Immune Parameters as Biomarkers of *Mycobacterium tuberculosis* Sterilization during Anti-tuberculosis Treatment

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Promoter: Prof. Gerhard Walzl

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Declaration

I hereby declare that this dissertation is my own original work and that I have not previously submitted it at any university for a degree.

Signature: Date: 1 August 2007

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Abstract

Setting

Study conducted in Tygerberg, Cape Town in South Africa.

Hypothesis

Host biomarkers associated with the antimycobacterial immune response during active infection with *M. tuberculosis* and during anti-tuberculosis chemotherapy are indicative of bacterial killing in the host and can be used in models to predict eventual treatment outcome.

Objectives

1. To investigate immune parameters that were selected in a biological context as biomarkers of the extent of disease and early response to anti-tuberculosis treatment.
2. To use selected immune parameters to characterise fast and slow responders to anti-tuberculosis therapy.

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Findings

Evaluation of cytokine multiplex fluorescent bead-based immunoassays as a screening tool in the search for biomarkers

The data showed that cytokine multiplex fluorescent bead-based immunoassays achieved acceptable recoveries to detect antigen-specific IFN-γ responses in whole blood supernatant making it attractive for biomarker screening. However, proper optimisation needs to be done and proper controls included when using these kits.

Markers of extent of disease

High levels of CRP at diagnosis were found to be associated with the presence of multiple cavities on chest X-rays. A high level of suPAR and sICAM-1 at diagnosis were associated with the extent of alveolar disease. Also significant were the associations between the level of granzyme B, LAG-3 at diagnosis and the size of the cavities. No significant associations were observed between sTNFRs or DR5 with the chest X-ray grading of tuberculosis disease.

Early classification of fast and slow responders to anti-tuberculosis treatment

After cross-validation classification, discriminant analysis (DA) and support vector machine (SVM) analysis of selected immune parameters (sICAM-1 CRP, granzyme B, suPAR, sTNFRs, LAG-3 and CD3dim/CD56+ (% of CD45+) resulted in a 75% to 100% correct classification of the fast responders and a 82% to 100% correct classification of the slow responders when using DA. For SVM, the correct classification of the fast responders ranged from 88% to 100%, and that for the slow responders ranged from 95% to 100%.

Differential gene expression in fast and slow responders to treatment

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Direct comparison of fast and slow responders showed that IL-4 transcripts were significantly higher in the fast responders at week one after initiation of treatment when compared to slow responders**.** IL-4δ2 was also differentially expressed. Although IL-4δ2 was significantly up-regulated in both fast and slow responders after one week of treatment compared to diagnosis, IL-4δ2 expression was more than two folds higher in slow responders than in fast responders**.** No significant differences between the fast and slow responders were observed in the expression of TGF-β, TGF-βRII, Foxp3 and GATA-3.

Conclusion

Predictive models for differential anti-tuberculous treatment responses combining host proteins are promising and should be included in larger prospective studies to find the optimal markers for inclusion into clinical trials of new drugs and for implementation into clinical practice.

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Opsomming

Ligging

Studie onderneem in Tygerberg, Kaapstad, Suid-Afrika.

Hipotese

Gasheerbiomerkers wat verband hou met die antimikobakteriële immuunrespons tydens aktiewe infeksie deur *M. tuberculosis* en tydens teentuberkulose chemoterapie dui op bakteriële doding in die gasheer en kan in modelle gebruik word om die uiteindelike uitkoms van die behandeling te voorspel.

Doelwitte

1. Om gekose immuunparameters in ’n biologiese konteks as biomerkers van die omvang van siekte en vroeë reaksie op behandeling te ondersoek.
2. Om gekose immuunparameters te gebruik om vinnige en stadige reageerders op teentuberkulosebehandeling te karakteriseer.

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Bevindings

Evaluering van die sitokien veelvuldige fluoresseer-pêrelbaseerde immuuntoets (*cytokine multiplex fluorescent bead-based immunoassays*) as 'n siftingsinstrument in die soeke na biomerkers

Die data het getoon dat die sitokien veelvuldige fluoresseer-pêrelgebaseerde immuuntoets in staat was om antigeenspesifieke IFN-γ-respons te meet wat dit aanloklik maak vir biomerkersifting. Sorgvuldige optimering moet egter gedoen word en behoorlike beheer moet ingesluit word wanneer hierdie stelle gebruik word.

Merkers van omvang van siekte

Hoë vlakke van CRP by diagnose is getoon om verband te hou met die teenwoordigheid van veelvoudige holtes op die pasiënte se borskas x-strale. Hoë vlakke van suPAR en sICAM-1 by diagnose was assosieer met die omvang van alveolêre siekte. Die assosiasie tussen die vlakke van granzyme B, LAG-3 by diagnose en die grootte van die holtes was ook betekenisvol. Daar was geen betekenisvolle assosiasies toe sTNFRs of DR5 en die borskas x-straalgradering van tuberkulosesiekte nie.

Vroeë klassifikasie van vinnige en stadige reageerders op teentuberkulosebehandeling

Ná klassifikasie op grond van kruisstawing het diskriminant-analise (DA) en ondersteuningsvektormasjiene (SVM) van geselekteerde immuunparameters (sICAM-1 CRP, gransiem B, suPAR, sTNFRs, LAG-3 en CD3dim/CD56+ (% van CD45+)) gelei tot 'n 75% tot 100% korrekte klassifikasie van die vinnige reageerders met DA en 'n 82% tot 100% korrekte klassifikasie van stadige reageerders. Vir SVM het die korrekte klassifikasie van vinnige reageerders gewissel van 88% tot 100%, en vir stadige reageerders het dit gewissel van 95% tot 100%.

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Differensiële geenuitdrukking in vinnige en stadige reageerders op behandeling

In vergelyking met die vlak by diagnose is die uitdrukkingsvlak van IL-4 in die vinnige reageerders betekenisvol opgereguleer met ’n faktor van 9.2 teen die eerste week ná die aanvang van behandeling, in kontras met die stadige reageerders. Daar was geen verskille tussen die vinnige en die stadige reageerders met betrekking tot die uitdrukking van TGF-β, TGF-βRII, Foxp3 en GATA-3 nie.

Gevolgtrekking

Voorspellende modelle vir differensiële tuberkulose behandelingsresponse wat gasheerproteïene kombineer, hou belofte in en behoort in groter prospektiewe studies ingesluit te word om die mees geskikte merkers te vind vir insluiting in kliniese proewe van nuwe middels en vir implementasie in kliniese praktyk.

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Abbreviation

Ag: Antigen

AIDS: Acquired immune deficiency syndrome

APC: Antigen-presenting cell

BM: Bone marrow

CD: Cluster designation (cluster of differentiation)

c**DNA**: copy deoxyribonucleic acid

CFU: colony forming unit

CMI: Cell-mediated immunity

CR: Complement receptor

CRP: C-reactive protein

CSF: Colony-stimulating factor

CTL: Cytotoxic T lymphocyte

DC: Dendritic cell

DTH: Delayed-type hypersensitivity

DR5: Death receptor 5

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

FACS: Fluorescence-activated cell sorter

FITC: Fluorescein isothiocyanate (a fluorochrome)

FOXP3: Forkhead box P3

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

G-CSF- Granulocyte colony stimulating factor

GM-CSF: Granulocyte-monocyte colony stimulating factor GVHD Graft-versus-

HIV: Human immunodeficiency virus

HLA: Human leukocyte antigen (MHC)

HPRT1: Hypoxanthine phosphoribosyltransferase 1

HRP: horseradish peroxidase

ICAM: Intercellular adhesion molecule

IFN: Interferon

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Ig: Immunoglobulin (antibody molecule)

IL: Interleukin

IP-10: Interferon-inducible protein

K cell: Killer cell

KO: knock out

LAG-3: Lymphocyte activation gene-3

LPS: Lipopolysaccharide (endotoxin)

mAb: Monoclonal antibody

MAP: Multi-analyte profiling

MCP: Macrophage chemotactic protein

MHC: Major histocompatibility complex

MIP (1): Macrophage inhibitory protein

mRNA: messenger ribonucleic acid

M. tuberculosis*: Mycobacterium tuberculosis*

NK: Natural killer cell

PAGE: Polyacrylamide gel electrophoresis

PBMC: peripheral blood mononuclear cell

PCR: Polymerase chain reaction

qRT-**PCR**: quantitative reverse transcription polymerase chain reaction

TB: Tuberculosis

s: Soluble

SDS: Sodium dodecyl sulphate

TGF-β: Tumor growth factor-beta

TGF-βR: Tumor growth factor receptor-beta receptor

T cell: thymus-derived lymphocyte

Th: Helper T cell

TNF-α: Tumor necrosis factor-alpha

T-reg cell: T-regulatory cell

uPAR: Urukinase plasminogen activator receptor

uPO: acidic ribosomal protein

VEGF: Vascular endothelial growth factor

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CHAPTER 1: Tuberculosis: Burden, Infection, Immunology and Treatment

Declaration:

The information contained in this chapter was used in:

1. A review article: **Correlates for disease progression and prognosis during concurrent HIV/TB infection**

Joel Fleury Djoba Siawaya,* Morten Ruhwald, Jesper Eugen-Olsen, Gerhard Walzl. *International Journal of Infectious Diseases* (2007) 11, 2890-2899.

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1.1 Burden of tuberculosis

Mycobacterium tuberculosis bacilli (*M. tuberculosis*), an obligate, aerobe-transmitted bacterium, is known for its ability to enter a dormant state under adverse metabolic conditions and delay its multiplication for years, which has helped its survival in humans (1).

Worldwide, the growing epidemic of TB is alarming. An estimated one third of the world population is infected with *M. tuberculosis* and at risk of developing the disease. The lifetime risk of progressing to active TB when latently infected with TB is about 5% (1). This escalates to over 10% per annum when co-infected with HIV (2). In addition, the risk of mortality due to HIV/TB co-infection is twice that of HIV infection alone (3). With more than eight million people progressing to active tuberculosis every year, and a death rate of about 25%, pulmonary tuberculosis is one of the most life-threatening human diseases. More than 90% of global TB cases and deaths occur in the developing world (4). In sub-Saharan Africa, over 1.5 million tuberculosis cases are declared per year (5). Based on the 2007 World Health Organization report, South Africa has one of the worst TB rates in the world, with a TB incidence of 600 cases per 100 000 persons and 270 178 new cases each year (6).

Despite the availability of anti-tuberculosis chemotherapy, which have been available for over half a century, the tuberculosis infection rate is not yet under control and increases each year (7;8). Several factors are involved in the rise of tuberculosis: the ability of the bacteria to subvert the host immune pathway, inadequate TB management programmes, poor treatment adherence by patients and the emergence of drug-resistant *M. tuberculosis* strains. It has been predicted that if TB control is not improved, one billion people will be newly infected with TB by 2020, over 150 million people will become diseased and 36 million will die (9).

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1.2 Infection, immunology and pathology of tuberculosis

1.2.1 Infection and primary response

Tuberculosis (TB) is a contagious disease. The germs are propelled into the air when infectious individuals sneeze, cough or spit. Although the inhalation of a small number of these germs by a person is sufficient to be infected, the likelihood of *M. tuberculosis* transmission is increased by the number of bacilli inhaled, the frequency of exposure, and presumably the general nutritional and immune status or immune profile of the exposed subject (10;11).

M. tuberculosis enters the body through the respiratory tract and reaches the lungs, the initial site of infection. The primary defence against *M. tuberculosis* in the lower respiratory tract involves alveolar macrophages (12). Macrophages engulf *M. tuberculosis*, through complement, mannose or scavenger receptors in a vacuole called the phagosome (13). After bacilli uptake, the macrophages induce phagosome-lysosome fusion and acidification through IFN-γ (14) and Ca^{2+}-dependent signalling mechanisms (15). The subsequent reduction in the intra-phagosomal pH and the production of nitrogen and oxygen radicals (16;17) lead to growth inhibition and killing of bacilli. Parallel to this, infected macrophages induce cell-mediated and adaptive immunity (12;13;18-21). The development of cell-mediated immunity (CMI) occurs within 14 to 42 days of infection (12). CMI is characterised by granuloma formation by activated macrophages and lymphocytes (12;13;20). T-cell stimulation through cytokine- (such as IFN-γ and TNF-α) mediated mechanisms increases the anti-mycobacterial activity of macrophages and enhances their ability to control the infection (22).

Neutrophils, natural killer cells (NK) and dendritic cells have been shown to be actively involved in immunity to tuberculosis. Studies have shown that neutrophils provide defensins for macrophage-mediated killing and can even bring about killing of *M. tuberculosis*

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(12;23). NK cells can directly kill the pathogens or may lyse *M. tuberculosis*-infected cells by inducing apoptosis (programmed cell death) (12;24). It has been shown that macrophage apoptosis results in reduced viability of the mycobacterium (25).

1.2.2 Cell-mediated immune response

The protective immune response to *M. tuberculosis* relies on CMI (26;27). The development of CMI occurs within 14 to 42 days of infection (12). CMI is characterised by the mobilisation of activated macrophages and lymphocytes into lesions, leading to granuloma formation (12;20). Infected macrophages and dendritic cells (DCs) process and present mycobacterial peptides to T-cells. T-cells, through cytokine- (IFN-γ and TNF-α) mediated mechanisms, increase the anti-mycobacterial activity of the macrophages and enhances their ability to control the infection (26;27). Dendritic cells are seen as crucial in the production of an effective adaptive immune response due to their ability to carry antigens to lymphoid tissue, where the interaction with CD4 T-cells occurs (28). A few weeks post-infection, the number of activated CD4+ and CD8+ T-cells in the lung-draining lymph nodes increases and these cells display an effector/memory phenotype (CD44hiCD45loCD62L-); approximately half of these cells are CD69+, designated to interact with antigen-presenting cells (APCs) through the major histocompatibility complex (MHC) I and II. CD4+ T-cells are major effector cells in CMI against *M. tuberculosis*, and their principal function is to support the intracellular killing of *M. tuberculosis* in infected macrophages by secreting cytokines such as IFN-γ and TNF-α, which are crucial for macrophage activation (12;18;19). CD8+ T-cells are actively involved in killing *M. tuberculosis*-infected macrophages (29). However, CD8+ T-cells may also contribute to IFN-γ-dependent macrophage activation, leading to intracellular killing of *M. tuberculosis* by macrophages (30).

In most people, CMI controls, but fails to completely clear, the infection. The bacilli

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can remain alive within the granuloma, in a state of latency that can persist for years without causing the disease. Nevertheless, progression to disease can happen any time upon reactivation due to the weakening of the immune system (1;19;31).

1.2.3 Immunopathology: The door to immune evasion

A successful host response to an invading pathogen requires precise coordination of the immune players (32). The recognition of a pathogen by phagocytes induces cell activation and cytokine and chemokine secretion, which lead to the establishment a cytokine-chemokine network (32). Through a complex process of regulation and cross-regulation, this network influences the interplay of immune effectors. Cytokines are double edged sword any disequilibrium of their intricate balance may lead to adverse outcome. The magnitude and type of cytokines produced, may boost host protective mechanisms but can also lead to tissue injury, fever and cachexia (33;34).

M. tuberculosis possesses numerous immune evasion strategies. Initially, *M. tuberculosis* persistence depends on its ability to resist the antimicrobial activities of alveolar macrophages. One of the crucial evasion strategies used by the bacilli is the induction of a powerful inflammatory response, leading to immunopathology, or alternatively the establishment of a compromising anti-inflammatory response, driven by the need of the host to prevent immune-mediated damage. Either mechanism may be exploited by *M. tuberculosis* to promote its survival and its transmission to neighbouring non-activated macrophages (35;36). Other *M. tuberculosis* evasion strategies include prevention of macrophage apoptosis and interference with the response to IFN-γ and with phagosome development (18), modulation of antigen presentation (19;37) and modulation of the secretion of oxygen radical scavengers (38). In addition, infected antigen-presenting cells (APCs) contribute to defective T-cell proliferation and function through the production of immunosuppressive cytokines

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(TGF-β, IL-10) (19;39) and the activation of CD4+ CD25+ regulatory T-cells, which have been reported to display immunosuppressive properties (40-42).

1. 3 Directly observed short course anti-tuberculosis therapy, follow up, evaluation and identification of patients at risk for treatment failure and relapse

The directly observed short course therapy (DOTS) is a six-month regimen divided into two phases, the intensive phase and the continuation phase. The intensive phase consists of two months of five-times-a-week isoniazid (INH), ethambutol (EMB), rifampin (RIF) and pyrazinamide (PZA). The continuation phase consists of four months of five-times-a-week INH and RIF. However, the transfer of patients from the intensive phase to the continuation phase depends on the conversion of their sputum direct smears to negative (Essential Drugs Programme South Africa 2003 Edition). The International Union Against Tuberculosis and Lung Disease (IUATLD) advises that patients who failed to convert to sputum smear or culture negative by the end of the intensive phase of treatment (slow responders) remain on the intensive phase of treatment for another month whereas, patients with negative sputum smear or culture after two month of intensive therapy (fast responders) are started on the continuation phase regimen.

Treatment failure occurs in 1 to 6% of drug susceptible patient that completed DOTS (43;44). These patients are more susceptible to developing drug resistance and may then become vectors of drug resistant strain transmission (45;46). Relapse into active tuberculosis after initial cure occurs in 2 to 7% of patients with drug-susceptible isolates treated with contemporary 6 month anti-tuberculosis therapy (47). The risk of relapse may be increased by adverse reactions to therapy (47-49) and by the presence of residual cavitations on chest radiography (49-51) at the end of treatment.

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The intensive phase of treatment is crucial in prevention of treatment failure, relapse and emergence of drug resistance. The presences of cavities on chest x-ray radiograph at diagnosis and a positive sputum smear or culture at the end of the intensive phase of treatment were shown to characterize patients at high risk for treatment failure or relapse (52;53). Different mechanisms may be responsible for the delay of sputum smear or culture conversion in patients during DOT. Bacterial burden and extensive cavitary disease at diagnosis (46;52), drug malabsorption (54;55) and metabolism (56) during treatment have been the reported causes of the differential response observed in patients during anti-tuberculosis treatment. Therefore it will be particularly important to identify patients at risk for poor response or predict the intensive phase outcome as early as possible after initiation of treatment. This will allow the appropriate measures such as drug regimen adjustment to be taken early to minimize treatment failure, relapse and the emergence of drug resistance.

Continuous monitoring of bacterial activity by sputum culture during the course of anti-TB chemotherapy is very useful in the assessment of treatment efficacy. A parameter has emerged with important potential in the assessment of patient progress during treatment: time to detection (TTD), also known as time to positivity (TTP). TTP represents the time to detectable growth of *M. tuberculosis* in culture. Hanna et al. (57) were amongst the first to observe that TTP of *M. tuberculosis* increased in samples of patients receiving anti-TB therapy and that no change in TTP correlated with poor response during treatment. Further evidence of the potential use of TTP as an early indicator of treatment effectiveness comes from the study by Epstein et al. (58) that showed that TTP of M. tuberculosis in sputum culture correlates with response to anti-TB therapy.

However, the use of *M. tuberculosis* culture-based tests for the assessment of TB treatment response have important limitations as a substantial proportion of HIV positive TB cases have a negative sputum smear at diagnosis (59;60). Moreover, these tests can take

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several weeks to achieve results (61) and they are of no benefit in HIV patients where extra-pulmonary TB frequently occurs. Cultures-based tests are also expensive and are often not available in resource constraint settings. Thus the identification of affordable and simple tests for host correlates of TB treatment efficacy would be of great importance for clinical management and for clinical trials of urgently needed new antituberculous drugs.

1.4 Looking into the future of anti-tuberculosis therapy - from diagnosing the disease to predicting treatment outcomes

Definitions

- *Biomarker:* It is a objectively measured and evaluated characteristic that is used as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Wikipedia).
- *Surrogate marker:* A biomarker intended to substitute for a clinical endpoint. The measurement of surrogate markers provides a way to test the effectiveness of a treatment (Wikipedia).

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The International Union Against Tuberculosis and Lung Disease (IUATLD) currently recommends the week eight of anti-tuberculosis (TB) treatment sputum status as a surrogate marker for the evaluation of response to therapy for patients undergoing directly observed short course anti-TB chemotherapy (DOTS) (62). Implementation of the IUATLD recommendations has certainly improved TB care, although there are limitations to the current evaluation process, mainly because two months is too long a wait to establish a favourable or poor response to anti-TB treatment and make a clinical decision. During this two-month period, the mycobacterium may have time to develop resistance to anti-TB drugs (63). In addition, the overall duration of therapy has serious implications for patient adherence to therapy and places a serious strain on healthcare systems servicing developing countries, as

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they struggle to afford full implementation of the recommended treatment programme (six months of directly observed therapy). The process of drug development and validation is also affected by the current evaluation process, especially when monitoring clinical drug trials of new drugs, and the two-month delay wastes resources and time. Current literature suggests that it may be possible not only to identify patients who require longer than six-month TB treatment regimens in order to prevent recurrence, but, more importantly, to identify the majority of patients who would only require a shorter antibiotic treatment course (47;64). If we were able to stratify TB patients at the time of diagnosis or shortly after the start of treatment into risk groups for recurrence and into groups requiring different durations of treatment, TB programmes might be able to concentrate their efforts on ensuring strict adherence to short (three to four month) treatment regimens in the vast majority of patients and to reserve longer treatment options for those with a higher risk of recurrence. To improve the effectiveness of therapeutic interventions for TB, it may be necessary to take a more individualised treatment approach or at least an approach based on stratification of patients according to a risk scale for adverse treatment response (delayed response, failed treatment or recurrence after cure). Such an approach requires appropriate biomarkers that are measurable early during treatment. For all these reasons the search for surrogate markers that can provide primary measurements of treatment effectiveness and clinical prognosis would be important. Facilitating the development and validation of new therapeutic strategies (the right treatment for the right patients at the right time), minimising drug tolerance and resistance due to sub-optimal treatment, and accelerating or shortening clinical trials of new anti-TB drugs could result from the use of such markers. The interest in finding such biomarkers is growing, judged by the emphasis placed on biomarker research by the World Health Organization (WHO), the European and Developing Countries Clinical Trials Partnership (EDCTP), and the Bill and Melinda Gates Foundation (BMGF).

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In the context of drug development, early bactericidal activity (EBA), which quantitatively assesses changes in colony forming units (CFU) in sputum during a few days of therapy, has emerged as a potential surrogate marker for drug sterilising activity. However, as highlighted by Burman (65), EBA has not proved efficient in predicting or identifying bacterial sterilisation, an essential component of anti-tuberculous drug regimens offered by key drugs like rifampicin and PZA. Nevertheless EBA may still be useful for the evaluation of the spectrum of activity of new drugs (66;67) and for the comparison of anti-TB regimens (68). Other measures such as sputum *M. tuberculosis* messenger RNA (69;70), sputum and serum cytokine levels (71) were shown to be promising as markers for response to anti-tuberculosis treatment and mycobacterial clearance. However, some concerns have been raised regarding the use of host markers such as cytokines as biomarkers for the assessment of therapy. The use of prognostic or diagnostic biomarkers traditionally requires such markers to be specifically associated with the targeted disease. The immune response, however, has many redundant mechanisms for specific diseases and such mechanisms are also involved in responses to multiple types of pathogens. This makes host biomarkers sensitivity and specificity challenging. It therefore is generally accepted that the model validating the end point should be designed on a set of markers rather than a single marker. In TB, for example, Brahmbhatt *et al*. (72) and Veenstra *et al*. (73) recently showed that this concept holds promise. Furthermore, with advances in technology and knowledge of physiological and pathogenic features of TB we can now embark on the targeted investigation of markers in biological context to assess their prognostic power for anti-TB therapy efficacy and outcome. More prospective studies need to be done to validate the long list of potential biomarkers. There is a need to screen candidate surrogate markers in smaller groups of patients before engaging in a large-scale validation, as suggested by Gosling *et al*. (74) for clinical trials. These prospective studies should be comprehensively designed and include clinically well-defined patients (e.g. extent of disease, time of culture conversion and time to positivity (TTP)

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with different treatment outcomes (e.g. culture positive or negative after two month of therapy, cured, treatment failed and relapse) in order to adequately screen biomarkers associated with different end points.

Candidate sets of biomarkers associated with clinical end points of interest should be validated in large-scale studies before being implemented for clinical use. In clinical settings, validated biomarkers should be applied with care, as other possible aetiologies, such as different infections, need to be ruled out or taken into consideration. Although the TB-biomarker field is challenging, pursuing this research area must be prioritised so that the healthcare system can better assess patient therapy, so that tools for cost-effective evaluation of new drugs can be found and for improved control of the TB pandemic.

1.5 Present study: Biomarkers for TB treatment response: objectives, and rational.

1.5.1 Objectives

The ultimate outcome for which predictive markers are needed is relapse after initial cure. However, well-characterized patients with relapse and with available biological samples for biomarker testing are rare. Therefore, the primary aims of this work were:

- To investigate targeted bio-molecules as surrogate markers for the extent of disease at treatment onset.
- To investigate targeted bio-molecules as predictive markers of early response to anti-tuberculosis therapy as defined by week eight Ziehl-Neelsen sputum smear microscopy or Bactec culture status.

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Both the extent of radiological disease prior to treatment and the early response during treatment are known determinants of treatment failure and relapse. It was anticipated that

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markers may be found that will be suitable for validation in future large multi-sites studies prior to the development of tests for implementation in clinical management and in clinical trial settings.

1.5.2 Hypothesis

The magnitude of changes in host parameters associated with the antimycobacterial immune response during active *M. tuberculosis* infection and during the course of anti-tuberculosis therapy is indicative of host-pathogen interactions and could be used to predict eventual treatment outcome.

1.6 Candidate biomarkers

Soluble urokinase plasminogen activator receptor (suPAR)

Urokinase plasminogen activator receptor (uPAR) is a cell-surface molecule to which urokinase plasminogen activator (uPA) binds prior to activation. uPA and its receptor (uPAR) are involved in different physiological processes, including tissue remodelling and cell adhesion, migration and invasion (75;76). The prognostic power of suPAR has already been demonstrated in some diseases. Regarding human immunodeficiency virus, there is evidence that suPAR is a strong prognosis indicator of disease progression and patient survival (77). In cancer patients, suPAR has shown itself to be valuable in the assessment of prognosis and tumour recurrence (78). In tuberculosis, Eugen-Olsen *et al*. demonstrated that suPAR concentrations were elevated in TB patients and associated with mortality (79). They suggested the potential use of suPAR as a marker of treatment efficacy, a claim that needs to be investigated further.

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Intracellular adhesion molecule type 1(ICAM-1)

ICAM is involved in immune cell recruitment and the maintenance of granuloma structure in the host response to TB. López Ramírez et *al* (80) showed that *M. tuberculosis* infected monocytes had increased and sutained expression of ICAM-1. They suggested that *M. tuberculosis* induced ICAM-1 up-regulation promotes the induction and maintenance of cell mediated immune response needed to clear the infection. Soluble ICAM-1 has also been reported to be a sensitive marker of tuberculosis activity and anti-tuberculosis drug action in humans (80;81). Many studies have demonstrated that the sICAM-1 levels of TB patients were significantly lower after treatment compared to the levels before treatment, making it a candidate marker for the therapy efficacy (80-82).

C-reactive protein (CRP)

During tuberculosis, host clearance of the pathogen may be dependent on its capacity to efficiently activate macrophages to bind and ingest *M. tuberculosis*, a process in which CRP is actively involved. CRP is known to interact with cell-surface receptors, resulting in pathogen opsonisation and enhanced phagocytosis (83-85). In tuberculosis infection, CRP has been reported to increase significantly in patients with active tuberculosis and is linked to extensive disease (86-89). Furthermore, CRP was shown to be very sensitive to treatment, with its level normalising within days of therapy (86;89). This makes CRP a good candidate marker for early evaluation of treatment response.

Lymphocyte activation gene 3 (LAG-3/CD223)

LAG-3 is selectively expressed in activated T- and NK-cells and has been reported to negatively regulate their function (90;91). The LAG-3 gene was showed to act as an immune system regulator that keeps the immune response under control through regulatory T-cell

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action and prevents an excessive and detrimental response (92). In the absence of LAG-3, the ability of regulatory T-cells to control the action of effectors T-cells was inhibited (92). Lienhardt *et al*. showed that patients with favourable treatment outcomes had higher levels of sLAG-3 two to three months after the initiation of anti-tuberculosis treatment and at the end of treatment (93). Also, Triebel *et al*. reported that sLAG-3 may be a prognostic marker in some forms of breast cancer (94). In the light of these observations, sLAG-3 certainly qualifies as a potential candidate biomarker for treatment response.

Granzyme B

Apoptosis of infected cells is believed to play a role in controlling TB infection (24). However, *M. tuberculosis*-induced T-cell apoptosis has been shown to reduce *M. tuberculosis*-stimulated IFN-γ and IL-2 production (12). Furthermore, granzyme B was identified by Gondek *et al*. as one of the key factors in immunosuppression mediated by CD 4+ CD 25+ T-regulatory cells (24;40). One way or another, the evaluation of the apoptosis rate is important, as it carries useful information on a magnitude of host-pathogen interactions. Because granzyme B is a cofactor in the apoptosis process (95), its level may be used to assess apoptosis and it would be interesting to explore its potential as a surrogate marker for early treatment evaluation.

Tumour necrosis factor (TNF)-α and its receptors

TNF-α is a pro-inflammatory cytokine secreted by macrophages, dendritic cells and T-cells. This cytokine is required to contain *M. tuberculosis* by walling off infection through the induction of granuloma formation (12;13;96). A further contribution of TNF-α in the control of *M. tuberculosis* resides in its ability to induce apoptosis of infected cells by binding to their cell-surface receptors (TNFRI and II) (97). However, in excess TNF-α leads to severe tissue destruction (13;98). The release of sTNFRs is seen as a regulatory mechanism aimed to keep

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TNF-α activity under control and limit TNF-α–associated immunopathology (97;99). Conversely, however, the release of TNFRs, which results in the inactivation of TNF-α, may lead to immune evasion by *M. tuberculosis* (99).

Experiments conducted on *M. tuberculosis*-infected macrophages revealed that antimycobacterial therapy significantly reduces the percentage of cells producing TNF-α (100). In addition, investigating the characteristics and clinical value of serum changes of pro-inflammatory cytokines and their receptors in patients with pulmonary tuberculosis and during anti-tuberculosis treatment, Tang *et al*. found that the serum levels of TNF-α, and soluble tumour necrosis factor receptors (sTNFRs), were significantly higher in both active and inactive tuberculosis groups. After two months' anti-tuberculosis treatment, the serum levels of TNF-α and sTNFRs were significantly lower than before therapy in 15 cases out of 17 (101). Juffermans *et al*. (34) also reported the modulation of TNF-α and showed that, during TB treatment, levels of sTNFRI, and sTNFRII were higher in patients with active TB than in healthy contacts and that sTNFRs declined in the patients during treatment. These findings set up TNF-α and sTNFRs as interesting candidates markers of treatment response.

Death receptor (DR)-5

DR5 is a member of the TNF-related apoptosis-inducing ligand (TRAIL) receptor family. Contrary to TNF-α induced apoptosis, TRAIL-DR5-induced apoptosis is much less toxic (102). To date, no study has yet investigated DR5 in the context of TB. Thus, investigating DR5 secretion during active TB and anti-tuberculosis treatment will give additional insight into the host immune response to TB.

Interleukin-4 (IL-4)

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IL-4 is secreted mostly by Th2-cells and its role in human tuberculosis is not fully understood, although excess production of IL-4 during tuberculosis is associated with a depressed Th1 response and might be detrimental to the host (13;103). Studies have reported an association between the level of IL-4 expression and disease severity (103),(104). Controversially it has also been suggested that IL-4 might play a role in protective immunity against TB, as Sugawara et *al*. (105) demonstrated that *M. tuberculosis* infected IL-4 knockout mice had high *M. tuberculosis* colony-forming unit (CFU). Also, there is evidence that increased IL-4 secretion enhances TNF-α toxicity (103) and promotes TNF-α-mediated apoptosis in *M. tuberculosis*-activated lymphocytes (106).

The discovery of IL-4δ2, an IL-4 splice variant and IL-4 antagonist, brought new insight of immune regulation and problems with its assessment. Commonly used immunoassays for IL-4 do not differentiate between IL-4 and its splice variant and it is now clear that IL-4 levels have to be interpreted together with IL-4δ2 expression levels. Studies have revealed increased expression of IL-4δ2 in healthy contacts (107) or individuals with latent TB contrarily to patients with active TB who showed low expression of IL-4δ2 and increased IL-4 expression (108). Also it has been reported that TB patients had greater levels of mRNA for both cytokines when compared to healthy controls and that only IL-4δ2 level increased in parallel with IFN-γ after anti-tuberculous treatment (109), which make both IL-4 and IL-4δ2 candidate markers for the evaluation of patients response to therapy.

Transforming growth factor beta (TGF-β) and its receptors type two

TGF-β is highly pleiotropic and is known to affect a number of cells of the immune system (110). It has been shown that the combined production of IL-10 and TGF-β may act to down-modulate host protective immunity to *M. tuberculosis* (20;42;111;112). TGF-β was reported to be present in the granulomatous lesions of TB patients and secreted by human

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monocytes after stimulation with *M. tuberculosis* (12;20). This cytokine has important anti-inflammatory effects, including deactivation of macrophage production of oxygen and nitric radicals, T-cell inhibition and proliferation and down regulation of IFN-γ (20;42;112-114). TGF-β RI and RII co-expression is required for cellular responses to TGF-β and may act to down-modulate host immunity. It has also been shown that inflammation signals like LPS or IFN-γ induce a down-modulation of TGF-R expression that is accompanied by a diminished ability of the cells to respond to TGF-β (111;112).

GATA-3 transcription factor

The occurrence of a Th2-type response in TB infection has always been considered as one of the factors diminishing the effectiveness of Th1 cells against *M. tuberculosis*. Thus we feel it is important to include Th2-type response markers in the list of candidate surrogate markers. Because analysis of cytokine protein levels in TB patients usually makes it difficult to demonstrate the presence of a Th2 response, the expression level of mRNA for GATA-3, which is a Th2 cell-specific transcription factor, should be the best way to establish the occurrence of a Th2-type response in TB patients and its prognosis value. In the literature, the GATA-3 transcription factor was reported to control Th2-specific cytokine expression and to function as a negative regulator of the development of Th1 cells independently of is ability to up regulate Th2-type cytokines (115;116). Interestingly, Hirsch CS et al showed the degree of suppression decreased with the time on treatment (114).

Forkhead box P3 (FOXP3)

FOXP3 is implicated in the development and function of CD4 + CD25+ regulatory T-cells and can be used as a marker of its activity. CD4+ CD25+ regulatory T-cells have been reported to be implicated in immunosuppression (40). It would be interesting to investigate FOXP3 expression in TB patients and assess its informative value in the anti-tuberculosis

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treatment response.

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CHAPTER 2: Materials and Methods

2.1 Study design

2.1.1 Setting

The study was conducted in the Cape Town metropolitan area in South Africa, where the prevalence of new smears or cultures positive for TB was 313/100 000 population per year between 1993 and 1998 (117).

2.1.1.1 Study population

For this study, 12 healthy community controls and 29 HIV-negative newly diagnosed sputum smear and Bactec culture-positive patients, infected with drug susceptible *M. tuberculosis*, were selected. Nine were excluded for either non-compliance with treatment, multidrug-resistant TB, refusal of HIV test or incomplete follow-up visits. Postero-anterior and lateral chest X-rays were taken of all the patients at the beginning of chemotherapy and graded for extent of disease. In addition, the time to positivity (TTP) for each patient at each time point was known.

2.1.1.2 Eligibility and exclusion criteria

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a) Eligibility criteria

- Two samples Ziehl-Neelsen sputum -positive smear microscopy for *M. tuberculosis*
- Two samples one Ziehl-Neelsen sputum -positive smear microscopy and one Bactec culture positive for *M. tuberculosis*
- One sputum sample that was both smear positive and culture positive for *M. tuberculosis*
- One Ziehl-Neelsen sputum -positive smear microscopy, in conjunction with a chest radiograph typical of pulmonary tuberculosis

b) Exclusion criteria

- Previous TB disease
- Any other mycobacterial disease
- HIV infection
- Refusal of HIV testing
- Drug-resistant tuberculosis at diagnosis
- Incomplete follow-up or not documented visits

2.1.1.3 Chest X-ray grading

Standard postero-anterior and lateral chest radiographs were taken prior to therapy and were read by a pulmonologist who was unaware of patient clinical history and using a standard method (118). The radiological extent of disease was graded according to the total lung area opacity in relation to the size of the right upper lobe or the size of one entire lung. The grading included the following categories: i) alveolar disease less that the right upper lobe area, ii) equal to the right upper lobe, iii) more than the right upper lobe and iv) more than one whole lung. Disease was considered to be moderate if the total area of radiological

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involvement was less than or equal to the right upper lobe and severe if more than the right upper lobe was affected. The presence, number and size of cavities were also graded independently from the total alveolar involvement.

2.1.1.4 Treatment protocol

The patients received the six-month directly observed short course anti-tuberculosis therapy recommended by the South African National Tuberculosis Programme (based on WHO TB guidelines) and were subjected to strict adherence control throughout the treatment. The drug regimen consisted of a weight-related fixed combination of isoniazid (320 mg/day to 400 mg/day), rifampin (480 mg/day to 600 mg/day), ethambutol (800 mg/day to 1 200 mg/day) and pyrazinamide (1 000 mg/day to 1 250 mg/day) during the two months that constituted the intensive phase, followed by rifampicin and isoniazid during the four months of the continuation phase.

2.1.1.5 Monitoring of bacteriology and classification of patients into treatment response and outcome groups

Mycobacterial culture was done using the automated BACTEC 12B liquid radiometric method (Becton-Dickinson, MD, Bethesda, USA). BACTEC 12B vials were incubated at 37 °C and the growth index read daily at an identical time to limit reading variability. The TTP was recorded as the number of days required for each vial to reach a growth index of ≥10. Cultures were monitored for 60 days before being classified as negative. Mycobacterial drug susceptibility testing for resistance to first- and second line drugs was done using the BACTEC method (ref: BACTEC 460TB Systems product and procedure manual, Becton-Dickinson Diagnostic Instrument Systems, Sparks, Md., 1996) at diagnosis and at the end of therapy. Direct sputum smear microscopy was performed using the Ziehl-Neelsen method.

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Sputum smear ZN stains and BACTEC cultures were done on the first and the third day of treatment and at week one, two, four, eight, thirteen and 26 after the initiation of chemotherapy. Patients were classified into fast or slow responders to chemotherapy based on their BACTEC culture status at week eight (culture positive/culture negative) after being started on treatment.

2.1.2 Selection of patients

The study finally included 12 healthy community controls and 20 HIV-negative active TB patients (11 males and nine females from 18 to 51 years old) with positive sputum smear and culture at diagnosis. After the intensive phase of chemotherapy (two months), Bactec sputum culture identified eight fast responders and 12 slow responders.

2.2 Evaluation of cytokine multiplex fluorescent bead-based immunoassays as screening tool for the search of biomarkers

The evaluation of three commercially available cytokines multiplex assays was carried out to establish their suitability for biomarkers screening and limits.

2.2.1 Definitions

Recovery: Ratio of the observed amount of cytokine compared to the expected known amount of cytokine in a sample, expressed as a percentage. An acceptable recovery falls within the range of 70 – 130 % *(Bio-Rad Principles of Curve Fitting for Multiplex Sandwich Immunoassays, Rev B*).

The following formula was used to calculate recovery:

$$\frac{\text{(Observed Concentration in spiked sample – Observed Concentration in unspiked sample) X 100}}{\text{Expected concentration (amount of recombinant cytokine used to spike sample)}}$$

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Linearity: Ratio of the observed amount of cytokine in diluted sample compared to the total amount of cytokine in undiluted sample, multiplied by the coefficient of dilution and expressed in percentage.

The following formula was used to calculate linearity:

$$\frac{\text{(Observed Concentration in diluted Sample) X (Coefficient of dilution) X 100}}{\text{Total Concentration of cytokine in undiluted sample}}$$

Recovery and linearity definitions and calculations were obtained from the RnD-Systems spike and recovery immunoassay sample validation protocol.

Reading: Reported fluorescence of the sample

Positive reading: Reported fluorescence of a sample that is above background fluorescence and corresponds to a positive concentration.

RP1: RP1 represents the fluorescence channel used for assay quantification. Low RP1 is the fluorescent channel recommended for quantification of a wide range of cytokines with a wide dynamic range of concentrations; whereas high RP1 is recommended for quantification of low concentrations of cytokines as it provides greater sensitivity.

5 PL-(parameters logistic) Regression Curve: A standard curve build upon a five parameters logistic equation and that corrects for asymmetry in the curve shape.

2.2.2 Methodology

This study followed an integrated methodology, comparing 3 commercially available multi-plex Luminex kits (Bio-Rad's Cytokine 17-plex kit; Linco Inc's 29-plex kit; and RnD

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System's Fluorokine-Multi Analytes Profiling (MAP) kit as well as the RnD Systems IFN-γ Quantikine ELISA kit. We used the following two approaches: **1)** Measurement of recombinant cytokines in serum and in unstimulated whole blood supernatant samples, each spiked with serial dilutions of the multiplex standard provided with the Luminex kits in order to calculate the recovery (accuracy) of the assay for each of the different cytokines; **2)** Measurement of native induced IFN-γ *in vitro* in whole blood supernatant and peripheral blood mononuclear cell (PBMC) culture supernatants where whole blood supernatant and PBMCs were stimulated with *Mycobacterium tuberculosis (M. tuberculosis)* antigens or Bacille Calmette Guerin (BCG). The following three studies were done:

Study 1: Comparison of the recovery of IFN-γ levels in serum and whole blood supernatant between Bio-Rad cytokine assays versus the RnD Systems Quantikine IFN-γ ELISA

Study 2: Bio-Rad 17-plex cytokine assay recovery study in whole blood supernatant

Study 3: Bio-Rad 17-plex, Linco 29-plex and RnD Systems fluorokine-(MAP) assay comparison study.

Studies 1 and 2 and the first part of study 3 made use of spiked (therefore known concentrations of recombinant cytokines) samples, with known concentrations of recombinant cytokines, to compare recovery and accuracy between the different kits. The second part of study 3 assesses not only the recovery of different kits but also the ability of the different Luminex kits to measure the concentration of IFN-γ in unmanipulated (unspiked) samples where IFN-γ levels were measured by ELISA as a gold standard test**.**

2.2.3 Study 1: Comparison of the Bio-plex (Bio-Rad) cytokine assay versus the RnD Systems Quantikine IFN-γ ELISA

Sample preparation

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The following samples were included:

1. Unspiked and spiked serum from a healthy laboratory donor diluted one in four with sample diluent as recommended by the manufacturer.
2. Unspiked and spiked unstimulated supernatant from a six-day whole blood culture assay diluted one in 10 in RPMI-1640 (GIBCO).
3. Unspiked and spiked unstimulated culture supernatant from a seven-day PBMC culture assay where PBMCs were resuspended at 1 x 10^6 cells/ml in RPMI-1640 (GIBCO) and 10 % AB serum (Sigma Aldrich).
4. Unspiked culture supernatant from whole blood and PBMC assays, as described above, stimulated with live BCG (SSI 241103 Statens Serum Institute, Denmark) for seven days.

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Controls included:

1. Standard diluent + RPMI-1640 (1:1) spiked with recombinant IFN-γ from the RnD Quantikine kit standard (control 1) and
2. Sample diluent + RPMI-1640 (1:1) spiked with recombinant IFN-γ from the RnD Quantikine kit standard (control 2)

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Spiking of samples and controls was performed at a concentration of 500 pg/ml with recombinant cytokine from the RnD IFN-γ Quantikine kit.

Bio-Rad human 17-plex assay (Study 1)

A Bio-Rad human cytokine 17-plex kit (cat# 171A11171) was carried out as per the manufacturer's instructions. Briefly, a standard vial from the Bio-Rad human cytokine 17-plex kit containing 500 000 pg of lyophilised recombinant multiplex standard (standard control # CO2722) was reconstituted in 0.5 ml of Bio-Rad standard diluent (cat # 171304000) to 250

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000 pg/ml. The reconstituted standard was further diluted one in ten to 25 000 pg/ml, and then serially diluted one in four to produce a nine-point standard curve ranging from 0.5 pg/ml to 25 000 pg/ml, either in matrix (i) kit standard diluent alone for the measurement of cytokines in serum (Standard curve 1), or in matrix (ii) kit standard diluent mixed 1:1 with RPMI-1640 (Sigma Aldrich cat # R0883) for the measurement of cytokines in culture supernatants (standard curve 2). The 25 000 pg/ml standard in matrix (i) (standard diluent alone) was also used to spike the samples and controls, as described below. The standard curve was run in duplicate, while samples and controls were run in singlet. A 50 µl volume of each sample, control or standard was added to a 96 well plate (provided with the kit) containing 50 µl of antibody coated fluorescent beads. Biotinylated secondary and streptavidin-PE antibodies were added to the plate with alternate incubation and washing steps. After the last wash step, 125 µl of wash buffer was added to the wells, the plate was incubated and subsequently read on the Bio-plex array reader, using a 5 PL regression curve to plot the standard curve. Samples and controls were read at both a low RP1 target setting (used to maximize assay sensitivity when the expected concentrations are below 3 200 pg/ml) and a high RP1 target setting (used for broad range concentrations) on the Bio-plex suspension array using a high throughput fluidics (HTF) system (cat# 171000005). Data was subsequently analysed using the Bio-plex manager software, version 3.

RnD Systems Quantikine IFN-γ ELISA (study 1)

The ELISA was done using the RnD Systems IFN-γ Quantikine ELISA kit (cat# DIF50) as per the manufacturer's instructions. Briefly, lyophilised Quantikine standard was reconstituted in distilled water and serially diluted one in two in kit standard diluent to produce a seven-point standard curve ranging from 15.6 pg/ml to 1000 pg/ml. Thereafter, 100 µl of assay diluent was added to designated wells in a 96-well polystyrene microplate (provided with the kit) coated with polyclonal antibody against IFN-γ, followed immediately

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by 100 µl of standard, sample or control. The standard curve, samples and controls were run in duplicate. The plate was incubated for two hours at room temperature, washed and thereafter 200 µl of horseradish peroxydase (HRP)-conjugated IFN-γ antibody followed by 200 µl of substrate solution was added to the wells, followed by another incubation period and washing step between the two additions. After 30 minutes of incubation, 50 µl of stop solution was added to the wells and the plate read at 450 nm, with the wavelength correction set at 570 nm, on a multi-detection microplate reader (Bio-Tek instruments Inc, part # 7081000). Sample concentrations were determined using the KC4 microplate data analysis software, version 3.34, revision 12.

2.2.4 Study 2: Bio-Rad human 17-plex assay recovery study

Bio-Rad human 17-plex assay (Study 2)

The Bio-Rad human cytokine 17-plex assay was carried out as per the manufacturer's instructions, with a few exceptions as stipulated below. The standard vial was reconstituted in unstimulated whole blood assay supernatant (one in ten whole blood with RPMI-1640 (GIBCO), incubated at 37˚C, 5% CO_2 for six days) and not in the standard diluent, as recommended by the manufacturer, This was done in order to ensure that the matrix used in the generation of the standard curve resembled that of the samples as closely as possible. Briefly, a nine-point standard curve was generated by performing serial dilutions of the reconstituted standard (lot # 5004060). In order to assess recovery, supernatant samples SN1, SN2 and SN3 were spiked at three different concentrations with recombinant cytokine using the Bio-plex kit standard. Samples were run in duplicate. In order to keep the matrix of the spiked samples as similar as possible to the matrix of the standard curve, the volume of reconstituted standard used to spike the samples in all experiments was kept to a minimum of 10 µl to minimize pippeting error.

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2.2.5 Study 3: Bio-plex, Linco-plex and RnD Systems fluorokine-(MAP) assay comparison study

Bio-Rad human 17-plex assay (Study 3)

The standard curve was generated and the assay conducted as described above in the section on the *Bio-Rad human 17-plex assay study 2*. Two sets of samples were used. The first set was generated using whole blood from 5 healthy laboratory donor diluted one in ten with RPMI-1640 with glutamax and stimulated with different *Mycobacterium tuberculosis* derived antigens, (generously donated by Tom Ottenhoff, Leiden University. See table 3.3 in the result section for the antigens names), and a phytohaemagglutinin (PHA)-stimulated positive control. Unstimulated culture supernatant served as a negative control. The second set of samples was generated from unstimulated whole blood culture as described above. Supernatants were harvested on day seven and spiked at five different concentrations with recombinant cytokine from the Bio-Rad standard (lot # 5004060, table 2.1), the results of which were used to calculate recovery. The assay was conducted and plate read as described before.

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Table 2.1: Expected concentrations of cytokines in the spiked supernatant samples (pg/ml) using the Bio-Rad assay. Whole blood supernatant samples were assessed for endogenous cytokine levels and spiked with known concentrations of cytokines using the Bio-Rad standard containing recombinant cytokines for the 17 different cytokines in the kit. The table represents the expected concentration after subtraction of the endogenous levels of cytokines

Spiked concentrations for cytokines in the Bio-Rad 17-plex kit									
	IFN–γ	**TNF–α**	**IL-1β**	**IL-2**	**IL-4**	**IL-5**	**IL-6**	**IL-7**	**IL-8**
Spike 1	2945.6	2100	4893.8	2754.2	1086.6	8061.7	5348	8532	5000
Spike 2	406.8	350	978.8	550.8	217.3	1612.3	1069.6	1706	1000
Spike 3	268.2	175	489.4	275.4	108.7	806	534.8	853	500
Spike 4	35.6	87.5	48.9	27.5	10.9	80.6	53.5	85	50
Spike 5	14.6	43.8	9.8	2.8	2.2	16.1	10.7	17	10
	IL-10	**IL-12p70**	**IL-13**	**IL-17**	**MCP1**	**MIP-1β**	**G-CSF**	**GM-CSF**	
Spike 1	7516.6	7497.2	1191.4	4321.3	5348	3963.4	3963.4	3963.4	
Spike 2	1503.3	1499.4	238.3	864.3	1069.6	792.7	792.7	792.7	
Spike 3	751.7	749.7	119.1	432.1	534.8	396.3	396.3	396.3	
Spike 4	75.2	75	11.9	43.2	53.5	39.6	39.6	39.6	
Spike 5	15	15	2.4	8.6	10.7	7.9	7.9	7.9	

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Linco human 29-plex assay (study 3)

A human 29-plex Linco Inc assay (cat no HCYTO -60-K-PMX29) was done according to manufacturer's instructions. Briefly, a standard curve ranging from 3.2 pg/ml to 10 000 pg/ml was generated by serial dilution of reconstituted standard. Two sets of samples were used, as described earlier, with the exception that the Linco Inc standard (provided with the kit) was used to spike unstimulated whole blood culture at final concentrations of 5000, 1000, 500, 50 and 10 pg/ml. The filter plate was blocked by pipetting 200 µl of assay buffer into each well. After 10 minutes the assay buffer was discarded by vacuum aspiration and 25 µl of assay diluent was added to the wells designated for the samples, while 25 µl of RPMI-1640 with Glutamax (GIBCO) was added to the wells designated for standards. According to the plate layout, 25 µl of either standard or sample was then added to the appropriate wells after which 25 µl of antibody coated fluorescent beads was added. Biotinylated secondary (detection) and Streptavidin-PE-labelled antibodies were then added to the plate respectively, with alternate incubation and washing steps. Finally 100 µl of sheath fluid was added to the wells and the plate read immediately on the Bio-plex array reader, at high and low RP1 targets, using a 5 PL regression curve.

RnD Systems fluorokine-(MAP) assay using human Base kits A and B (study 3)

The assay was done according to the manufacturer's instructions. Briefly, the standard curves for the RnD System fluorokine-(MAP) human base kits A (cat # LUH000) and B (cat # LUH001) were generated by reconstitution in standard diluent provided with the kit. Samples included the same set of antigen-stimulated whole blood culture supernatants used for the Bio-plex and LINCO-plex assays described earlier, as well as serum (diluted one in four) and whole blood supernatant spiked at six different concentrations with recombinant cytokine from the RnD System's standard (Part # 895531, lot # 238222 and Part # 895546, lot #

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238223 [base kit A] and Part # 892794, lot # 233020 [base kit B], table 2.2). An eight-point standard curve, with each cytokine spanning its own unique specific range, was generated and 50 µl of each standard and sample were added to a 96-well plate containing fluorescent antibody coated beads. After alternate incubation and washing steps, detection and PE-labelled secondary antibodies were added and the plate read on the Bio-plex array reader, at a low RP1 target, using a 5 PL regression curve.

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Table 2.2: Expected Concentrations of Cytokines in the spiked supernatant and serum samples (pg/ml) using the RnD assay. Whole blood supernatant and serum samples from a healthy control were assessed for endogenous cytokine levels and spiked with known concentration of cytokines using recombinant cytokine from the RnD Systems fluorokine-(MAP) 13-plex standard containing all 13 cytokines. The table represents the expected concentration after subtraction of the endogenous levels of cytokines

Spiked concentrations for cytokines in the RnD Systems MAP Base Kit A							
	IFN- γ	**TNF-α**	**IL-1α**	**IL-1rα**	**IL-4**	**IL-6**	**IL-8**
Spike 1	950	1400	1200	2650	1200	1750	1800
Spike 2	475	700	600	1325	600	875	900
Spike 3	237.5	350	300	662.5	300	437.5	450
Spike 4	118.8	175	150	331.3	150	218.8	225
Spike 5	59.4	87.5	75	165.6	75	109.4	112.5
Spike 6	14.8	21.87	18.75	41.4	18.75	27.34	28.12

Spiked concentrations for cytokines in the RnD Systems MAP Base Kit A				Spiked concentrations for cytokines in the RnD Systems MAP Base Kit B	
	IL-10	**MCP1**	**MIP-1α**	**MIP-1β**	**IL-12p70**	**IP 10**
Spike 1	1300	1000	4500	2100	19000	1400
Spike 2	650	500	2250	1050	9500	700
Spike 3	325	250	1125	525	4750	350
Spike 4	162.5	125	562.5	262.5	2375	175
Spike 5	81.3	62.5	281.3	131.3	1187.5	87.5
Spike 6	20.31	15.62	70.3	32.81	593.8	21.87

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RnD Systems Quantikine ELISA (study 3)

The same *M. tuberculosis* derived antigens- and PHA-stimulated samples used for the Bio-Rad human 17-plex assay (study 3) and Linco human 29-plex assay were also assessed by IFN-γ ELISA. The assay was performed as described for the RnD-system Quantikine ELISA test 1 as per manuacturer's instructions.

Statistics: Bio-plex, Linco-plex and RnD Systems fluorokine-(MAP) assay comparison (Study 3)

The correlation between the concentrations of cytokines as measured by the different Immunoassays was assessed by the mean of intra-class correlation coefficients and the Pearson product-moment correlation coefficient. The analysis was done using STATISTICA (version 7).

2.3 Soluble biomarker analysis

2.3.1 Serum integrity testing

Blood samples were taken from all the patients before they were started on anti-tuberculosis chemotherapy (diagnosis) and at week 1 in month 1, 2, 3 and 6 after the initiation of chemotherapy. Serum was separated by centrifugation (2500 rpm at 4^{o}C for 7 min) and aliquots of 500 µl stored at -80^{o}C until the day of experiment. The samples were collected between – give dates- and assays performed in –give month and year. Prior analysis, the serum integrity was verified by running the samples on a polyacrylamide one-dimension (1D) and two-dimension (2D) gels to confirm that proteins degradation had not occurred.

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2.3.2 Enzyme-linked immunoassay (ELISA)

Using ELISA kits from different manufacturers, serum samples were analysed for suPAR (Virogate), sICAM-1 (RnD Systems), CRP (Bender MedSytems), LAG-3 (Apotech) and Granzyme B (Bender MedSytems). Assays were conducted according to the manufacturers' instructions. Briefly, the method entails that coating antibody (primary antibody) against the target protein is adsorbed onto microwell plates. The target protein present in the sample binds to antibodies adsorbed to the microwells, and a biotin-conjugated secondary antibody specific for the target protein is added and binds to the target protein captured by the primary antibody and incubated. After incubation, the unbound biotin-conjugated secondary antibody is removed in a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated antibody. After incubation, unbound Streptavidin-HRP is removed during a wash step and substrate solution reactive with HRP is added to the wells. A coloured product is formed in proportion to the amount of target protein present in the sample. The reaction is terminated by adding acid and absorbance is measured at 450 nm, with wavelength correction set at 65 nm. The target protein concentration in the samples is determined by plotting their absorption at 450 nm against a standard curve prepared from serial dilutions of the recombinant protein of interest.

2.3.3 Multi-analyte profiling (MAP) assays

The human death receptors 3-plex (Biosource) and the Cytokines/Chemokines 30-plex (LINCO Research Millipore) kits were used for the analysis of soluble TNFRI, TNFRII and DR5 levels in a biological context. Assays were conducted according to the manufacturers' instructions. Each bead set was coated with capture antibody specific for one analyte. The target protein in the sample binds to the bead-coated antibody. Captured analyte is detected using a biotinylated detection antibody and streptavidin-(PE) phycoerythrin and read on the

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Bio-plex analyser (Bio-Rad), which is a dual laser, flow-based sorting and detection platform. One laser is bead-specific and determines which cytokine is being detected. The other laser determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound.

2.4. Immunophenotyping by flow cytometry

The immunophenotyping by flow cytometry was performed by Veenstra Hanne from our group. This data was used in an attempt to improve the power of the predictive models as it was conducted on the same cases as the soluble marker investigations.

Reagents

- Fluochrome-labelled monoclonal antibodies (MAb) CD45-PerCP, CD3-PE, CD4-FITC, CD8-FITC, CD19-FITC, CD56-FITC, and γδTCR-FITC (BD-Pharmingen),
- Vα24-PE (Beckman Coulter)
- Saponin (Sigma)
- Polyethylene glycol 4000 (PEG) (Merck)

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Sodium heparin anti-coagulated whole blood with was washed with phosphate-buffered saline (PBS), suspended in 100µL of 0.1% bovine serum albumin (BSA), 0.05% sodium azide in PBS and added to the required antibody mixtures. Cells were washed after 20 minutes at 4°C and red blood cells (RBCs) lysed using cold PBS containing 0.05% saponin, 0.05% sodium azide and 3% PEG. Following centrifugation at 700xg the cell pellets were fixed in 4% formaldehyde in PBS. Samples were analysed using the Becton-Dickinson FACS Calibur and CellQuest software. Lymphocytes were gated in a CD45-PerCP versus Side Scatter plot (10 000 events in this gate were acquired) and these were further analyzed for expression of CD3 and CD4 (or CD8, CD19, CD56, γδTCR) in the FL1 and FL2 channels respectively. The lymphocyte sums calculated were all between 95 and 100% *(Methods from* 'THE

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INVESTIGATION OF PERIPHERAL BLOOD CELLULAR IMMUNE RESPONSES DURING INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS', PhD thesis by Veenstra H, 2006).*

2.5 Gene expression analysis

2.5.1 Messenger ribonucleic acid (mRNA) extraction and integrity test

One volume (5 ml) of freshly drawn blood (at diagnosis and week 1 in month 1, 2 and 6) was added to ten volumes of guanidinium isothiocyanate and triton (GICT)-stabilisation reagent (Roche) to achieve cell lysis and stabilisation of the nucleic acids. Nucleic acid-stabilised blood samples were stored at -80^{o}C until the extraction of mRNA

RNA was isolated from *ex vivo* blood samples using the mRNA isolation kits for blood and bone marrow (Roche). The isolation was done according to the manufacturer's instructions. Magnetic glass particles (MGPs) were added to blood lysate and bound to total nucleic acids during incubation. Following incubation, MGPs were separated by centrifugation (1100-x g) and unbound elements were removed (by wash). Nucleic acids were eluted by incubation in MGP elution buffer, separated from MGPs (using a magnetic separator) and transferred into fresh tubes. RNA was then isolated from the total nucleic acids by means of biotin-labelled oligo (dT) and streptavidin-coated magnetic particles (SMPs). SMPs were separated using a magnetic separator and unbound elements were washed out. RNA was eluted in nuclease-free water (Ambion) and concentrated using sodium acetate and ethanol. After centrifugation and an ethanol wash in RNase inhibitor superase (Ambion), the pellet was resuspended in "RNA secure" (Ambion), which is an RNA suspension solution. RNA purity and concentration were assessed using the nanospectrophotometer. RNA integrity was verified by reverse transcription of extracted mRNA was into clonal deoxyribonucleic

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acid (cDNA) and amplification of the β-actin gene (Ambion RT-PCR kit). The PCR products were run on a 1% agarose gel.

2.5.2 Reverse transcription of mRNA into clones of deoxyribonucleic acid (cDNA)

Reverse transcription was carried out with the reverse transcription kit (QIAGEN) according to the supplier's specifications. RNA samples were first incubated in genomic (g)-wipe-out buffer for two minute at 42^{o}C. After gDNA elimination, RNA was reverse transcribed using reverse transcriptase, RT buffer and RT primers provided with the kit. Reactions took place at 42 ^{o}C and were inactivated at 95 ^{o}C. cDNA purity and concentration were assessed using a nanospectrophotometer.

2.5.3 Quantitative real-time PCR

The expression of genes of interest was quantified relatively using the Light Cycler (Roche) and the Quantitec SYBER green polymerase chain reaction (PCR) kit (QIAGEN). PCRs were conducted according to the manufacturer's instructions. The targeted genes were IL-4, IL-4δ2, TGF-β, TGF-βRII, Foxp3 and GATA-3. Primers used for the amplification of IL-4* (both IL-4 alternative transcripts) TGF-β, TGF-βRII, Foxp3 and GATA-3 were manufactured by QIAGEN. Because QIAGEN IL-4* primers did not differentiate between IL-4 and IL-4 splice variant (IL-4δ2) specifically design IL-4 and IL-4δ2 primers were used in an additional experiment (119). The sequences of primers used for the amplification of target and house keeping genes are in table 2.3. Some manufacturer could not disclose the sequences of primers we used to amplify our target genes. Therefore, for these genes, instead of the primers sequences the reference sequence identification (RefSeqs-ID) are shown in table 2.3. The housekeeping genes used as reference genes for the normalisation of data included β-actin (QIAGEN primers), GAPDH (Integrated DNA Technology (IDT) primers), HPRT1 (IDT primers) and uPO (IDT primers) (table 2.3 for the primers sequences). Although validated

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primers were used, the amplification efficiency of each primer pair was assessed. This was done through a titration series of amplicon from a patient. PCRs consisted of a 20 µl reaction including, 10 µl of SYBER green master mix, 2 µl (0.1 µg) of cDNA, 2 µl (05 µM) of primers and 6 µl of RNase-free water. Data were analysed using the REST 2005 program from Corbett Life Science.

Table 2.3: Sequences of primers used for the amplification of target and house keeping genes. When the primers sequences could not be disclosed by the manufacture the code of target genes they amplified were used instead in the table.

Targeted genes Primers sequences or Reference sequences ID	
IL-4*	NM_000589, NM_172348
IL-4	**Forward**: 5'-CGA GTT GAC CGT AAC AGA CAT-3'; **Reverse**:5' CGT CTT TAG CCT TTC CAA GAA G-3'
IL-4δ2	**Forward**: 5'-CAG AGC AGA AGA ACA CAA CTG-3'; **Reverse**: 5'-GTC TTT AGC CTT TCC AAG AAG-3'
TGF–β, and	NM_000660
TGF-βRII,	NM_003242
Foxp3	NM_014009
GATA-3	NM_001002295, NM_002051
House keeping Genes	
β-actin	NM_001101
GAPDH (1)	**Forward**: 5'-ACC CAC TCC TCC ACC TTT G -3'; **Reverse**: 5'-CTC TTG TGC TCT TGC TGG G -3'
GAPDH (2)	**Forward**: 5'-TGA AGG TCG GTG TGA ACG GAT TTG GC -3'; **Reverse**: 5'-CAT GTA GGC CAT GAG GTC CAC CAC -3'
HPRT1	**Forward**: 5'-TGA CAC TGG CAA AAC AATGCA-3'; **Reverse**: 5'-GGT CCT TTT CAC CAG CAA GCT-3'
uPO	**Forward**: 5'-GCA ATG TTG CCA GTG TCT GT-3'; **Reverse**: 5'-GCC TTG ACC TTT TCA GCA AG-3'

(*) = Primers used amplified both IL-4 and IL4δ2

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2.6 Statistical analysis

The statistical analysis of the results in this thesis was done in collaboration with the Stellenbosch University Centre for Statistical Consultation.

2.6.1 Analysis on soluble biomarker

All on the soluble markers were done using STATISTICA software version 7. Statistical analysis of the immune response between and within groups (slow responders and fast responders) at diagnosis, week one, week five, month two and at the end of chemotherapy (month six) was done using repeated measures of analysis of variance. The difference between the patients and the controls was analysed using one-way ANOVA. The association between the immune parameters and the extent of disease was assessed using the Mann Whitney U-test. A p-value of $p < 0.05$ was judged significant. The predictive power of selected immune markers for sputum culture status at month two was assessed by general discriminant analysis (GDA) and support vector machines (SVM).

2.6.2 Analysis on gene expression

The differences in targeted mRNA expression between groups and between diagnosis and week one after initiation of treatment were analysed using REST 2005 software. REST 2005 is a strong randomisation test that determines whether there is a significant difference between two groups. The program takes into account reaction efficiency and normalisation. The program performs 50,000 random reallocations of samples between the two groups, and it is based on the principle that: "If any perceived variation between the groups is due only to chance, then it will be possible to randomly swap values between the two groups and not see a

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greater difference than what was initially seen between the groups" (**adapted from REST 2005 manual**).

2.7 Ethics

All patients involved in the study received and signed a consent form. The study was approved by the local health committees representing the people in the areas and the Ethics Committee of the Faculty of Health Sciences, Stellenbosch University, the Ethics Committee of Tygerberg Hospital and the Head of Health, City of Cape Town.

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CHAPTER 3: Evaluation of Cytokine Multiplex Fluorescent Bead-based Immunoassays as a Screening Tool for the Search of Biomarkers

3.1 Introduction

Cytokines are important modulators of immune response pathways (120-122). Cytokine expression profiling (CEP) has become a popular and established method for the identification and characterisation of disease-associated immune responses (123-126). Previously, CEP was a laborious process requiring substantial sample volumes when multiple cytokines were under investigation. However, CEP methodology has been revolutionised by the recent introduction of Luminex technology, a bead based assays allowing the measurement of up to 100 different analytes in a 50 µl volume sample (127).

The reduced sample volume and time-saving advantages of the Luminex system have made it an attractive method for large-scale cross-sectional, association or cohort studies which investigate the host immune response (128-131). Khan *et al*. (132) have comparatively assessed multiplex kits from LINCO Research, Bio-Rad Laboratories, RnD Systems and Biosource International and compared them to an enzyme-linked immunosorbant assay (ELISA). The comparison was based on the measurement of a sample of five cytokines (serum samples from healthy individuals intravenously injected with endotoxin). They

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reported that the cytokine concentrations, as measured by the different kits, showed similar trends, although the absolute concentrations measured were different.

There are also a number of reports validating Luminex systems. These studies often use the approach of a single sample type, focusing on either serum (132;133), plasma (134;135) or culture supernatants (136;137). However, the number of independent published articles comparing the performance of different Luminex kits across different sample types is limited (138), as limited is the number of cytokines evaluated by the kits.

The present study not only has the advantage of combining a head-to-head comparison of different kits and assays on their ability to measure cytokine levels in different types of sample, but is also the first independent study to comparatively assess the recovery of each cytokine by the commercially available Bio-Rad 17-plex, LINCO 29-plex and RnD Fluorokine-(MAP) base kits A and B (13 cytokines total) with reference to instrument settings and calibration.

3.2 Results

3.2.1 Bio-Rad human 17-plex assay vs. RnD Quantikine IFN-γ ELISA (study 1)

The comparative assessment of IFN-γ ELISA and IFN-γ measurements from the Bio-Rad human 17-plex kit was done using serum and PBMC supernatant samples unspiked and spiked at 500 pg/ml (table 3.1). Overall, the ELISA showed better recoveries than Bio-Rad's human 17-plex. No IFN-γ was detected by either the Bio-Rad assay or the ELISA in the unspiked, unstimulated supernatant. The baseline level of IFN-γ in unspiked serum, as measured by the ELISA, was nil, whereas the Bio-Rad showed a reading of 257 pg/ml. The spiked control recoveries for the ELISA were 117% (control 1) and 116% (control 2) compared to 55.9% (control 1) and 31.17 % (control 2) for the Bio-Rad human 17-plex assay.

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The recoveries of the spiked serum samples were 119.6 % for the ELISA and 30.4 % for Bio-Rad human 17-plex. IFN-γ spiked whole blood supernatant, as measured by the ELISA, showed a recovery of 121.8%, whereas the Bio-Rad kit recovery was 45.7.5%. The linearity of both assays was assessed by measuring the concentration of IFN-γ in undiluted and diluted BCG-stimulated PBMC supernatant with a dilution factor of ½. Both assays showed similar linearity, with a range of 93.46% to 110%, as measured by ELISA, compared to 70.5% to 94.43% as measured by the Bio-Rad human 17-plex.

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Table 3.1: IFN-γ based comparison of Bio-Rad human 17-plex and ELISA (RnD System). Serum, non-stimulated supernatant and BCG stimulated supernatant from a PBMC assay were used to comparatively assess the performance of the Bio-Rad human 17-plex. Pre-diluted serum and non-stimulated supernatant were spiked at 500pg/ml to assess the recovery. The linearity was assessed by measuring the level of IFN-γ in undiluted and diluted BCG stimulated culture supernatant.

Samples and controls Spiked at 500 pg/ml	**Recovery%**	
	ELISA	Bio-Rad /Bio-plex
Serum	119.6	30
WBA	121.8	45.75
PBMC	113.8	8.76
Control 1	117	55.9
Control 2	116	31.17
BCG stimulated supernatant from PBMC assay Linearity study	**Linearity%**	
	ELISA	Bio-Rad /Bio-plex
PBMC 1 (healthy volunteer 1)	93.46	94.43
PBMC 2 (healthy volunteer 2)	110	70.5

3.2.2 Recovery of the Bio-Rad human 17-plex assays (study 2)

The recovery of the Bio-Rad human 17-plex was assessed using spiked whole blood culture supernatants from three healthy individuals and each of the supernatants was spiked at three different concentrations (table 3.2). Generally, a lack of accuracy was observed as illustrated in table 3.2. At a high RP1 target, 21% of positive readings were in the recovery range of 70 to 130%, whereas only 12.4% were within that range when samples were read at a low RP1 target. The individual recovery ranges are summarised in table 3.2.

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Table 3. 2: Bio-Rad human 17-plex expected and observed cytokine concentrations and recovery (Study 2). Unstimulated whole blood culture supernatant samples from a healthy volunteer were spiked at three different concentrations. Samples were analysed on the Bio-plex system instrument and the recovery of each cytokine in the panel assessed. In the table are represented the expected concentrations after subtraction of the endogenous levels of cytokines

		High RP1 target		Low RP1 target	
	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)	Observed (pg/ml)	Recovery (%)
IFN-gamma	1797.5	OOR>	NA	9316	518.3
	179.7	600.25	334	491.23	273.36
	18.0	40.36	224.2	27.1	150
TNF-alpha	10351.4	8424.8	81.4	16605.5	160.4
	1035.1	2212	213.7	1582.7	152.9
	103.5	178.7	172.6	138.7	134.0
IL-1beta	3132.0	4082.4	130.3	10131.7	323.5
	313.2	1800.33	574.8	421.8	134.7
	31.3	56.6	180.7	49.4	157.7
IL-2	1762.7	1882.8	106.8	2895.7	164.3
	176.3	363.8	206.4	302.2	171.4
	17.6	40.3	228.7	32.6	185.0
IL-4	695.4	707.1	101.7	OOR>	NA
	69.5	202.8	291.6	164.7	236.8
	7.0	22.2	319.2	15.3	220.0
IL-5	5159.5	8582.8	166.3	13496	261.6
	516.0	2727.2	528.6	739.7	143.4
	51.6	96.4	186.8	79.5	154.1
L-6	3422.8	2817	82.3	8190.6	239.3
	342.3	893.2	261.0	550.6	160.9
	34.2	107.9	315.3	60.4	176.5
IL-7	5460.6	9190	168.3	10819.1	198.1
	546.1	1392	254.9	1032	189.0
	54.6	145.1	265.8	98.6	180.6
IL-8	3672.0	3702.8	100.8	5661.5	154.2
	367.2	861.3	234.6	613	166.9
	36.7	89	242.4	39.1	106.5
IL-10	4810.6	4540.7	94.4	7128.86	148.2
	481.1	1348.9	280.4	732.23	152.2
	48.1	98.43	204.6	75.53	157.0
IL-12-p70	4798.2	4641.6	96.7	88036	1834.8
	479.8	1191.7	248.4	942	196.3
	48.0	114	237.6	68.4	142.6
IL-13	762.5	1116.1	146.4	1401	183.7
	76.3	169.7	222.6	140.1	183.7
	7.6	15.7	205.8	14.1	184.8
IL-17	2765.6	3074.8	111.2	9773.8	353.4
	276.6	940.6	340.1	791.9	286.3
	27.7	110.2	398.4	70.6	255.2
GM-CSF	2552.7	4100.5	160.6	4716.7	184.8
	255.3	597.3	234.0	478.6	187.5
	25.5	55.1	215.8	42	164.5
G-CSF	2536.6	21494.94	847.4	3861	152.2
	253.7	564.8	222.7	431.6	170.1
	25.4	50.1	197.5	40.2	158.5
MCP-1	3412.6	5958.1	174.6	7755.5	227.3
	341.3	700.5	205.3	595.1	174.4
	34.1	16.7	48.9	7.5	22.0
MIP-beta	2544.7	1962.6	77.1	6157.1	242.0
	254.5	636.56	250.2	382.1	150.2
	25.4	47.5	186.7	44.9	176.4

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3. 2. 3 Bio-Rad human 17-plex, LINCO 29-plex and RnD Systems MAP base kit A and B recoveries study and comparison.

3.2.3.1 Bio-Rad human 17-plex assay (test 3)

In this study, recovery of the Bio-Rad human 17-plex was assessed for five different concentrations of individual cytokines. Fluorescence was read both at high and low RP1 targets, with 85 readings made for each RP1 target. A total of 65 readings out of 85 were positive for the low RP1 target (see Figure 3.1), with 54% of these positive readings (41.2% of the total readings) falling within the acceptable recovery range of 70 to 130%. There were 62 positive readings out of 85 at the high RP1 target, with 61% of these (44.7% of the total readings) falling within the acceptable recovery range of 70 to 130%. The cytokines IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-12p70, IL-13, IL-17, GM-CSF and MCP-1 measured most accurately when samples were read at the high RP1 target, whereas measurements of IL-5, IL-10, G-CSF and MIP-1β showed better recoveries when samples were read at a low RP1 target. Interfering interactions in the samples presumably led to falsely increased, and signal inhibition for, IL-8 detection, which resulted in out-of-range readings for four out of the five assessed concentrations. The recoveries of the individual cytokines included in the Bio-Rad human 17-plex panel are shown in Figure 3.2.

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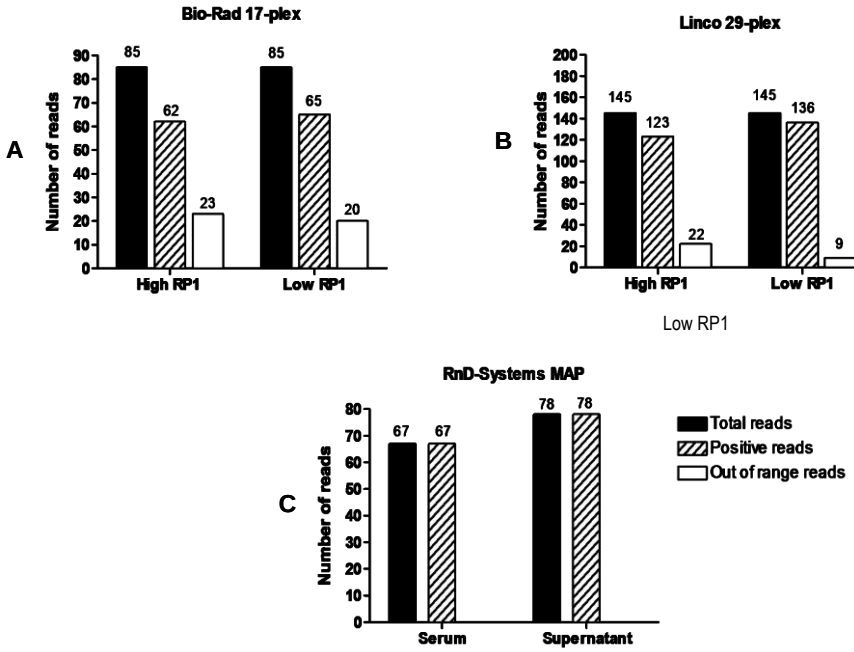

Figure 3.1. The total, positive and out of range readings for Bio-Rad's human 17-plex, LINCO's 29-plex and RnD-System's MAP 13-plex assays. Total reads or readings, positive reads (reads that gave a concentration within the standard curve range) and out of range reads are illustrated. **(A):** Bio-Rad 17-plex kit total, positive and out of range readings. Un-stimulated whole blood culture supernatants from a healthy donor was spiked at 5 different concentrations ranging from 8532-2.2pg/ml with standard containing the 17 different recombinant cytokines from the Bio-Rad kit. Samples were then read on the Bio-plex instrument at low and high RP1 targets. **(B)** LINCO 29-plex kit total, positive and out of range readings. Un-stimulated whole blood culture supernatants from a healthy donor were spiked at 5 different concentrations ranging from 5000-10 pg/ml with standard containing the 29 different recombinant cytokines from the LINCO-Inc kit. Samples were then read on the Bio-plex instrument at low and high RP1 targets. **(C)** RnD Systems MAP 13-plex kit's total, positive and out of range readings. Un-stimulated whole blood culture supernatant and serum from a healthy donor were spiked at 5 or 6 different concentrations ranging from 2650-14.8 pg/ml with standard from the 13 different recombinant cytokines from the RnD System's base kits A and B. Samples were then read on the Bio-plex instrument at low RP1 targets.

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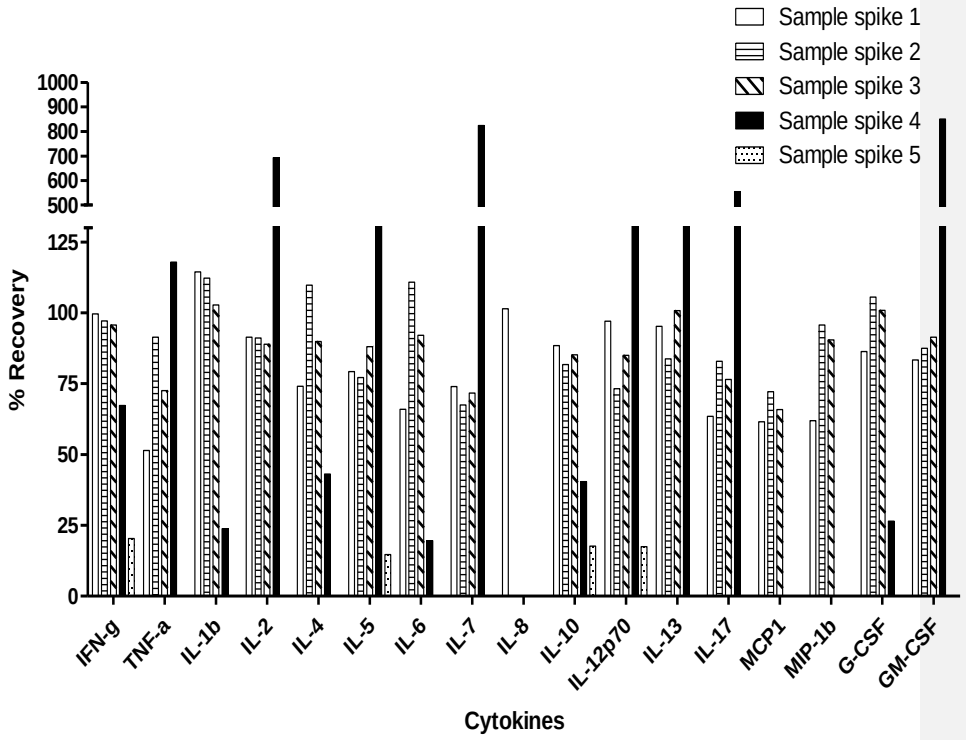

Figure 3.2. **Recoveries of the Bio-Rad 17-plex assay (study 3).** Un-stimulated whole blood culture supernatant samples from a healthy donor were spiked at five different concentrations (8532-2.2 pg/ml) with the standard from Bio-Rad 17-plex kit. Samples were assayed in duplicate and read at high and low RP1 targets on the Bio-plex system instrument. Recoveries were calculated for each of the cytokines in the panels for each of the spiked concentrations. The Figure shows the best recovery obtained for each individual cytokine.

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3.2.3.2 LINCO human 29-plex assay

The recovery of the cytokines forming part of the LINCO-Inc 29-plex panel were assessed at five different concentrations (5 000, 1 000, 500, 50 and 10 pg/ml), and read at high and low RP1 targets. The test showed an acceptable performance. A total of 145 readings were made at each RP1 target and 123 of these fell within the detection range when read at high RP1, compared to 136 out of 145 when read at the low RP1 target (Figure 3.1). Of the positive readings, 78.4% (66.2% of the total readings) read at the high RP1 target had recoveries falling within the acceptable range of 70 to 130%, whereas approximately 70% of positive readings (65.7% of the total readings) made at a low RP1 target achieved this acceptable recovery. Measurements of IFN-γ, IL-1β, IL-4, IL-6, IL-7, IP-10, MCP-1 and G-CSF were found to be most accurate when the plate was read at a high RP1 target, with recoveries falling between 70 to 130%, whereas those for TNF-α, IL-1α, IL-1rα, IL-2, IL-5, IL-10, IL-12p40, IL-12p70, IL-13, Fractalkine, MIP-1α, MIP-1β, GM-CSF, TGF-α, sCD40L, VEGF, Eotaxine and EGF were most accurate when read at a low RP1 target . IL-15 and IL-17 showed similar recoveries at both high and low RP1 targets. The level of false signal was very high for IL-8. The coefficients of variation between the measurement at high RP1 and low RP1 targets were less than 5% when applicable (when both high and low RP1 showed positive readings). The recoveries of the individual cytokines included in the LINCO 29-plex panel are shown in Figure 3.3.

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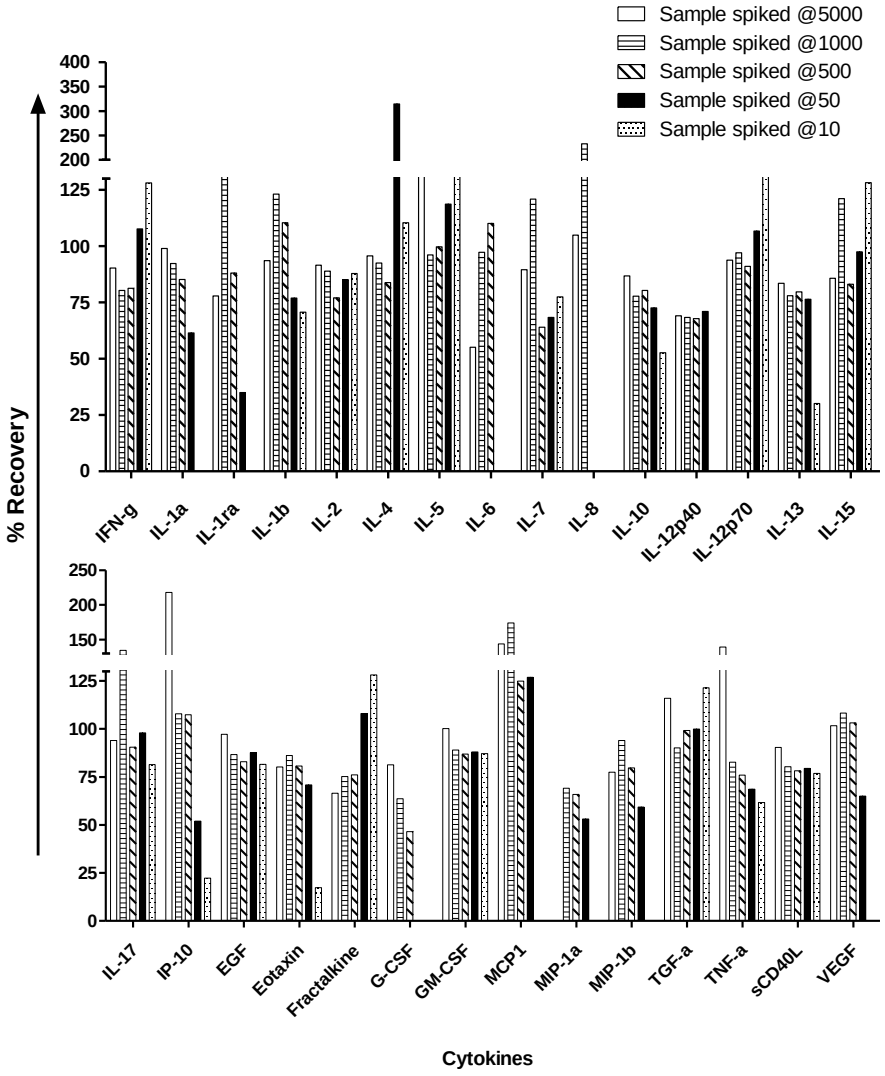

Figure 3.3: **Recoveries of the LINCO 29-plex assay.** Un-stimulated whole blood culture supernatant samples from a healthy donor were spiked at five different concentrations ranging from 5000-10pg/ml with the standards from the LINCO 29-plex kit. Samples were assayed in duplicate and read at high and low RP1 targets on the Bio-plex system instrument. Recoveries were calculated for each of the cytokines in the panels for each of the spiked concentrations. The Figure shows the best recovery obtained for each individual cytokine.

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3.2.3.3 RnD Systems Fluorokine-MAP assay

The recoveries of 13 cytokines measured in whole blood culture supernatant and serum samples were assessed at six different concentrations (*Table 2.2 Material and Methods section* using the RnD Systems Fluorokine-MAP assay. A total of 78 readings were made using whole blood culture supernatant and 67 from serum samples. All whole blood supernatant and serum sample readings were positive and within the standard curve range (Figure 3.1). A total of 67% of whole blood supernatant samples achieved recoveries within 70 to 130%, compared to approximately 56% of the serum samples. Details of individual cytokine recoveries are shown in Figure 3.4. The recoveries of control spiked standard diluents ranged from 53.3% to 103.8% for whole blood supernatant samples and from 66.5% to 130% for serum samples.

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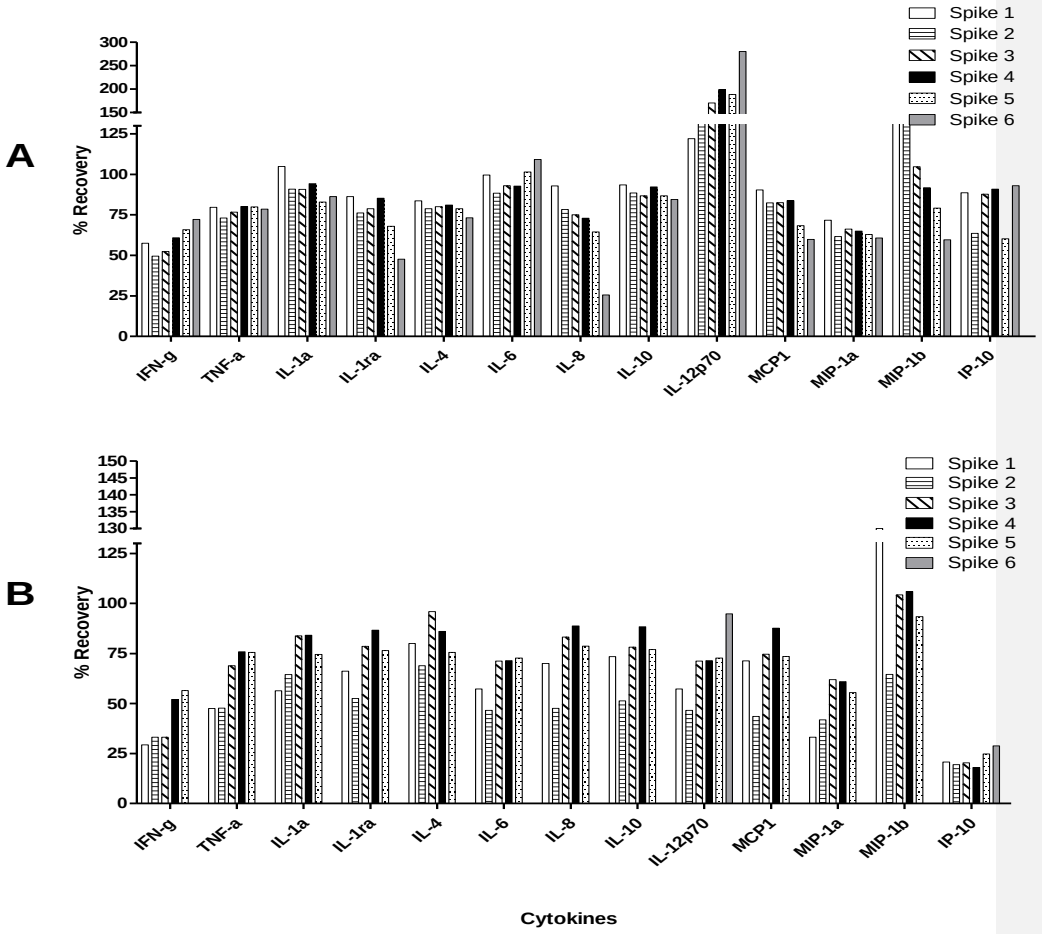

Figure 3.4: Recoveries of RnD System's Fluorokine-MAP 13-plex base kits A and B. **(A)** Un-stimulated whole blood culture supernatant samples from a healthy donor were spiked at six different concentrations (2650-29.7 pg/ml) with the standards from RnD-System Fluorokine-MAP base kits A and B. Samples were assayed in duplicate, read at a low RP1 target on the Bio-plex system instrument and recoveries calculated for each of the cytokines in the panel. The Figure shows the individual cytokine's best recovery obtained. **(B)** Serum samples from a healthy donor were spiked at five or six different concentrations (2650-43.8 pg/ml) with the standards from RnD-System's Fluorokine-MAP base kits A and B. Samples were assayed in duplicate, read at a low RP1 target on the Bio-plex system instrument and recoveries calculated for each of the cytokines in the panel. The Figure shows the individual cytokine's best recovery obtained.

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3.2.3.4 RnD Systems ELISA

The RnD System's IFN-γ ELISA was used as a gold standard against which the different luminex kits where compared for their reliability in measuring cytokine concentrations in biological samples. The ELISA was chosen as a golden standard standard due to the very good recovery values achieved with this kit (table 3.1). Samples tested included antigen-stimulated samples with their negative and positive controls. As expected, the negative control showed a very low level of IFN-γ, whereas the positive control and antigen-stimulated samples showed higher levels of IFN- γ (table 3.2).

3.2.3.5 Bio-Rad 17-plex, LINCO 29-plex, RnD Systems Fluorokine-MAP and RnD Systems ELISA: IFN-γ-based comparison

The concentration of IFN-γ, as measured in stimulated whole blood culture supernatant by the LINCO-plex assay, RnD Systems Fluorokine-MAP assay and RnD Systems ELISA, followed the same trend. In general, poor correlations were observed between the Bio-Rad 17-plex assay and the other assays (table 3.3 and Figure 3.5.). The intra-class correlation coefficients (ICC) of agreement and the Pearson product-moment correlation coefficient (r) between the different luminex kits and the RnD Systems ELISA were respectively 0.64 (ICC) and 0.75 (r) for the correlation between LINCO 29-plex and RnD Systems ELISA., The correlation analysis between RnD Systems Fluorokine-MAP/RnD Systems ELISA gave an ICC of agreement of 0.1 and a Pearson correlation coefficient (r) of 0.99. Whereas the analysis of the correlation between the Bio-Rad 17-plex kit and the RnD Systems ELISA measurement gave an ICC of agreement of -0.01 and a Pearson correlation coefficient (r) of -0.09 and The correlation between the different luminex kit measurements for the cytokines present in the three kit panels is shown in table 3.4.

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Table 3.3. **IFN-γ-based comparison of ELISA, LINCO 29-plex, Bio-Rad human 17-plex and RnD Systems Fluorokine-MAP assays**. Supernatant was generated using whole blood from a healthy subjects (1-5) in six-day assays stimulated with eight different antigens and a phytohaemagglutinin (PHA)-stimulated positive control.

	RnD Systems ELISA (pg/ml)	LINCO 29-plex (pg/ml)	Bio-Rad human 17-plex (pg/ml)	RnD Systems Fluorokine-MAP (pg/ml)
Whole blood supernatant				
PHA-stimulated	1731.38	451.56	64.7	86.83
Negative control	0	1.39	0	0
ESAT-CFP-10 (1)	1181.6	969.12	undetected	Not measured
ESAT-CFP-10 (2)	908.83	950.62	2.14	Not measured
ESAT-6 (2)	839.42	536.92	193.53	Not measured
RV1115 (4)	489.1	247	49.08	30.26
ESAT-6 (3)	235.67	125.97	104.94	17.65
TB10.4 (5)	118.9	133.82	172.19	Not measured
RV1115 (3)	57.5	55.63	undetected	1.56
RV1073 (5)	23.78	0.44	99.2	0.28

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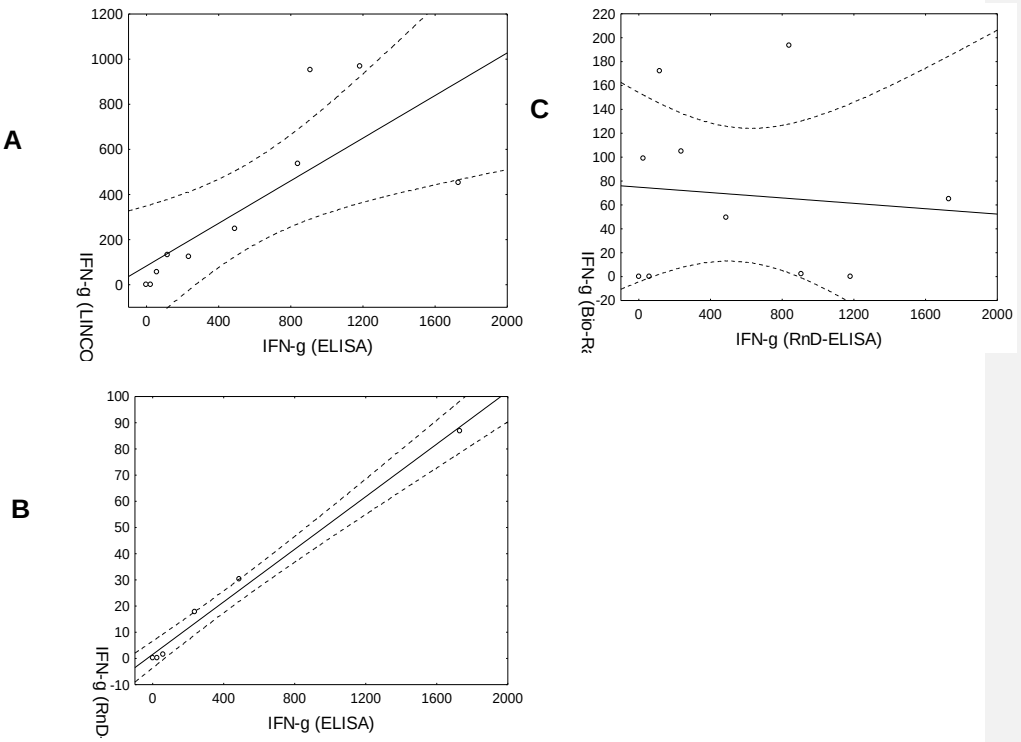

Figure 3.5: **IFN-γ-based correlation between ELISA, LINCO 29-plex, Bio-Rad 17-plex and RnD Systems Fluorokine-MAP-13-plex assays.** IFN-γ concentration was measured in stimulated whole blood culture supernatant using the LINCO 29-plex assay, RnD Systems Fluorokine-MAP-13-plex assay, Bio-Rad 17-plex assay and RnD Systems IFN-γ ELISA. **(A)** Scartterplot of the correlation between the LINCO 29-plex assay and RnD Systems ELISA. A positive correlation was observed between LINCO 29-plex assay and RnD Systems ELISA (r = 0.75; ICC of agreement = 0.64). **(B)** Scartterplot of the correlation between the RnD Systems Fluorokine-MAP 13-plex assay and RnD Systems ELISA. A positive correlation was observed between RnD Systems Fluorokine-MAP and RnD Systems ELISA (r = 0.99; ICC of agreement = 0.1). **(C)** Scartterplot of the correlation between the Bio-Rad 17-plex assay and RnD Systems ELISA. A negative correlation was observed between the Bio-Rad 17-plex assay and RnD Systems ELISA (r = - 0.09; ICC of agreement = - 0.01).

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Table 3.4: **Correlation between ELISA, LINCO 29-plex, Bio-Rad 17-plex and RnD Systems Fluorokine-MAP 13-plex assays**. Whole blood from a healthy donor were stimulated with *M. tuberculosis* derived antigens and with phytohaemagglutinin (PHA) control in six day assays. The table shows the intra-class correlation coefficients (ICC) of agreement and consistency as well as Pearson correlation coefficients (r) between measurements obtained with the different luminex kits and the ELISA.

	Rater 1	Rater 2	ICC agreement	ICC consistency	r
IFN–γ	RnD System ELISA	LINCO 29-plex	0.638	0.67	0.75
	RnD System ELISA	Bio-Rad 17-plex	-0.014	-0.02	-0.1
	RnD System ELISA	RnD-System MAP	0.1	0.1	0.99
	LINCO 29-plex	Bio-Rad 17-plex	-0.06	-0.085	-0.2
	LINCO 29-plex	RnD-System MAP	0.26	0.36	0.97
	Bio-Rad 17-plex	RnD-System MAP	0.18	0.21	0.22
IL-2	LINCO 29-plex	Bio-Rad 17-plex	0.56	0.65	0.71
IL-4	LINCO 29-plex	Bio-Rad 17-plex	0.098	0.17	0.23
	LINCO 29-plex	RnD-System MAP	-0.005	-0.01	-0.3
	Bio-Rad 17-plex	RnD-System MAP	0.002	0.0064	0.23
IL-6	LINCO 29-plex	Bio-Rad 17-plex	-0.05	-0.07	-0.1
	LINCO 29-plex	RnD-System MAP	0.77	0.74	0.88
	Bio-Rad 17-plex	RnD-System MAP	-0.08	-0.075	-0.1
IL-8	LINCO 29-plex	Bio-Rad 17-plex	0.017	0.03	0.04
	LINCO 29-plex	RnD-System MAP	0.3	0.56	0.73
	Bio-Rad 17-plex	RnD-System MAP	-0.046	-0.042	-0
IL-10	LINCO 29-plex	Bio-Rad 17-plex	-0.02	-0.02	-0
	LINCO 29-plex	RnD-System MAP	0.007	0.014	0.68
	Bio-Rad 17-plex	RnD-System MAP	0.004	0.007	0.45
GM-CFS	LINCO 29-plex	Bio-Rad 17-plex	-0.014	-0.021	-0.2
TNF-α	LINCO 29-plex	Bio-Rad 17-plex	-0.06	-0.067	-0.1
	LINCO 29-plex	RnD-System MAP	0.9	0.9	0.91
	Bio-Rad 17-plex	RnD-System MAP	0.42	0.38	0.38
IL-1β	LINCO 29-plex	Bio-Rad 17-plex	-0.27	-0.27	-0.3
IL-5	LINCO 29-plex	Bio-Rad 17-plex	-0.1	-0.093	-0.1
	LINCO 29-plex	RnD-System MAP	-0.046	-0.038	-0.1
	Bio-Rad 17-plex	RnD-System MAP	-0.18	-0.15	-0.5
IL-13	LINCO 29-plex	Bio-Rad 17-plex	-0.14	-0.138	-0.2
IL-17	LINCO 29-plex	Bio-Rad 17-plex	-0.1	-0.11	-0.2
MCP-1 (MCAF)	LINCO 29-plex	Bio-Rad 17-plex	0.5	0.48	0.48
	LINCO 29-plex	RnD-System MAP	0.02	0.043	0.62
	Bio-Rad 17-plex	RnD-System MAP	-0.008	-0.018	-0.2
MIP-1β	LINCO 29-plex	Bio-Rad 17-plex	0.04	0.037	0.05
	LINCO 29-plex	RnD-System MAP	0.25	0.25	0.73
	Bio-Rad 17-plex	RnD-System MAP	0.27	0.26	0.43
MIP-1α	LINCO 29-plex	RnD-System MAP	0.72	0.78	0.95
IL-7	LINCO 29-plex	Bio-Rad 17-plex	0.11	0.25	0.36
IL-12	LINCO 29-plex	Bio-Rad 17-plex	-0.16	-0.14	-0.1
G-CSF	LINCO 29-plex	Bio-Rad 17-plex	0.004	0.005	0.01
IL1-α	LINCO 29-plex	RnD-System MAP	0.33	0.49	0.97
IL-1rα	LINCO 29-plex	RnD-System MAP	0.23	0.39	0.85

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3.3 Discussion

This study evaluated three commercially available cytokine multiplex bead immunoassays from Bio-Rad, LINCO Inc and RnD Systems. The results suggest that, for the particular samples tested in this study, the LINCO Inc human 29-plex and the RnD Systems Fluorokine-MAP assays were the most accurate for the measurement of cytokine concentrations in whole blood culture supernatant and achieved good recovery ranges for most cytokines whereas the performance of the Bio-Rad human 17-plex assay was suboptimal.

The first comparative study, between the Bio-Rad human 17-plex assay and RnD Systems ELISA, was based on the measurement of IFN-γ in spiked samples (serum and supernatant) and revealed that the recovery achievable by the Bio-Rad human 17-plex assay kit was less than half of that obtained by the ELISA. Further comparison, including the Bio-Rad human 17-plex assay, LINCO 29-plex assay, RnD Systems Fluorokine-MAP assay and ELISA, was made based on IFN-γ responses in antigen-stimulated whole blood culture supernatant. It was found that all assays were capable of differentiating the positive and negative controls. Moreover, they were able to efficiently pick up the antigens' specific IFN-γ response when applicable, with the exception of the Bio-Rad human 17-plex assay, where IFN-γ levels in two of the antigen-stimulated samples (ESAT-CFP-10 and Rv1115) went undetected.

Concentrations of IFN-γ measured by the LINCO 29-plex assay, RnD Systems Fluorokine-MAP assay and ELISA seemed to be correlated, but results obtained using the Bio-plex assay correlated poorly with values obtained using the other three assays. This contradicts a study by Khan *et al.* (132), who showed that cytokine levels measured by the

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Bio-plex assay have similar trends to the LINCO-plex and ELISA measurements. Very similar to the findings by DuPont *et al*. (139) was a very strong correlation between the level of IFN-γ measured by ELISA and the LINCO-plex kit in whole blood culture supernatant found in this study.

Although recoveries should ideally not fall outside the acceptable range, this can be considered useable if the recovery remains constant across different sample types and dilutions. This was not the case when measuring cytokines using the Bio-Rad kits used during this study. Therefore it would be impossible to compensate for any inaccuracy evident in any one sample matrix. Discrepancies observed using the Bio-plex kits may be partly explained by the presence of interfering proteins such as heterophilic antibodies (140;141). De Jager *et al. (142)* have described methods to avoid heterophilic antibody interference in plasma and synovial fluid that improved the performance of the multiplex immunoassay. However, any manipulation of samples in clinical studies is not necessarily advantageous due to possible unforeseen effects on the results. The fact that LINCO Inc and RnD Systems kits had reasonable accuracy in this study suggests that other mechanisms may play a role in the Bio-Rad test kit.

It will therefore be wise to perform careful optimisation and validation of any commercial multiplex cytokine assays prior to large-scale clinical studies, as the quality controls supplied with the kits to measure standard curve integrity can only guarantee the accuracy or sensitivity of the assay if they are reconstituted and measured in the same matrix type as the samples investigated. Matrix effects appear to play a major role in assay performance and the type of sample tested may therefore have serious effects on assay performance. It is clear that the theoretical capabilities of this new technology cannot be fully achieved in practice (134). Researchers using these kits should include replicates of samples as well as negative and positive (low, medium and high) controls with known concentrations

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of the cytokines of interest to be confident about the results obtained. Furthermore, controls should be included that reflect both the diluents used to reconstitute the standard supplied with the kit and the sample matrix tested in order to account for possible matrix effects. This will allow the assessment of linearity and recovery and aid in the choice of best standard curve regression and optimal calibration (143).

3.5 Conclusion

In conclusion, the most appropriate use for multiplex cytokine assays currently would be as a screening tool, for the selection of candidate biomarkers characteristic of disease-associated immune responses. Promising candidates can then be validated using a method that has been proven reliable, such as high-sensitivity ELISAs.

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CHAPTER 4: Sample integrity

4.1 Introduction

Sample integrity is a very critical factor for the reliability of biological test results. Considering that the samples to be used for this study had been stored for almost four years, it was essential to assess serum for protein integrity and *ex vivo* blood for presence of non-degraded mRNA.

4.2 Study design

Ex vivo blood samples from TB patients who had been excluded from the study due to non-compliance or presence of drug resistant isolates and of whom the serum and nucleic acids had been stored and stabilised exactly like the samples for the main study and for the same period were randomly selected and tested for protein and mRNA integrity.

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4.3 Results and discussion

4.3.1 Serum

Serum samples were run on polyacrylamide one-dimension (1D) and two-dimension (2D) gels. The gels showed no smear, indicating that the proteins had not been degraded (see Figures 4.1 and 4.2) and that the samples were fit for protein analysis.

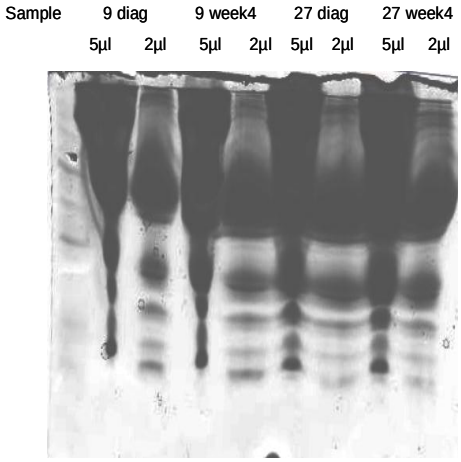

Figure 4.1: Coomassie-stained 1D SDS-PAGE gel of the serum protein from TB patients in the study.
No smear is observed on the gel image, thus protein degradation had not occurred.

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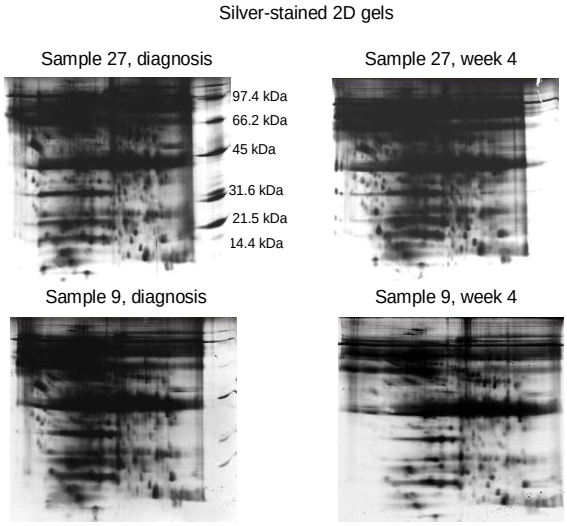

Figure 4.2: **Silver-stained 2D gels of the serum protein from TB patients in the study**. No smear is observed on gel image, thus protein degradation had not occurred.

4.3.2 Nucleic acid-stabilised *ex vivo* blood

Extracted mRNA from frozen blood was converted into clonal deoxyribonucleic acid (cDNA) and the housekeeping gene (β-actin) was amplified (RT-PCR). The PCR products were run on a 1% agarose gel. This gel showed clear amplification of the gene, revealing mRNA of a good quality (see Figure 4.3).

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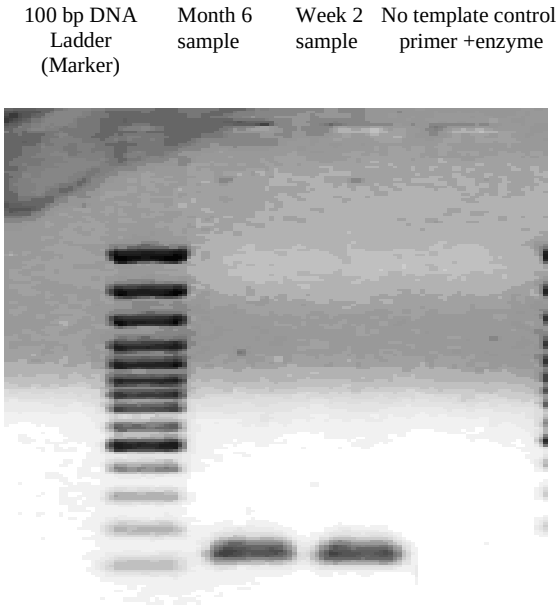

Figure 4.3: 1% agarose gel of RT-PCR product of mRNA from TB patients in the study. The β-actin gene was nicely amplified in the test samples, revealing the integrity of the mRNA.

4.4 Conclusion

The assessment of the integrity of the serum- and nucleic acid-stabilised *ex vivo* blood samples showed that samples were still of a good quality and reliable for proteomic and transcriptomic studies respectively.

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CHAPTER 5: Evaluation of Biomarkers in a Biological Context as Measures of Efficacy and Prognostic Tools in Early Response During Anti-tuberculosis Treatment

Part 1: Soluble Immune Markers

Declaration

The information contained in this chapter was presented as an oral presentation at the 5th European Congress on Tropical Medicine and International Health and the Abstract was published in the *European Journal of Tropical Medicine and International Health*.

Reference: **J.F. DJOBA SIAWAYA***, N.B. Bapela, H. Veenstra, M. Kidd, N. Beyers, P. van Helden and G. Walzl. **Combined Immune Parameters and X-Ray Data in Early Prognosis of Month 2 Sputum Culture Conversion.** *European Journal Tropical Medicine and International Health. Abstract O27-07, Volume 12, Supplement 1, May 2007.*

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5.1 Introduction

Directly observed treatment short course (DOTS) is currently the most effective means of combating tuberculosis (TB)(144-146) and consists of a six month drug regimen divided into a four-drug intensive phase of two months, followed by a two-drug continuation phase of four months (147). The International Union Against Tuberculosis and Lung Disease (IUATLD) recommends sputum smear or culture status after two months of treatment to evaluate early response to chemotherapy (62). The implications of failure of sputum conversion at this time point include a continuation of the intensive phase and sputum culture with drug sensitivity testing to exclude drug resistance, which is not part of routine management in many national TB control programs in developing countries due to cost implications. However, a two month waiting period before the first indications of treatment efficacy is very long and has implications for individual patients, control programs and clinical trials of new drugs. Moreover, the bacteria have time to adapt and develop drug tolerance(148) and resistance(149;150) during ineffective therapy.

The overall duration of therapy may adversely affect patient adherence to treatment and places a serious strain on healthcare systems in developing countries, as they struggle to afford full implementation of the recommended treatment programme. Current literature suggests that it may be possible not only to identify patients who require longer than six-month TB treatment regimens in order to prevent recurrence, but to identify the majority of patients who would require a standard antibiotic treatment course (47;64). Stratification of TB patients at the time of diagnosis or shortly after the start of treatment into risk groups for treatment response, including relapse, might enable TB programmes to concentrate their efforts on ensuring strict adherence to shorter (three to four months) treatment regimens in many patients and to reserve longer treatment options for those with a higher risk of relapse. Such markers may also decrease the required sample size of clinical trials by ensuring equality

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across treatment arms and would thereby facilitate the development and validation of new therapeutic strategies. Markers of treatment effect may also have a major impact on clinical trials of new drugs as they would shorten the duration of such trials.

Cavitatory lung disease at diagnosis and a positive sputum culture at the end of the intensive phase of treatment have been shown to characterize patients at high risk for treatment failure or relapse (52;53). However, the equipment and skills to perform radiological grading of the extent of disease is not universally available in areas with a high TB prevalence and the delay after start of treatment before month two sputum culture results become available decrease the usefulness of these markers. Markers that are measurable by simple tests like ELISA and that correlate with i) the extent of pulmonary TB disease and ii) predict the intensive phase treatment outcome as early as possible would therefore have significant advantages.

Only a few studies have focused on immune correlates for early identification of slow and fast responders during the course of anti-TB chemotherapy. Veenstra *et al.*(151) and Brahmbhatt *et al.*(152) demonstrated that immune parameters could be integrated in mathematical models for the early prediction of week eight sputum culture conversion. The present study follows the same approach by investigating the relationship between host marker expression and radiological extent of disease and examines the predictive value of host immune parameters on week eight sputum culture conversion. These parameters were selected from the literature and are chosen for their biological context (34;79;81;82;93;153-155). We show that individual markers correlate with the radiological extent of disease and a combination of markers has a high predictive ability for month two treatment response.

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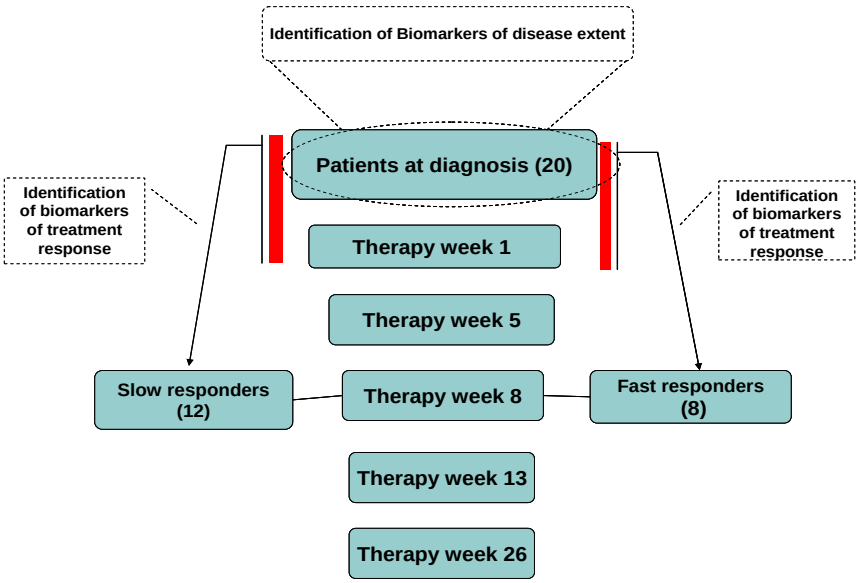
5.2 Study design

The sampled population consisted of 20 active TB patients. After the intensive phase of chemotherapy (two months), the sputum smear test identified 14 fast responders (sputum smear negative) and six slow responders (sputum smear positive), whereas the Bactec sputum culture pointed out eight fast responders (sputum culture smear negative) and 12 slow responders (sputum culture positive). It was decided to use the Bactec sputum culture status at month two as the end point for sputum sterilisation and early response to chemotherapy, as sputum culture is currently the most sensitive method for the identification of *M. tuberculosis* in sputum (156). The parameters under investigation included sICAM, CRP, suPAR, LAG-3, granzyme B, sTNFRs, DR5 and $CD3^{dim}/CD56^{+}$ NKT cells, previously described by Veenstra *et al*. (151). sICAM, CRP, suPAR, LAG-3, Granzyme B, sTNFRs and DR5 were screened for surrogates of extent of disease. In addition, the parameters were combined in logarithmic model for the prediction of the outcome of the intensive phase of treatment.

Study flow diagram



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5.3 Results

5.3.1 Chest X-ray radiography

Chest X-rays revealed the presence of cavities in 19 of the 20 patients investigated; 14 patients had multiple cavities; nine patients had cavity sizes above 4 mm and 11 patients below 4 mm; seven patients showed bilateral and 11 unilateral disease; 11 patients had radiologically extensive disease (greater that a single apical lobe segment).

Cavity distribution and alveolar disease distribution among slow and fast responders to therapy

A total of 67% of the slow responders and 75% of the fast responders had multiple cavities; 50% of the slow responders and 37% of the fast responders had extensive alveolar disease. 58% of the slow responders and 25% of the fast responders had cavities with a size greater than 4 mm (Figure 5.1).

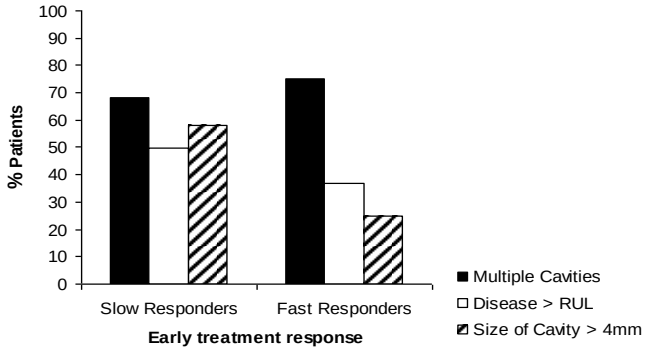

Figure 5.1. **Characteristics of cavities and extent of pulmonary infiltrates in slow and fast responders to early tuberculosis treatment.** Chest x-rays were taken between -2 or +2 months of diagnosis with smear positive pulmonary tuberculosis and were blindly analysed for cavities and extent of alveolar disease. The % of slow and fast responders presenting with key radiological findings is shown. RUL: right upper lobe as reference area for total extent of radiological involvement.

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5.3.2 Immune parameter profiles at diagnosis and extent of pulmonary TB disease

The concentration of suPAR, sICAM, CRP, LAG-3, granzyme B, sTNFRs and DR5 were measured in all patients at diagnosis and correlated with radiological extent of disease. The association between immune parameters and chest x-ray grading was assessed using the Mann Whitney test. The significant findings are shown in Figure 5.2. High levels of CRP at diagnosis were found to be associated with the presence of multiple cavities ($p \leq 0.05$). The concentration of suPAR and sICAM-1 at diagnosis were significantly higher in patients with extensive alveolar disease ($p \leq 0.05$). Patients with cavity sizes greater than 4mm had significantly higher concentrations of granzyme B ($p < 0.01$) and sLAG-3 ($p < 0.05$) at diagnosis compared to patients with cavity sizes of less than 4mm. No significant associations were found between the markers sTNFRs and DR5 and radiological grading.

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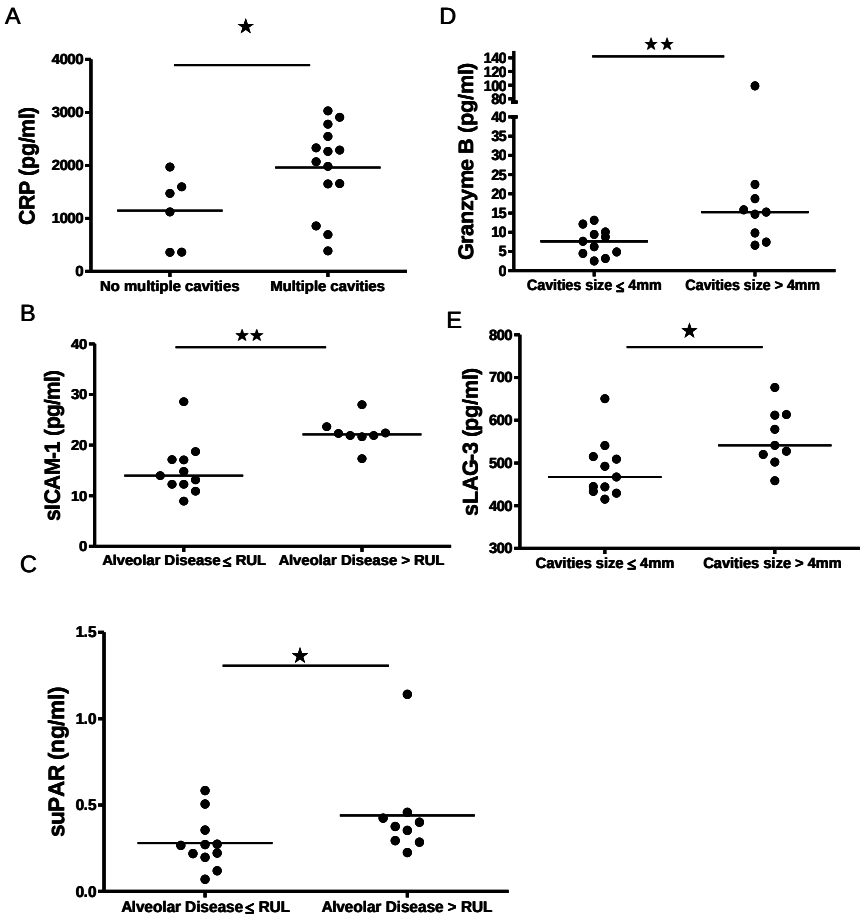

Figure 5.2: Serum level of CRP, sICAM-1, suPAR, Granzyme B and sLAG-3 in patients with different TB disease presentation on chest-x-ray radiography. Chest x-rays were taken between -2 or +2 months of diagnosis with smear positive pulmonary tuberculosis and were blindly analysed for cavities and extent of alveolar disease. (**A**) Serum levels of CRP at diagnosis is shown in TB patients with and without the presence of multiple cavities on chest x-rays. (**B**) Serum levels of sICAM-1 at diagnosis and (**C**) serum levels of suPAR at diagnosis are shown in TB patients with radiological extent of disease ≤ and > RUL., (**D**) Serum levels of granzyme B and (**E**) serum levels of sLAG-3 at diagnosis were measured in TB patients with cavity sizes ≤ and > than 4mm., RUL: right upper lobe (single apical lobe segment) as reference area for total extent of radiological involvement.

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5.3.3 Profile of fast and slow responders for selected immune parameters

suPAR

Both fast and slow responders had significantly higher levels of suPAR at diagnosis compared to controls (p-value ≤ 0.0005) (Figure 5.3a). Anti-tuberculous treatment significantly modulates the levels of suPAR. No significant differences were found between patients of either group and controls at the end of treatment (Figure 5.2a). The modulation of suPAR in fast and slow responders during therapy followed the same pattern (Figure 5.3a) and no statistically significant differences were observed between fast and slow responders during the course of therapy. In patients of both groups suPAR concentration decreased significantly after one week of treatment (p-value ≤ 0.05) and by the end of treatment were not different from control levels (Figure 5.3a).

sICAM-1

Both fast and slow responders had significantly higher levels of sICAM at diagnosis compared to controls (p ≤ 0.0005) (Figure 5.3b). sICAM-1 concentrations showed little change in both groups until the thirteenth week of treatment and only declined at the end of treatment where the differences between patients and controls were no longer significant (Figure 5.3b). Direct comparison of fast and slow responders showed no significant differences (Figure 5.3b). By the end of treatment, only slow responders presented with a significant lower sICAM-1 level compared to diagnosis (p ≤ 0.0001) (Figure 5.3b).

CRP

Both fast and slow responders had significantly higher levels of CRP at diagnosis compared to controls (p ≤ 0.005) (Figure 5.3c). After initiation of treatment (week one, five and thirteen) and at the end of treatment no significant differences were found between

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patients and controls (Figure 5.3c). In patients of both groups, CRP dropped and was significantly lower at week one, week five, week thirteen and at the end of treatment when compared to levels at diagnosis in the same responder group ($p \leq 0.0005$) (Figure 5.3c).

sLAG-3

Concentrations of sLAG-3 were not significantly different in patients compared to controls at diagnosis; at week five the level of sLAG-3 in fast responders dropped below control levels, ($p \leq 0.005$) (Figure 5.3d). By week thirteen, both fast and slow responders had significantly lower concentrations of LAG-3 compared to controls Fast and slow responders had similar level of sLAG-3 at the end of treatment to controls (Figure 5.3d). No significant differences were observed in sLAG-3 profiles between fast and slow responders. At the end of treatment sLAG-3 increased in both responder groups to the levels seen at diagnosis (Figure 5.3d).

Granzyme B

No difference was seen in the levels of granzyme B between either responder groups at any time point and controls (Figure5. 3e). Slow responders had higher concentrations of granzyme B than fast responders throughout treatment but the differences did not reach statistical significance (Figure 5.3e). In all patients, granzyme B concentrations remained relatively constant during the intensive phase of therapy and declined at treatment end (Figure 5.3e).

sTNFRI

No significant changes were seen between fast responders, slow responders and controls for the concentrations of sTNFRI at any time point (Figure 5.3f) and there were no

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significant differences between responder groups. In fast responders, TNFRI concentrations were significantly higher at week one (W1) than at diagnosis ($p \leq 0.005$) (Figure 5.3f).

sTNFRII

Fast responders presented with significantly higher levels of sTNFRII than controls throughout treatment ($p \leq 0.005$) except at week thirteen. No significant differences were observed between slow responders and controls. Fast responders had higher TNFRII concentrations throughout treatment than slow responders but the difference observed did not reach statistical significance (Figure 5.3g).

DR5

sDR5 levels were very low to undetectable in both fast and slow responders at diagnosis. Controls had significantly higher concentrations of sDR5 than patients at diagnosis ($p \leq 0.0005$) (Figure 3h). By the end of treatment, sDR5 had increased compared to diagnosis and approximated control levels (Figure 5.3h). During the first thirteen weeks (three months) of treatment, sDR5 was found to be very low in patients (irrespective of their month two status) and increased significantly at the end of treatment ($p \leq 0.0001$) (Figure 5.3h).

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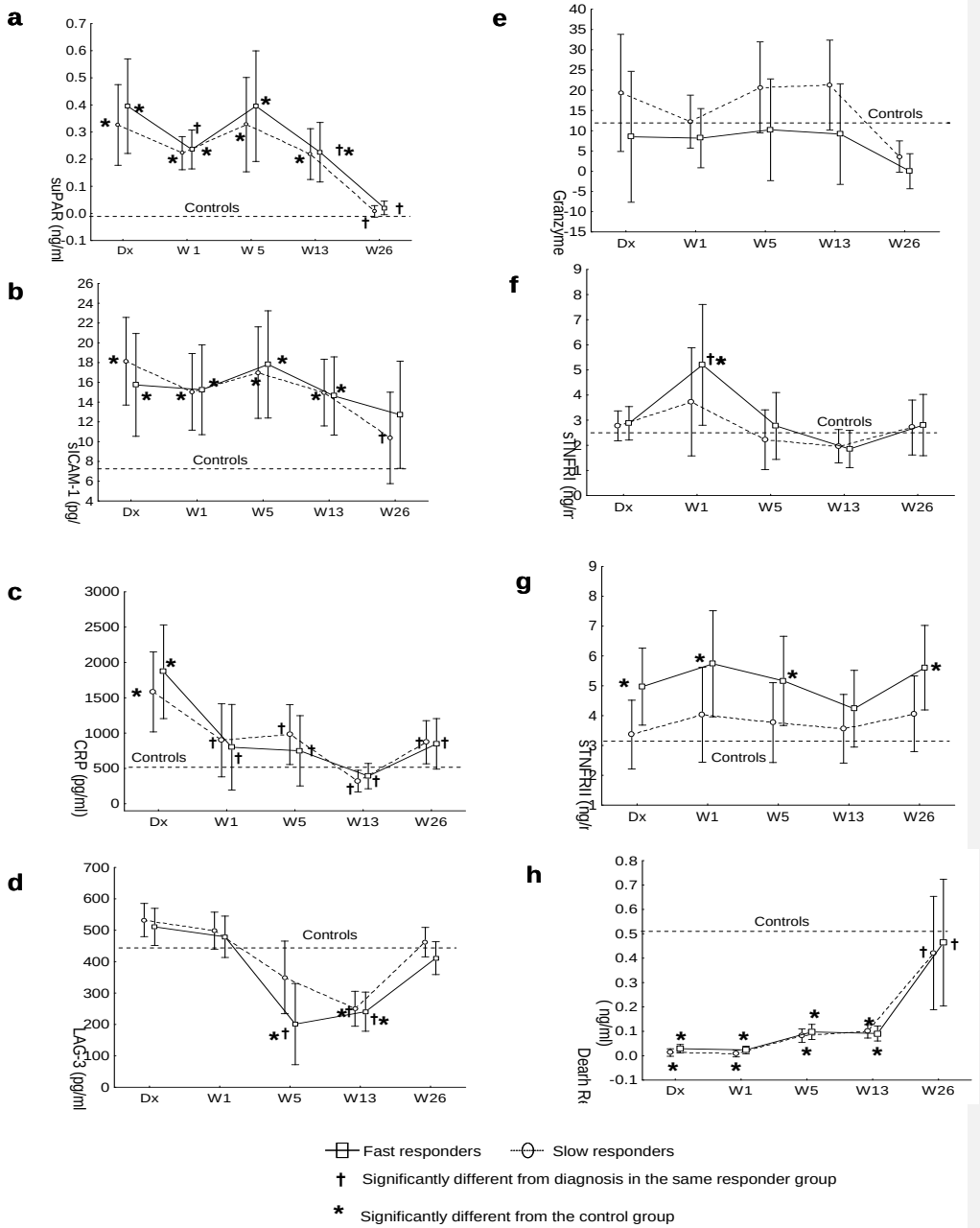

Figure 5.3: Levels of soluble host markers in serum of controls and TB patients with fast and slow treatment response. Blood was collected pre-treatment and at week 1, 5, 13, and 26 of treatment and ELISAs performed**. (a)** CRP, **(b)** sICAM-1, **(c)** suPAR, **(d)** sLAG-3, **(e)** granzyme B, **(f)** sTNFRI, **(g)** sTNFRII and **(h)** DR5 is shown. Control levels are represented by the horizontal dotted line.

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5.3.4 Correlation between immune parameters and bacterial load as measured by the time to positivity

The time to positivity (TTP) represents the time to detectable growth of *M. tuberculosis* in liquid culture (BACTEC of MGIT methods) and it is believed to be a measure of bacterial burden, where early growth detection in culture indicates a high bacterial load whereas delayed growth indicates low bacterial load. This part of the study assessed the correlation between immune parameters (CRP, sICAM-1, suPAR, sLAG-3, granzyme B, sTNFRs and DR5) and the time to positivity at diagnosis, week one and week five of treatment. The correlation between the different analytes and the TTP were established using the Spearman's rank correlation test, which is a test for correlation between sequences of pairs of values. No significant correlation was seen between the TTP and any of the immune parameters investigated.

5.3.5 Early identification of fast and slow responders to anti-tuberculosis treatment

Analysis including all immune parameters investigated

Although individually the level of the markers investigated were not significantly different between the fast and the slow responders, the best set of variables with strong predictive power for month two sputum sterilization was subsequently determined using two different classification techniques, namely discriminant analysis (DA) and support vector machines analysis (SVM). A best subsets approach was used for each technique, where all combinations of variables were evaluated using leave-one-out cross validation. Due to the small sample size no single “best” model for each technique may apply, but all models that gave reasonable cross validation results were investigated and variables identified that appeared repeatedly in the models, these variables are presented in table 5.1. From this table

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we see that sICAM-1 at diagnosis, CD3dim/CD56+ (% of CD45+) at diagnosis, CD3dim/CD56+ (% of CD45+) at week one and the difference in CD3dim/CD56+ (% of CD45+) between diagnosis and week one were the variables accentuated by both techniques. Cross validation classification results ranged from 75% to 100% correct classification for fast responders using DA and 82% to 100% for slow responders. For SVM, the correct classification results for the fast responders ranged from 88% to 100% and for the slow responders it ranged from 95% to 100%. The combinations of markers giving the best correct classification of both the fast and the slow responders are shown in table 5.2.

Analysis including soluble parameters only

The best set of variables with strong predictive power for month two sputum sterilization was determined as described above, only this time CD3dim CD56+ NKT-cells were excluded from the analysis. Results for cross validation classification using DA identified sICAM-1 (at diagnosis and week one), and CRP (at diagnosis and week one) as the best set of variables with predictive power for month two sputum culture conversion with 62.5% correct classification for fast responders and 83.3% correct classification for slow responders. The SVM identified sTNFRI (week one) Granzyme B (diagnosis) and LAG-3 (diagnosis) as the best set of variables with predictive power for month two sputum culture conversion with 87.5% correct classification for fast responders, and 72.7% correct classification for slow responders.

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Table 5.1. **Diagnosis and week one measurements entered in general discriminative and support vector machine analysis highest predictive models**. Discriminant analysis and support vector machine analysis models gave 75% to 100% and 88% to 100% correct classification of patients into response groups respectively.

Parameters	General discriminative analysis # times entered out of 6 models	Support vector machine Analysis # times entered out of 21 models
sICAM-1 @ diagnosis	6	20
CD3dim/CD56+ NKT cells (% of CD45+) @ diagnosis	4	13
CD3dim/CD56+ NKT cells (% of CD45+) @ week 1	4	13
CD3dim/CD56+ NKT cells (% of CD45+) between diagnosis and week1	4	12
CRP @ diagnosis	3	3
CRP @ week1	3	1
sICAM-1 week1	3	1
Granzyme B diagnosis	Not entered	4
sTNFRII @ diagnosis	Not entered	4
sTNFRII @ week 1	Not entered	4
sTNFRI @ diagnosis	Not entered	3
suPAR-diagnosis	Not entered	3
LAG-3 diagnosis	Not entered	3
Granzyme B week 1	Not entered	1
sTNFRI week 1	Not entered	1
LAG-3 week1	Not entered	1

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Table 5.2. General Discriminant Analysis and Support Vector Machine Analysis best classification of fast and slow responders to therapy. Parameters were measured at diagnosis (Dx) and at week one (W1) after initiation of treatment and included C-reactive protein (CRP), soluble intercellular adhesion molecule-1 (sICAM-1), soluble urokinase plasminogen activator receptor (suPAR), soluble lymphocyte activation gene-3 (sLAG-3), granzyme B, tumour necrosis factor receptor one and two (sTNFR I & II), death receptors 5 (DR5), CD3dim/CD56+ NKT-cells and chest X-ray grading

General Discriminant Analysis		
	Fast responders Correct Classification after Cross Validation	Slow responders Correct Classification after Cross Validation
sICAM-1 Dx;| CRP-Dx | CD3dim/CD56+% -Dx | CD3dim/CD56+% W1	87.50%	100%
Support Vector Machine Analysis		
sICAM-1.Dx | CRP.Dx | CRP.W1 | CD3dim/CD56+% Dx	100%	100%
sICAM-1.Dx | CRP.Dx | Granzyme B.Dx | CD3dim/CD56+% Dx	100%	100%

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5.4 Discussion and conclusion

Currently, ZN sputum smear and sputum culture conversion at month two after initiation of therapy are the only validated methods (157) for the evaluation of early treatment efficacy but both approaches have serious limitations. Sputum smear analysis lacks sensitivity and it takes several weeks before a sputum culture can be reported as negative (60 days was used in the present study). Other microbiological markers of treatment response, including Ag85 in sputum (158-160), need further validation. Host immune biomarkers may offer an alternative to microbiological readouts to assess early treatment efficacy. Here we show that combination of biomarkers measured before and one week after initiation of therapy hold promise as predictive markers for early treatment response.

The function and the potential application of CRP,(161;162) sICAM,(81;154) suPAR,(79) LAG-3,(93) granzyme B(155) and sTNFRs(34;153;163) as single markers of disease activity and progression in *M. tuberculosis* infection and other diseases are well documented in the literature but these markers do not have a sufficient predictive ability on an individual level. Furthermore, few studies have included combinations of potential markers (151;152). The markers in this study were selected for their reported association with active TB and can be linked to different phases of host immune responses against TB. They include proteins associated with the acute phase inflammatory response (CRP), proteins associated with cell adhesion, stimulation and migration (sICAM-1; suPAR), proteins involved with regulatory T cell activation (sLAG-3) and apoptosis (granzyme B; sTNFRs; sDR5).

The present study also shows that serological biomarkers such as CRP, sICAM-1 granzyme B, sLAG-3 and suPAR correlate with radiological extent of pulmonary tuberculosis prior to treatment initiation, which is consistent with previous reports.(89;164;165) It is furthermore known that the extent of pulmonary disease as assessed by chest radiography

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affects not only the time of sputum smear or culture conversion during anti-tuberculosis treatment but also relapse rates after cure at the end of 6-month therapy.(47;52;166;167) Therefore, baseline levels of CRP, sICAM-1 granzyme B, sLAG-3 and suPAR could be useful in ensuring a greater level of homogeneity of patient and aid in the stratification of patient groups entering clinical trials.

Although the direct comparison of soluble immune parameters in fast and slow responders showed no statistically significant differences at any time point, changes over time after initiation of treatment pointed to significant differences between fast and slow responders to treatment.

Compared to control levels, both patient groups had significantly higher pre-treatment levels of suPAR, sICAM-1 and CRP but only fast responders had increased sTNFRII levels suggesting an enhance pro-inflammatory response. Both responder groups had markedly suppressed levels of sDR5. By the end of the six month treatment period all marker levels had returned to control levels, except sTNFRII levels in fast responders. CRP levels normalised after one week of treatment but suPAR, sICAM-1 and DR5 levels only did so by week 26.

Significant changes from pre-treatment levels occurred late during treatment for suPAR, sICAM-1 (slow responders only) and DR5 but already after one week for CRP. The rapid decrease of CRP, an acute phase protein involved in pathogen opsonization has previously been reported (161) and might be due to a drop in mycobacterial load soon after initiation of treatment. The prolonged increase in sICAM-1 and suPAR might indicate a continued mobilization of effectors cells to the site of infection to clear persistent mycobacteria. The delayed resolution of inflammatory markers has also been previously reported by Chan et al(168) regarding interleukin 2 receptor levels in active TB patients during treatment.

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sLAG-3 levels in patients were not different from controls at diagnosis or week 26 but dropped sharply, although temporarily, to below the levels in non-diseased individuals at week five and 13 in fast responders and at week 13 in slow responders. LAG-3 protein acts as an immune system regulator via regulatory T cells and prevents excessive and detrimental responses.(92) In absence of LAG-3 regulatory T cells fail to control effector T cells.(92) The kinetics of LAG-3 production in this study suggests that immune regulation sets in after control of mycobacterial infection has been achieved and this may happen earlier in fast responders.

TNFR-induced signalling promotes activation, differentiation, survival and death of different cell types, including lymphocytes.(169;170) TNFRI signalling directly promotes macrophage activation, granuloma formation,(171) cytotoxicity and apoptosis of infected cells(170), whereas TNFRII signalling is important for thymocytes and cytotoxic T cell proliferation (172), immune regulation(170) and counters suppressive activity of regulatory T cells (173). In excess, TNFRII may promote apoptosis by potentiating TNF/TNFR-1 interactions (174;175). Therefore, levels of sTNFRs released during an infection reflect the state of immune activation. The interplay between activating and suppressive mechanisms may also reflect successful treatment. Fast responders demonstrated an earlier suppression of circulating LAG-3 than slow responders and concentrations of sTNFRII were higher than in controls throughout treatment. sTNFRI levels also increased significantly at week one of treatment only in fast responders. Together these markers suggest that fast responders have a more activated immune response during the early treatment period. The earlier LAG-3 suppression may be in keeping with this concept as counter-regulatory mechanisms may become appropriate after successful control of pathogens by antibiotics.

This study is one of the first to describe the profile of sDR5 in TB patients during treatment. DR5 interaction with tumor necrosis factor–related apoptosis-inducing ligand

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(TRAIL) mediates apoptosis (176;177). No differences between responder phenotypes were found but the pronounced low levels of this marker during treatment until a sharp increase to control levels at week 26, suggested that some apoptotic mechanism may be down-regulated during active disease and return to normal once the pathogens have been cleared by chemotherapy. The suppressed DR5 dependant pathways observed here may be a host mechanism to limit apoptosis-associated toxicity (178).

Although no single serological marker was found to clearly differentiate fast from slow responders when the two groups were directly compared, logarithmic models combining markers at diagnosis and one week after initiation of treatment gave decent predictions of sputum culture conversion after 8 weeks of treatment. The observations made in this study are of relevance since, the sputum culture status at the end of the intensive phase of treatment is linked to relapse rates(53;166). Furthermore it is encouraging that the markers with predictive ability also correlate with the extent of disease on x-rays, another validated risk factor for relapse (52;166;179).

When CD3dim/CD56 + NK T-cells were not included for the classification analysis, the two different classification techniques used to classify responder phenotypes in this study identified different markers as important contributors to the models. This could suggest that the classification techniques are not accurate or that the number of cases used is inadequate. However, as all markers used in this study are non-specific and their levels reflect general levels of inflammation, the concept of predictive models using combinations of non-specific host markers of chronic inflammation may still remain valid. The challenge for the future will be to identify the strongest markers that contribute to such models. It is also unclear how concurrent illness, including HIV infection, would affect the validity of such models.

The host markers in this study may provide only an indirect indication of drug effect.

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As successful chemotherapy decreases the bacterial load, the host immune response, which was previously activated but overwhelmed by uncontrolled active infection, is allowed to return to a normal state when suppressive immune mechanisms gradually turn off effectors responses. The different rates at which these secondary responses take place in fast and slow responders may provide important predictive information. In the present study all patients received the same standard therapy. An important step in the evaluation of promising predictive markers will include testing in clinical trials with different treatment regimens where marker expression will have to be correlated with differential microbacterial outcomes.

The aim of TB drug biomarker discovery will include the identification of markers that can aid in the shortening of clinical trials of new anti-tuberculosis drug candidates and, thereby offset some of the challenges that face new TB drug development. Currently, the success of chemotherapy for TB relies on monitoring of recurrence in the first two years after completed treatment. This means that clinical trials for new anti-TB drug candidates have a long duration, which, against the background of a high failure rate of new drug candidates entering Phase II and III testing, provides a serious disincentive to pharmaceutical industry focus on this important area, particularly given the low profit margins due to predominance of TB in resource-poor settings. Although the present study did not investigate markers for relapse, surrogate or predictive biomarkers that provide an early indication of drug efficacy would significantly decrease the cost of clinical trials and accelerate development of new antibiotics for TB treatment. Additionally, biomarkers may allow stratification of treatment regimens with possibly shortening of treatment in a majority of patients that may not require a six month treatment period and intensified regimens in those with increased risk for poor response and relapse.

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In conclusion, this study shows that the concept of biomarkers for the prediction of differential treatment responses using combinations of host markers holds promise and requires further prospective investigation to attain an optimal set of markers.

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CHAPTER 6: Evaluation of Biomarkers in a Biological Context as Measures of Efficacy and Prognostic Tools in Early Response to Anti-tuberculosis Treatment

Part 2: Differential Expression of Selected Immune Genes

6.1 Introduction

Gene expression under specific conditions may be useful in establishing and understanding the nature of physiological alteration and linking this to a clinical phenotype. Mistry and et *al* (180) showed that the host gene expression patterns can discriminate between active, latent, recurrent, and cured tuberculosis. Importantly, the discrimination between recurrent and cured tuberculosis could be established at the time subjects were healthy. Furthermore, investigating adjunctive recombinant human interleukin-2 immunotherapy in multi drug-resistant TB subjects, findings by Johnson B et *al* (181) suggested that differential genes expression may provide an indication of antimicrobial response.

The present study investigated, in fast and slow responders, the expression of genes for which the protein products have been shown to be part of antimycobacterial immunity and immunopathology. The targeted genes included: IL-4, IL-4δ2, TGF-β, TGF-βRII, Foxp3 and GATA-3.

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6.2 Study design

mRNA was extracted from nucleic acid-stabilised blood samples from twelve fast and eight slow responders to anti-tuberculosis treatment taken at diagnosis and at week one after initiation of treatment. The mRNA was reverse transcribed into cDNA and the expression of the targeted genes was analysed by mean of quantitative real-time PCR. The mRNA expression data from the target analytes was normalised using the expression of housekeeping genes as described in Materials and Methods

6.3 Results and discussion

6. 3. 1. IL-4 and IL-4δ2 mRNA expression in the fast and slow responders during treatment

Background: The role of IL-4 in human tuberculosis is not fully understood yet. Although excess production of IL-4 during tuberculosis is associated with a depressed Th1 response (13;103) and increased disease severity (103;104), it has also been suggested that IL-4 might play a role in protective immunity against TB (105;182), as IL-4 up-regulates the expression of complement receptors and mannose receptors in general (182). Studies on IL-4 KO mice have generated contradictory data. Sugawara et *al.* (105) demonstrated that *M. tuberculosis* infected IL-4 knockout mice had higher *M. tuberculosis* colony-forming units compared to wild type mice, and therefore that IL-4 was required for the defence against mycobacterial infection. Conversely, a study done by Hernandez-Pando et *al*. (103) on IL-4 KO mice showed that the absence of IL-4 led to diminished bacterial growth. Furthermore, with the discovery of an IL-4 alternative spliced variant (IL-4δ2) which has been shown to be an IL-4 antagonist (183), it is believed that the balance between IL-4 and IL-4δ2 may be important in the regulation of IL-4 effects (184). Therefore, the study of IL-4 should also include IL-4δ2 to

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allow a more complete understanding of IL-4 biology as studies have shown that IL-4δ2 competes very effectively with IL-4 for their common binding sites.

IL-4 mRNA expression in fast and slow responders using primers that amplified both IL-4 and IL-4δ2 mRNA reversed transcripts.

The comparison of IL-4 mRNA expression (no discrimination between the different types of IL-4) at diagnosis (p-value = 0.23) and at week one (p-value = 0.06) showed no statistically significant differences between fast and slow responders (table 6.1). However, the analysis of IL-4 mRNA expression change over time within each group showed that the changes in IL4 mRNA transcripts in the fast responders between diagnosis and week one were significant (p-value = 0.018) and up regulated by a factor of 7.68. No significant changes were observed in IL4 mRNA transcription in the slow responders between diagnosis and week one after the treatment was initiated (table 6. 2).

Quantification of IL-4 mRNA expression in fast and slow responders using IL-4 specific primers and IL-4δ2 specific primers.

The use of primers specific for IL-4 and IL-4δ2 showed no statistically significant differences in IL-4 and IL-4δ2 between fast and slow responders at diagnosis. At week one only IL-4 mRNA transcription showed significantly different transcription between groups with the fast responders showing significantly higher level of IL-4 mRNA transcripts than the slow responders (p-value = 0.038; table 6.1). IL-4δ2 was transcription was 2.13 times higher in the fast responders compared to the slow responders but the statistical analysis showed no significance (p-value = 0.083).

The changes from diagnosis to week one of treatment for IL-4 and IL-4δ2 mRNA transcription in the individual groups showed that slow responders significantly down

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regulated IL-4 by a factor of 0.06 (p-value = 0.002) whereas IL-4δ2 mRNA transcription was significantly up regulated by a factor of 47.32 (p-value = 0.0001) (table 6.2). The simultaneous down regulation of IL-4 mRNA transcripts and up regulation of IL-4δ2 mRNA transcripts in the slow responders may have resulted in the absence of significant changes in IL-4 mRNA transcription at week one in experiments with primers that amplified both the reverse transcripts of IL-4 and IL-4δ2. In the fast responders no significant changes in IL-4 transcription was observed between diagnosis and week one (p-value = 0.69). However IL-4δ2 transcription was significantly up regulated by a factor of 18.93 (p-value = 0.026) after one week of treatment (table 6.2). Thus the significant changes in IL-4 mRNA transcripts observed in fast responders between diagnosis and week one when non-specific primers were used was most probably due to the contribution of IL-4δ2 mRNA transcripts.

The biological explanation of the up regulation of IL-4δ2 resulting in a decrease of the IL4/IL-4δ2 ratio (Figure 6.1) from 176 to 2.96 in the fast responders and from 212 to 1.1 in the slow responders after one week of treatment (Figure 6.1a-b) may lie in a drop in bacterial load which could allow the immune system to adopt immune regulatory mechanisms. It is not clear whether the differences in IL-4 transcript regulation described above are a consequence of or mechanistically involved in the rate of sputum culture conversion. A certain level of IL-4 may be required to sustain complement receptors and mannose receptor expression and promote phagocyte killing of bacteria in parallel to drug associated killing. The requirement of IL-4 for the defense against mycobacterial infection has been shown in the past (105). Another possible explanation could be that higher levels of IL- 4 in the fast responders were the consequence of increased apoptosis that occurred in this group as suggested by the findings of Veenstra H et *al* (185). Veenstra H et *al* postulated that "IL-4 was secreted by cells as autocrine/paracrine growth factor and that the secretion ceases at the onset of apoptosis, leading to accumulation of IL-4. Interestingly from a potential biomarkers prospect, the

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changes in of IL-4 and IL-4δ2 regulation during early treatment and the differences between the responder groups may position these analytes as potential valuable predictor of treatment response that need to be investigated further in large scale studies.

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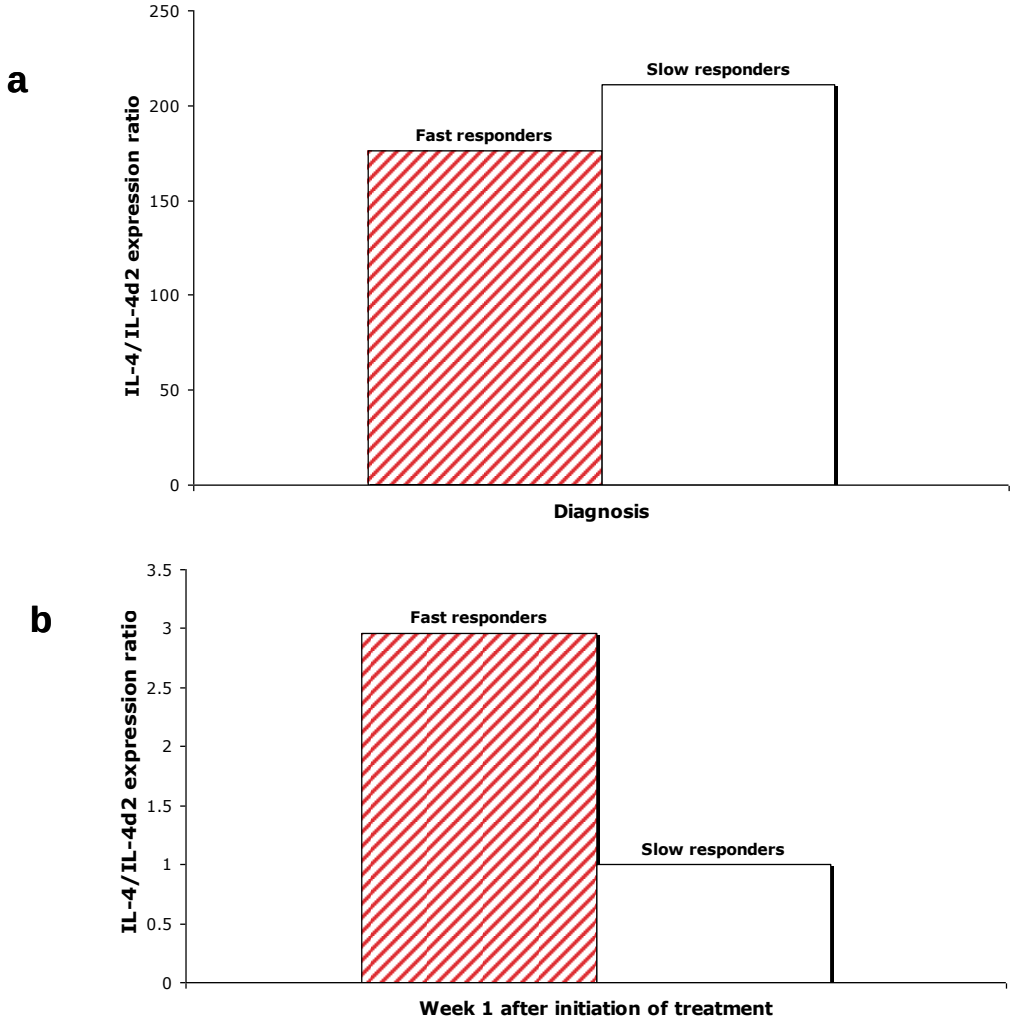

Figure 6.1: (a-b) IL4/IL-4δ2 expression ratio at diagnosis and at week one after initiation of treatment. IL4/IL-4δ2 ratio declined from 176 to 2.96 in the fast responders and from 212 to 1.1 in the slow responders after one week of treatment

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6. 3. 2. TGF-β and TGF-βRII mRNA expression between fast and slow responders during treatment.

TGF-β has been documented to impair host protective immunity to *Mtb* (110-112). TGF-βRII expression is required for cellular responses to TGF-β and may also inhibit host immunity. In addition, it has been shown that the Lipopolysaccharide (LPS) inflammatory-induced response or IFN-γ decreases TGF-βRII expression, which is accompanied by a diminished ability of the cells to respond to TGF-β (186). Patients with active TB have increased expression of TGF-β mRNA (187). Furthermore, the level of TGF-β and TGF-βRII expression in active TB patients were shown to be significantly higher compared to cured patient (112). Interestingly, Hirsch CS et al showed the degree of suppression decreased with the time on treatment (114)

In the present study, no differences in the expression levels of TGF-β or TGF-βRII were found between the fast and slow responders to treatment at diagnosis (p-value [TGF-β] = 0.793; p-value [TGF-βRII] = 0.545) or at week one (p-value [TGF-β] = 0.22; p-value [TGF-βRII] = 0.2) (table 6.1). No significant changes in TGF-β or TGF-βRII expression were observed between diagnosis and week one in either the fast or the slow responders (table 6.2). The data suggest that TGF-β and TGF-βRII expression pre-treatment and early after the start of treatment did not capture the events that influenced week eight sputum culture conversion.

6. 3. 3. FOXP3 mRNA expression in fast and slow responders during treatment.

Foxp3 is a transcription factor expressed by CD4+ CD25+ regulatory T-cells and these cells have been shown to be involved in immune suppression (40;42). Robert T et al (119) have recently shown that mycobacterium associated immunosuppression in active TB patients

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was most likely mediated by naturally occurring regulatory T cells. To investigate the association of regulatory T-cells with month two sputum culture conversion, we measured the expression levels of FOXP3 in the fast and slow responders at diagnosis and at week one after initiation of therapy.

No difference in the expression level of FOXP3 was found between the fast and slow responders to treatment at diagnosis (p-value = 0.88) or at week one (p-value = 0.17) (table 6.1). Fast responders showed an increase in FOXP3 by a factor of 5.03 (p-value = 0.11) after one week of treatment, whereas the slow responders showed only a 1.95 factor increase (p-value = 0.34) (table 6.2). However, the changes in FOXP3 expression recorded in the fast or slow responders were not statistically significant most probably due the large standard error in FOXP3 expression recorded in the groups. Nevertheless, FOXP3 as a potential predictor of week eight sputum culture conversion cannot be ruled out completely. Further studies including more patients and additional time points should be considered.

6. 3. 4. GATA-3 mRNA expression in fast and slow responders to treatment

GATA-3 is a Th2 cell-specific transcription factor that has been reported to control Th2-specific cytokine expression and to function as a negative regulator of the development of Th1 cells independently of its ability to up regulate Th2-type cytokines (115;116;188). The presence of Th2 type responses in patients with active TB has been shown to be associated with the radiological extent of pulmonary disease (189). As the extent of disease is a risk factor for delayed sputum conversion in TB patients under treatment, GATA-3 expression at diagnosis or at week one after the initiation of anti-tuberculosis therapy was investigated.

No significant differences between fast and slow responders at diagnosis (p-value = 0.52) or week one (p-value = 0.065) (table 6.1) were found. Although the differences between fast and slow responders were not statistically significant, GATA-3 mRNA expression was

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3.84 fold higher in the fast responders than in the slow responders at week one (p-value = 0.06). Fast responders showed an increase in GATA-3 by a factor of 3.61 (p-value = 0.13) after one week of treatment, whereas slow responders had a 1.5 factor increase (p-value = 0.42) (table 6.2). These changes were not statistically significant but large standard errors in FOXP3 expression could affect the significance.

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Table 6. 1: **Differential mRNA expression between the fast and the slow responders during treatment (diagnosis and week one after initiation of treatment)**. The expression factor in the table represent the the level of expression of the transcripts in the fast responders when compared to the slow responders.

	Diagnosis n = 20			Week one n = 20		
Analytes	**Mean Expression factor**	**Standard error**	**p-value**	**Mean Expression factor**	**Standard error**	**p-value**
IL-4*	0.396 (lower regulation in fast responders)	0.038 - 3.37	0.23	2.12 (higher regulation in fast responders)	0.82 - 4.83	0.06
IL-4	6.7 (higher regulation in fast responders)	0.05 - 4400	0.24	1.75 ((higher regulation in fast responders)	0.9 – 2.94	0.038 ⊕
IL-4δ2	0.68 (lower regulation in fast responders)	0.029 – 8.9	0.45	2.13 (higher regulation in fast responders)	0.58 – 8.3	0.083
TGF-β	1.01	0.12 - 6.83	0.793	2.58 (higher regulation in fast responders)	0.139 – 26.	0.22
TGF-βRII	1.350 (higher regulation in fast)	0.13 - 13.48	0.545	3.4 (higher regulation in fast responders)	0.21 -71.42	0.2
FOXP3	0.892 (lower regulation in fast responders)	0.088 - 8.17	0.880	2.7 (higher regulation in fast responders)	0.5 - 15.17	0.17
GATA-3	1.387 (higher regulation in fast)	0.178 - 12.3	0.520	3.84 (higher regulation in fast responders)	0.75 -28.92	0.06

(*) = Primers used amplified both IL-4 and IL4δ2 ⊕ = Significant p-value

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Table.6.2. mRNA expression changes between diagnosis and week one after initiation of treatment

	Fast Responders n = 8			Slow Responders n = 12		
Analytes	**Mean Expression factor**	**Standard error**	**p-value**	**Mean Expression factor**	**Standard error**	**p-value**
IL-4*	7.68 (up regulated at week one)	1.5 - 36.96	0.018⊕	2 (up regulated)	0.37 -11.16	0.3
IL-4	0.71 (down regulated)	0.11 – 7.13	0.69	0.06 (down-regulated)	0.26 – 0.98	0.002 ⊕
IL-4δ2	18.93 (up regulated)	0.6 – 488.3	0.026⊕	47.32. (up regulated)	4.01–703.4	0.0001 ⊕
TGF-β	2 (up regulated)	0.091 - 22.5	0.4	0.94	0.12 - 6.61	0.74
TGF-βRII	2.25 (up regulated)	0.17 - 43.41	0.39	1.1	0.087 -14.4	0.71
FOXP3	5.03 (up regulated)	0.51 - 43.98	0.11	1.95 (up regulated)	0.25 - 21.1	0.34
GATA-3	3.61.(up regulated)	0.34 - 29.18	0.13	1.5 (up regulated)	0.18 - 13.4	0.42

(*) = Primers used amplified both IL-4 and IL4δ2 ⊕ = Significant p-value

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6.3.5 Genes expression and extent of tuberculosis as defined by chest x-ray radiography

Previous studies have reported Th2 type response mainly IL-4 (190) to be associated with the extent of disease. However, in the present study no associations between Th2 type response as measured by IL-4 and GATA-3 mRNA transcription and chest x-ray presentations of the disease were found (table 6.3). Other immune parameter that play a role in TB immunopathology namely IL-4δ2, TGF-β, TGF-βRII, and Foxp3 were also investigated for their association with chest x-ray presentations of the disease. The data showed no association between chest x-ray radiography grading of pulmonary extent of disease and the immune parameter investigated

6. 1. Conclusion

This study showed that fast and slow responders had differential gene expression following initiation of treatment. This observation suggests that treatment induced changes in mycobacterial infection are associated with measurable physiological changes in the host that may carry information on treatment outcome as postulated in the starting hypothesis of this study. Further investigations are needed to elucidate the underlying causes of the differences observed between fast and slow responders during anti-tuberculosis treatment.

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CHAPTER 7: General Discussion, Future Work and Implementations

7.1 General discussion

Despite the implementation of DOTS and its relatively good coverage (global coverage: 89%), the incidence of tuberculosis worldwide remains a cause for concern (6). Therefore it is necessary to develop new strategies for the development of anti-tuberculosis drugs and the treatment of tuberculosis. The current standard treatment strategy do not allow for stratification of patients into different treatment arms and the majority of patients may be receiving excessively long treatment regimens whereas a minority with an increased risk for poor outcome are receiving inadequate treatment regimens. The exceptions are patients with previous tuberculosis who receive additional drugs and follow a longer treatment duration that cases with a first episode. Currently no particular emphasis is placed on the extent of disease at diagnosis, although failure to sterilise the sputum culture and multi-drug resistance have been shown to be associated significantly with cavitary tuberculosis (46).

Furthermore, the only evaluation of treatment response comes after the intensive phase of therapy, which is two months after the initiation of treatment. The relatively long time between treatment initiation and the first evaluation of its effectiveness raises some concerns, as two-month suboptimal treatment may increase the risk for the development of drug

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tolerance or resistance in some patients (46;148;149). Thus biomarkers of extent of tuberculosis disease and treatment effectiveness that can easily be measurable in resource constrained settings early after the onset of therapy may therefore have a significant clinical impact by decreasing the risk of the development of drug tolerance and resistance, as these biomarker will allow the identification of patients at increased risk for poor treatment outcome.

New multiplex assays based on the Luminex X-map technology, which in theory enable the simultaneous measurement of up to 200 plasma proteins, may become important tools in the ongoing search for biomarkers. This thesis evaluated commercially available kits using this technology and shows that these assays are able to perform with reasonable accuracy and that this technology may hold promise in the targeted search for biomarkers. However, proper optimisation needs to be performed and adequate controls need to be included.

This thesis also demonstrated that the extent of pulmonary tuberculosis disease affects the profile of the patient immune parameters (granzyme B, CRP, sICAM-1, sLAG-3, suPAR and sTNFRI) at diagnosis. It was also demonstrated that the direct comparison of the fast and slow responders on the basis of the selected immune parameters showed no statistically significant differences. However, comparison of different responder groups with healthy community controls unmasked certain differences between them, as demonstrated by the level of suPAR, sICAM-1, sLAG-3, sTNFRII and sTNFRI for selected time points. This may indicate that larger numbers of cases are needed to increase the power of the studies. One other important observation in the study was that even small changes that are not perceived as significant by conventional statistical analysis were able to segregate fast from slow responders with high accuracy when combined in multivariant logarithmic models. Moreover, mRNA transcription analysis of selected genes showed that the fast and slow responders had

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differential gene expression following the initiation of treatment. It was evident that the fast responders had overall enhanced levels of immune regulatory markers after introduction of treatment compared to slow responders. The findings made in this study support the concept that non-specific biomarkers for TB treatment response hold promise and that the underlying differential response to antimycobacterial therapy results in differences in immune reactions that can be used to identify patient with a high risk for a poor treatment outcome. This is of great significance as this proof of concept will most certainly motivate researches on TB biomarkers discovery and ultimately lead to the development and validation of new therapeutic strategies that will allow the right treatment for the right patients at the right time.

7.2 Future work and implementations

7.2.1 Goals and Objectives

The goal of future studies would be to discover stronger host biomarkers for human tuberculosis disease status and treatment outcome. The hypothesis underlying such a project is that the molecular composition of the host's body fluids, which can be characterised by modern bioanalytical techniques, reflects disease status and response to drug therapy. A broad-spectrum molecular analysis of body fluid samples from well-characterised TB patients and advanced analytical capabilities is being planned. The objectives will be to identify predictive biomarkers of cure versus relapse, to verify and validate biomarkers highlighted in this thesis and to select biomarkers for assay development.

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7.2.2 Project Design

Sample collection at Stellenbosch University (SU)

Three hundred and thirteen adult HIV-negative new TB patients were enrolled prior to directly-observed short course (six month) treatment. Samples were collected longitudinally during treatment and for two years following therapy, and carefully catalogued and stored. This sample collection is believed to be a unique resource and includes plasma, serum, urine, live *Mycobacterium tuberculosis* (*Mtb*)-stimulated whole blood culture supernatant and corresponding RNA and *ex vivo* RNA. The patients can be stratified into treatment responder phenotypes: fast (48%) or slow responders (52%) at month two of therapy; cured (n = 211) or failed treatment (n = 5) after six months of therapy; or recurrence (n = 22) within 30 months of diagnosis. For the proposed study, samples from 19 patients with recurrence and 153 without recurrence are available (Figure 8.1). The time point of sputum smear or culture conversion and time-to-positivity (TTP) in BACTEC culture at different times during therapy are known for each patient. Patients with drug-resistant TB, poor treatment adherence and HIV infection were excluded from the full study. This sample collection will be mined for molecular signals that predict outcome and thereby generate sufficient preliminary data to facilitate future biomarker research and justify more extensive prospective studies.

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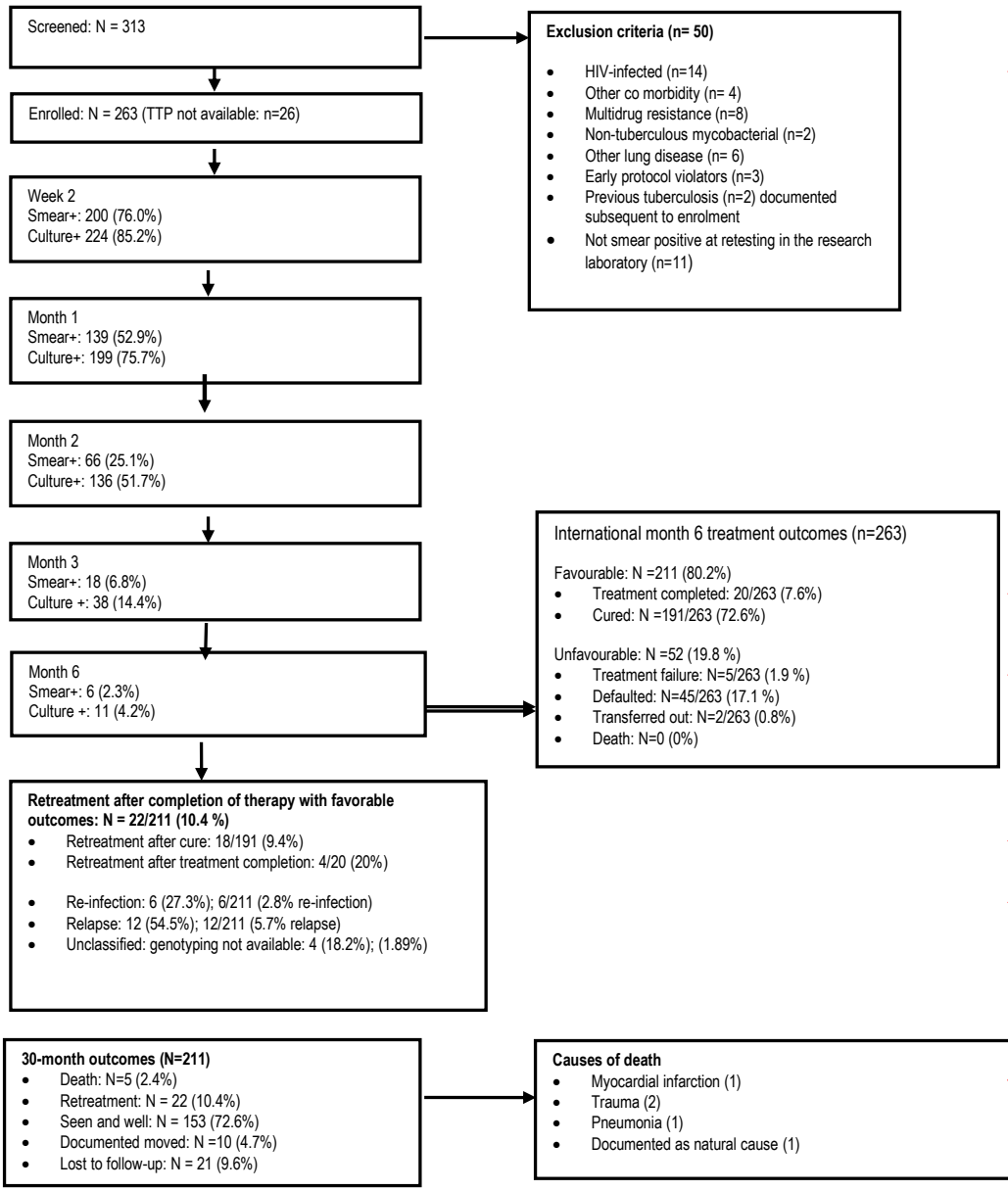

Figure 8.1: Overview of sputum smear and culture conversion and outcomes at 30 months in new smear-positive pulmonary tuberculosis patients (n=263 All smear and culture results are reported as the number of patients who attended follow-up visits, who could produce sputum and for whom results were available. Missing data were as follows: week 2 smear (14), week 2 culture (21), month 1 smear (18), month 1 culture (34), month 2 smear (21), month 2 culture (32), month 3 smear (31), month 3 culture (80), month 6 smear (44), and month 6 culture (67).

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Patients selection

Sixty representative patients will be selected (with a full complement of sample sets) in a case cohort study design with the following groups: 1) 12 patients with relapse of TB within two years of bacteriological cure after standard six-month anti-TB therapy, and 2) 40 randomly-selected patients who were cured after six months of therapy and did not develop relapse with TB within two years of treatment completion. All the patients will be well-characterised, with documented drug-sensitive *Mtb* strain infection and good treatment adherence. Both patient groups will include patients with different smear, culture and TTP status at different time points. This approach will allow the correlation of marker expression with active, and subsequently inactive, disease.

Plasma, serum, *ex vivo* RNA, urine, *Mtb*-stimulated whole blood culture supernatant and corresponding RNA will be used. Time points for analysis will include pre-treatment, week one, week two, week four, week twenty-six (end) of treatment and week fifty two (six months after treatment completion).

Analytical platforms

a) Targeted marker analysis

Promising biomarker such as suPAR, sICAM-1, CRP, sLAG-3 and IL-4 that were identified by the present study plus TNF-α, IFN-γ, IL-1α, IL-1β, IL-1rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IP-10, G-CSF, GM-CSF, MCP-1, MIP-1α, MIP-1β, TGF-α, sCD40L, EGF, VEGF, Eotaxine and Fractalkine will be measured by ELISA and multiplex cytokine arrays in serum and *Mtb*-stimulated whole blood culture supernatant.

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b) Metabolomic and proteomic analysis of plasma and urine samples

In collaboration with experts in the field specialised mass spectrometry, chromatography and nuclear magnetic resonance spectroscopy platforms will be used to measure and identify biological molecules present in the plasma and urine samples.

c) Transcriptomic analysis of ex vivo *RNA and of RNA from live* Mtb*-stimulated whole blood culture*

In collaboration with experts in the field gene expression profiles will be characterised by hybridisation to Affymetrix Human Genome U133 Plus 2 microarrays. RNA from *ex vivo* blood samples will be tested to assess gene mRNA expression changes between diagnosis and week one after initiation of treatment expression, allowing comparison with results from analyses of serum or plasma proteins.

d) Statistical analysis and interpretation of transcript, protein and metabolite datasets

In collaboration with experts in the field data processing and statistical analysis approaches will be customised for the types of molecular data generated by the measurement technologies. Initially, univariate analysis to identify molecular measurements that are statistically related to the clinical measures of interest will be used. Specialised and distinct analysis modules to identify biomarkers for diagnosis, prognosis, treatment response prediction and patient monitoring, including predictive statistical modelling, exploratory clustering and correlation networks, will be used to evaluate the association of each measured molecule, alone (univariate) or in combination with other molecules (multivariate), with the desired outcome.

Expected outcomes and implementations

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The project will make use of a unique sample collection obtained during treatment from well-characterised TB patients who were then followed up for two years following cure. Although the number of relapsed patients is relatively small (n = 12), based on power calculations performed on simulated datasets, the prospects for discovering predictive markers for cure versus recurrence are good. In addition, the project will have sufficient power to test whether surrogate markers for bacteriological cure can be identified by a combination of 'omics' approaches and accompanying advanced statistical modelling.

In addition, the project will lead to important insights into the design issues for future prospective studies of biomarkers in this field, including the types of biological samples that prove to be useful for this purpose. Another benefit will be biomarkers that can aid in shortening clinical trials of new anti-tuberculosis drug candidates, and thereby facilitate the ultimate approval of new drug therapies. Because evaluating the success of chemotherapy for TB currently relies on monitoring of recurrence in the first two years after treatment is complete, clinical trials for new anti-TB drug candidates have a long duration which, against the background of a high failure rate of new drug candidates entering Phase II and III testing, provides a serious disincentive to pharmaceutical industry focus in this important area. A further disincentive for new anti-TB drug development is the high incidence of TB in resource-poor settings, which means that pharmaceutical companies selling anti-TB drugs operate in markets with low profit margins. Surrogate biomarkers that provide an early indication of drug efficacy, or predictive biomarkers for cure versus recurrence, would significantly decrease the cost of clinical trials, accelerate development of new anti-TB therapies and, ultimately, increase their availability.

New drugs and shorter treatment regimens are urgently needed, as about nine million people worldwide develop active tuberculosis annually and the provision of directly-observed, six-month therapy places an unacceptably high burden on TB control programmes in

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developing countries, where the disease is most prevalent. Also, new drugs are essential to address the growing number of multi-drug resistant (MDR) and extensively drug resistant (XDR) strains that are emerging worldwide

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Reference List

(1) Cardona PJ, Ruiz-Manzano J. On the nature of Mycobacterium tuberculosis-latent bacilli. Eur Respir J 2004 Dec 1;24(6):1044-51.

(2) Moreno S, Baraia-Etxaburu J, Bouza E, Parras F, Perez-Tascon M, Miralles P, et al. Risk for developing tuberculosis among anergic patients infected with HIV. Ann of Intern Med 1993;119:194-8.

(3) World Health Organization. Tuberculosis. World Health Organization Media Center 2005;Fact sheet N°104.

(4) World Health Organization. Treatment of Tuberculosis Guidelines for national programmes. World Health Organization 2003;Third edition:11.

(5) Excler JL. State of the art of new vaccines Research & Development : Initiative for Vaccine Research. Geneva: World Health Organization; 2003 Apr.

(6) World Health Organisation. WHO, Global Tuberculosis Control: Surveillance, Planning, Financing. 2007.

(7) Samson Kironde. Tuberculosis. South African Health Review 2000; 335-50.

(8) Dolin P.J, Raviglione MC, and Kochi A. Global tubercolosis incidence and mortality during 1990-2000. Bull WHO 1994;72:213-20.

(9) World Health Organization. World Health Organization fact sheet. Geneva, Switzerland: World Health Organization; 2002.

(10) American Thoracic Society, Centers for Disease Control and Prevention. Diagnostic Standards and Classification of Tuberculosis in Adults and Children . Am J Respir Crit Care Med 2000 Apr 1;161(4):1376-95.

(11) Hudelson P. Gender differentials in tuberculosis: the role socio-economic and cultural factors. Tuber and Lung Disease 1996;77:391-400.

(12) Raja A. Immunology of tuberculosis. Indian J Med Res 2004 Oct;120:213-32.

(13) van Crevel R, Ottenhoff THM, van der Meer JWM. Innate Immunity to Mycobacterium tuberculosis. Clin Microbiol Rev 2002 Apr 1;15(2):294-309.

(14) Hostetter JM, Steadham EM, Haynes JS, Bailey TB, Cheville NF. Cytokine effects on maturation of the phagosomes containing Mycobacteria avium subspecies paratuberculosis in J774 cells. FEMS Immunol Med Microbiol 2002;34(2):127-34.

Stellenbosch University http://scholar.sun.ac.za

(15) Malik ZA, Denning GM, Kusner DJ. Inhibition of Ca2+ Signaling by Mycobacterium tuberculosis Is Associated with Reduced Phagosome-Lysosome Fusion and Increased Survival within Human Macrophages. J Exp Med 2000 Jan 17;191(2):287-302.

(16) Thoma-Uszynski S, Stenger S, Takeuchi O, Ochoa MT, Engele M, Sieling PA, et al. Induction of Direct Antimicrobial Activity Through Mammalian Toll-Like Receptors. Science 2001 Feb 23;291(5508):1544-7.

(17) Adams LB, Dinauer MC, Morgenstern DE, J L Krahenbuhl. Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to Mycobacterium tuberculosis using transgenic mice. Tuber Lung Dis 1997;78(5-6):237-46.

(18) Russell DG. Mycobacterium tuberculosis: here today, and here tomorrow. Nat Rev Mol Cell Biol 2001 Aug;2(2001):569-86.

(19) Young D, Hussell T, Gordon Dougan. Chronic bacteria infections: living with unwanted guests. Nat Rev Immunol 2002 Nov;3(2002):1026-32.

(20) Toossi Z, Ellner JJ. Host Response to Mycobacterium Tuberculosis. Frontiers in Bioscience 1998 Jul;3(1998):133-40.

(21) Orne IM, Cooper AM. Cytokine/chemokine cascades in immunity to tuberculosis. Immunol Today 1999 Jul;20(7):307-12.

(22) Kaufmann SHE. Protection against tuberculosis: cytokines, T cells, and macrophages. Ann Rheum Dis 2002 Nov 1;61(90002):54ii-58.

(23) Fulton S A, Reba S M, Martin T D, Boom W H. Neutrophil-Mediated Mycobacteriocidal Immunity in Lung during Mycobacterium bovis BCG infection in C57BL/6 Mice. Infect Immun 2002 Sep;70(9):5322-7.

(24) Lieberman J. THE ABCS OF GRANULE-MEDIATED CYTOTOXICITY: NEW WEAPONS IN THE ARSENAL. Nat Rev Immunol 2005 May;3:361-70.

(25) Molloy A, Laochumroonvorapong P, Kaplan G. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin. J Exp Med 1994;180:1499-509.

(26) Kaufmann S H E. How Can Immunology Contribute to the Control of Tuberculosis **?** Nat Rev Immunol 2001 Oct;1(2001):20-30.

(27) Kaufmann S H E. Protection against tuberculosis: cytokines, T cells, and macrophages. Ann Rheum Dis 2002 Nov 1;61(90002):54ii-58.

(28) Marino S, Pawar S, Fuller C.L, Reinhart T.A, Flynn J.L, and Kirschner D.E. Dendritic Cell Trafficking and Antigen Presentation in the Human Immune Response to Mycobacterium tuberculosis. J Immunol 2004;173:494-506.

(29) Sud D, Bigbee C, Flynn J.L, Kirschner D.E. Contribution of CD8 T Cells to Control of Mycobacterium tuberculosis Infection. J Immunol 2006;176:4296-314.

Stellenbosch University http://scholar.sun.ac.za

(30) Carranza C, Juarez E, Torres M, Ellner JJ, Sada E, Schwander SK. Mycobacterium tuberculosis Growth Control by Lung Macrophages and CD8 Cells from Patient Contacts. Am J Respir Crit Care Med 2006 Jan 15;173(2):238-45.

(31) Scanga CA, Mohan VP, Joseph H, Yu K, Chan J, Flynn JL. Reactivation of latent tuberculosis: variations on the Cornell murine model. Infect Immun 1999;67(9):4531-8.

(32) Frankenstein Z, Alon U, Cohen I. The immune-body cytokine network defines a social architecture of cell interactions. Biology Direct 2006;1(1):32.

(33) Zganiacz A, Santosuosso M, Wang J, Yang T, Chen L, Anzulovic M, et al. TNF-alpha is a critical negative regulator of type 1 immune activation during intracellular bacterial infection. J Clin Invest 2004;113(2004):401-13.

(34) Juffermans NP, Verbon A, van Deventer SJ, van Deutekom H, Speelman P, van der Poll T. Tumor Necrosis Factor and Interleukin-1 Inhibitors as Markers of Disease Activity of Tuberculosis. Am J Respir Crit Care Med 1998 Apr 1;157(4):1328-31.

(35) Balcewicz-Sablinska MK, Keane J, Kornfeld H, Remold HG. Pathogenic Mycobacterium tuberculosis Evades Apoptosis of Host Macrophages by Release of TNF-R2, Resulting in Inactivation of TNF-{alpha}. The journal of Immunology 1998 Sep 1;161(5):2636-41.

(36) Engele M, Castiglione K, Schwerdtner N, Wagner M, Bolcskei P, Rollinghoff M, et al. Induction of TNF in Human Alveolar Macrophages As a Potential Evasion Mechanism of Virulent Mycobacterium tuberculosis. The journal of Immunology 2002 Feb 1;168(3):1328-37.

(37) Boom WH, Canaday DH, Fulton SA, Gehring AJ, Rojas RE, Torres M. Human immunity to M. tuberculosis: T cell subsets and antigen processing. Tuberculosis 2003;83(1-3):98-106.

(38) Wanchu A, Bhatnagar A, Khullar M, Sud A, Bambery P, Singh S. Antitubercular therapy decreases nitric oxide production in HIV/TB coinfected patients. BMC Infectious Diseases 2002;2(1):15.

(39) de la Barrera S, Aleman M, Musella R, Schierloh P, Pasquinelli V, Garcia V, et al. IL-10 down-regulates costimulatory molecules on Mycobacterium tuberculosis-pulsed macrophages and impairs the lytic activity of CD4 and CD8 CTL in tuberculosis patients. 2004.

(40) Gondek DC, LipFan Lu, Quezada SA, Sakaguchi S, Noelle RJ. Cutting Edge:Contact-mediated suppression by CD4+ CD25+ regulatory cells involve a granzyme B-dependent, perforine-independent mechanism. J Immunol 2005;174:1783-6.

(41) Hornef MW, Wick MJ, Rhen M, Normark S. Bacterial strategies for overcoming host innate and adaptive immune response. Nat Immunol 2002 Nov;3(2002):1033-40.

Stellenbosch University http://scholar.sun.ac.za

(42) Nakamura K, Kitani A, Strober W. Cell Contact-dependent Immunosuppression by CD4+CD25+ Regulatory T Cells Is Mediated by Cell Surface-bound Transforming Growth Factor {beta}. J Exp Med 2001 Sep 4;194(5):629-44.

(43) Mawer C, Ignatenko NV, Wares DF, Strelis AK, Golubchikova VT, Yanova GV, et al. Comparison of the effectiveness of WHO short-course chemotherapy and standard Russian antituberculous regimens in Tomsk, western Siberia. The Lancet 2001 Aug 11;358(9280):445-9.

(44) Chadha SL, Bhagi RP. TREATMENT OUTCOME IN TUBERCULOSIS PATIENTS PLACED UNDER DIRECTLY OBSERVED TREATMENT SHORT COURSE (DOTS) - A COHORT STUDY. Ind J Tub 2000;47:155-8.

(45) Sonnenberg P, Murray J, Shearer S, Glynn JR, Kambashi B, Godfrey-Faussett P. Tuberculosis treatment failure and drug resistance--same strain or reinfection? Trans R Soc Trop Med Hyg 2000;94(6):603-7.

(46) Kritski AL, Rodrigues de Jesus LS, Andrade MK, Werneck-Barroso E, Vieira MA, Haffner A, et al. Retreatment tuberculosis cases. Factors associated with drug resistance and adverse outcomes. Chest 1997 May 1;111(5):1162-7.

(47) Hong Kong Chest Service/British Medical research council. Controlled Trial of 2, 4, and 6 Months of Pyrazinamide in 6-Month, Three-Times-Weekly Regimens for Smear-positive Pulmonary Tuberculosis, Including an Assessment of a Combined Preparation of Isoniazid, Rifampin, and Pyrazinamide. Amer Rev Resp Dis 1990;143:700-6.

(48) Combs DL, O' Brien RJ, Geiter LJ. USPHS Tuberculosis Short-Course Chemotherapy Trial 21: Effectiveness, Toxicity, and Acceptability. Ann of Intern Med 1990;112(6):397-406.

(49) Tam CM, Chan SL, Kam KM GR, Mitchison DA. Rifapentine and isoniazid in the continuation phase of a 6-month regimen. Final report at 5 years: prognostic value of various measures. Int J Tuberc Lung Dis 2002;6(1):3-10.

(50) Sonnenberg P, Murray J, Glynn JR, Shearer S, Kambashi B, Godfrey-Faussett P. HIV-1 and recurrence, relapse, and reinfection of tuberculosis after cure: a cohort study in South African mineworkers. Lancet 2001;358(1687):1693.

(51) Chang KC, Leung CC, Tam CM. Risk factors for defaulting from anti-tuberculosis treatment under directly observed treatment in Hong Kong. Int J Tuberc Lung Dis 2004;8(12):1492-8.

(52) Guler M, Unsal E, Dursun B, AydIn O, Capan N. Factors influencing sputum smear and culture conversion time among patients with new case pulmonary tuberculosis. International Journal of Clinical Practice 2007;61(2):231-5.

(53) Xu WG, Gao Z, Fan BD. Follow-up observation on relapse of smear negative pulmonary tuberculosis after short-course chemotherapy. Zhonghua Jie He He Hu Xi Za Zhi 26[2], 74-76. 2003.
Ref Type: Abstract

Stellenbosch University http://scholar.sun.ac.za

(54) Ray J, Gardiner I, Marriott D. Managing antituberculosis drug therapy by therapeutic drug monitoring of rifampicin and isoniazid. Internal Medicine Journal 2003;33(5-6):229-34.

(55) Mehta JB, Shantaveerapa H, Byrd RP, Jr., Morton SE, Fountain F, Roy TM. Utility of Rifampin Blood Levels in the Treatment and Follow-up of Active Pulmonary Tuberculosis in Patients who Were Slow to Respond to Routine Directly Observed Therapy. Chest 2001 Nov 1;120(5):1520-4.

(56) Wilkinson GR. Drug Metabolism and Variability among Patients in Drug Response. N Engl J Med 2005 May 26;352(21):2211-21.

(57) Hanna B, Walters SB, Heller PA, et al. Time to culture detection of mycobacterium tuberculosis as a possible index of patient progress and outcome. 1995 Sep; San Francisco, Interscience Conference on Antimicrobial Agents & Chemotherapy, 2007 p. 17-20.

(58) Epstein MD, Schluger NW, Davidow AL, Bonk S, Rom WN, Hanna B. Time to detection of Mycobacterium tuberculosis in sputum culture correlates with outcome in patients receiving treatment for pulmonary tuberculosis. Chest 1998 Feb 1;113(2):379-86.

(59) Colebunders R, Bastian I. A review of the diagnosis and treatment of smear-negative pulmonary tuberculosis. Int J Tuberc Lung Dis 2000;4(97):107.

(60) Hargreaves NJ, Kadzakumanja O, Phiri S, Nyangulu DS, SalaniponiFM, Harries AD, et al. What causes smear-negative pulmonary tuberculosis in Malawi, an area of high HIV seroprevalence? Int J Tuberc Lung Dis 2001 2001;5:113-22.

(61) Kapoor SC. The difficult diagnosis. Indian J Tuberc 1998;45:129-30.

(62) Enarson D E, Rieder H L, Arnadottir T, Trebucq A. Management Of Tuberculosis A Guide For Low Income Countries. Fifth ed. France: IUATLD.; 2000.

(63) Sharma K S, and Mohan A. Multidrug-Resistant Tuberculosis. Chest 130[1], 261-272. 2006.
Ref Type: Journal (Full)

(64) Balasubramanian R, Sivasubramanian S, Vijayan VK, Ramachandran R, Jawahar MS, Paramasivan CN, et al. Five year results of a 3-month and two 5-month regimen for the treatment of sputum-positive pulmonary tuberculosis in South India. Tubercle 1990;71:253-8.

(65) Burman WJ. The Hunt for the Elusive Surrogate Marker of Sterilizing Activity in Tuberculosis Treatment. Am J Respir Crit Care Med 2003;167:1299-300.

(66) Gosling RD, Heifets L, Gillespie SH. A multicentre comparison of a novel surrogate marker for determining the specific potency of anti-tuberculosis drugs. J Antimicrob Chemother 2003 Sep 1;52(3):473-6.

Stellenbosch University http://scholar.sun.ac.za

(67) SIRGEL FRED, BOTHA FRED, PARKIN DONA, VANádeáWAL BERN, SCHALL ROBE, DONALD PETE, et al. The Early Bactericidal Activity of Ciprofloxacin in Patients with Pulmonary Tuberculosis. Am J Respir Crit Care Med 1997 Sep 1;156(3):901-5.

(68) Gillespie SH, Charalambous BM. A novel method for evaluating the antimicrobial activity of tuberculosis treatment regimens. The International Journal of Tuberculosis and Lung Disease 2003 Jul;7:684-9.

(69) DESJARDIN LE, PERKINS MD, WOLSKI KATH, HAUN SHIR, TEIXEIRA LUCI, CHEN YING, et al. Measurement of Sputum Mycobacterium tuberculosis Messenger RNA as a Surrogate for Response to Chemotherapy. Am J Respir Crit Care Med 1999 Jul 1;160(1):203-10.

(70) Moore DF, Curry JI, Knott CA, Jonas V. Amplification of rRNA for assessment of treatment response of pulmonary tuberculosis patients during antimicrobial therapy. J Clin Microbiol 1996 Jul 1;34(7):1745-9.

(71) Ribeiro-Rodrigues R, Resende Co T, JOHNSON JL, Ribeiro F, Palaci M, Sa RT, et al. Sputum Cytokine Levels in Patients with Pulmonary Tuberculosis as Early Markers of Mycobacterial Clearance. Clin Diagn Lab Immunol 2002 Jul 1;9(4):818-23.

(72) Brahmbhatt S, Black GF, Carroll NM, Beyers N, Salker F, Kidd M, et al. Immune markers measured before treatment predict outcome of intensive phase tuberculosis therapy. Clin Exp Immunol 2006 Nov;146(2):243-52.

(73) Veenstra H , Baumann R , Carroll NM , Lukey PT, Kidd M, Beyers N, et al. Changes in leukocyte and lymphocyte subsets during tuberculosis treatment; prominence of CD3dim CD56+ NKT cells in fast treatment responders. Clin Exp Immunol 2006;145:252-60.

(74) Gosling RD, Heifets L, Gillespie SH. A multicentre comparisonof a novel surrogate marker for determining the specific potency of anti-tuberculosis drugs. J Antimicrob Chemother 2003;52:473-6.

(75) Trigwell S, Wood L, Jones P. Soluble Urokinase Receptor Promotes Cell Adhesion and Requires Tyrosine-92 for Activation of p56/59hck. Biochemical and Biophysical Research Communications 2000 Nov 19;278(2):440-6.

(76) Resnati M, Pallavicini I, Wang JM, Oppenheim J, Serhan CN, Romano M, et al. The fibrinolytic receptor for urokinase activates the G protein-coupled chemotactic receptor FPRL1/LXA4R. Proc Natl Acad Sci U S A 2002;99(3):1359-64.

(77) Sidenius N, Sier CFM, Ullum H, Pedersen BK, Lepri AC, Blasi F, et al. Serum level of soluble urokinase-type plasminogen activator is a strong independant predictor of suvival in human immunodeficiency virus infection. Blood 2000 Dec;96(13):4091-5.

(78) Stephens RW, Pedersen AN, Nielsen HJ, Hamers MJ, Hoyer-Hansen G, Ronne E, et al. ELISA determination of soluble urokinase receptor in blood from healthy donors and cancer patients. Clin Chem 1997;43(10):1868-76.

Stellenbosch University http://scholar.sun.ac.za

(79) Eugen-Olsen J, Gustafson P, Sidenius N, Fischer TK, Parner J, Aaby P, et al. The serum level of soluble urokinase receptor is elevated in tuberculosis patients and predicts mortality during treatment: a community study from Guinea-Bissau. The International Journal of Tuberculosis and Lung Disease 2002;6(8):686-92.

(80) Lopez Ramirez GM, Rom WN, Ciotoli C, Talbot A, Martiniuk F, Cronstein B, et al. Mycobacterium tuberculosis alters expression of adhesion molecules on monocytic cells. Infect Immun 1994 Jun 1;62(6):2515-20.

(81) Demir T, Yalçinoz C, Keskinel I, Demiröz F, Yildirim N. sICAM-1 as a serum marker in the diagnosis and follow-up of treatment of pulmonary tuberculosis. The International Journal of Tuberculosis and Lung Disease 2002 Feb;6(2):155-9.

(82) Lai CK, Wong KC, Chan CH, Ho SS, Chung SY, Haskard DO, et al. Circulating adhesion molecules in tuberculosis. Clin Exp Immunol 1993 Dec;94(3):522-6.

(83) Schultz DR, Arnold PI. Properties of four acute phase proteins: C-reactive protein, serum amyloid a protein, [alpha]1-acid glycoprotein, and fibrinogen. Seminars in Arthritis and Rheumatism 1990 Dec;20(3):129-47.

(84) Mold C, Gresham HD, Du Clos TW. Serum Amyloid P Component and C-Reactive Protein Mediate Phagocytosis Through Murine Fc{{gamma}}Rs. The journal of Immunology 2001 Jan 15;166(2):1200-5.

(85) DE BEAUFORT AJ, LANGERMANS JAM, MATZE-VAN DER LANS AM, HIEMSTRA PS, VOSSEN JM, VAN FURTH R. Difference in Binding of Killed and Live Streptococcus pneumoniae Serotypes by C-Reactive Protein. Scandinavian Journal of Immunology 1997;46(6):597-600.

(86) Köksal D, Ünsal E, Aksaray S, Güvener E, Sipit T. The relation of serum interleukin-6 and C-reactive protein to clinical parameters in pulmonary tuberculosis. Eur Respir J 2004;24(supplement 48):35s.

(87) Baynes R, Bezwoda W, Bothwell T, .Khan Q, Mansoor N. The non-immune inflammation response: serial changes in plasma iron, iron-binding capacity, lactoferin and C-reactive protein. Scand J Clin lab Invest 1986;46(7):695-704.

(88) Lee JH CJ. Changes of plasma interleukin-1 receptor antagonist, interleukin-8 and other serologic markers during chemotherapy in patients with active pulmonary tuberculosis. Korean Journal of Internal Medecine 2003 Sep;18(3):138-45.

(89) Plit ML, Anderson R, van Rensburg CEJ, Page-Shipp L, Blott JA, Fresen JL, et al. Inluence of antimicrobial chemotherapy on spirometric parameters and pro-inflammatory indices in severe pulmonary tuberculosis. Eur Respir J 1998;12:351-6.

(90) Baixeras E, Huard B, Miossec C, Jitsukawa S, Martin M, Hercend T, et al. Characterization of the lymphocyte activation gene 3-encoded protein. A new ligand for human leukocyte antigen class II antigens. J Exp Med 1992 Aug 1;176(2):327-37.

Stellenbosch University http://scholar.sun.ac.za

(91) Huard B, Prigent P, Pages F, Bruniquel D, Triebel F. T cell major histocompatibility complex class II molecules down-regulate CD4+ T cell clone responses following LAG-3 binding. Eur J Immunol 1996;26(5):1180-6.

(92) Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in Regulatory T Cells. Immunity 2004 Oct;21(4):503-13.

(93) Lienhardt C, Azzurri A, Amedei A, Fielding K, Sillah J, Sow O.Y, et al. Active tuberculosis in Africa is associated with reduced Th1 and increased Th2 activity *in vivo*. Eur J Immunol 2002;32:1605-13.

(94) Triebel F, Hacene K, Pichon MF. A soluble lymphocyte activation gene-3 (sLAG-3) protein as a prognostic factor in human breast cancer expressing estrogen or progesterone receptors. Cancer Letters 2006 Apr 8;235(1):147-53.

(95) Waterhouse NJ, Sedelies KA, Clarke CJ. Granzyme B; the chalk-mark of cytotoxic lymphocyte. J Transl Med 2004;2(1):36.

(96) Prabha C, Jalapathy KV, Matsa RP, Das SD. Role of TNF-alpha in host immune response in tuberculosis pleuritis. Curr Sci 2003 Sep 5;8(2003):639-42.

(97) Shibata J, Goto H, Arisawa T, Niwa Y, Hayakawa T, Nakayama A, et al. Regulation of tumour necrosis factor (TNF) induced apoptosis by soluble TNF receptors in Helicobacter pylori infection. Gut 1999 Jul 1;45(1):24-31.

(98) Mohan VP, Scanga CA, Yu K, Scott HM, Tanaka KE, Tsang E, et al. Effects of Tumor Necrosis Factor Alpha on Host Immune Response in Chronic Persistent Tuberculosis: Possible Role for Limiting Pathology. Infect Immun 2001 Mar 1;69(3):1847-55.

(99) Gerain J, Lienard D, Pampallona S, Baumgartner M, Ruegg C, Buurman WA, et al. SYSTEMIC RELEASE OF SOLUBLE TNF RECEPTORS AFTER HIGH-DOSE TNF IN ISOLATED LIMB PERFUSION. Cytokine 1997 Dec;9(12):1034-42.

(100) Gil D, Garcia L F, Rojas R. Modulation of macrophage apoptosis by antimycobacterial therapy: physiological role of apoptosis in the control of Mycobacterium tuberculosis. Toxicol Appl Pharmacol 2003;190:111-9.

(101) Tang S, Xiao H, Fan Y, Wu F, Zhang Z, Li H, et al. Changes of proinflammatory cytokines and their receptors in serum from patients with pulmonary tuberculosis. Zhonghua Jie He He Hu Xi Za Zhi 2002 May;25(6):325-9.

(102) Nagane M, Pan G, Weddle JJ, Dixit VM, Cavenee WK, Huang H-JS. Increased Death Receptor 5 Expression by Chemotherapeutic Agents in Human Gliomas Causes Synergistic Cytotoxicity with Tumor Necrosis Factor-related Apoptosis-inducing Ligand in Vitro and in Vivo. Cancer Res 2000 Feb 1;60(4):847-53.

(103) Hernandez-Pando R, Aguilar D, Hernandez MLG, Orozco H, Rook G. Pulmonary tuberculosis in BALB/c mice with nonfunctional IL-4 genes: changes in the inflammatory effects of TNF- α and in the regulation of fibrosis. Eur J Immunol 2004;34:174-83.

Stellenbosch University http://scholar.sun.ac.za

(104) Rook GA, Hernandez-Pando R, Dheda K, Teng Seah G. IL-4 in tuberculosis: implications for vaccine design. Trends Immunol 2004 Sep;25:483-8.

(105) Sugawara I, Yamada H, Mizuno S, Iwakura Y. IL-4 Is Required for Defense against Mycobacterial Infection. Microbiol Immunol 2000;44(12):971-9.

(106) Seah G.T, and Rook G.A.W. IL-4 Influences Apoptosis of Mycobacterium-Reactive Lymphocytes in the Presence of TNF-α. J Immunol 2006;167:1230-7.

(107) Fletcher HA, Owiafe P, Jeffries D, Hill P, Rook GAW, Zumla A, et al. Increased expression of mRNA encoding interleukin (IL)-4 and its splice variant IL-4delta2 in cells from contacts of Mycobacterium tuberculosis, in the absence of in vitro stimulation. Immunology 2004;112(4):669-73.

(108) Demissie A, Abebe M, Aseffa A, Rook G, Fletcher H, Zumla A, et al. Healthy Individuals That Control a Latent Infection with Mycobacterium tuberculosis Express High Levels of Th1 Cytokines and the IL-4 Antagonist IL-4δ2. J Immunol 2004;172(6938):6943.

(109) Dheda K, Chang JS, Breen RA, Kim LU, Haddock JA, Huggett JF, et al. In vivo and in vitro studies of a novel cytokine, interleukin 4delta2, in pulmonary tuberculosis. Am J Respir Crit Care Med 2005;172(4):501-8.

(110) de Visser KE, Kast WM. Effects of TGF-beta on the immune system: implications for cancer immunotherapy. Leukemia 1999;13(8):1188-99.

(111) Cottrez F, Groux H. Regulation of TGF-beta Response During T Cell Activation Is Modulated by IL-10. J Immunol 2001;167(2001):773-8.

(112) Bonecini-Almeida MG, Ho JL, Boechat N, Huard RC, Chitale S, Doo H, et al. Down-Modulation of Lung Immune Responses by Interleukin-10 and Transforming Growth Factor {beta} (TGF-{beta}) and Analysis of TGF-{beta} Receptors I and II in Active Tuberculosis. Infect Immun 2004 May 1;72(5):2628-34.

(113) Hirsch CS, Yoneda T, Averill L, Ellner JJ, Toossi Z. Enhancement of intracellular growth of Mycobacterium tuberculosis in human monocytes by transforming growth factor-beta 1. J Infect Dis 1994 Nov;170(1994):1229-37.

(114) Hirsch CS, Hussain R, Toossi Z, Dawood G, Shahid F, Ellner JJ. Cross-modulation by transforming growth factor beta áin human tuberculosis: Suppression of antigen-driven blastogenesis and interferon gamma áproduction. PNAS 1996 Apr 16;93(8):3193-8.

(115) João PB, Anjana Rao. Molecular Regulation of Cytokines Gene Expression During the Immune Response. J Clin Immunol 1999;19(2):98-108.

(116) Murphy KM, Reiner SL. The lineage decisions of helper T-cells. Nat Rev Immunol 2002 Feb 2;(2002):933-43.

(117) Verver S, Warren RM, Beyers N, Richardson M, van der Spuy GD, Borgdorff MW, et al. Rate of Reinfection Tuberculosis after Successful Treatment Is Higher than Rate of New Tuberculosis. Am J Respir Crit Care Med 2005 Jun 15;171(12):1430-5.

Stellenbosch University http://scholar.sun.ac.za

(118) Simon G. Radiology in epidemiological studies and some therapeutic trials. 1966. British Medical Journal 1966;5512:491-4.

(119) Roberts T, Beyers N, Aguirre A, Walzl G. Immunosuppression during active tuberculosis is characterized by decreased interferon- gamma production and CD25 expression with elevated forkhead box P3, transforming growth factor- beta , and interleukin-4 mRNA levels. J Infect Dis 2007 Mar 15;195(6):870-8.

(120) Finkelman F.D, Shea-Donohue T, Jon Goldhill, Sullivan C.A, Morris S.C, Madden K.B, et al. CYTOKINE REGULATION OF HOST DEFENSE AGAINST PARASITIC GASTROINTESTINAL NEMATODES:Lessons from Studies with Rodent Models. Annu Rev Immunol 1997;15:505-33.

(121) Strieter R.M, Belperio J.A, Keane M.P. Cytokines in innate host defense in the lung. J Clin Invest 2002 Mar;109(6):699-705.

(122) Stenger S, Röllinghoff M. Role of cytokines in the innate immune response to intracellular pathogens. Ann Rheum Dis 2001 2001;60:43-6.

(123) Jiang B, Snipes-Magaldi L, Dennehy P, Keyserling H, Holman RC, Bresee J, et al. Cytokines as mediators for or effectors against rotavirus disease in children. Clin Diagn Lab Immunol 2003 Nov;10(6):995-1001.

(124) Hutchinson KL, Villinger F, Miranda ME, Ksiazek TG, Peters CJ, Rollin PE. Multiplex analysis of cytokines in the blood of cynomolgus macaques naturally infected with Ebola virus (Reston serotype). J Med Virol 2001 Nov;65(3):561-6.

(125) Enitan D.Carrol, Alistair P.J.Thomson, Ashley P.Jones, Graham Jeffers, and C.Anthony Hart. A predominantly anti-inflammatory cytokine profile is associated with disease severity in meningococcal sepsis. Intensive care Med 2005;30(10):1415-9.

(126) Dieli F, Singh M, Spallek R, Romano A, Titone L, Sireci G, et al. Change of Th0 to Th1 Cell-Cytokine Profile Following Tuberculosis Chemotherapy. Scand J Immunol 2000;52:96-102.

(127) Probst M.C.O, Rothe G, Schmitz G. Bead-Based Multiplex Analysis. Laboratoriums Medizin 2003 Jun;27(5-6):182-7.

(128) Maggio M, Blackford A, Taub D, Carducci M, Ble A, Metter EJ, et al. Circulating inflammatory cytokine expression in men with prostate cancer undergoing androgen deprivation therapy. J Androl 2006;27(6):725-8.

(129) Coutinho HM, McGarvey ST, Acosta LP, Manalo DL, Langdon GC, Leenstra T, et al. Nutritional status and serum cytokine profiles in children, adolescents, and young adults with Schistosoma japonicum-associated hepatic fibrosis, in Leyte, Philippines. J Infect Dis 2005 Aug;192(3):528-36.

(130) Probst M.C.O, Rothe G, Schmitz G. Bead-Based Multiplex Analysis. Laboratoriums Medizin 2003 Jun;27(5-6):182-7.

Stellenbosch University http://scholar.sun.ac.za

(131) Opalka D, Lachman C.E, MacMullen S.A, Kathrin U.Jansen, Judith F.Smith, Narendra Chirmule, et al. Simultaneous Quantitation of Antibodies to Neutralizing Epitopes on Virus-Like Particles for Human Papillomavirus Types 6, 11, 16, and 18 by a Multiplexed Luminex Assay. Clin Diagn Lab Immunol 2003 Jan;10(1):108-15.

(132) Khan S.S, Smith M.S, Reda D, Suffredini A.F, and McCoy JrJP. Multiplex Bead Array Assays for Detection of Soluble Cytokines: Comparisons of Sensitivity and Quantitative Values Among Kits From Multiple Manufacturers. Cytometry 2004;61B(1):35-9.

(133) Ray CA, Bowsher RR, Smith WC, Devanarayan V, Willey MB, Brandt JT, et al. Development, validation, and implementation of a multiplex immunoassay for the simultaneous determination of five cytokines in human serum. J Pharm Biomed Anal 2005 Jan;36(5):1037-44.

(134) Liu M .Y, Xydakis A.M, Hoogeveen R.C, Jones P.H, E.O'Brian Smith, Nelson K.W, et al. Multiplexed Analysis of Biomarkers Related to Obesity and the Metabolic Syndrome in Human Plasma, Using the Luminex-100 System. Clin Chem 2005;51(7):1102-9.

(135) Kofoed K, VestSchneider U, Scheel T, Andersen O, and Eugen-Olsen J. Development and Validation of a Multiplex Add-On Assay for Sepsis Biomarkers Using xMAP Technology. Clin Chem 2006;52(7):1284-93.

(136) dupont NC, Wang K, Wadhwa PD, Culhane JF, Nelson EL. Validation and comparison of luminex multiplex cytokine analysis kits with ELISA: Determinations of a panel of nine cytokines in clinical sample culture supernatants. J Reprod Immunol 2005 Aug;66(2):175-91.

(137) Hildesheim A., Ryan R.L., Rinehart E., Nayak S., Wallace D., Castle P.E., et al. Simultaneous measurement of several cytokines using small volumes of biospecimens. Cancer Epidemiol Biomarkers Prev 2002 Nov;11(11):1477-84.

(138) Prabhakar U, Eirikis E, Reddy M, Silvestro E, Spitz S, Pendley C 2nd, et al. Validation and comparative analysis of a multiplexed assay for the simultaneous quantitative measurement of Th1/Th2 cytokines in human serum and human peripheral blood mononuclear cell culture supernatants. J Immunol Methods 2004;291(1-2):27-38.

(139) dupont NC, Wang K, Wadhwa PD, Culhane JF, Nelson EL. Validation and comparison of luminex multiplex cytokine analysis kits with ELISA: Determinations of a panel of nine cytokines in clinical sample culture supernatants. J Reprod Immunol 2005 Aug;66(2):175-91.

(140) Martins TB, B.M.Pasi, C.M.Litwin, and H.R.Hill. Heterophile Antibody Interference in a Multiplexed Fluorescent Microsphere Immunoassay for Quantitation of Cytokines in Human Serum. Clin Diagn Lab Immunol, 2004;11:325-9.

(141) Kathryn L.Kellar, Rizwan R.Kalwar, Kimberly A.Dubois, Dennis Crouse, William D.Chafin, and Beth-Ellen Kane. Multiplexed Fluorescent Bead-Based Immunoassays for Quantitation of Human Cytokines in Serum and Culture Supernatants. Cytometry 2001;45:27-36.

Stellenbosch University http://scholar.sun.ac.za

(142) de Jager W., Prakken B.J., Bijlsma J.W.J, Wietse Kuisa, Ger T.Rijkers. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. J Immunol Methods 2005;300:124-35.

(143) de Jager W., Ger T.Rijker. Solid-phase and bead-based cytokine immunoassay: A comparison. Methods 2006;38:294-303.

(144) American Thoracic Society, The Centers For Disease Control And Prevention, and Infectious Diseases Society of America. Treatment of Tuberculosis: Recommendations Reports. Morbidity and Mortality Weekly Report 2003 Jun 20;52(RR-11):2.

(145) Caminero Luna JA. Tuberculosis guide for specialist physicians. International Union Against Tuberculosis and Lung Disease 2004;23.

(146) Jasmer RM, Seaman CB, Gonzalez LC, Kawamura LM, Osmond DH, Daley CL. Tuberculosis Treatment Outcomes: Directly Observed Therapy Compared with Self-Administered Therapy. Am J Respir Crit Care Med 2004 Sep 1;170(5):561-6.

(147) Schluger NW, Harkin T, Rom W. Principles of Therapy of Tuberculosis in the Modern Era. First edition ed. Boston, MA: Little, Brown and Company; 1996.

(148) Wallis RS, Patil S, CHeon SH, Edmonds K, Phillips M, Perkins MD, et al. Drug Tolerance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 1999;43(11):2600-6.

(149) March F, Garriga X, Rodriguez P, Moreno C, Garrigo M, Coll P, et al. Acquired drug resistance in Mycobacterium tuberculosis isolates recovered from compliant patients with human immunodeficiency virus-associated tuberculosis. Clinical Infectious Diseases 1997 Nov;25(5):1044-7.

(150) Deoskar RB, Sengupta B, Rajan KE, Barthwa S, Falleiro J, Sharma SK. Study of Drug Resistant Pulmonary Tuberculosis. MJAFI 2005; 61:245-8.

(151) Veenstra H, Baumann R, Carroll NM, Lukey PT, Kidd M, Beyers N, et al. Changes in leucocyte and lymphocyte subsets during tuberculosis treatment; prominence of CD3dimCD56+ natural killer T cells in fast treatment responders. Clinical & Experimental Immunology 2006;145(2):252-60.

(152) Brahmbhatt S, Black GF, Carroll NM, Beyers N, Salker F, Kidd M, et al. Immune markers measured before treatment predict outcome of intensive phase tuberculosis therapy. Clinical & Experimental Immunology 2006;146(2):243-52.

(153) Lawn SD, Rudolph D, Wiktor S, oulibaly D, ckah A, Lal RB. Tuberculosis (TB) and HIV infection are independently associated with elevated serum concentrations of tumour necrosis factor receptor type 1 and beta-2-microglobulin, respectively. Clinical and Experimental Immunology 122, 79-84. 2000.
Ref Type: Journal (Full)

(154) Saltini C, Colizzi V. Soluble immunological markers of disease activity in tuberculosis. Eur Respir J 1999 Sep;14(3):485.

Stellenbosch University http://scholar.sun.ac.za

(155) Cooper AM, D'Souza C, Frank AA, Orme IM. The course of Mycobacterium tuberculosis infection in the lungs of mice lacking expression of either perforin- or granzyme-mediated cytolytic mechanisms. Infect Immun 1997 Apr 1;65(4):1317-20.

(156) Lawson L, Yassin MA, Ramsay A, Olajide I, Thacher TD, Davies PDO, et al. Microbiological validation of smear microscopy after sputum digestion with bleach; a step closer to a one-stop diagnosis of pulmonary tuberculosis. Tuberculosis 2006 Jan;86(1):34-40.

(157) Wallis RS. Surrogate markers to assess new therapies for drug-resistant tuberculosis. Expert Rev Anti Infect Ther 2007 Apr;5(: 2007 Apr;5(2):163-8.

(158) Hellyer TJ, DesJardin LE, Teixeira L, Perkins MD, Cave MD, Eisenach KD. Detection of Viable Mycobacterium tuberculosis by Reverse Transcriptase-Strand Displacement Amplification of mRNA. J Clin Microbiol 1999 Mar 1;37(3):518-23.

(159) Dheda K, Chang JS, Breen RA, Haddock JA, Lipman MC, Kim LU, et al. Expression of a novel cytokine, IL-4delta2, in HIV and HIV-tuberculosis co-infection. AIDS 2005;19(15):1601-6.

(160) Wallis RS, Perkins M, Phillips M, Joloba M, Demchuk B, Namale A, et al. Induction of the antigen 85 complex of Mycobacterium tuberculosis in sputum: a determinant of outcome in pulmonary tuberculosis treatment. J Infect Dis 1998;178(4):1115-21.

(161) de Beer FC, Nel AE, Gie RP, Donald PR, Strachan AF. Serum amyloid A protein and C-reactive protein levels in pulmonary tuberculosis: relationship to amyloidosis. Thorax 1984;39(3):196-200.

(162) Bajaj G, Rattan A, Ahmad P. Prognostic value of 'C' reactive protein in tuberculosis. Indian Pediatr 1989;26(10):1010-3.

(163) Nokta M, Rossero R, Loesch K, Pollard RB. Kinetics of tumor necrosis factor alpha and soluble TNFRII in HIV-infected patients treated with a triple combination of stavudine, didanosine, and hydroxyurea. AIDS Res Hum Retroviruses 1997;13(18):1633-88.

(164) MUKAE H, ASHITANI Ji, TOKOJIMA M, IHI T, KOHNO S, Matsukura S. Elevated levels of circulating adhesion molecules in patients with active pulmonary tuberculosis. Respirology 2003;8(3):326-31.

(165) Shijubo N, Imai K, Nakanishi F, Yachi A, Abe S. Elevated concentrations of circulating ICAM-1 in far advanced and miliary tuberculosis. Am Rev Respir Dis 1993;148(5):1298-301.

(166) Chang KC, Leung CC, Yew WW, Ho SC, Tam CM. A Nested Case-Control Study on Treatment-related Risk Factors for Early Relapse of Tuberculosis. Am J Respir Crit Care Med 2004;170:1124-30.

(167) Panjabi R, Comstock GW, Golub JE. Recurrent tuberculosis and its risk factors: adequately treated patients are still at high risk [Review Article]. The International Journal of Tuberculosis and Lung Disease 2007 Aug;11:828-37.

Stellenbosch University http://scholar.sun.ac.za

(168) Chan CH, Lai CK, Leung JC, Ho AS, Lai KN. Elevated interleukin-2 receptor level in patients with active pulmonary tuberculosis and the changes following anti-tuberculosis chemotherapy. Eur Respir J 1995 Jan 1;8(1):70-3.

(169) Chan FK-M, Siegel RM, Lenardo MJ. Signaling by the TNF Receptor Superfamily and T Cell Homeostasis. Immunity 2000 Oct;13(4):419-22.

(170) Hehlgans T, Pfeffer K. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. Immunology 2005;115(1):1-20.

(171) Jacobs M, Brown N, Allie N, Chetty K, Ryffel B. Tumor Necrosis Factor Receptor 2 Plays a Minor Role for Mycobacterial Immunity. Pathobiology 2000;68:68-75.

(172) Kim E Y, Priatel J J, Teh SJ, and Teh HS. TNF Receptor Type 2 (p75) Functions as a Costimulator for Antigen-Driven T Cell Responses In Vivo. J Immunol 2006;176(2):1026-35.

(173) Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. Blood 2006 Jul 1;108(1):253-61.

(174) Chan FK, Lenardo MJ. A crucial role for p80 TNF-R2 in amplifying p60 TNF-R1 apoptosis signals in T lymphocytes. Eur J Immunol 2000;30(2):652-60.

(175) Grell M, Zimmermann G, Gottfried E, Chen C M, Grünwald U, Huang D C, et al. Induction of cell death by tumour necrosis factor (TNF) receptor 2, CD40 and CD30: a role for TNF-R1 activation by endogenous membrane-anchored TNF. EMBO J 1999;18(11): 3034-43.

(176) Mori T, Doi R, Toyoda E, Koizumi M, Ito D, Kami K, et al. Regulation of the resistance to TRAIL-induced apoptosis as a new strategy for pancreatic cancer. Surgery 2005 Jul;138(1):71-7.

(177) de Jong S, Timmer T, Heijenbrok FJ, de Vries EG. Death receptor ligands, in particular TRAIL, to overcome drug resistance. Cancer Metastasis Rev 2001;20(1-2):51-6.

(178) Corazza N, Brumatti G, Schaer C, Cima I, Wasem C, Brunner T. TRAIL and immunity: more than a license to kill tumor cells. Cell Death Differ 2004 Dec;11(Suppl 2):S122-5.

(179) Rifapentine and isoniazid once a week versus rifampicin and isoniazid twice a week for treatment of drug-susceptible pulmonary tuberculosis in HIV-negative patients: a randomised clinical trial. The Lancet 2002 Aug 17;360(9332):528-34.

(180) Mistry R, Cliff JM, Clayton CL, Beyers N, Mohamed YS, Wilson PA, et al. Gene-expression patterns in whole blood identify subjects at risk for recurrent tuberculosis. J Infect Dis 2007;195(3):357-65.

Stellenbosch University http://scholar.sun.ac.za

(181) Johnson BJ, Estrada I, Shen Z, Ress S, Willcox P, Colston MJ, et al. Differential Gene Expression in Response to Adjunctive Recombinant Human Interleukin-2 Immunotherapy in Multidrug-Resistant Tuberculosis Patients. Infect Immun 1998 Jun 1;66(6):2426-33.

(182) Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J Exp Med 1992 Jul 1;176(1):287-92.

(183) Vasiliev AM, Vasilenko RN, Kulikova NL, Andreev SM, Chikileva IO, Puchkova GY, et al. Structural and functional properties of IL-4delta2, an alternative splice variant of human IL-4. J Proteome Res 2003;2(3):273-81.

(184) Atamas SP, Choi J, Yurovsky VV, White B. An alternative splice variant of human IL-4, IL-4 delta 2, inhibits IL- 4-stimulated T cell proliferation. The journal of Immunology 1996 Jan 15;156(2):435-41.

(185) Veenstra H, Baumann R, Lukey PT, Beyers N, van Helden P, Walzl G. High level of intracellular IL-4 are expressed in circulating apoptotic T cells in patients with tuberculosis and in community controls. Clin Exp Immunol 2007;Accepted for publication.

(186) Khalil POeal. Regulation of the effects of TGF-beta1 by activation of latent TGF-beta1 and differential expression of TGF-beta receptors (TGF-betaR-I and TGF-beta R-II) in idiopathic pulmonary fibrosis. Thorax 2001;26(2001):907-45.

(187) Toossi Z, Gogate P, Shiratsuchi H, Young T, Ellner JJ. Enhanced production of TGF-beta by blood monocytes from patients with active tuberculosis and presence of TGF-beta in tuberculous granulomatous lung lesions. The journal of Immunology 1995 Jan 1;154(1):465-73.

(188) Zhu J, Yamane H, Cote-Sierra J, Guo L, Paul WE. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell Res 0 AD;16(1):3-10.

(189) Seah GT, Scott GM, Rook GA. Type 2 cytokine gene activation and its relationship to extent of disease in patients with tuberculosis. J Infect Dis 2000 Jan;181(1):385-9.

(190) Seah GT, Rook GAW. High Levels of mRNA Encoding IL-4 in Unstimulated Peripheral Blood Mononuclear Cells from Tuberculosis Patients Revealed by Quantitative Nested Reverse Transcriptase-Polymerase Chain Reaction; Correlations with Serum IgE Levels. Scandinavian Journal of Infectious Diseases 2001;33(12 supp 1):49-52.