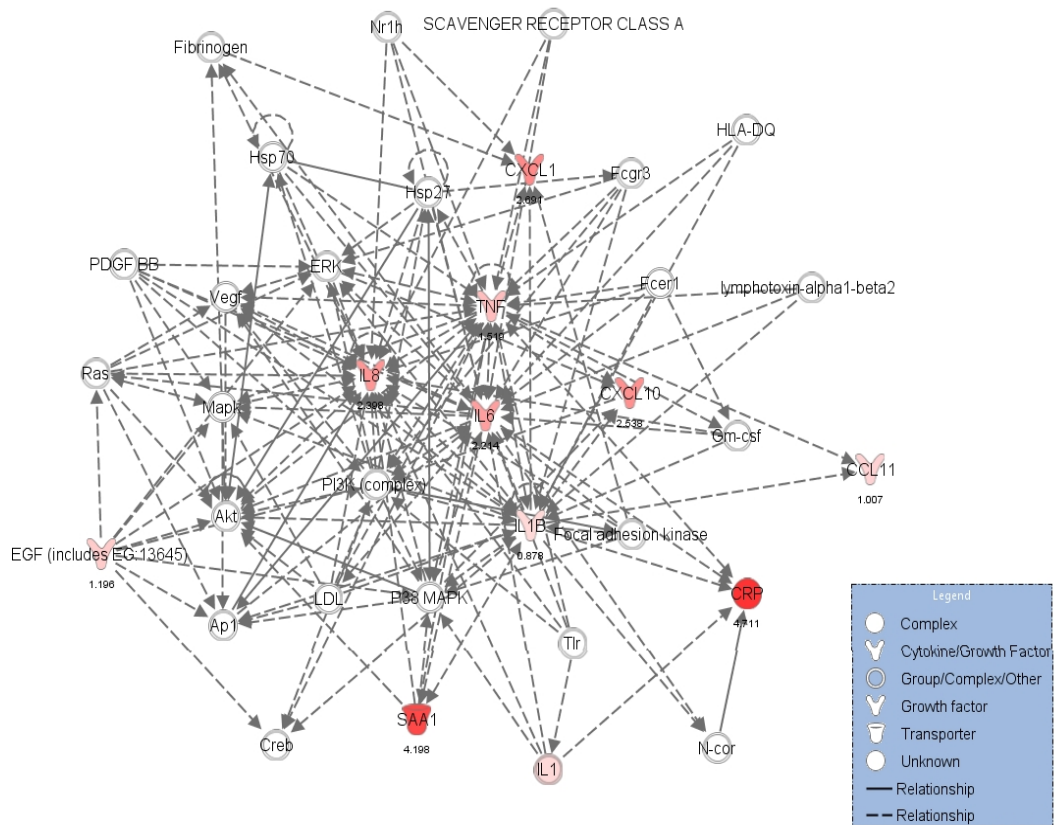


Figure 3.5 Ingenuity network analyses for specific host markers which show a significant decrease at week 1 of treatment: The main pathway that was identified in this network was the IL-17 and the acute phase response signalling pathway. The diagram shows interactions between measured and unmeasured, putative molecules in this pathway. Red indicates measured markers that are down regulated at week 1

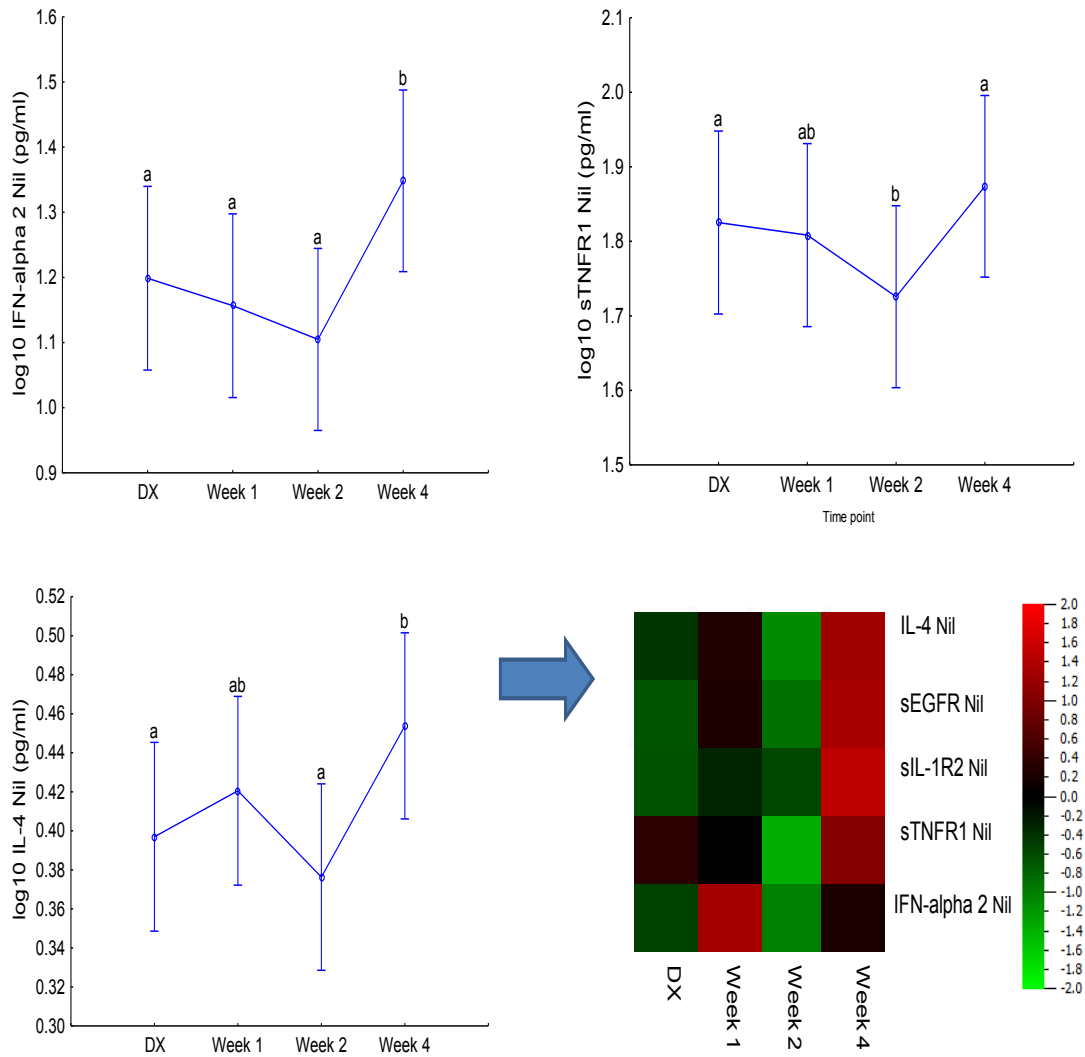


Figure 3.6 Host Markers showing a significant increase at week 4: Markers were grouped according to response patterns (only markers with significant changes are shown). The heatmap was created using mean log values for each time point using the QluCore Explorer Software where red indicates high expression and green indicates low expression. The line graphs are representatives for the response pattern that are shown. The data was analysed by one-way, repeated measures ANOVA with LSD post-hoc test. Different letters on the line graphs indicate that they were significantly different from each other with a p value of ≤ 0.05 . Values with the same letters are not statistically different from each other.

Table 3.5 The Ingenuity Pathway analysis software illustrates the disease, molecular function and the pathway for the set of host markers which expressed a significant increase at week 4

Response Pattern	Disease Function	Molecular Function	Pathways
A significant increase at week 4	<ul style="list-style-type: none"> Inflammatory response and infectious disease 	<ul style="list-style-type: none"> Cellular Development Cellular function and maintenance Cell Death 	<ul style="list-style-type: none"> The role of cytokines mediating communication between immune cells

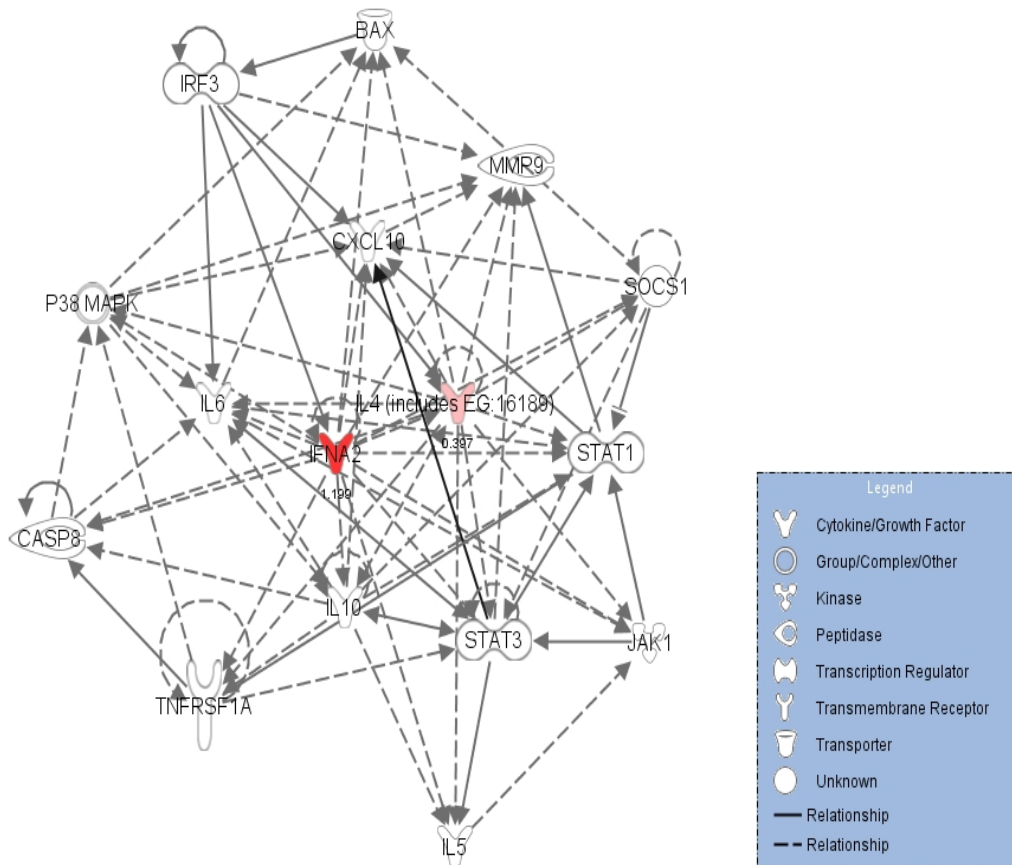


Figure 3.7 Ingenuity network analyses for specific host markers which show a significant increase at week 4 of treatment: The role that these host markers play is mediating communication between immune cells. The diagram shows interactions between measured and unmeasured, putative molecules in this pathway. Red indicates measured markers that are up regulated at week 4

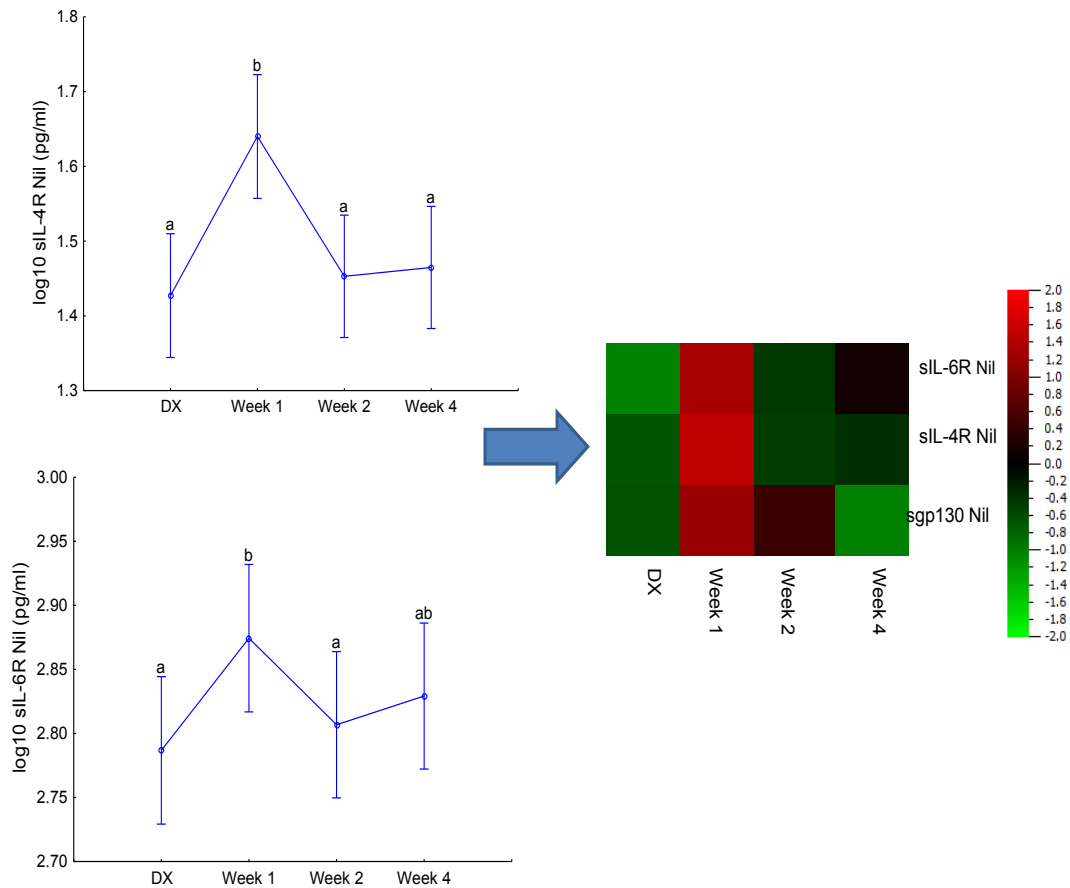


Figure 3.8 Host Markers showing transient increase at week 1: Markers were grouped according to response patterns (only markers with significant changes are shown). The heatmap was created using mean log values for each time point using the QluCore Explorer Software where red indicates high expression and green indicates low expression. The line graphs are representatives for the response pattern that are shown. The data was analysed by one-way, repeated measures ANOVA with LSD post-hoc test. Different letters on the line graphs indicate that they were significantly different from each other with a p value of ≤ 0.05 . Values with the same letters are not statistically different from each other

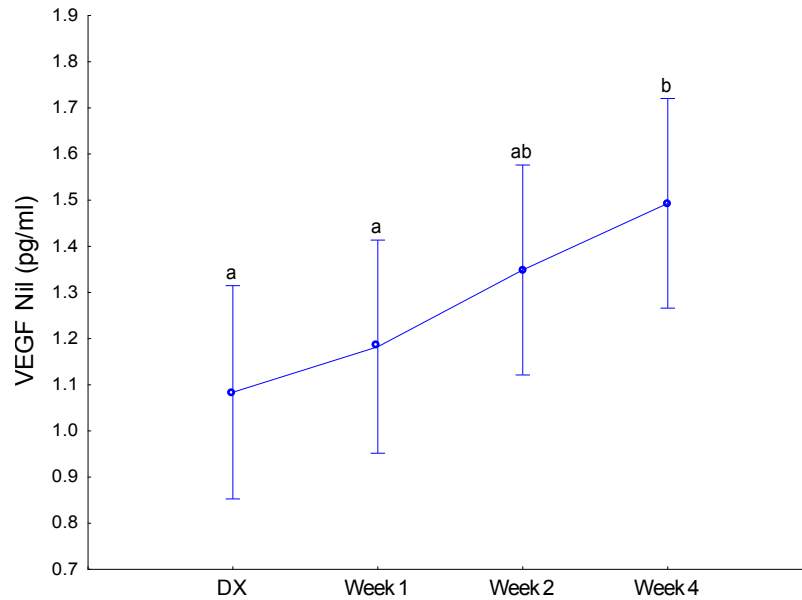


Figure 3.9 The unstimulated levels of VEGF detected in patients with active TB on treatment. The data was analysed with one-way, repeated measures ANOVA with LSD post-hoc test. Different letters on the line graphs indicate that they were significantly different from each other with a p value of ≤ 0.05 . Values with the same letters are not statistically different from each other.

3.3.3 Cytokine profiles in fast and slow responders to TB treatment

The repeated measures analysis showed no significant differences in cytokine production by fast and slow responders during anti-TB treatment.

Further analyses of the data using GDA investigated multi-variable models (with a maximum of 4 variables) with the ability to classify fast and slow responders (month 2 sputum culture results) at the onset of anti-TB treatment and 1, 2, 4 weeks as measurements

The most frequently incorporated markers into the top 20 four-variable models were identified. The host marker $sgp130_{Nil}$ was the most frequently occurring diagnosis marker that discriminated between fast and slow responders (Figure 3.10). The combination of $IFN\text{-}\alpha\ 2_{Nil}$, $sgp130_{Nil}$, $sgp130_{Ag\text{-}Nil}$, $sIL\text{-}4R_{Nil}$ was the top performing models. It classified fast responders with an accuracy of 88.9% and slow responders with an accuracy of 93.8% and in a resubstitution classification matrix with 88.9% and 87.5% respectively after leave-one-out cross validation.

$CRP_{Ag\text{-}Nil}$ was the most frequently occurring week 1 marker which could discriminate fast and slow responders (Figure 3.11). The combinations of week 1 levels of $IL\text{-}12p40_{Nil}$, $CRP_{Ag\text{-}Nil}$, $IL\text{-}12p70_{Ag\text{-}Nil}$ and $sVEGFR1_{Ag\text{-}Nil}$ classified fast and slow responders with an accuracy of 100% and 93.7% respectively in a resubstitution classification matrix and with 88.9% and 87.5% respectively after leave-one-out cross validation.

The host marker $sgp130_{Nil}$ was the most frequently occurring week 2 marker which could discriminate fast and slow responders (Figure 3.12). The combinations of week 2 $SAP\ A_{Ag\text{-}Nil}$, $IL\text{-}12p70_{Nil}$, $sgp130_{Nil}$ and $sTNFR1_{Ag\text{-}Nil}$ classified fast and slow responders with an accuracy of 100% and 93.7% respectively in a resubstitution classification matrix and with 90% and 87.5% respectively after leave-one-out cross validation.

$SAP\ A_{Nil}$ was the most frequently occurring week 4 markers which could discriminate fast and slow responders (Figure 3.13). The combinations of week 4 $IFN\ \alpha\ 2_{Nil}$, $MCP\text{-}1_{Ag\text{-}Nil}$, $MCP\text{-}3_{Ag\text{-}Nil}$ and $sVEGFR3_{Nil}$ classified fast and slow responders with an accuracy of 100% and 70% respectively in a resubstitution classification matrix and 70% and 93.7% respectively after leave-one-out cross validation.

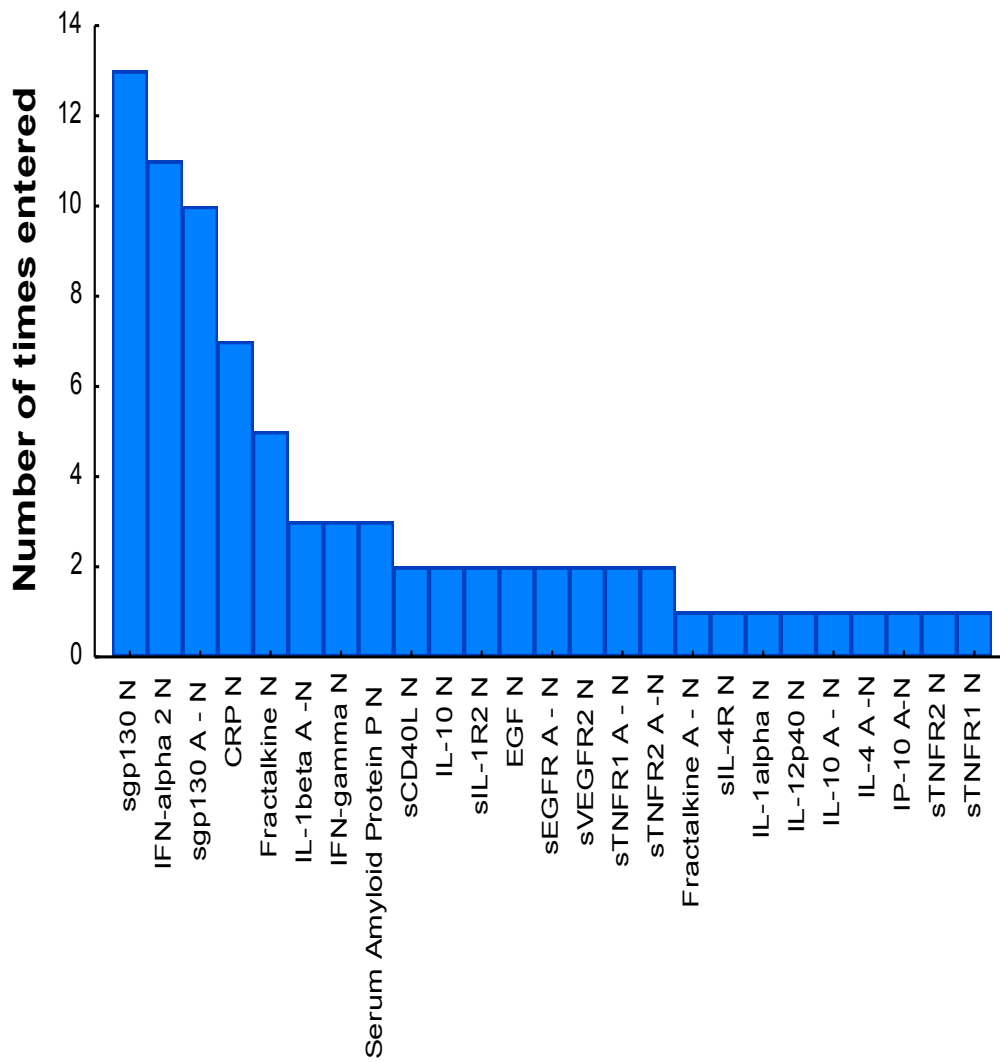


Figure 3.10 Frequency of individual analytes in top models for discriminating between fast and slow responders at pre-treatment. The columns represent the number of inclusions of individual markers into the most accurate four-analyte models by GDA analysis (20 models)

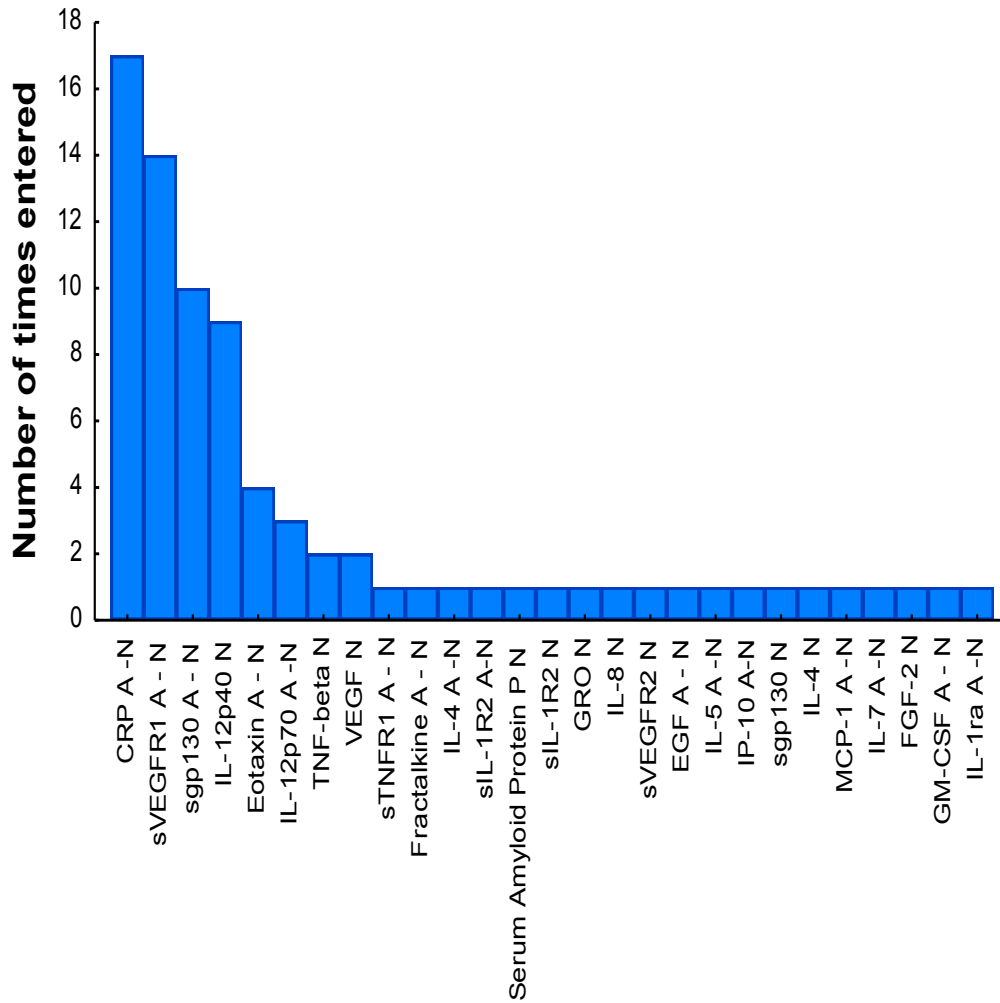


Figure 3.11 Frequency of individual analytes in top models for discriminating between fast and slow responders at week 1. The columns represent the number of inclusions of individual markers into the most accurate four-analyte models by GDA analysis (20 models)

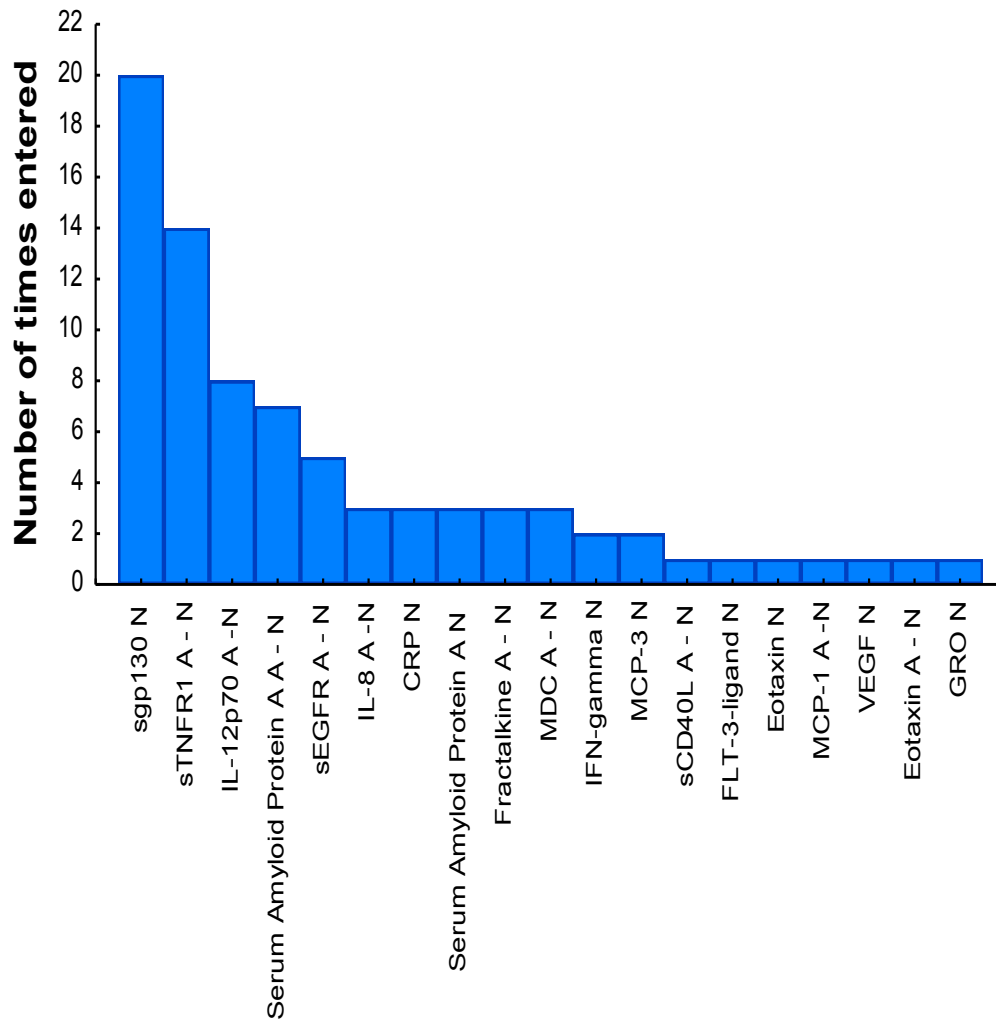


Figure 3.12 Frequency of individual analytes in top models for discriminating between fast and slow responders at week 2. The columns represent the number of inclusions of individual markers into the most accurate four-analyte models by GDA analysis (20 models)

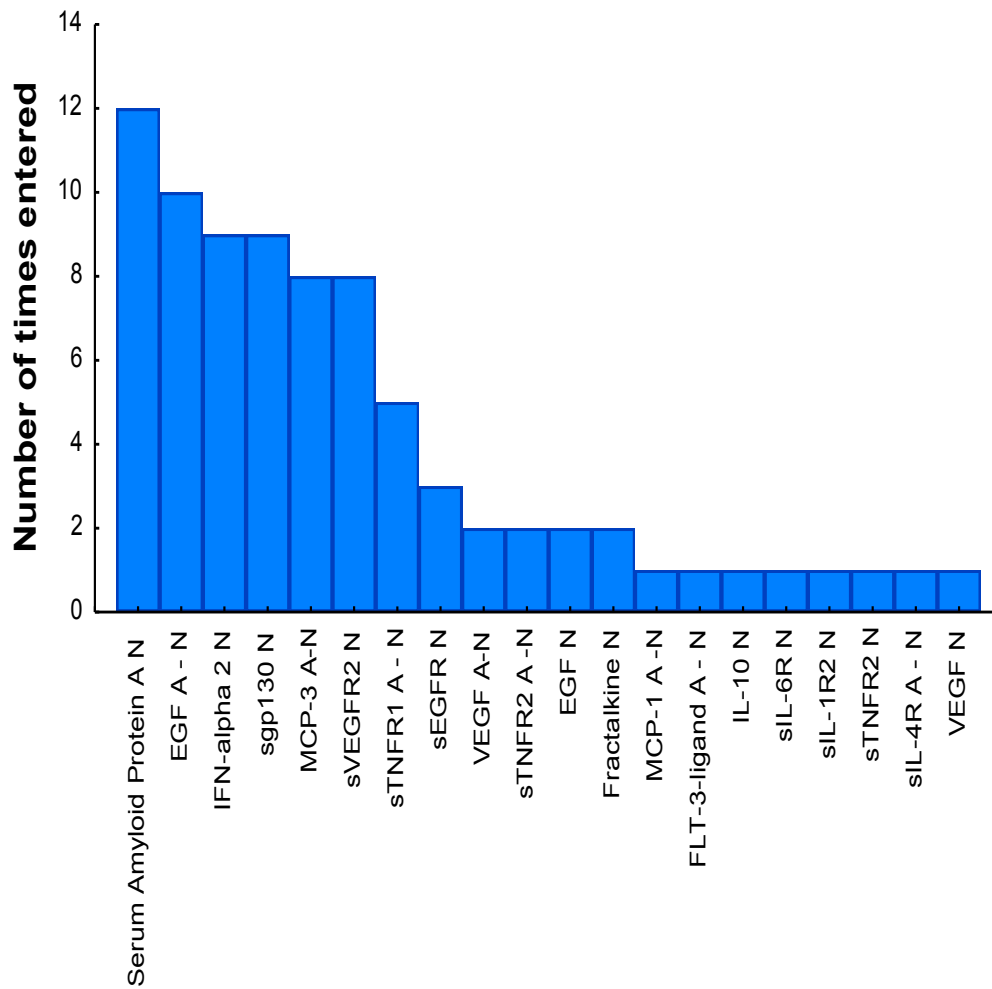


Figure 3.13 Frequency of individual analytes in top models for discriminating between fast and slow responders at week 4. The columns represent the number of inclusions of individual markers into the most accurate four-analyte models by GDA analysis (20 models)

3.4 Discussion

Previous studies have reported that levels of several host markers in plasma/serum are affected by various inflammatory disorders and diseases. In this study we screened the profiles of 57 host markers in unstimulated and antigen (live *M.tb*) stimulated whole blood culture supernatants before and during the first 4 weeks of anti-tuberculosis treatment in participants with active TB, to determine if this long-term antigen stimulated sample is suitable for discovery of biomarkers for early treatment response. All active participants used for the purpose of this study showed a positive response to treatment resulting in complete clinical remission. The markers that show significant changes during treatment would subsequently be candidates for further evaluation in TB treatment response studies.

The results of this study reveal that during the early phase of treatment, multiple markers monitored in unstimulated participant samples became altered in their response. Conversely, in stimulated participant samples there was no effect seen in the response to the majority of these markers. Chemokine GRO and pro-inflammatory IL-1beta were the only markers that showed changes in antigen stimulated responses (with unstimulated values subtracted).

We also demonstrated that the host markers expressed different response patterns during early treatment. These included gradual decreases, decreases by week 1, increases at week 4, transient increases at week 1 or an ongoing increase during the four weeks of treatment. We used Ingenuity Pathway Analysis to explain the response patterns in a biological context.

The chemokines (MCP-3, Fractalkine) and the inflammatory cytokine TNF-beta and sCD40 ligand levels showed a gradual decrease over the four weeks of treatment. The acute phase proteins (CRP, SAP P, SAP A, IL-6), pro and anti-inflammatory cytokines (TNF- α , IL-1beta, IL-1ra, sIL-2alpha, sTNFR1) and the chemokines (IL-8, IP-10, Eotaxin, GRO) were highly abundant before the initiation of treatment and significantly decreased in the first week of treatment. The response of both cytokine/chemokine groups decreased during the first four weeks of treatment possibly do so due to the therapy-induced reduction of the bacterial load.¹²⁵ The decrease in antigen presentation maybe associated with decreased bacterial load, which in turn reduces cell to cell signalling which leads to a reduction of pro-inflammatory cytokines, regulatory cytokines and chemokines.

The anti-inflammatory cytokine IFN- α 2, the Th1 suppressive cytokine IL-4 and the soluble receptor (sTNFR1, sEGFR and sIL-1R2) levels increase by week 4. As bacterial numbers decline, the immune system may attempt to restore homeostasis by inhibiting the function of pro-inflammatory cytokines like TNF and IL-1 by secreting soluble receptors. IL-4 now inhibits the Th1 response, preventing immunopathology.³⁸ Other soluble receptors (sIL-4R, sIL-6R, sgp130) showed transient increases at week 1 and may also be a reflection of a transient need to inhibit the pro-inflammatory processes that marked the stage of uncontrolled bacterial replication. Once those inflammatory reactions are regulated, the levels of the soluble receptors return to baseline.

VEGF was the only host marker that increased during treatment. VEGF is produced by macrophages and is a major mediator of angiogenesis and vascular permeability.^{126–129} Its unclear what the role of VEGF plays in the pathogenesis of TB but past studies have revealed that VEGF may play a role in the neovascularisation in granulomatous tissue which causes chronic inflammation associated with pulmonary damage.¹³⁰ Some previous studies have shown elevated levels of VEGF in plasma of active TB patients and a decrease during TB treatment.^{131,132} However, our study found an increase in VEGF during early TB therapy and is in keeping with another study which revealed that high serum levels of VEGF may be associated with the process of healing which requires the formation of new blood vessels to assist in tissue regeneration.¹³³ Furthermore, here we measured cytokine levels in 7-day culture supernatants and not in *ex vivo* samples like serum. For this reason, it is possible that levels of individual cytokines will be differentially affected by degradation of some cytokines, uptake by receptors or even continued production in culture.

The marker, GRO and IL-1beta were 2 out of the 57 markers to produce a response in antigen stimulated cultures.

IL-1 beta is a pro-inflammatory cytokine with important functions in immunological control of infectious diseases like *M.tb*.¹³⁴ IL-1 beta increases have been described in previous studies in those with active TB.^{135,136} Dlugovitzky D *et al* reported increased IL-1 beta production when peripheral blood mononuclear cells (PBMC) were stimulated with whole sonicated *M.tb*.¹³⁷ In this present study the levels of IL-1 beta decreased with treatment. These results are similar to those of Tang and colleagues¹³⁸ who showed that the levels of IL-beta were enhanced in pulmonary tuberculosis and declined with chemotherapy.

GRO is a chemokine which is expressed by lung epithelium, fibroblasts, endothelial cells and alveolar macrophages. Several studies *in vivo* and *in vitro* suggest that GRO acts via the receptor CXCR2 and is an important mediator of neutrophil chemotaxis. GRO has been shown to be elevated in several inflammatory conditions and cancers.¹³⁹⁻¹⁴¹ It has shown therapeutic potential in clinical trials of chronic obstructive pulmonary disease (COPD).¹⁴² GRO has not been identified as a host marker in tuberculosis but in this study we found decreasing levels during anti-tuberculosis treatment, possibly reflecting a diminishing need for neutrophils as infection is gradually controlled.

The comparison of fast and slow responders to treatment showed no significant difference between the two groups for any of the host markers. Additionally, the multi-variant analysis performed on the onset of treatment and week 1, 2 and 4. Results showed that pre-treatment levels of sgp130_{Nil}, IFN-alpha 2_{Nil}, levels of CRP_{Ag-Nil}, sVEGFR1 at week 1, levels of sgp130_{Nil}, sTNFR1_{Ag-Nil} at week 2 and levels of SAP_{ANil}, EGF_{Ag-Nil} at week 4 was able to predict week 8 sputum culture.

The main limitation in our study is that a longer follow up period during treatment is needed in order to understand the effects of treatment on the levels of the different host markers that showed significant changes during treatment.

3.5 Conclusion

In conclusion, antigen stimulated responses appear less promising than several markers in unstimulated cultures, which should rather be assessed *ex vivo* in serum or plasma. This would reduce the need for work in a BSL III facility, would reduce the risk to laboratory workers that working with live *M.tb* poses, would allow much more rapid results and would avoid the artefacts that are undoubtedly associated with unstimulated 7-day culture. Future evaluations of these markers in different outcomes of TB treatment, including relapse and failed treatment, need to be conducted. In addition, the change in response from baseline to the end of treatment should be determined as this may prove to be helpful when conducting *M.tb* stimulated assays.

CHAPTER 4:

The utility of Quantiferon-TB Gold In Tube (QFT- GIT) based short-term whole blood culture for the identification of *M.tb*-specific host markers for early treatment response

4.1 Introduction

Whole blood assay can be used to measure antigen-specific T cell responses.^{143, 144} This assay offers the advantage that many cytokines and therefore bio-signatures for different outcomes can be assessed. This may be of benefit in trials of new TB drugs or vaccines and may also provide insights into disease pathogenesis.¹⁴⁵ In Chapter 3 a whole blood assay using live *M.tb* was used and the majority of host markers showed early treatment changes in the unstimulated cultures with only two markers showing changes in antigen stimulated cultures. The whole blood assay in general has several advantages, as it requires a much smaller volume of sample compared to other assays and the T cells are maintained in an environment more similar to that found *in vivo*¹⁴⁶ but the use of live *M.tb* imposes several disadvantages such as biohazard issues for laboratory workers and the 7-day lag time for results.¹⁴⁶

QFT-IT, one of the IGRA tests, offers several significant advantages over a 7-day, live *M.tb* stimulated whole blood assay. QFT-IT is a highly standardized diagnostic test for the diagnosis of latent *M.tb* infection. They are *in vitro* assays which measure sensitization to *M.tb* antigens by evaluating IFN- γ response in whole blood samples after overnight culture.¹⁴⁷ The QFT-IT uses the ELISA to measure the amount of IFN- γ released in response to *M.tb* specific antigens (ESAT-6, CFP-10 and TB 7.7).⁸¹ The IGRAs have been extensively studied and results have shown that they are as sensitive as, and more specific than the TST.^{148,149} There are many advantages of using the IGRAs rather than the TST for diagnosis of LTBI: they require a single patient visit and are not affected by prior BCG vaccination which decreases false-positive responses, results can be obtained within 24 hours and small volumes of blood are required, which has been particularly useful when studying children.^{150,151} The major limitation with this assay, as is the case for TST, is the inability to distinguish between latent and active TB disease.¹⁵²

In previous studies it has been observed that IGRAs have limited clinical utility as a marker of treatment efficacy as the reports have been very inconsistent as many indicate an increase,¹⁴⁷ decrease^{153,154} or no change¹⁵⁵ in IFN- γ levels during treatment. IGRAs have also shown to have limited utility in a high burden HIV prevalent settings.¹⁵³

It also has been reported that the adaptation of the QFT-IT test can be used to discriminate between active and latent TB by screening multiple biomarkers other than IFN- γ in the culture supernatant.^{116,156–158}

The aim of this study was to evaluate if QFT -IT can be adapted to detect an array of host markers which could show early changes in treatment response and to determine if the changes in host markers during early treatment correlate with sputum culture conversion at week 8.

The information that is acquired in this study will guide us to assess this sample type for its utility as a TB treatment biomarker discovery tool.

4.2 Study Design and Methods

(i) Micronutrient Study

In this study the sample population consisted of 19 TB participants who were all successfully cured at month 6. These participants were randomly selected from 77 patients in the placebo arm of the Micronutrient study as described in Chapter 2 (Figure 4.1). All these participants started anti-tuberculosis treatment at study entry after the first sample was collected.

Briefly, 1ml of blood was drawn directly into the three QFT vacutainer tubes (Victoria, Australia). The tubes were incubated for 16-24 hours at 37°C and plasma was harvested and frozen in multiple aliquots at -80°C. The aliquot was used for the QFT IT ELISA and another set was used to measure the levels of 57 markers in unstimulated and antigen stimulated supernatants using the Luminex technology.

For all analysis the unstimulated, antigen stimulated and *M.tb* specific marker levels (background subtracted from antigen stimulated levels) were used as separate variables during data analysis in order to access the changes in baseline unstimulated levels.

(ii) Controls

To compare the levels of the markers in TB participants we used historical data from healthy community controls (non-TB diseased participants). Levels of IL-2, IL-1ra, IL-4, IL-5, EGF, IL-6, IL-7, TGF-alpha, IL-8, IL-12p70, IL-13, IL-15, IFN- γ , TNF- α , MCP-1, sCD40L, MIP-1 α , MIP-1 beta, IL-1 α , GM-CSF, G-CSF, VEGF, IP-10, SAP P, CRP and SAP A were available from 34 community controls from another biomarker study. These results have been published by Chegou *et al.*, 2009.¹³⁵ Pair-wise comparisons of healthy control levels with each of the three participant time points (diagnosis, week 2 and week 8) was done with one-way ANOVA.

Samples from the three on-treatment time points were compared using one-way, repeated measures ANOVA with LSD post-hoc test (refer to chapter 2.3.2). The QluCore and Ingenuity Pathway Core Analysis were also used to analyse this data set. Analysis of the immune response between fast and slow responders was performed using variance estimation, precision and comparative analysis including the LSD-test. The predicative power of the measured analytes for sputum culture status at week 8 was assessed by GDA.

Although the main aim of the study was to evaluate treatment response, comparison of levels before and during early treatment with levels in healthy controls was deemed necessary as our data set did not include measures after completed treatment and as we therefore could not deduce whether levels could be anticipated to change further at later time points in therapy or after completion of therapy. Due to the cost of these tests the analyte levels were not repeated in healthy controls for the present study.

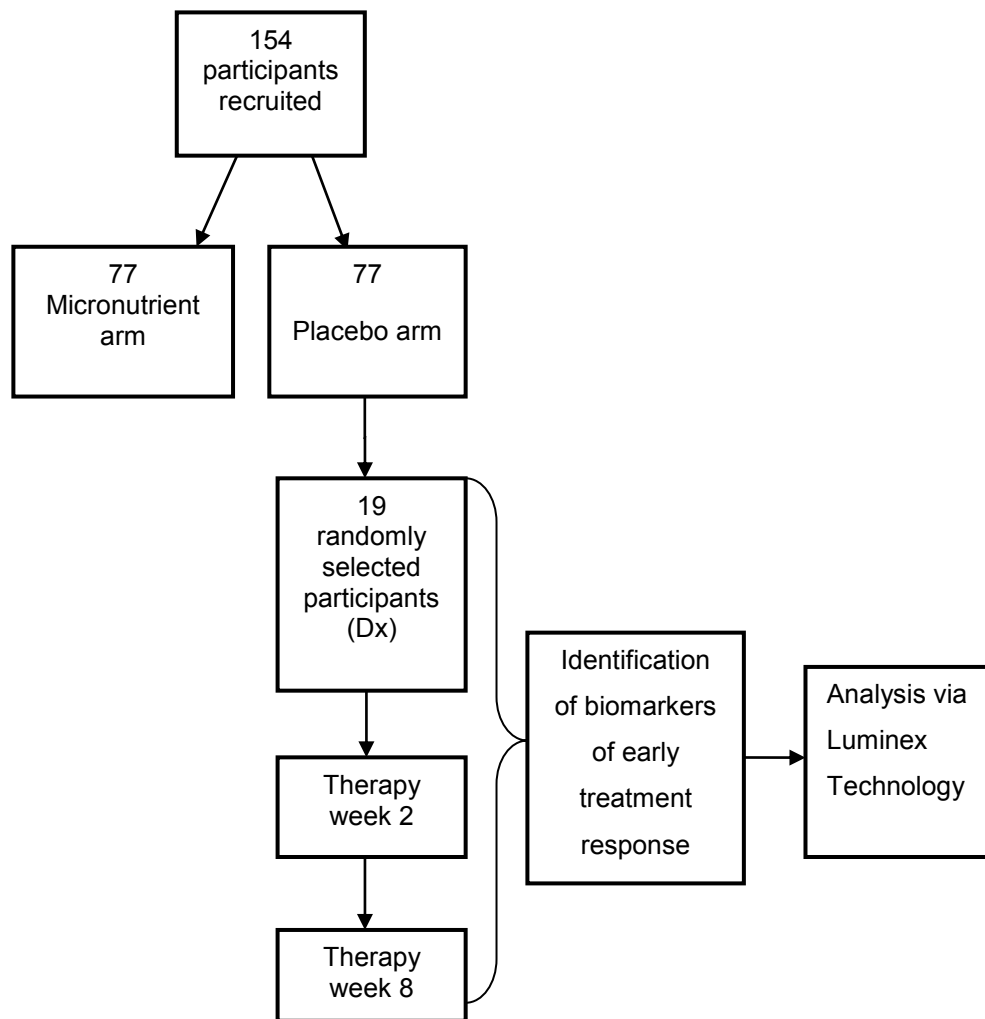


Figure 4.1 Flow diagram and summary of study design. 154 participants were initially recruited for the Micronutrient study. 77 participants received placebo and 77 were on micronutrient supplementation. Nineteen participants from the placebo group were randomly selected and their QFT-GIT supernatants were used in the present study for the identification of host biomarkers of early treatment using Luminex technology

4.3 Results

4.3.1 Clinical and Microbiological markers of treatment response

Of the 19 participants who were cured after 26 weeks of standard DOTS, 11 (57.9%) were male and 8 (42.1%) were female. They ranged between the ages of 18 to 59 years.

The clinical characteristics of the study participants are demonstrated in Table 4.1

The sputum M.tb culture results revealed that 18 (94.7%) were positive at baseline and 11 (57.9%) were still positive at week 8 of treatment.

The sputum smear for acid fast bacilli results showed that 18 (94.7%) were positive at baseline and 9 (47.4%) were still positive at week 8 of treatment. Significant differences were observed in the smear positivity rates between week 2 and 8 ($p < 0.01$) and between diagnosis and week 8 ($p < 0.01$).

At baseline 14 (73.7%) and at week 8 of treatment 16 (84.2%) of the study participants were QFT-IT positive (using the manufactures cut-off values). There were no significant differences observed in the QFT-IT results across the time points.

Although all the participants were cured at month 6 (end of treatment), 11 (57.9%) were still culture positive at week 8 and 9 (47.36%) were still smear positive. The culture status at week 8 was used to stratify patients into slow and fast treatment responders. Eleven slow responders and eight fast responders were identified.

TTP could not be used as a continuous variable, as the NHLS which performed the cultures, did not inoculate the MGIT tubes with a consistent sputum volume. Stratification into treatment response groups was therefore based on the dichotomous variable MGIT positive or negative.

Of the 34 community controls that were recruited in Chegou *et al.*, 2009 study, 14 (41%) were male and 20 (59%) were female. The mean age was 31.8 years; they ranged between the ages of 11 to 60 years. The mean TST reading was 22.8mm with a range of between 0.0-46.0mm. The percentage of QFT-IT positivity in these participants was 73.5%.

Table 4.1: Clinical characteristics of study participants

	DX	Week 2	Week 8	P 1	P 2	P 3
Culture						
Positive, n (%)	18(94.7)	17(89.5)	11(57.9)	0.615	0.137	0.049
Smear						
Positive, n (%)	18(94.7)	17(89.5)	9(47.4)	0.615	<0.01	<0.01
QFT-IT						
Positive, n (%)	14(73.7)	14(73.7)	16(84.2)	0.714	0.462	0.318

The proportion of test positive participants at each time point was compared using the WINPEPI software (www.brixtonhealth.com/pepi4windows.html). P 1 represents the p value for comparison between diagnosis and Week 2, P 2 for comparison between Week 2 and Week 8 and P 3 between Diagnosis and Week 8. The significant p values are shown in **bold** in the table.

4.3.2 The comparison of community controls and TB participants at the onset of anti-TB treatment, week 2 and 8

The levels of IL-2, IL-1ra, IL-4, IL-5, EGF, IL-6, IL-7, TGF-alpha, IL-8, IL-12p70, IL-13, IL-15, IFN- γ , TNF- α , MCP-1, sCD40L, MIP-1 α , MIP-1 beta, G-CSF, VEGF, IP-10, GM-CSF, EGF, SAP P, CRP and SAP A of the community controls at baseline were compared with the levels of TB patients at diagnosis, week 2 and week 8 on anti-tuberculosis treatment.

(i) No significant difference between controls and TB participants

The levels of pro and anti-inflammatory cytokines, chemokines, growth factors and acute phase proteins i.e. IL-2_{Nil}, IL-2_{Ag-Nil}, IL-4_{Nil}, IL-5_{Nil}, IL-5_{Ag-Nil}, IL-6_{Nil}, IL-6_{Ag-Nil}, IL-7_{Nil}, IL-7_{Ag-Nil}, IL-13_{Nil}, IL-15_{Nil}, IL-15_{Ag-Nil}, IFN- γ _{Nil}, IFN- γ _{Ag-Nil}, IL-12p70_{Nil}, GM-CSF_{Nil}, TNF- α _{Ag-Nil}, TGF- α _{Ag-Nil}, MCP-1_{Ag-Nil}, sCD40L_{Ag-Nil}, MIP-1 beta_{Ag-Nil}, G-CSF_{Ag-Nil}, VEGF_{Ag-Nil}, GM-CSF_{Nil}, GM-CSF_{Ag-Nil}, SAP P_{Ag-Nil}, SAP A_{Nil} and CRP_{Nil}, showed no significant differences between the community controls and TB patients at anytime point (not shown).

(ii) Significant baseline differences between controls and patients only at baseline (normalization of levels by week 2 of treatment)

The levels of Th2 cytokines, IL-4_{Ag-Nil} and IL-13_{Ag-Nil}, a chemokine, MIP-1 α _{Ag-Nil}, and two acute phase proteins, CRP_{Ag-Nil} and SAP A_{Ag-Nil} showed a significant difference at baseline when compared with controls (Figure 4.2)

(iii) Significant baseline and week 2 differences between controls and patients with no significant differences at week 8 (normalization of levels by week 8 of treatment)

The levels of anti-inflammatory cytokines, growth factors and acute phase proteins i.e. IL-1ra_{Ag-Nil}, IL-1alpha_{Ag-Nil}, VEGF_{Ag-Nil} and SAP P_{Nil} respectively showed significant differences at baseline and week 2 when compared to controls (Figure 4.3).

(v) Significant differences across all time points when compared to controls (no normalization of levels during observation period)

The levels of growth factors, chemokines, pro-inflammatory, anti-inflammatory and acute phase proteins i.e. EGF_{Nil}, EGF_{Ag-Nil}, G-CSF_{Nil}, IP-10_{Nil}, IP-10_{Ag-Nil}, IL-8_{Nil}, IL-8_{Ag-Nil}, MCP-1_{Nil}, sCD40L_{Nil}, MIP-1 α _{Nil}, MIP-1 beta_{Nil}, IL-6_{Nil}, IL-12p70_{Ag-Nil}, IL-15_{Nil}, TNF- α _{Nil}, TGF- α _{Nil}, IL-1 alpha_{Nil}, CRP_{Nil} and sCD40L_{Nil} were significantly different at all time points (baseline, week 2 and week 8) in TB patients when compared to controls (Figure 4.4 A, B and C)

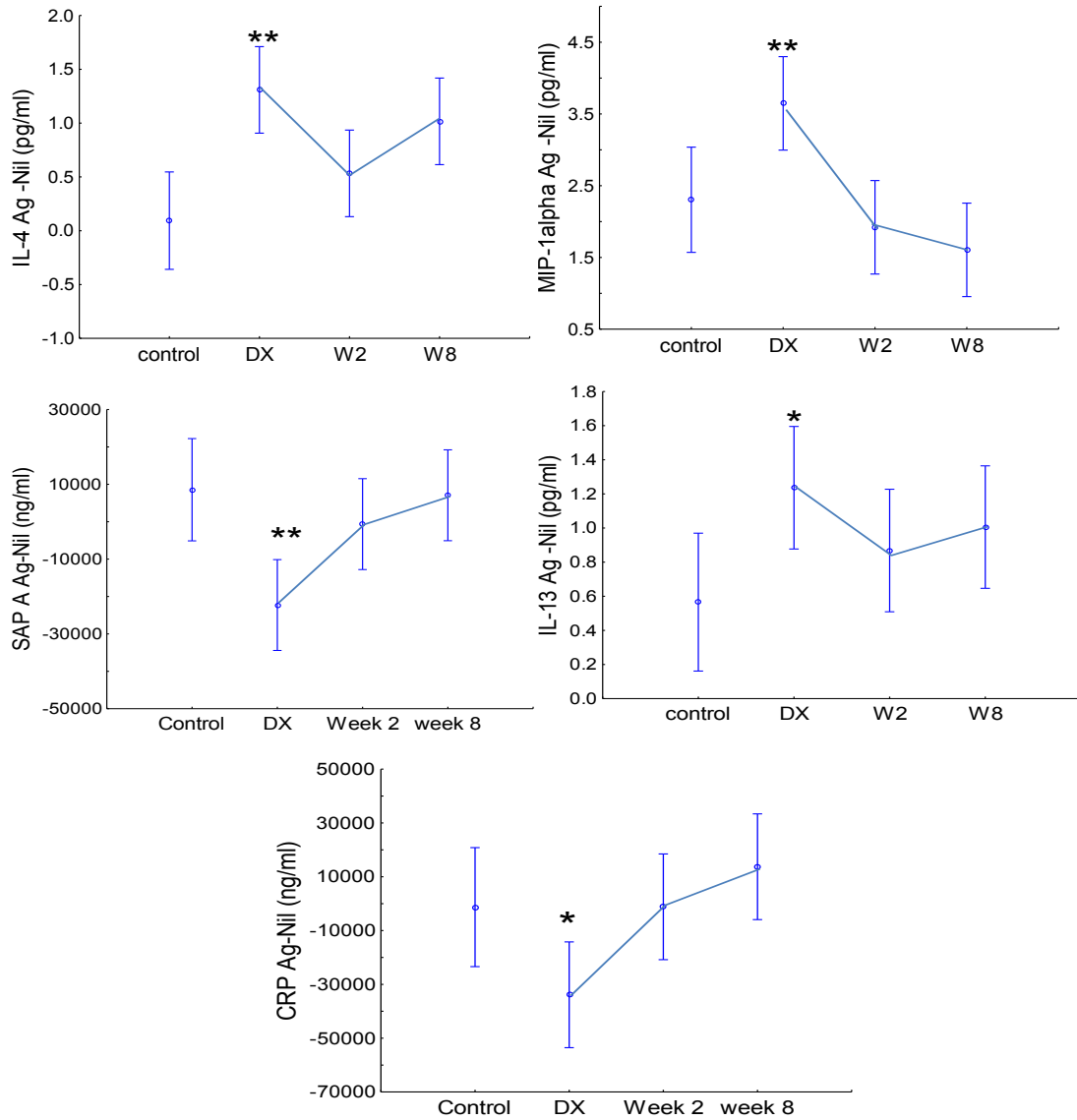


Figure 4.2 Mean concentrations of host markers in the Quantiferon supernatants that show significant differences at diagnosis between controls and TB patients. The vertical bars denote 95% confidence levels. The significant p values are shown for significant differences between the control group and the TB patients at the different time points: * $p < 0.05$, ** $p < 0.01$. Pair-wise comparisons of healthy control levels with each of the three participant time points (diagnosis, week 2 and week 8) was done with one-way ANOVA.

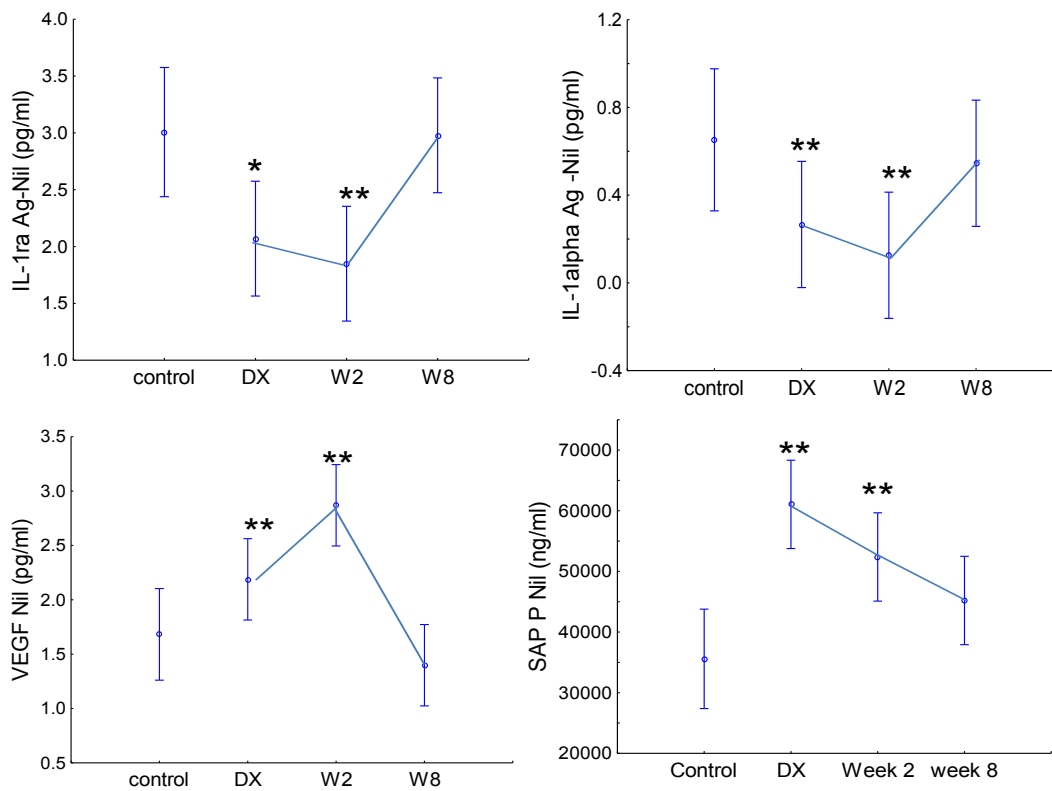


Figure 4.3 Mean concentrations of host markers in the Quantiferon supernatants that show significant differences at diagnosis and/or week 2 between controls and TB patients. The vertical bars denote 95% confidence levels. The significant p values are shown for significant differences between the control group and the TB patients at the different time points: * p < 0.05, ** p < 0.01. Pair-wise comparisons of healthy control levels with each of the three participant time points (diagnosis, week 2 and week 8) was done with one-way ANOVA.

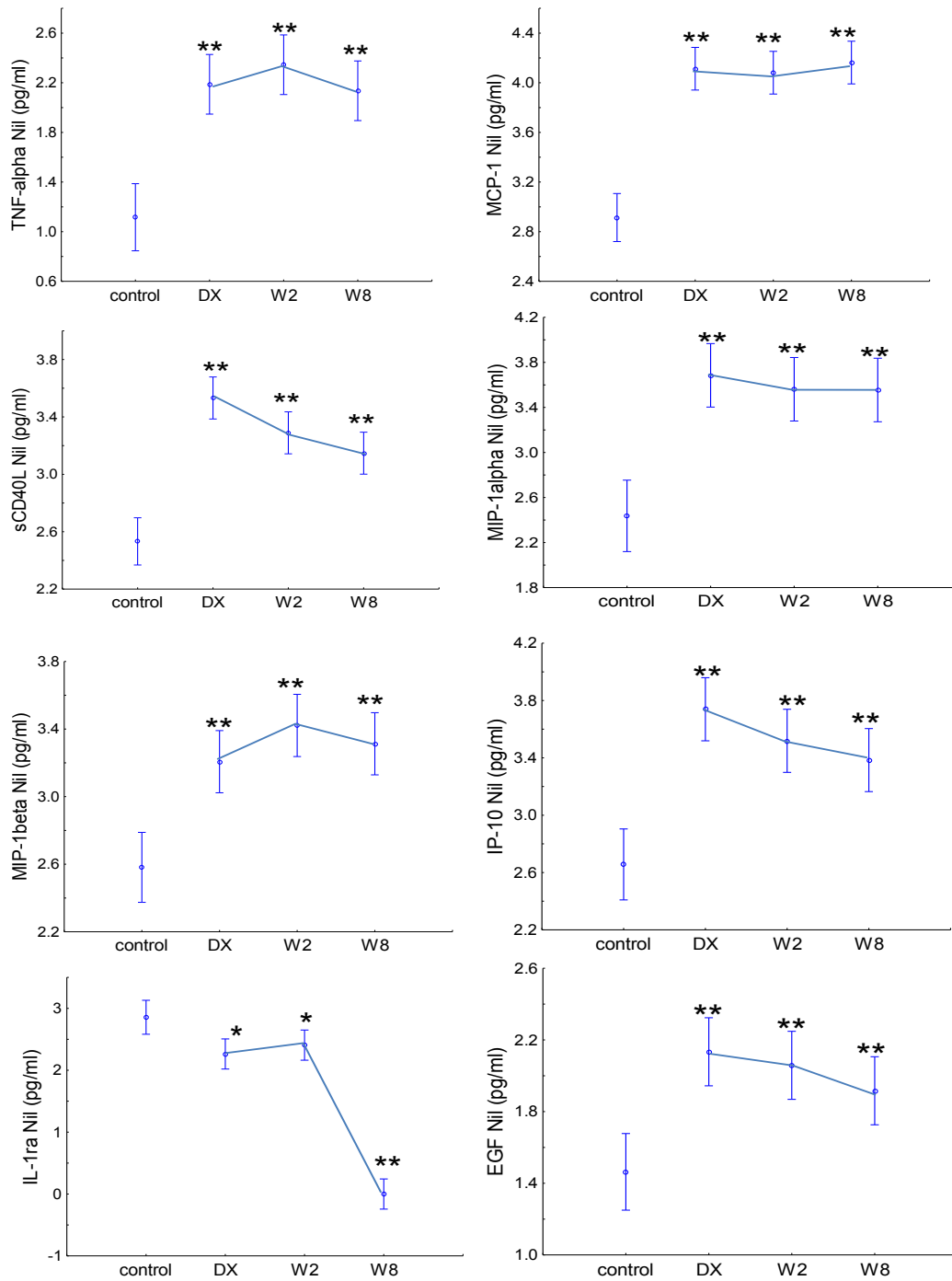


Figure 4.4 (A) Unstimulated mean concentrations of host markers in the Quantiferon supernatants that show significant differences across all time points when compared to controls. The vertical bars denote 95% confidence levels. The significant p values are shown for significant differences between the control group and the TB patients at the different time points: * $p < 0.05$, ** $p < 0.01$. Pair-wise comparisons of healthy control levels with each of the three participant time points (diagnosis, week 2 and week 8) was done with one-way ANOVA.

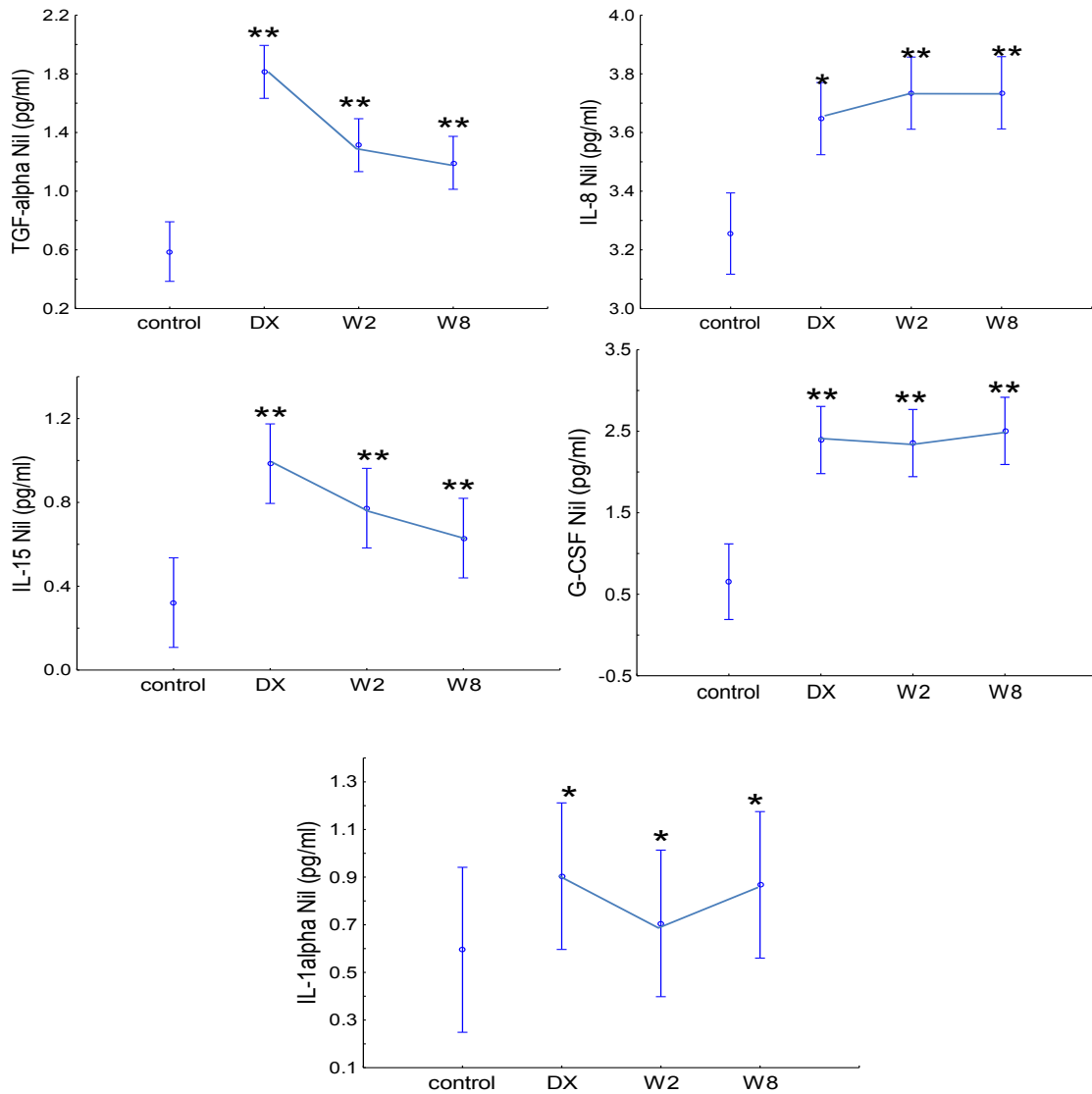


Figure 4.4 (B) Unstimulated mean concentrations of host markers in the Quantiferon supernatants that show significant differences across all time points when compared to controls. The vertical bars denote 95% confidence levels. The significant p values are shown for significant differences between the control group and the TB patients at the different time points: * p<0.05, **p<0.01. Pair-wise comparisons of healthy control levels with each of the three participant time points (diagnosis, week 2 and week 8) was done with one-way ANOVA.

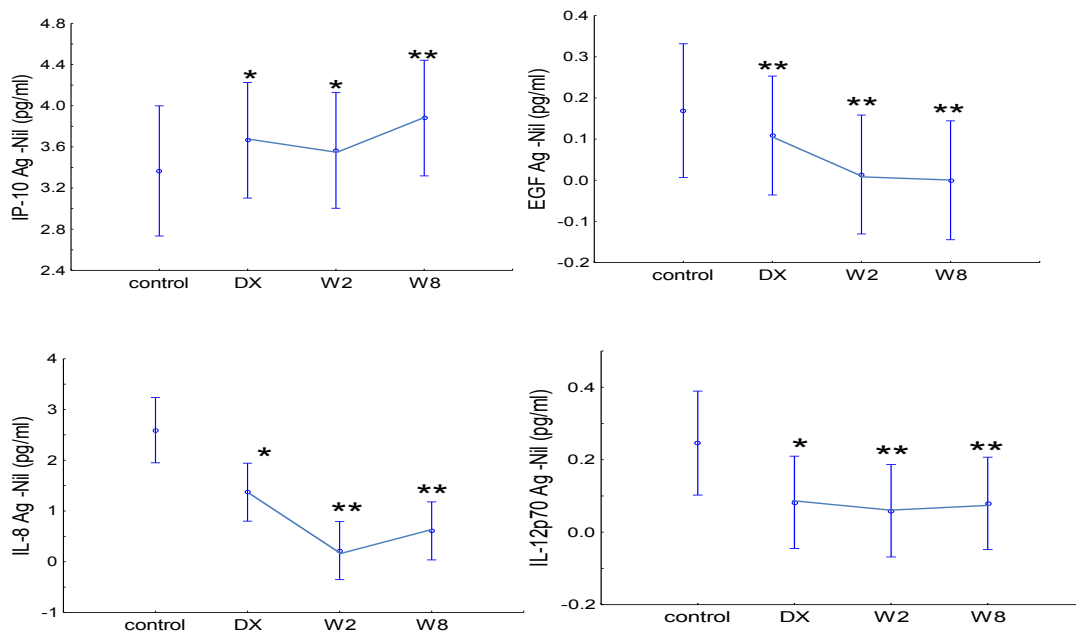


Figure 4.4 (C) Antigen stimulated (background subtracted from antigen stimulated levels) mean concentrations of host markers in the Quantiferon supernatants that show significant differences across all time points when compared to controls. The vertical bars denote 95% confidence levels. The significant p values are shown for significant differences between the control group and the TB patients at the different time points: *p<0.05, **p<0.01. Pair-wise comparisons of healthy control levels with each of the three participant time points (diagnosis, week 2 and week 8) was done with one-way ANOVA.

4.3.3 Response patterns observed in levels of host markers in participants with active tuberculosis at the onset of anti-TB treatment, week 2 and 8

The levels of 57 host markers were investigated in 19 study subjects with tuberculosis. The host markers that were investigated consisted of chemokines/cytokines, growth factors, soluble receptors and acute phase proteins.

Out of the 57 markers that were used in this study, FLT-3 ligand, GM-CSF, IL-1 alpha, IL-3, IL-9, IL-15, IL-17, IL-5, IL-12p70, sCD30, sEGFR, sgp130, sIL-1R1, sIL-1R2, sVEGFR2 were present at very low to undetectable concentrations.

Eotaxin, FGF-2, G-CSF, IL-12p40, MCP-1, MCP-3, MIP-1alpha, IL-1 beta, IL-10, TGF- α , IL-13, IL-7, IFN- γ , TNF- α , sIL-2Ralpha, sIL-4R, sIL-6R, sRAGE, sTNFR1, sTNFR2, VEGFR1 and VEGFR2 were present at detectable concentrations but there were no significant differences between the different time points.

The levels of 17 markers changed during the course of treatment. These markers could be organised into four categories as shown below. Mean log transformed values for the different time points are represented in Table 4.2.

(i) Host markers with a gradual decrease during the first 8 weeks of treatment

The unstimulated levels of chemokines, acute phase proteins and growth factors i.e. SAP P ($p=0.01$), EGF ($p=0.03$), SAP A ($p<0.001$), CRP ($p<0.001$), IP-10 ($p<0.001$), sCD40L ($p<0.001$) and MDC_{Ag-Nil} ($p=0.07$) were highest at pre-treatment levels and dropped significantly during treatment (Figure 4.5 A). According to Ingenuity Pathway Analysis these host markers are involved in the inflammatory response to infectious diseases mediating acute phase response signalling. These markers also play a role in cell to cell signalling and interaction, antigen presentation, cellular function, cellular maintenance and movement (Figure 4.6).

(ii) Host marker with a transient decrease at week 2

The unstimulated levels of chemokines and anti-inflammatory cytokines i.e. fractalkine ($p=0.01$), IFN-alpha 2 ($p=0.03$), IL-7 ($p<0.001$), IL-4 ($p<0.001$) and IL-4_{Ag-Nil} ($p<0.05$) had transiently decreased levels at week 2 (Figure 4.5 B). These markers are involved in inflammation and mediation of communication between immune cells. The molecular and cellular functions include cellular movement, cell to cell signalling and interaction and cellular growth and proliferation (Figure 4.7).

(iii) Host markers with a transient increase at week 2

These markers included the unstimulated levels of chemokines and inflammatory cytokines i.e. MIP-1 beta ($p=0.07$), MDC ($p<0.001$), GRO ($p=0.03$) and TNF-beta ($p<0.001$) (Figures 4.5 C). These markers are involved in inflammation and mediate differential regulation of cytokine production in macrophages and T helper cells by IL-17 A and IL-17 F. These markers' main cellular and molecular functions include cell-to-cell signalling and interaction, cellular movement and molecular transport (Figure 4.8)

(iv) Host markers with a significant decrease at week 8

These markers included the unstimulated levels of IL-1ra ($p<0.001$) and VEGF ($p<0.001$) (Figure 4.9). Heat maps and Ingenuity Pathway core analysis were only done for groups of four or more markers expressing the same pattern.

Table 4.2: Mean log transformed values of host markers in TB participants at onset of treatment, week 2 and 8

Cytokine	Diagnosis	Week 2	Week 8
SAP P _{Nil}	4.79 ± 0.15	4.71 ± 0.11	4.49 ± 0.54**
EGF _{Nil}	2.13 ± 0.45	2.06 ± 0.32	1.92 ± 0.56*
SAP A _{Nil}	5.60 ± 0.75	4.78 ± 1.04**	3.74 ± 0.93***
CRP _{Nil}	5.99 ± 0.32	5.46 ± 0.32**	4.82 ± 0.92***
IP-10 _{Nil}	3.74 ± 0.46	3.52 ± 0.48**	3.38 ± 0.51***
sCD40L _{Nil}	3.53 ± 0.25	3.29 ± 0.36	3.15 ± 0.30
MDC _{Ag-Nil}	0.86 ± 1.17	0.37 ± 0.79*	0.21 ± 0.55*
Fractalkine _{Nil}	2.23 ± 0.64	1.61 ± 1.09**	2.29 ± 0.69
IFN-alpha 2 _{Nil}	2.01 ± 1.34	1.29 ± 1.00*	2.15 ± 1.07
IL-7 _{Nil}	0.52 ± 0.36	0.17 ± 0.36***	0.40 ± 0.27*
IL-4 _{Nil}	2.01 ± 1.13	0.80 ± 1.01***	1.83 ± 0.88
IL-4 _{Ag-Nil}	1.31 ± 1.03	0.53 ± 0.76*	1.02 ± 1.08
MIP-1beta _{Nil}	3.21 ± 0.45	3.42 ± 0.35*	3.31 ± 0.38
MDC _{Nil}	2.96 ± 0.21	3.15 ± 0.20***	3.06 ± 0.19**
GRO _{Nil}	3.55 ± 1.07	4.10 ± 0.25*	4.00 ± 0.22
TNF-beta _{Nil}	1.63 ± 0.30	2.27 ± 0.62***	1.74 ± 0.32
IL-1ra _{Nil}	2.26 ± 0.73	2.41 ± 0.58	0 ± 0***
VEGF _{Nil}	2.19 ± 0.92	2.87 ± 0.64*	1.40 ± 1.08**

Values are expressed as mean ± standard error of the mean (SEM). The p values are shown are for differences between diagnosis and the different time points. * p<0.05, **p<0.01, ***p<0.001

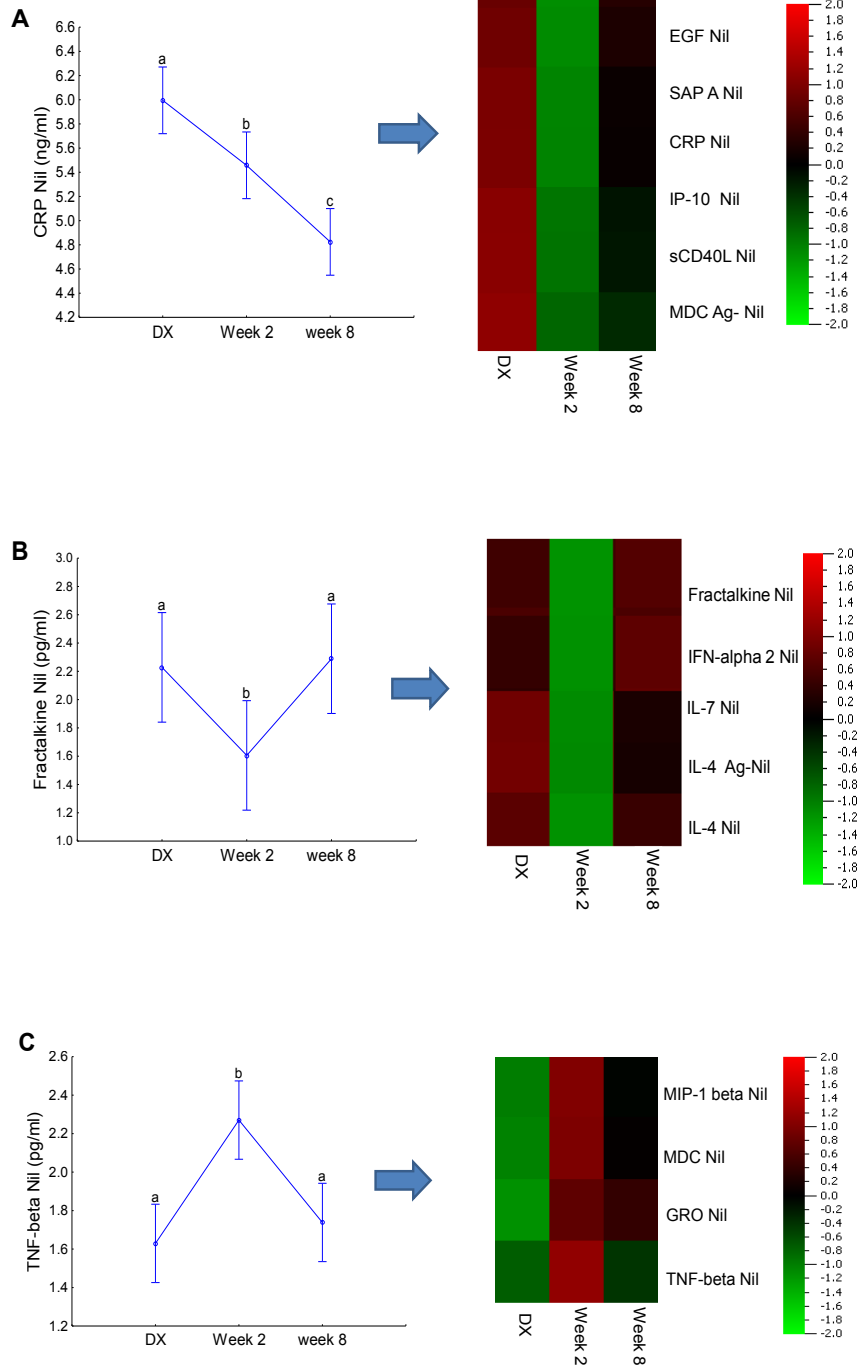


Figure 4.5 The Quantiferon host marker expression patterns during TB treatment: The Quantiferon supernatant cytokine levels were determined pre-treatment (Dx) and at week 2 and 8 of treatment in 19 active tuberculosis patients. Markers were grouped according to response patterns (only markers with significant changes during treatment are shown): **(A)** Host markers showing a gradual decrease during the first 8 weeks of treatment. **(B)** Host markers showing a transient increase at week 2. **(C)** Host markers showing a transient decrease at week 2. The heat map was created using mean log values for each time point using the Qlucore Explorer Software where red indicates high expression and green indicates low expression. The line graphs are for one representative marker of the response patterns. Different letters on the line graphs indicate that they are significantly different from each other with the p value <0.05.

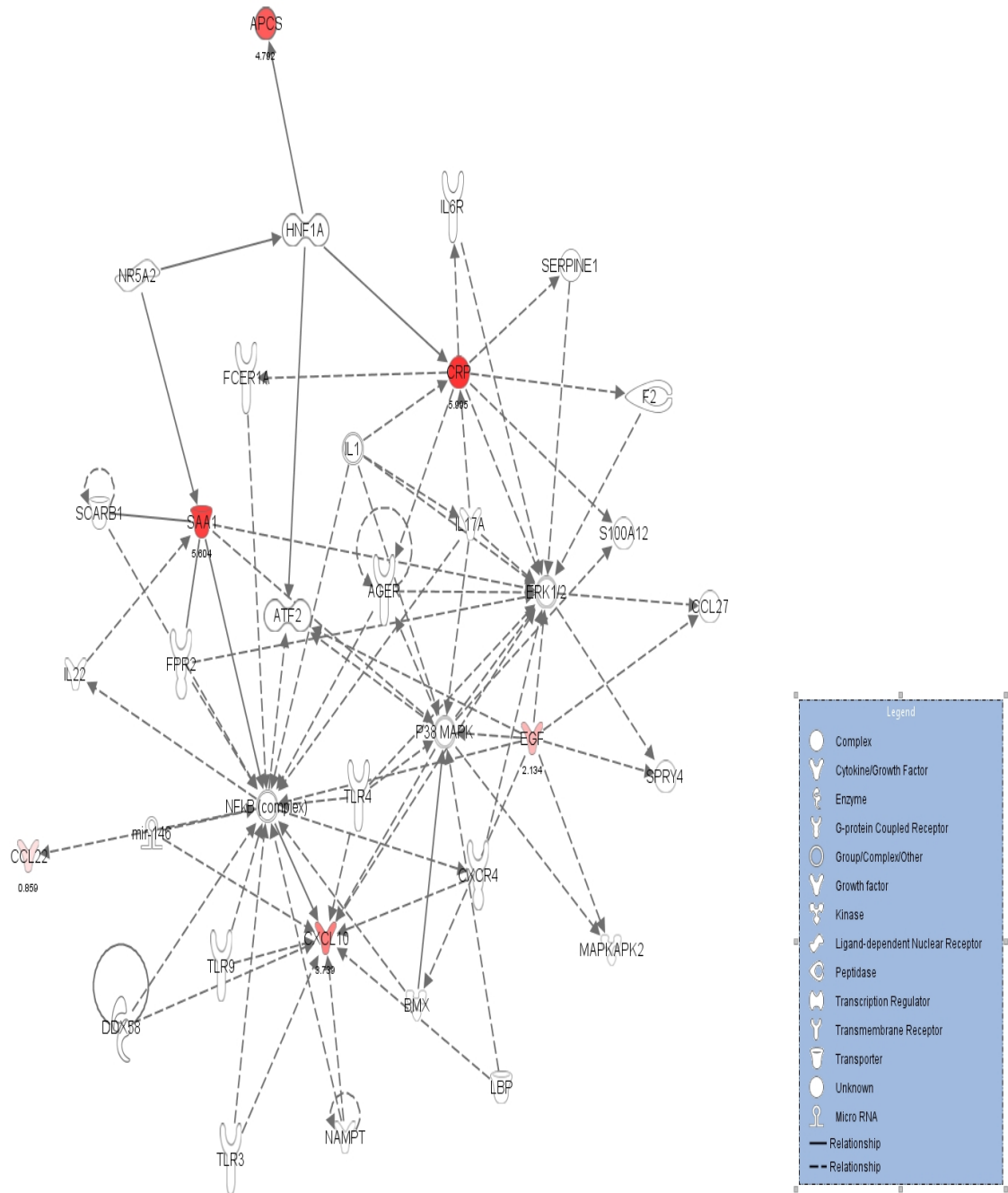


Figure 4.6 Ingenuity network analysis for host markers with a continuous decrease during the 8 week observation period. These host markers mediate acute phase response signalling. The diagram shows interactions between measured and unmeasured, putative molecules in the pathway. Red indicated measured markers with decreasing levels during treatment.

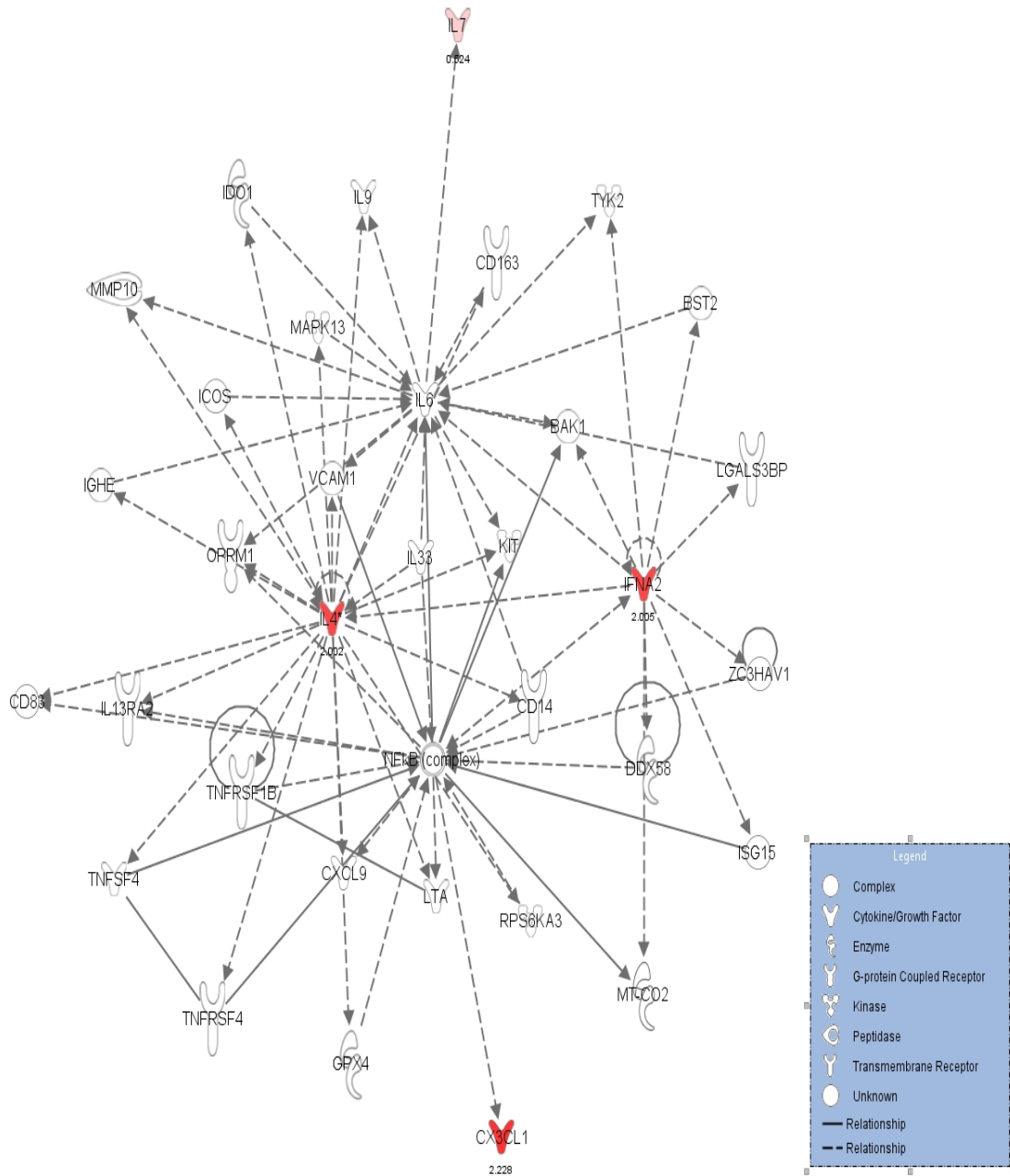


Figure 4.7 Ingenuity network analysis for host markers with a transient decrease at week 2: The role that these markers play is to mediate communication between immune cells. The diagram shows interactions between measured and unmeasured, putative molecules in the pathway. Red indicated measured markers that are down regulated at week 2.

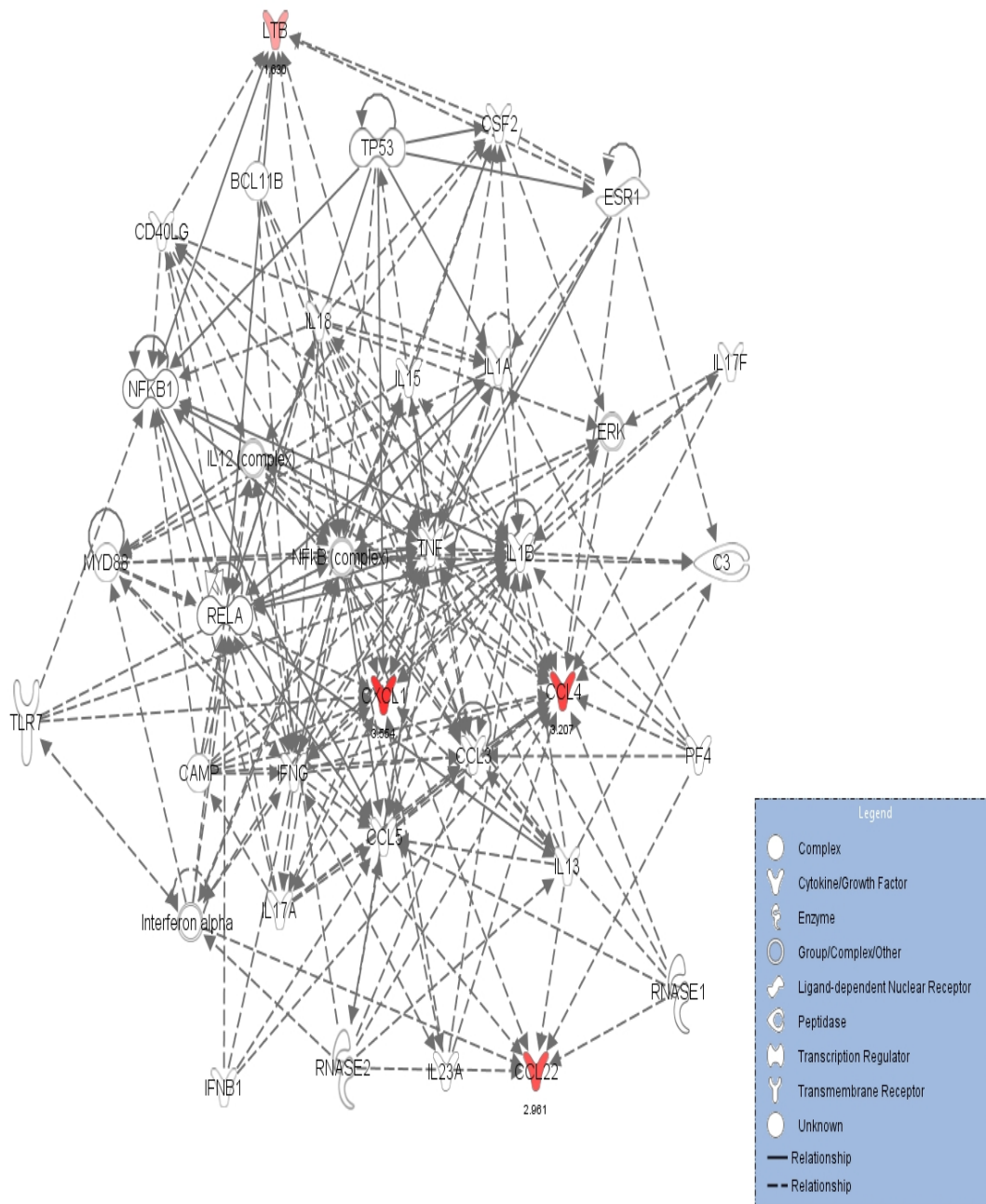


Figure 4.8 Ingenuity network for host markers with transient increases at week 2: These markers mediate differential regulation of cytokine production in macrophages and T helper cells by IL-17A and IL-17F. The diagram shows interactions between measured and unmeasured, putative molecules in the pathway. Red indicated measured markers with transient increases at week 2.

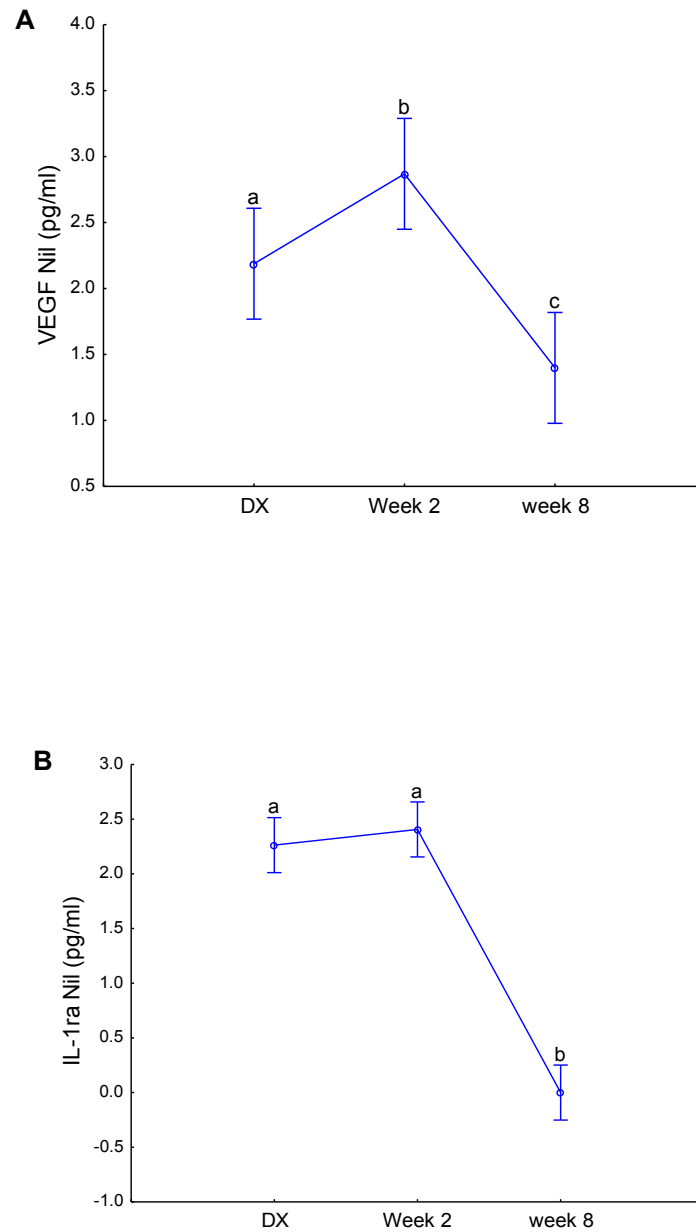


Figure 4.9 Mean levels of cytokines in QFT-GIT supernatants from TB patients at Diagnosis, Week 2 and Week 8 of treatment that respond with a significant decrease at week 8 (A and B): This data was analysed by mixed model repeated measures ANOVA (L.S means, 95% confidence interval (CI)). Different letters on the line graphs indicate that they are significantly different from each other with the p value < 0.05.

4.3.4 Cytokine profiles in fast and slow responders to TB treatment

The repeated-measures analysis showed significant differences in cytokine secretion by fast and slow responders in antigen stimulated supernatants (background subtracted from antigen stimulated levels) of IL-13 ($p=0.02$) and IL-1 beta ($p=0.04$). The LSD test, which allowed comparison between fast and slow responders at specific time points, revealed that there was a significant difference in the cytokine levels between these two groups. Analysis of pre-treatment levels showed that fast responders had significantly higher levels of IL-13 ($p=0.04$) when compared with slow responders (Figure 4.10). The analysis of pre-treatment levels demonstrated that slow responders had much higher levels of IL-1beta ($p<0.01$) when compared to fast responders. (Figure 4.11).

We further analysed the data using GDA to identify multi-variable models (with a maximum of 4 variables) with the ability to classify fast and slow responders (month 2 sputum culture results) using pre-treatment, week 2 and week 8 measurements.

The most frequently incorporated markers into the top 20 four-variable models were identified. The host marker IL-1beta_{Ag-Nil} was the most frequently occurring marker which could discriminate fast and slow responders at pre-treatment (Figure 4.12). The combinations of G-CSF_{Ag-Nil}, MIP-1beta_{Ag-Nil}, IL-1beta_{Ag-Nil} and sIL-4R_{Ag-Nil} classified fast responders and slow responders with an accuracy of 100% in a resubstitution classification matrix and with 100 % after leave-one-out cross validation

CRP_{Nil} and IL-1ra_{Nil} were the most frequently occurring markers which could discriminate fast and slow responders at week 2 (Figure 4.13). The combinations of IL-1ra_{Nil}, IL-9_{Nil}, CRP_{Nil} and sTNFR1_{Ag-Nil} classified fast responders and slow responders with an accuracy of 100% in a resubstitution classification matrix and with 100% after leave-one-out cross validation.

Fractalkine_{Ag-Nil} was the most frequently occurring marker which could discriminate fast and slow responders at week 8 (Figure 4.14). The combinations of Fractalkine_{Ag-Nil}, IP-10_{Ag-Nil}, TNF-beta_{Ag-Nil} and CRP_{Ag-Nil} classified fast responders and slow responders with an accuracy of 100% in a resubstitution classification matrix and with 100% and 90% respectively after leave-one-out cross validation.

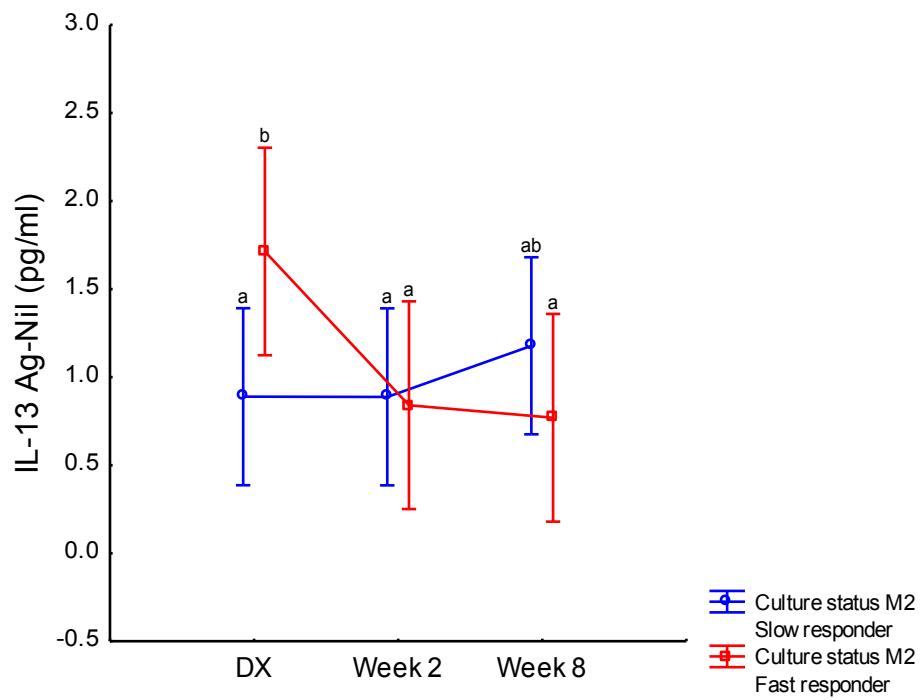


Figure 4.10 Mean levels of IL-13_{Ag-Nil} in QFT-GIT supernatants in TB patients at diagnosis, week 2 and week 8 of treatment. The vertical bars denote 95% CI. Different letters on the line graphs indicate that they are significantly different from each other with the p value < 0.05.

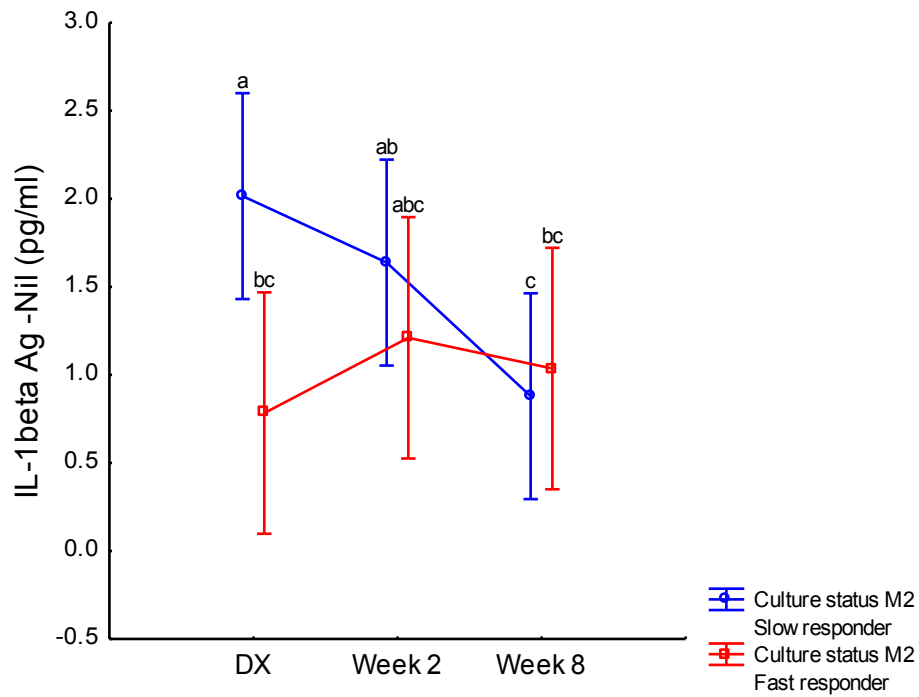


Figure 4.11 Mean levels of IL-1beta_{Ag-Nii} in QFT-GIT supernatants in TB patients at diagnosis, week 2 and week 8 of TB treatment. The vertical bars denote 95% CI. Different letters on the line graphs indicate that they are significantly different from each other with the p value <0.05.

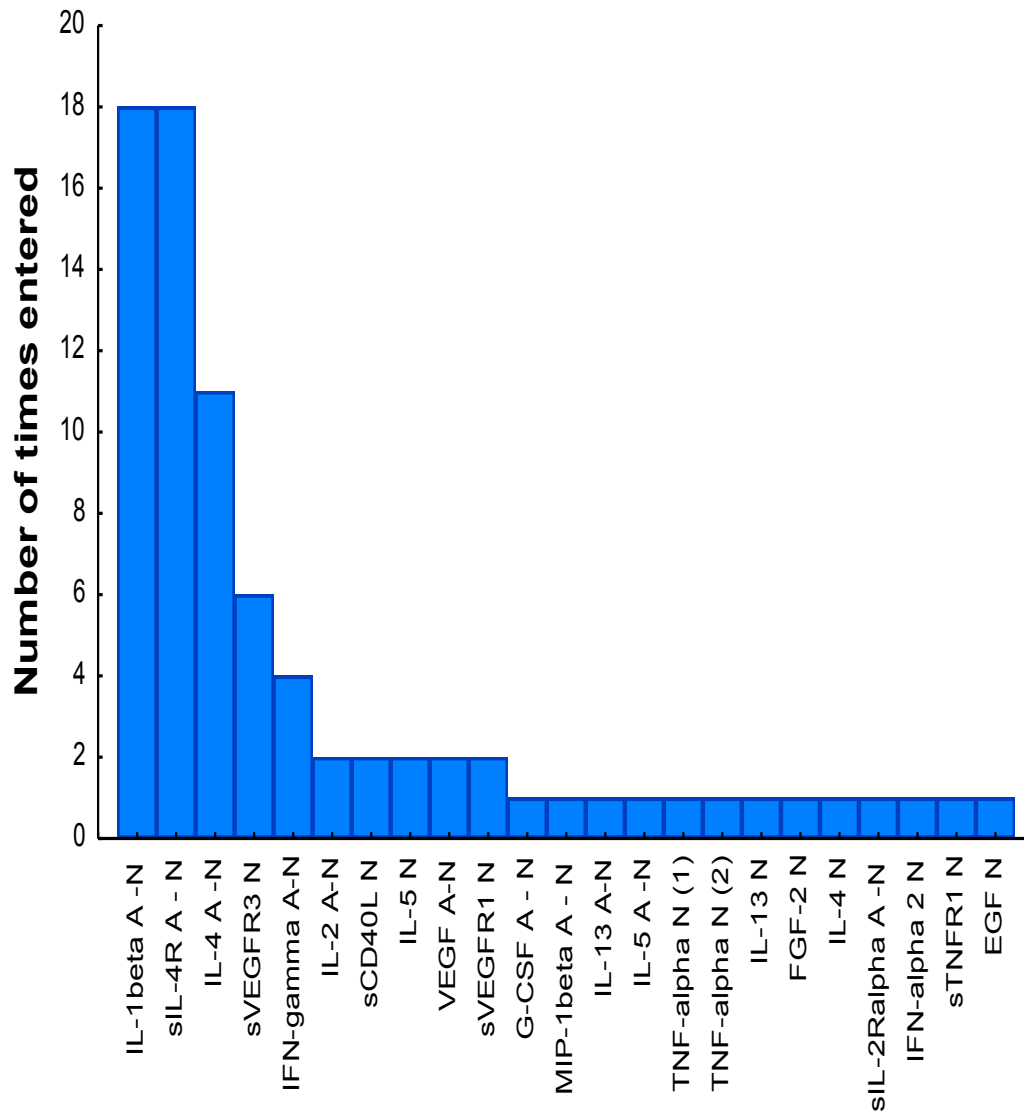


Figure 4.12 Frequency of individual analytes in top models for discriminating between fast and slow responders at pre-treatment. The columns represent the number of inclusions of individual markers into the most accurate four-analyte models by GDA analysis (20 models)

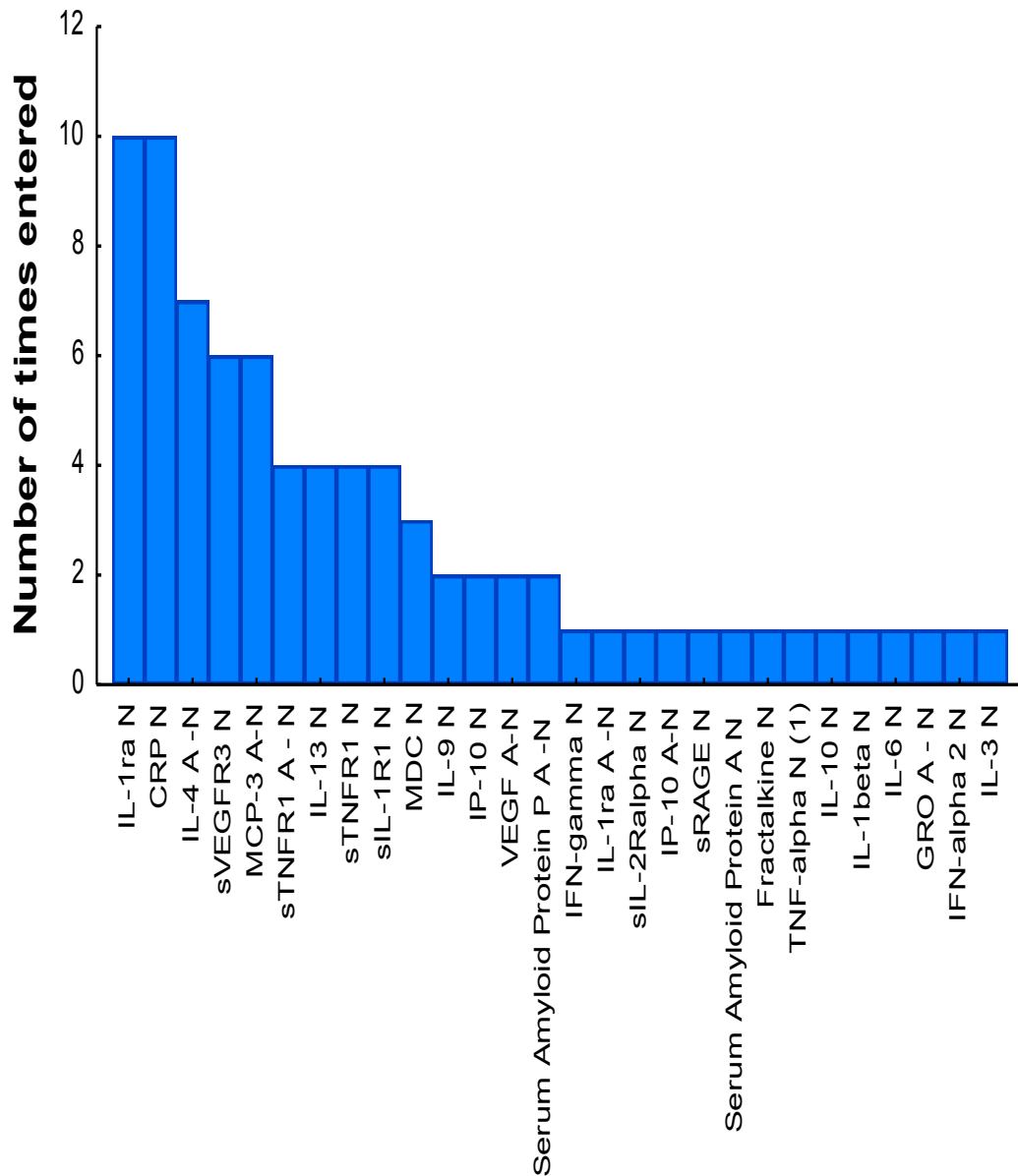


Figure 4.13 Frequency of individual analytes in top models for discriminating between fast and slow responders at week 2. The columns represent the number of inclusions of individual markers into the most accurate four-analyte models by GDA analysis (20 models)

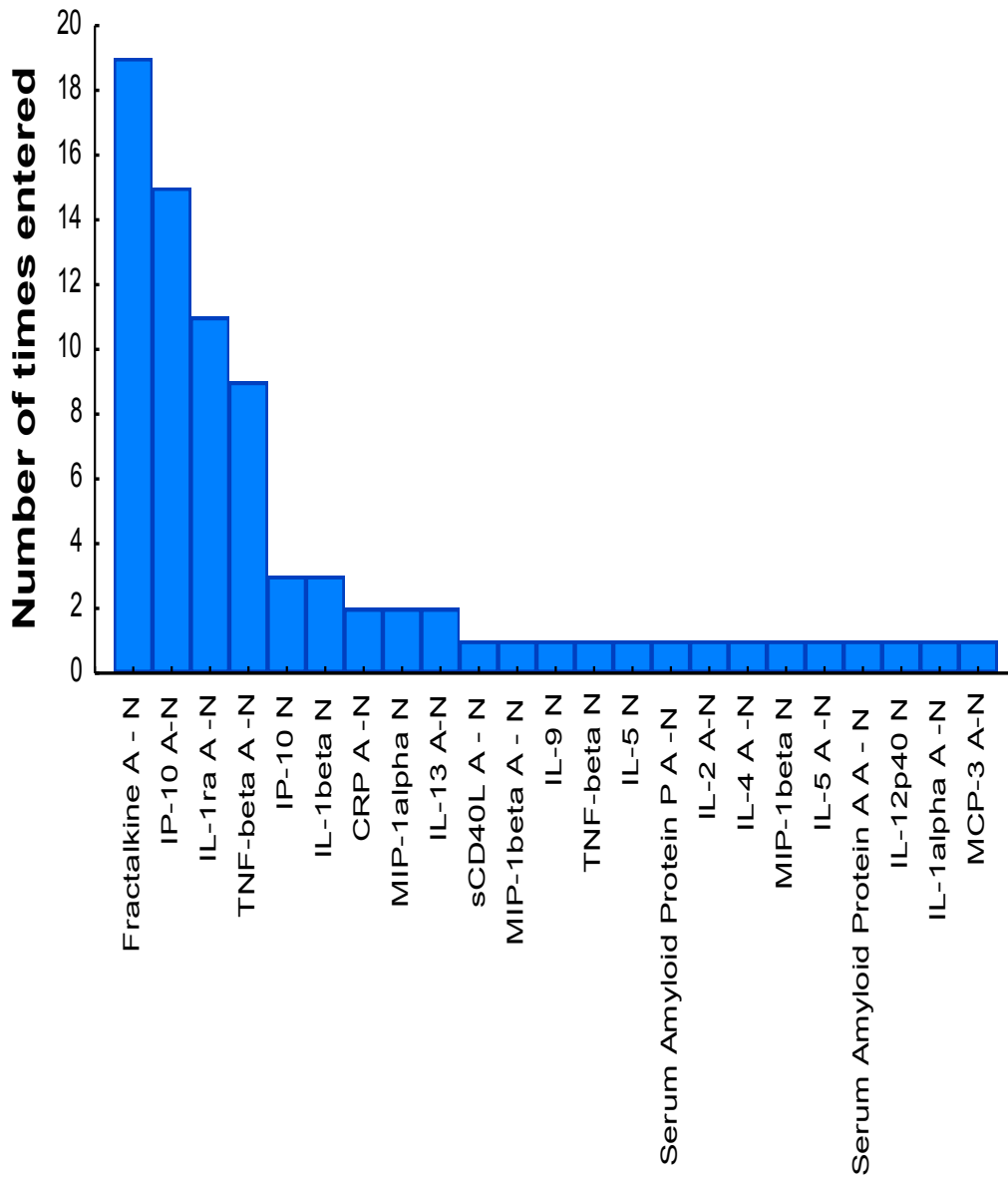


Figure 4.14 Frequency of individual analytes in top models for discriminating between fast and slow responders at week 8. The columns represent the number of inclusions of individual markers into the most accurate four-analyte models by GDA analysis (20 models)

4.3.5 Comparison of antigen stimulated levels in short term QFT-IT assay with the 7-day whole blood assay.

We investigated the responses in antigen specific samples during early treatment in the 7-day whole blood culture (chapter 3) and the QFT-IT assay (present chapter). IL-1 beta and GRO were the only host markers which showed early changes in the antigen stimulated samples in the 7-day whole blood assay and MDC and IL-4 showed antigen specific changes in QFT-IT assay. These two assays were compared at the two common time points, diagnosis and week 2. GRO and MDC expressed higher mean levels in the 7-day whole blood assay compared to the QFT-IT assay. The baseline level of GRO in the 7-day whole blood assay was significantly different from the baseline level in the QFT-IT ($p < 0.001$). The baseline and week 2 levels of MDC was significantly different ($p < 0.01$ and $p < 0.001$ respectively) (Figure 4.15).

IL-4 and IL-1 beta were expressed at higher mean levels in the QFT-IT assay when compared to the live 7-day whole blood assay. The baseline and week 2 levels of IL-1 beta ($p < 0.05$ and $p < 0.001$ respectively) and IL-4 ($p < 0.001$ and $p < 0.05$ respectively) were significantly different at baseline and week 2 (Figure 4.15).

Generally, Nil levels of cytokines were higher in QFT than in the 7-day assay (not shown) but as discussed before these cytokines may have to be measured in *ex vivo* samples like serum or plasma rather than in cultured assays.

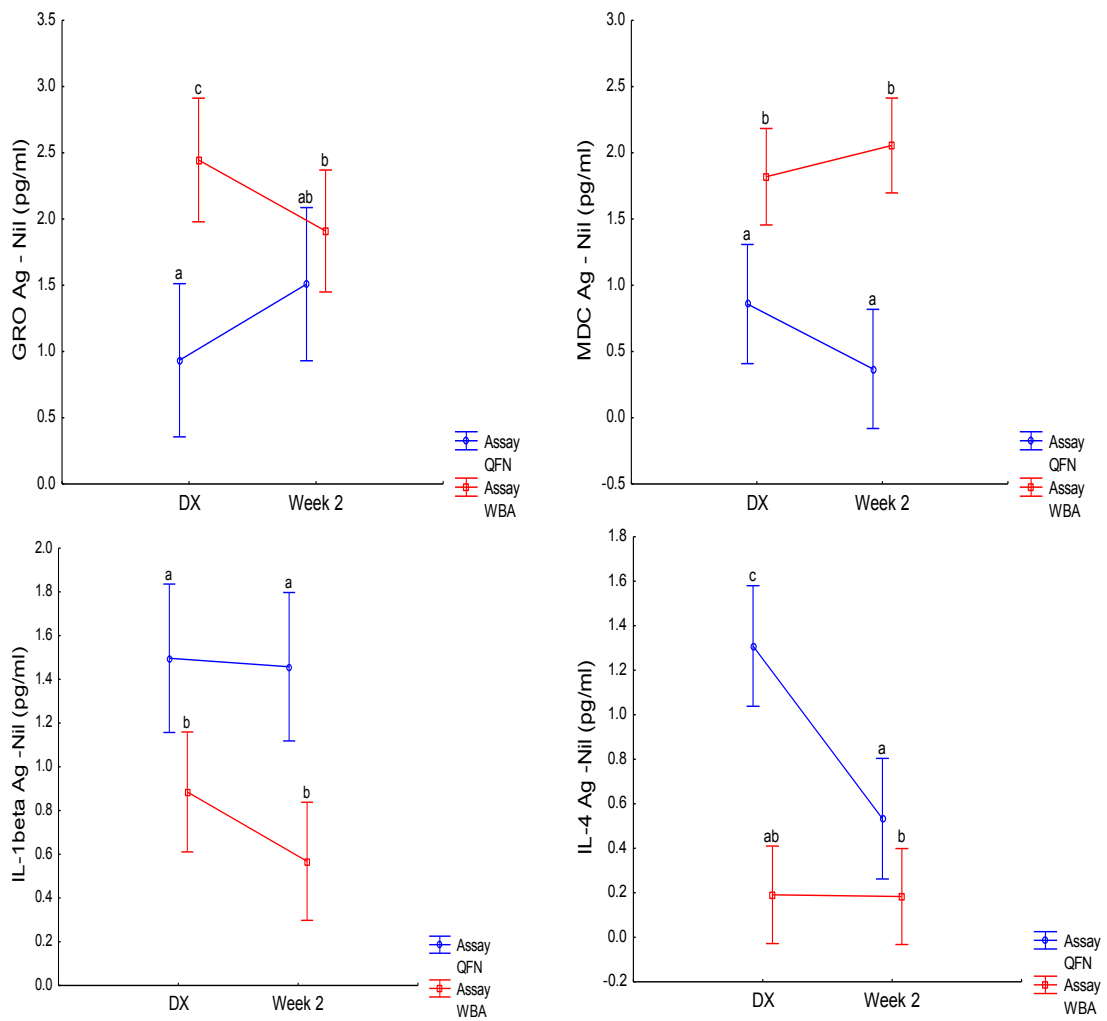


Figure 4.15 Comparison of the QFT-IT and whole blood assay at DX and week 2: QFT-IT produced higher mean levels of: IL-1beta and IL-4 when compared to the whole blood assay. The live 7 day whole blood assay produced higher levels of MDC and GRO when compared to the QFT-IT assay.

4.4 Discussion

Previous studies have adapted the use of QFT-IT and have identified multiple biomarkers which can differentiate between active and latent TB infection.

In this study we screened the profiles of 57 host markers in unstimulated and stimulated QFT-IT supernatants before and during the first eight weeks of treatment in participants with active TB to determine which host markers change significantly during early treatment and therefore could be potential candidates for treatment response biomarkers. A highly standardized assay like the QFT would have the advantage that it could be used in different clinical trial sites across the world with storage of assay supernatants for subsequent biomarker discovery. The markers that have shown significant changes during early treatment could be further evaluated in TB treatment response studies.

The major finding of this work is that multiple biomarkers were detected and showed significant changes in the unstimulated supernatants during early treatment compared to the majority of antigen stimulated supernatants that did not change significantly. However, unstimulated marker levels should rather be measured in *ex vivo* samples like plasma or serum to avoid artefacts due to degradation of proteins or due to binding to soluble or cell-bound receptors. The only markers whose antigen stimulated levels (antigen minus nil) change during treatment were the chemokine MDC and the anti-inflammatory cytokine IL-4. We also demonstrated that the host markers change with different response patterns during early treatment. These responses include gradual decreases, transient decreases by week 2, transient increases by week 2 and a significant increase by week 8 during the eight weeks of treatment.

Some cytokine levels in TB patients were statistically significantly different at different time points and between patients and controls whereas these response patterns were of uncertain biological significance. This may be due to the small number of participants or may be due to varying levels of inflammation during the early stages of treatment. Some cytokines showed no treatment response during the observation period and were not different from control values and are therefore unlikely to add benefit to treatment monitoring. Other markers show more promise for monitoring treatment response. The group of cytokines that are expressed at significantly lower or higher levels than in controls and that change by week two of treatment may be valuable early treatment response markers and warrant further investigation. The markers which remain significantly different from control values during the 8 weeks of

observation would in all likelihood return to baseline levels later during treatment and further studies should investigate whether such presumed late changes are indicative of achieving sterilizing cure.

The levels of acute phase proteins (SAP P, CRP, and SAP A), chemokines (IP-10 and MDC) and growth factors (EGF) and sCD40L (which is released by platelets and triggers the release of inflammatory mediators¹⁵⁹) decreased gradually over the eight weeks of treatment. The disease function associated with these markers is broadly defined as 'inflammatory responses' by the IPA. The cellular and molecular function associated with these markers includes, antigen presentation, general cellular functions, cell to cell signalling. The decrease in these functions may be related to the decline in the inflammatory response that is associated with the reduction in bacterial load due to therapy.¹⁶⁰ As bacterial numbers decline the need for a highly active inflammatory response, antigen presentation and communication between cells decreases too.

The levels of inflammatory cytokines (IFN-alpha 2, IL-4 and IL-7) and the chemokine (Fractalkine) are transiently decreased at week 2, which leads to a decrease in inflammation, cellular movement and cell to cell signalling. Transient changes are generally not considered to be direct treatment effects but in this case, it may indicate transient immune regulation. As bacterial numbers fall during the phase of rapid bacterial killing, the immune system responds by reprogramming its functions. However, as the battle against the infection is by no means won at this stage and as a significant healing process needs to take place these markers return to the high pre-treatment levels after the initial dramatic fall of pathogen numbers.

Similarly, the transient increase of markers may also be due to the need to reset immune responses as discussed in the previous paragraph. The inflammatory cytokine (TNF-beta) and chemokines (MDC, MIP-1beta and GRO) transiently increase by week 2 and this could also be a reflection of an early recovery of the immune response that takes place when antibiotic treatment alleviates immune suppression induced by overwhelming numbers of mycobacteria.¹²

VEGF and IL-1ra showed a significant decrease by week 8. VEGF, an angiogenesis mediator produced by macrophages has been shown to participate in wound healing and repairing of tissue.⁹⁷ Previous studies have shown elevated levels of VEGF in plasma of active TB patients and decreased during anti-tuberculosis treatment.^{97,161} This was also observed in this study. IL-1RA is a natural antagonist of IL-1 produced by macrophages.¹⁶² Several studies have reported elevated levels of IL-1ra in

pulmonary tuberculosis and levels decline during treatment.^{158,163} The levels of IL-1ra were shown to decrease in our study.

The markers, MDC and IL-4 were 2 out of the 57 markers to produce a response in antigen stimulated cultures. MDC is a chemokine that attracts activated T cells and is produced by macrophages and DC within secondary lymphoid tissues.¹⁶⁴ Arias M *et al.*, 2007 reported increased MDC production when whole blood was stimulated with *M.tb* specific antigens.¹⁶⁵ In this present study the levels of MDC decreased with treatment.

The comparison of fast and slow responders to treatment showed that the levels of IL-13_{Ag-Nil} were found to be significantly higher in fast responders at the baseline level when compared to slow responders. IL-13 is produced by TH2 cells and is a mediator in allergic inflammation and disease.¹⁶⁶ The role of IL-13 in *M.tb* is not well defined. Harris *et al.*, 2007 shows that IL-13 contributes to autophagy-mediated killing of *M.tb* in human and murine macrophages.¹⁶⁷ In a study done by Siawaya *et al.* in 2009 high levels of IL-13 were reported in fast responders compared to slow responders which corresponds with our study.⁹⁷ Concentrations of IL-1 beta_{Ag-Nil} showed significantly higher levels in slow responders very early during treatment; this could suggest that increased anti-inflammatory response that accompany more extensive disease or a lack of early response to chemotherapy may lead to a delay of sputum conversion in patients.¹⁶⁸

Multi-variant analysis was performed on markers measured at the onset of treatment and week 2 and 8. Pre-treatment levels of IL-1beta_{Nil} and sII-4R_{Nil}, levels of IL-1ra_{Nil}, CRP_{Nil} at week 2 and levels of fractalkine_{Nil} and IP-10_{Nil} at week 8 were able to predict week 8 sputum culture outcome with 100% accuracy but this result should be interpreted with caution due to the small number of study participants.

Out of the 57 markers that were evaluated in both assays only four markers showed antigen stimulated responses, IL-1beta and GRO in the 7 day whole blood assay and MDC and IL-4 in the QFT-IT assay. We compared these two assays at the two common time points, diagnosis and week 2 and it displayed that QFT-IT expressed much higher mean levels of IL-1beta and IL-4 compared to the 7-day assay. MDC and GRO expressed higher levels in the 7 day assay when compared to the QFT-IT assay. These differences could be contributed to these assays have different requirements when using whole blood and different incubation periods.¹⁴⁷ The QFT-

IT and the 7 day whole blood assay have shown not to be good replacements of serum and plasma for the discovery of host markers for early treatment.

The main limitation in this study, apart from the small number of participants, is that a longer follow up period during treatment was not available to follow the changed in host marker levels during the important sterilizing phase of treatment. However, the early changes remain important for the rapid evaluation of treatment success and for the evaluation of new drugs. This study sheds new light on the potential of some host markers in this context.

4.5 Conclusion

In conclusion, our preliminary results suggest that only a few of the 57 investigated host markers change during the first 8 weeks of treatment in QFT-IT antigen stimulated whole blood supernatants. Antigen stimulated responses appear less promising than several markers in unstimulated cultures, which should be assessed *ex vivo* in serum or plasma. Further studies of these markers in different outcome of TB treatment need to be conducted. In addition, further changes between week 8 and the end of treatment should be investigated as some markers did not reach control levels during the first 8 weeks of therapy in the present study.

CHAPTER 5:

Summary and Conclusion

The only accepted measure of early TB treatment response comes two months after the initiation of treatment at the end of the intensive phase of therapy. This long lag time before non-response to treatment is not recognized and allows ongoing tissue destruction, continued spread of organisms and even the potential development of new drug resistance in patients on suboptimal treatment regimens. Baseline measurements may allow stratification of patients into clinical trial arms with similar treatment needs. This would reduce the required size of clinical trial groups and thereby decrease the costs in clinical trials.

The choice of QFT-IT supernatants as sample types for the discovery of new biomarkers was based on the fact that this is a highly standardized commercial test with validated *M.tb* antigens and incubation times. Any new markers that are discovered have the potential to be translated into clinical use by making use of the well-developed QFT system where only the measurement of the new target analytes needs to be developed appropriately. Such an assay may be much more practical than the 7-day live *M.tb* assay with its bio-hazard issues for laboratory staff and requirement for a BSL facility.

This thesis evaluated the measurement of multiplex cytokine arrays in supernatants from two different *M.tb* antigen stimulated culture methods. Firstly a 7-day, live *M.tb* stimulated whole blood assay was evaluated and secondly the commercially available QFT-IT test tube system was assessed to investigate if the stimulation of *M.tb* antigens from TB patients undergoing TB treatment results in the production of host markers which can predict early treatment response.

Neither of the whole blood assays resulted in convincing antigen specific treatment responses. Many biomarkers in the unstimulated supernatants, however, showed early changes with distinct response patterns. Unstimulated culture responses should rather be assessed in *ex vivo* samples like serum or plasma due to possible artefacts due to degradation or uptake by cells in the cultures.

The comparison of host marker levels between controls (albeit historical data) and patients at the different time points in the QFT-IT assay showed that some markers levels did not recover to normal values during the observation period and these markers may need further evaluation in future studies. The group of cytokines that are expressed at significantly lower or higher levels than in controls and that change

by week two of treatment may be valuable early treatment response markers and warrant further investigation. The markers which remain significantly different from control values during the 8 weeks of observation would in all likelihood return to baseline levels later during treatment and further studies should investigate whether such presumed late changes are indicative of achieving sterilizing cure.

When looking at different responder groups (fast and slow responders according to week 8 culture status) the analytes in the live *M.tb* assay showed no statically significant differences. However in the QFT-IT assay IL-1beta_{Ag-Nil} and IL-13_{Ag-Nil} showed significant differences between fast and slow responders at pre-treatment levels. In this respect antigen-specific cytokine production may well have the potential to differentiate between responder phenotypes and may warrant further investigation. The GDA analysis to predict week 8 culture status performed with accuracy between 70-100% but these results should be interpreted with caution due to the small number of participants in both studies. These markers would also have to be tested in different outcomes of TB treatment, including relapse and failed treatment.

In conclusion, antigen-specific responses showed only limited potential for early TB treatment response monitoring but may have potential in differentiating between treatment outcomes. The QFT-IT samples do not appear to be equivalent to live *M.tb* stimulated 7-day whole blood assays.

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