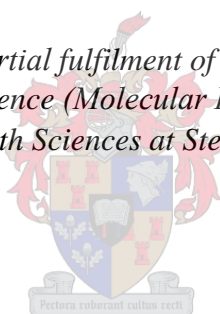# Bio-profiling of TB patients with and without Type 2 Diabetes before and during anti-tuberculosis drug (ATD) therapy

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

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March 2017

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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March 2017

ABSTRACT

Background

Patients with type 2 diabetes (DM2) are three times more likely to acquire active tuberculosis (TB) compared to otherwise healthy people. TB-DM2 comorbidity is characterized by poor TB treatment outcomes, increased risk of failure and relapse. The exact mechanisms of increased susceptibility of diabetics to TB are not well understood, but it is thought that hyperglycaemia is associated with an impairment of the innate and adaptive immune response.

Aim

To assess soluble (cytokines) and cellular (T-cell subsets) immunological markers of treatment response in TB patients with and without DM2 and their association with glycaemic control.

Materials and Methods

Serum cytokine concentrations were measured by means of the Luminex assay in 14 TB and 11 TB-DM2 patients at Baseline, Month 2 and Month 6. The HO-1 levels were measured from serum samples by means of ELISA in 40 TB and 20 TB-DM2 patients at Baseline, Week 2, Month 2 and Month 6. The frequency of different T-cell subsets (central memory, naïve, effector memory and terminally differentiated effector memory T cells) was established in 8 healthy controls, 13 DM2, 18 TB and 23 TB-DM2 patients at Baseline, Month 2 and Month 6 using flow cytometry.

Results

Throughout treatment pro- and anti-inflammatory cytokine concentrations were increased in serum of TB-DM2 patients when compared to TB patients. Fibrinogen and Procalcitonin were higher in TB patients compared to the TB-DM2 patients at the end of treatment. Various cytokines (IL-1β, IL-4, IL-6, IL-7, IL-8, IL-9, G-CSF, GM-CSF, MCP-1(MCAF) and IFN-γ) and growth factors (VEGF and PDGF) exhibited positive correlation at baseline with HbA1c and random blood glucose, respectively. The frequencies of $CD4^+$ naïve T cells increased from Baseline and Month 2 in TB patients. In contrast $CD4^+$ and $CD8^+$ naïve T cells decreased over time in the TB-DM2 patients. $CD4^+$ T CM cells increased over time in TB-DM2 patients. Activated $CD8^+$ naïve T cells increased over time in both TB and TB-DM2 patients while the terminally differentiated effector memory T cells decreased in both groups of study patients over time. No significant changes were observed in any $CD8^+$ T cell subset in both groups of study patients.

Conclusion

The present study reveals that TB-DM2 patients have altered cytokine production at baseline and throughout treatment which may be linked to chronic inflammation associated with obesity and diabetes. Glycaemic control displays an influence in cytokine production shown in TB-DM2 patients. The frequencies of T cell subsets is altered in TB-DM2 patients and changes throughout treatment. These results show that TB-DM2 patients are characterised by changes in the adaptive immune system, which may contribute to poor treatment outcomes.

OPSOMMING

Agtergrond

Pasiënte met tipe 2-diabetes (DM2) is drie keer meer geneig om aktiewe tuberkulose (TB) te ontwikkel in vergelyking met andersins gesonde mense. TB-DM2 komorbiditeit word gekenmerk deur swak TB-behandelingsuitkomste, verhoogde risiko van mislukking en terugval. Die presiese meganisme van verhoogde vatbaarheid van diabete vir TB word nie goed verstaan nie, maar dit word vermoed dat hiperglisemie ge-assosieer is met 'n beskadiging van die ingebore en aanpasbare immuunrespons.

Doel

Om oplosbare (sitokiene) en sellulêre (T-sel deelversamelings) immunologiese merkers van behandelingsrespons in TB-pasiënte met en sonder DM2 en hul verbintenis met glisemiese beheer te evalueer.

Materiale en Metodes

Serum sitokien-konsentrasies is gemeet deur middel van die Luminex-toets in 14 TB en 11 TB-DM2 pasiënte by die basislyn, Maand 2 en Maand 6. Die HO-1 vlakke is gemeet in serum monsters deur middel van ELISA in 40 TB en 20 TB-DM2 pasiënte by die basislyn, Week 2, Maand 2 en Maand 6. Die frekwensie van die verskillende T-sel deelversamelings (sentrale geheue, naïef, effektor geheue en terminaal gedifferensieerde effektor geheue T selle) is bepaal in 8 gesonde kontroles, 13 DM2, 18 TB en 23 TB-DM2 pasiënte by die basislyn, Maand 2 en Maand 6 met behulp van vloeisitometrie.

Resultate

Dwarsdeur behandeling is pro- en anti-inflammatoriese sitokien-konsentrasies verhoog in serum van TB- DM2 pasiënte in vergelyking met TB-pasiënte. Fibrinogeen en PCT was hoër in TB-pasiënte as in die TB-DM2 pasiënte aan die einde van die behandeling. Verskeie sitokiene (IL-1β, IL-4, IL-6, IL-7, IL-8, IL-9, G-CSF, GM-CSF, MCP-1 (MCAF) en IFN-γ) en groeifaktore (VEGF en PDGF) toon 'n positiewe korrelasie by basislyn met HbA1c en ewekansige bloedglukose, onderskeidelik. Die frekwensies van CD4$^+$ naïewe T- selle neem toe van die basislyn tot Maand 2 in TB-pasiënte. In teenstelling neem die CD4$^+$ en CD8$^+$ naïewe T selle af oor tyd in die TB-DM2 pasiënte. CD4$^+$ T CM selle verhoog oor tyd in TB-DM2 pasiënte. Geaktiveerde CD8$^+$ naïewe T selle verhoog oor tyd in beide TB en TB-DM2 pasiënte terwyl die terminaal gedifferensieerde effektor geheue T selle afneem

in beide groepe studie-pasiënte oor tyd. Geen beduidende verandering is waargeneem in enige CD8$^+$ T sel deelversameling in beide groepe studie-pasiënte.

Gevolgtrekking

Die huidige studie toon dat TB-DM2 pasiënte veranderde sitokien produksie by basislyn en dwarsdeur behandeling het wat gekoppel kan word aan chroniese inflammasie geassosieer met vetsug en diabetes. Glisemiese beheer het 'n invloed op sitokien produksie in TB-DM2 pasiënte getoon. Die frekwensies van T-sel deelversamelings is verander in TB-DM2 pasiënte en verandering geskiet regdeur behandeling. Hierdie resultate dui daarop dat TB-DM2 pasiënte gekenmerk word deur veranderinge in die aanpasbare immuunstelsel, wat kan bydra tot swak behandelingsuitkomste.

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It has been a tough journey throughout my study and without the support of many, I would not have been able to finish this work. I sincerely hope to be able to express my gratitude with the following words:

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LIST OF ABBREVIATIONS

A2M	α-2-macroglogulin
AFB	Acid- Fast Bacilli
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AUC	Area under the curve
BCG	Bacillus Calmettee Guérin
BL	Baseline
BMI	Body mass index
CCL5	Rantes
CRP	C-reactive protein
CYP2C9	Cytochrome P450 2C9
DM1	Type 1 Diabetes Mellitus
DM2	Type 2 Diabetes Mellitus
DOTS	Directly observed treatment short-course
EU	European Union
FFA	Free fatty acids
FFA	Free fatty acids
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte monocyte stimulating factor
HbA1c	Glycated Haemoglobin
HIV	Human Immunodeficiency Virus
HO-1	Heme Oxygenase 1
HREC	Human Research Ethics Committee
IDF	International Diabetes Federations
IFN	Interferon
IGRA	Interferon-gamma release assay
IL	Interleukin
INH	Isoniazid
IP	Interferon inducible protein
LTBI	Latent TB Infection
M.tb	Mycobacterium tuberculosis

M2	Month 2
M6	Month 6
MCP-1(MCAF)	Monocyte chemotactic protein
MDR	Multi drug resistant
MDR-TB	Multi-drug resistance TB
MFI	Median fluorescent intensity
MGIT	Mycobacterial Growth Indicator Tube
MIP	Macrophage inflammatory protein
OADC	Oleic Acid albumin Dextrose Catalase
PANTA	Polymyxin B-amphotericin B-nalidixic acid-trimethoprim-azlocillin
PAS	Para-amino salicylate
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCT	Procalcitonin
PDGF	Platelet-derived growth factor
PZA	pyrazinamide
SAA	Serum amyloid protein A
SAP	Serum amyloid protein P
SSA	Sub-Saharan Africa
TB	Tuberculosis
TCM	Central memory T cell
TEM	Effector memory T cell
TEMRA	Terminally differentiated effector memory T cell
TNF	Tumour necrosis factor
Tpa	Tissue-type plasminogen activator
TTP	Time to positivity
VEGF	Vascular endothelial growth factor
W2	Week 2
WHO	World Health Organization
XDR-TB	Extensively-drug resistant TB
ZN	Ziehl-Neelsen

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CHAPTER 1

Introduction

1.1 History of tuberculosis and drug treatment

Throughout history, tuberculosis (TB) has been one of the pandemics known to mankind, with records dating back to ancient Chinese and Egyptian manuscripts together with fossil data demonstrating TB disease in early hominins such as the *Homo erectus* (1). The term "tuberculosis" was coined by a German naturalist, Johann Lukas Schönlein in 1834 describing the condition by its tubercles (2). *Mycobacterium tuberculosis* (*M.tb*), the causative agent of TB, was later discovered in 1882 by Robert Koch and mainly infects alveolar macrophages resulting in the pathogenesis of the disease (3). During the 19th and early 20th century, research had put more focus on TB than any other disease as autopsies became more common. With the discovery of antibiotics, TB declined in the 1950s. Since then, little progress has been made to better elucidate the early and late stage of TB disease and transmission of infection. In spite of years of research, TB continues to be of an important bacterial infection globally (4). One of the turning points in the history of medicine was the discovery of antimicrobial chemotherapy in the 20th century (5).

With the invention of para-amino salicylate (PAS) and streptomycin by Lehmann as well as Schatz and Waksman respectively in the mid-1940s, the search for a cure ended. In the early 1950s, more effective drugs such as pyrazinamide (PZA) and isoniazid (INH) were introduced making TB more treatable (5). Crucial properties of these anti-TB drugs were the ability to prevent drug resistance as well as the ability to exhibit antibacterial activity (5). Anticipated by Ehrlich, the combination of various drug therapies became common when discovered that "under the influence of two different medicines the danger of rendering the parasites immune, which naturally would be a very great obstacle in connection with further treatment, is apparently greatly minimized" (5).

1.2 Tuberculosis: From latent to chronic infection

The survival and maintenance of the bacterium depends on its ability to develop resistance against the host cell defence systems inducing survival, proliferation as well as persistence within the infected cell (6). This is achieved by the production of various virulence factors by the bacterium that potentiates bacterial adherence and invasion into host cell. *M.tb* is transmitted between people by

aerosol droplets containing the bacterium which are inhaled and distally deposited in the lung alveoli (7). It is an intracellular bacterium which predominately infects alveolar macrophages, however, can infect various cell types such as myeloid dendritic cells, neutrophils (8) and alveolar epithelial type II pneumocytes which are abundantly found in the lung alveoli more so than macrophages (7,9). Furthermore, dendritic cells are crucial at the early stages of infection due to their enhanced antigen presentation and are key players in T cell activation specific to *M.tb* antigens. The cellular mediated response aids in preventing bacterial growth as well as dissemination (10). Protective immunological agents such as cytokines and effector molecules (granulysin, perforin and granzymes) are also produced by T cells specific to *M.tb*.

The state of latent infection can be accomplished either by spontaneous resolution of primary TB or by prompt control of *M.tb* growth in the lungs before the commencement of the disease (5). In response to *M.tb* infection, a vigorous cell-mediated immune response is initiated to halt the progression of the disease. This mainly limits the invasion of the lung parenchyma and local draining of lymph nodes by the bacteria, which collectively is called the Ghon complex. However, complete removal of the bacteria is a slow and difficult process to attain. Unfortunately, latent TB can be reactivated after years of persistence resulting in a progressive disease which can be actively transmitted. The underlying mechanisms responsible for reactivation to TB remain unclear, however, it is known that if the host's immune function is compromised, the risk for developing tuberculosis reactivation is increased (11). Latently infected individuals contain a reservoir that is critical for tubercle bacillus survival. Due to compromised immune system resulting in the development of active disease, the ability to persist in and transmit amongst populations renders the eradication of *M.tb* difficult.

1.3 Treatment regimen

Currently antimicrobial drugs are more accessible and treatment is inexpensive, however, lasts too long (6 months) (12). The increase in incidence of extensively-drug resistant TB (XDR-TB) and multi-drug resistance TB (MDR-TB) brings about further challenges in treatment, either by increased costs of treatment or by rendering it ineffective due to poor adherence. With the current drug treatment accompanied by patient compliance, there is still an increased number of multi drug resistant (MDR) in many areas globally (4). As expected, implementation of such regimens in developing countries becomes difficult especially in areas with ineffective public health infrastructure (12). The appearance of human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS)

over the past decades has added a further burden to TB control. Incident cases of TB and HIV co-infections continue to increase due to improper health care systems, particularly in sub-Saharan Africa. Furthermore, TB and HIV drug-drug interactions complicate things further. For host protection against *M.tb*, an effective and reliable vaccine unfortunately remains to be developed (11). This may be due to the complex life cycle of the bacterium in the host which induces various immunological responses that are not completely characterized bringing about undefined biochemical and molecular signature for protecting host against infection.

1.3.1 Directly observed treatment short-course (DOTS) program

At present, there are various medications available for TB treatment (13). However, majority of them have adverse effects. Currently the main treatment regime consists of a cocktail of four drugs taken for two months (Isoniazid, Pyrazinamide, Rifampin and Ethambutol) followed by the administration of two drugs (Isoniazid and Rifampin) for four or five months depending on disease severity as well as treatment response. This extended treatment length which adds to the demanding nature of the treatment (taken on a daily basis), often results in poor patient compliance (14). The DOTS program, introduced by WHO, is an internationally recommended strategy in TB management. The DOTS program consists of five distinct elements: financial and political commitment, quality diagnosis by use of sputum-smear microscopy, standardized treatment provided with direct and supportive observation, high quality and effective drug supply as well as evaluation of treatment. The DOTS program has shown to be effective especially for non-resistant TB strains depending on earlier diagnosis however, not for MDR or XDR-TB. TB is a manageable disease when treated at an early stage; however the pathophysiology is very complex (15). For one to acquire the infection it primarily depends on exogenous factors but reactivation of the disease is largely influenced by a compromised immune system.

1.3.2 Bacillus Calmettee Guérin (BCG) vaccine

Developed by Camille Guérin and Albert Calmette in the 1900s, the bacillus Calmette Guérin (BCG) was initially dispensed to a toddler in 1921 (12). The BCG was developed by a frequent passage of virulent, *Mycobacterium bovis* (*M.bovis*), which is a causative agent of TB in cattle. By the use of primitive in vitro culture methods, an attenuated strain was developed which continues to be effective in inducing protective immunity. In humans, BCG proved to be safe and efficacious and has become one of the vaccines highly administered (12). However BCG, currently the only vaccine used against

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TB, is only able to protect infants from miliary TB, but is unable to protect adults from pulmonary TB (4). Furthermore, in various geographical settings the efficacy of BCG differs. However, developing vaccines specific for intracellular pathogens such as *M.tb* continues to be a challenge. Host protection involves recruitment of cell mediated response of the immune system facilitated by T cells, which is particularly challenging to achieve (12). Therefore there is an urgent need for more effective therapeutics and prophylactic measures to reduce the risk of TB globally (4).

1.4 Epidemiology and immunology of TB

Through the advent of multidrug resistance and acquired immunodeficiency syndrome (AIDS) as being a global health threat, TB disease has become of greater public health importance in both developed as well as underdeveloped countries (18) with an estimated amount of 8.7 million TB incident cases and 1.4 million deaths occurred in 2011 (19). A third of the world's population has been estimated to have been infected with *M.tb* hence TB continues to be a key source of morbidity and mortality globally (20) with HIV infection and malnutrition being well-established risk factors (21). Asia and Africa have the highest TB burden and both China and India account for approximately 40% of the global TB cases (22). At present, diabetes mellitus and TB are of a global public health importance, particularly in the Sub-Saharan Africa (SSA) caused by the convergence of both non-communicable and communicable diseases (23). In 2014, approximately 9.6 million active TB cases were reported where 1.5 million deaths occurred. TB incident rate in South Africa is > 250-fold higher (834 cases per 100,000 population annually) than the United States (3 cases per 100,000 population annually) (Figure 1.1) (24).
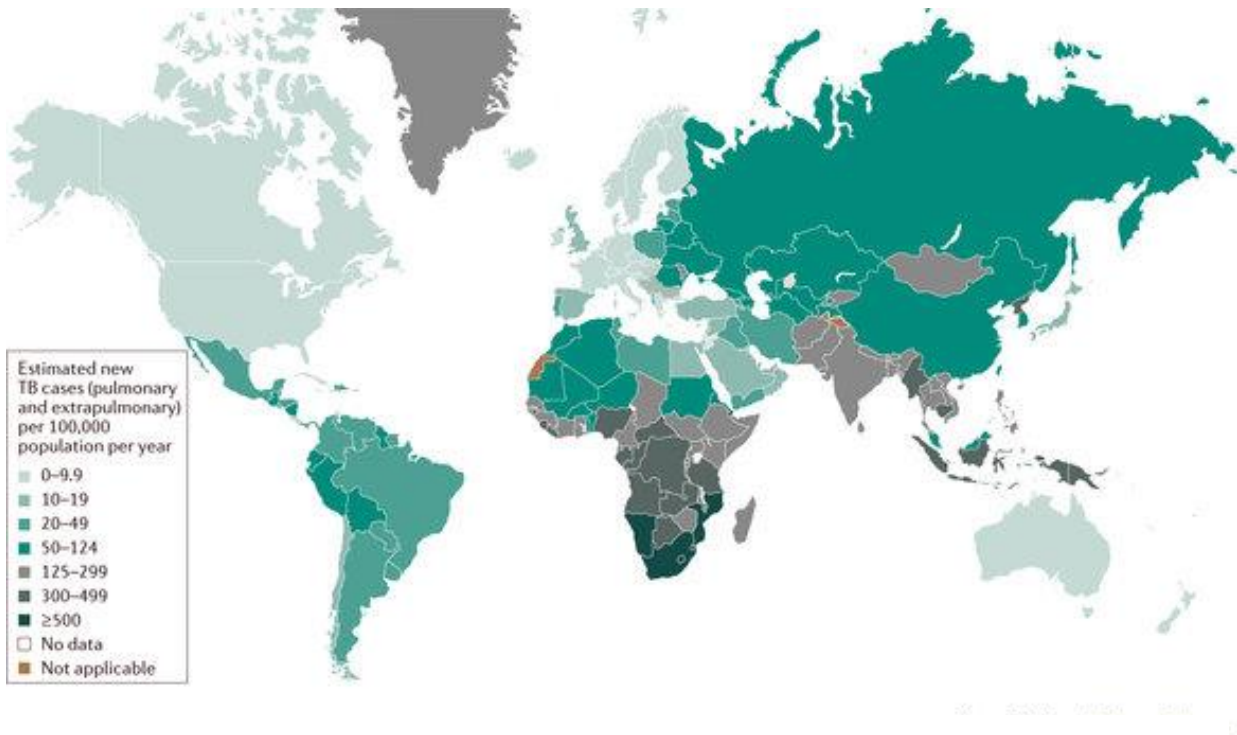
Figure 1.1 Global active TB incidence rate. Low active TB incident rates (< 10 cases per 100,000 annually) are situated in developed countries such as Canada, Australia, Europe, New Zealand and the United States. By contrast, high active TB incident rates are situated in undeveloped countries especially those situated in Africa (24).
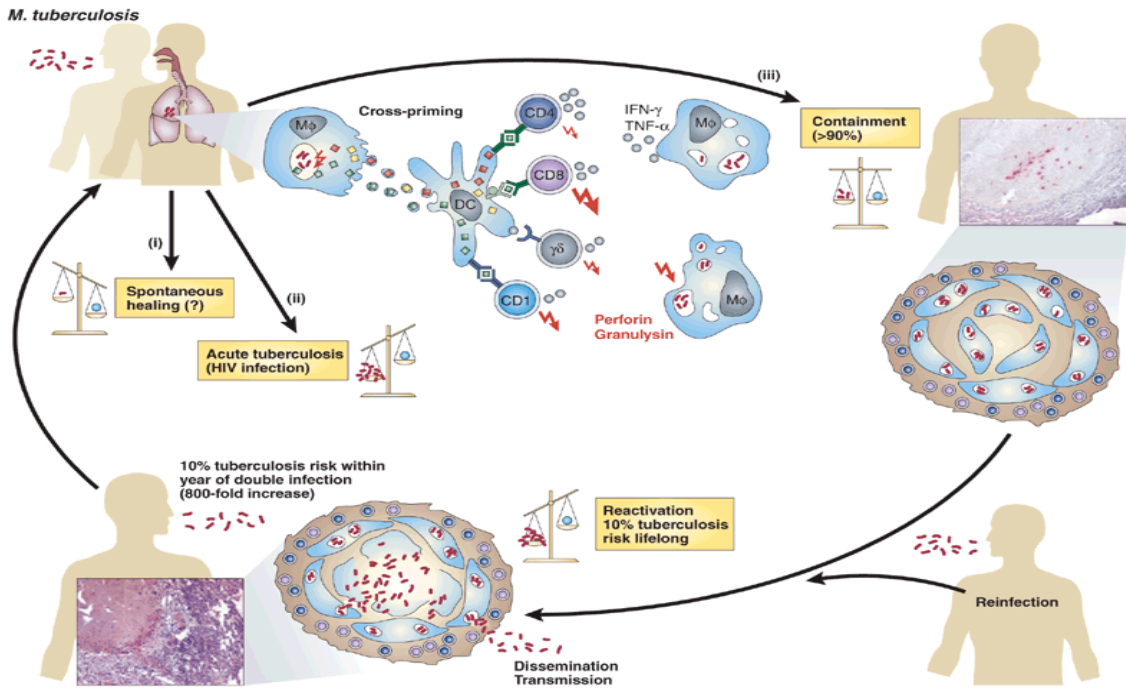
Figure 1.2 Human immunology and underlying mechanism of TB. Aerosol *M.tb* droplets are inhaled. The inhaled bacterium may either **(i)** be immediately destroyed by the immune system in the lungs however this rarely occurs; **(ii)** transforms into an active disease which usually occurs in individuals who are immunocompromised; **(iii)** remain dormant and is contained inside a granuloma rendering the individual as latently infected. *M.tb* in active TB individuals is not contained therefore the bacterium can be transmitted. Alveolar macrophages engulf the inhaled *M.tb* droplets where their antigens are presented to different T cell populations in the draining lymph nodes. Antigen specific T cells (CD1-restricted T cells, γδT cells, CD8+ T cells and CD4+ T cells) play a crucial role in host protection against M.tb. Production of TNF-α and IFN-γ activate macrophages which may aid in the eradication of the bacterium by means of granulysin and perforin (25).

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1.4.1 Pathogenesis of TB

As *M.tb* enters the alveolar space, the bacterium encounters the alveolar macrophages (Figure 1.2) (25). This process initiates the immune response where these macrophages produce inflammatory cytokines and chemokines serving as signals to other immune cells. Protection of the bacterium is due to a recalcitrant cell wall rich in glycolipids which results in a unique staining features. Therefore, infected macrophages do not eliminate the bacterium but serve as a bacterial transporter, conveying the bacterium from the lung parenchyma and drained to the lymph nodes (26). Replication of the bacterium occurs within the resident lung macrophages or alveolar macrophages. Induction of these signals cause the resident dendritic cells and monocyte-derived macrophages to migrate to the site of infection in the lungs. *M.tb* infected dendritic cells migrate to the regional lymph nodes. In the lymph node, $CD8^+$ and $CD4^+$ T cells priming against mycobacterial antigens occur (Figure 1.2) (25). T cells play a crucial role in the host protection against TB and their interaction with infected macrophages aids in the control of the infection (27). These T cells proliferate and migrate back to the lungs to the site of infection, seemingly due to responses by chemokine signals. The formation of a granuloma occurs when the T cells (along with B cells) and macrophages migrate to site of infection. Apart from macrophages and T cells; dendritic cells, fibroblasts, B cells, stromal cells as well as endothelial cells also forms part of the granuloma. The granuloma mainly contains the bacterium for years, provided that the host remains immunocompetent. Inside the granuloma, the bacterium is deprived from nutrients as well as oxygen however survives in a state of dormancy (26). Despite the control of the infection by the immune system, this does not necessarily result in sterilization. Once *M.tb* gains entry into the vacuoles of the macrophages, the protective immunity against the mycobacterium is reliant on the interaction between host cells and T cells (25). Most (90%) LTBI individuals effectively inhibit mycobacterial growth and remain latently infected during their lifetime (53). The rest of the LTBI individuals (10%) ultimately progress to active TB of which commonly occurs in the lung. For this bacterium-host balance to be attained, initially intracellular growth of inhaled *M.tb* is permitted in alveolar cells (macrophages, dendritic cells, phagocytes) in the alveolar space. The increased *M.tb* burden orchestrates the development of adaptive immunity from innate immunity. This activates localized T helper 1 (Th1) inflammatory response and recruits natural killer T cells, monocytes and lymphocytes to form the granuloma that inhibits bacterial growth. Dendritic cells also transport *M.tb* for antigen presentation to T lymphocytes in the regional thoracic nodes from the lungs. T lymphocytes then differentiate into *M.tb*-specific memory and effector T cells (53). Increased TB treatment failure rate in patients with the TB-DM2 comorbidity is rarely due to increased rate of *M.tb* drug resistance or treatment incompliance (54) .

Immunity to *M.tb* depends on several factors, including cytokines, chemokines, antibodies, macrophages, neutrophils, several T-cell subsets, and specific patterns of T-cell migration (30). Interferon-γ (IFN-γ), interleukin-12 (IL-12) as well as tumour necrosis factor-α (TNF-α) are crucial cytokines in bacterial control (28). Immune response to *M.tb* is orchestrated by cytokines of the adaptive and innate immune system of which frequent initiation of cellular type 1 immunity occurs (30). Due to anti-tuberculosis drug (ATD) therapy, altered cytokine concentration mediated by cell signalling, may occur which eventually leads to the recovery of TB disease (7, 10). To provide effective strategies that will ensure appropriate prophylactic and immunotherapeutic regimens, one has to better elucidate the immune response in immunocompromised (HIV co-infected, DM2, malnutrition, etc.) individuals exposed prior to tubercle bacilli (18). The immune response to mycobacteria is a proverbial double-edged sword with one edge battling infection and the other triggering tissue pathology (29). The risk of TB can be stratified into the risk of infection, which occurs in 30% of household contacts, followed by the risk of progression to TB, which occurs in 5-10% of those infected later in their lifetime (30).

1.5 Epidemiology and immunology of Diabetes Mellitus (DM)

Diabetes Mellitus (DM) is a group of metabolic disorders associated with prolonged high blood glucose (31). There are 2 types of DM (type 1 and type 2) of which the most common form of DM is type 2 (90%) and is the leading cause of the diabetes epidemic (31). Type 1 diabetes (DM1) is an autoimmune disorder where a progressive β-cell death occurs resulting in loss of insulin production. Type 2 diabetes (DM2) which is often associated with obesity, the body becomes resistant to insulin (37). In 2011, 366 million people were affected by diabetes where by 2030 this amount will increase to 552 million, according to the International Diabetes Federations (IDF). Most individuals with DM2 are from the ages of 40 to 59 years, and 4.6 million deaths have occurred in this group in 2011 alone (31). Approximately 8.2% adults (20 -79 years) that is 387 million people were diagnosed with diabetes in 2014 when compared to 382 million people in 2013. This number was projected to increase as far as 592 million in 2035. With approximately 46% undiagnosed cases, the increased risk to the development of complicated diabetes is yet ignored. Approximately 47% adults (40-59 years) had diabetes of whom 77% of them live in low and middle income countries. Globally, approximately 4.9 million people deaths had occurred in 2014 (32).

DM2 is the result of impaired glucose tolerance caused by insulin resistance followed by exhaustion of islet β-cell (33). In individuals with impaired glucose tolerance there are various host-related,

environmental and genetic factors that contribute in the progression of insulin resistance to DM2. Individuals with DM2 contain a gross impaired activation, chemotaxis, phagocytosis and antigen presentation by alveolar macrophages, T-lymphocytes and monocytes (34). There is further reduced phagocytosis by polymorphonuclear leukocytes (34, 35). This dysfunctional network is associated with the degree of hyperglycaemia. In this case of *M.tb*, further impairments occur such as cytokine production as well as eradication of pathogen. Hence the overall cell mediated immunity that is crucial in the protection against *M.tb*, is completely dysfunctional in individuals with DM2.

1.6 The double burden of DM2 and TB

One of the major leading examples of the interaction between a metabolic disorder and infectious disease is the association of DM2 with pulmonary tuberculosis (PTB) (36). DM2 is a chronic metabolic disorder which is non-transmissible (37). Both types of diabetes are characterized by dysfunctional metabolism, especially carbohydrate metabolism, as well as dysfunctional pancreatic β-cells (37, 38). Due to altered lipid levels, glucose and protein metabolism, diabetes results in defective micro and macro vascular circulation which sequentially is associated with the disease complications such as peripheral neuropathy, cardiovascular complications, retinopathy, delayed wound healing and nephropathy (37). Although the immunological mechanisms in increased susceptibility to *M.tb* in DM2 patients is still unknown, it has been shown that TB-DM2 patients experience altered innate and adaptive immune response (35). In *M.tb*-naïve individuals, macrophage function is altered during DM2. Furthermore, TB-DM2 patients are found to have decreased phagocytic and chemotactic activity as well as polarization of activated macrophages (18, 29). At the site of infection in the lung, altered alveolar macrophage phenotype are described as being correlated with DM2 status. In TB-DM2 patients, delayed activation of alveolar macrophages occur as determined by decreased expression of activation markers and low production of ROS (29). In a study conducted by Kumar *et al.* diminished type 1 and type 17 cytokine responses were found in latent TB patients with DM2 when compared to latently infected individuals (35).

In 2007 and 2008, the double burden of TB and DM2 became one of global interest amongst researchers (22). Two systematic reviews indicated a role of DM2 in developing active TB and cohort studies showed a relative risk of 3.1 (95% CI 2.3 to 4.3) and case control studies indicated odd ratios of 1.2 to 7.8 (39, 40). The increased risk of TB can be caused by both type 1 and type 2 DM, however DM2 contributes approximately 90% or more of the DM2 cases globally (22). DM2 and TB comorbidity is a public health burden. In 2012, the TB-DM2 incident cases in adults globally were

estimated to be about 15% with the actual number of TB-DM2 adult cases being 1 042 000 (41). This is similar to what is observed during HIV-associated TB. In the United States, 23.1% of individuals over 60 years of age have DM2 which is the seventh leading cause of death (41). DM2 not only increases the risk of acquiring active TB by 3 fold, but also increases the severity of the disease as well as the risk of treatment failures, relapse or death (42, 43). Particularly in people from low and middle classed countries where TB is prevalent, DM2 may be the result of an increased prevalence to TB disease (20, 44). HIV/AIDS has been known to be a greater risk factor for TB on an individual scale as compared to DM (45). However, due to the DM2 pandemic, its effect on TB is as equal as or even greater than HIV. In populations with a heightened HIV infection burden, the effect of DM2 may be masked by HIV (15). In relation to HIV infected individuals where poor TB outcomes are associated with decreased CD4 counts, the additional TB risk and poor TB outcome in individuals with DM2 is related to chronic hyperglycaemia (46). During chronic hyperglycaemia, defects in both innate and adaptive immune systems cause inefficient clearing of the pathogen.

1.7 Mechanisms underlying the increased susceptibility to TB in individuals with DM2

Patients affected by both TB and DM2 are characterised by poor TB treatment outcomes (20). The data presented in a prospective cohort study by Baker *et al*, showed that patients with DM2 and TB have more severe clinical manifestations, delayed sputum conversion and a higher probability of treatment failure, recurrence and relapse (20). TB also worsens glycemic control as part of the stress induced transient hyperglycemia associated with acute infections and makes the control of DM2 difficult (47). TB disease can lead to both transient hyperglycemia and chronic weight loss (47) and maintenance of blood sugar levels, at normal or near normal levels, is one of the primary goals in patient care (48). In a study conducted by Martinez *et al*, mice with chronic hyperglycaemia were susceptible to TB indicated by an increased bacterial burden (52). Poorly controlled diabetes may impair the cell-mediated immune response and neutrophil function and hyperglycaemia alone may provide a better environment for bacterial growth and increased virulence of various microorganisms (21). In a study conducted by Faurholt-Jepsen *et al*, TB-DM2 patients had elevated neutrophil granulocytes and increased acute phase response which could possibly be due to stress induced hyperglycaemia (21). Severe bacterial status on sputum microscopy and culture has been associated with poorly controlled hyperglycaemia (50) which may indicate the influence of hyperglycaemia on bacterial growth.

With regard to the possible effects of DM2 on the presentation and outcome of TB, recent data are scarcer. In the era before insulin therapy, patients with DM appeared to be doomed to die of pulmonary TB if they survived the diabetic coma (49). After the introduction of insulin in 1922, TB remained a serious and deadly threat to patients with DM, but with effective anti-TB treatment, their prognosis improved dramatically (49). Wang and colleagues, who conducted a study in Taiwan, found that diabetic patients were more likely to present with cavitary nodules (50). The causes were not clear, but the tendency probably implies that although diabetic hosts are able to develop granulomas and caseous necrosis, they are unable to eradicate mycobacteria due to abnormal innate effector cells such as macrophages. Moreover, sputum bacteriology results reveal that diabetics have a higher prevalence of acid fast bacilli (AFB) smear-positive cases compared to non-diabetic PTB patients (51). Cavitary disease is also associated with a larger population of bacilli. Immunosuppression induced by DM2 may be responsible for the atypical images and higher bacillary load in PTB patients with DM2. It is known that DM2 causes a decrement in lymphocyte activity and a diminution in the number of monocytes and macrophages, with abnormalities in their chemotactic and phagocytic activities. Moreover, DM2 also causes dysfunction of polymorphonuclear leukocytes, with a reduction in their bactericidal activity (51). Chronic DM2 mice had a delayed innate immune response in the presence of *M.tb*. This ultimately delayed bacterium transfer to draining lymph node as well as priming of adaptive immune response, is crucial for inhibiting bacterial growth (52).

1.7.1 DM2 associated with poor TB treatment outcomes

DM2 is known to predispose an individual to develop both primary and reactivated TB (37). With the global increase in the burden of the TB-DM2 comorbidity, the convergence between the two epidemics in relation to TB treatment imply that diabetes could increase the number of people infected as well as increase the need for anti-TB retreatment regimens (20). Irrespective of the improvement of clinical and public health care systems in the management of TB and DM2, not much is known about the biological as well as the immunological mechanisms that clarify this susceptibility (43). Various factors which include those associated with hyperglycaemia and insulin resistance as well as those that affect the function of the immune cells, may have an influence on the increased susceptibility to TB in the TB-DM2 comorbidity.

DM2 is thought to change the host immune defence against TB (Figure 1.3), ultimately resulting in an increased baseline (BL) mycobacterial burden as well as a longer period of culture conversion with treatment (36). Exposure to *M.tb* in healthy individuals without DM2 results in either no infection,

M.tb infection or primary TB (53). Risk to TB reactivation during their lifetime is 10% amongst those infected. Amongst those infected, treatment outcomes may either be successful, treatment failure or death. However, TB relapse may occur amongst those seemingly cured. LTBI infected individuals are not completely immune to all TB strains and therefore re-exposure to different strain results in re-infection. DM2 appears to influence TB pathogenesis as indicated by the bold arrows as "DM" (Figure 1.3). Immunity to *M.tb* in patients with the TB and DM2 comorbidity differs from DM2 patients. TB-DM2 patients experience delayed T cell priming, decreased IFN-γ production as well as dysfunctional phagocytosis. TB-DM2 patients have increased anti-inflammatory IL-10 cytokine, Th1 and Th17 cell-mediated response specific to *M.tb* (53). Treatment failures may be due to the extent of the TB disease, dysfunctional immune response in TB-DM2 patients or decreased anti-TB drug concentrations in TB-DM2 patients (54).
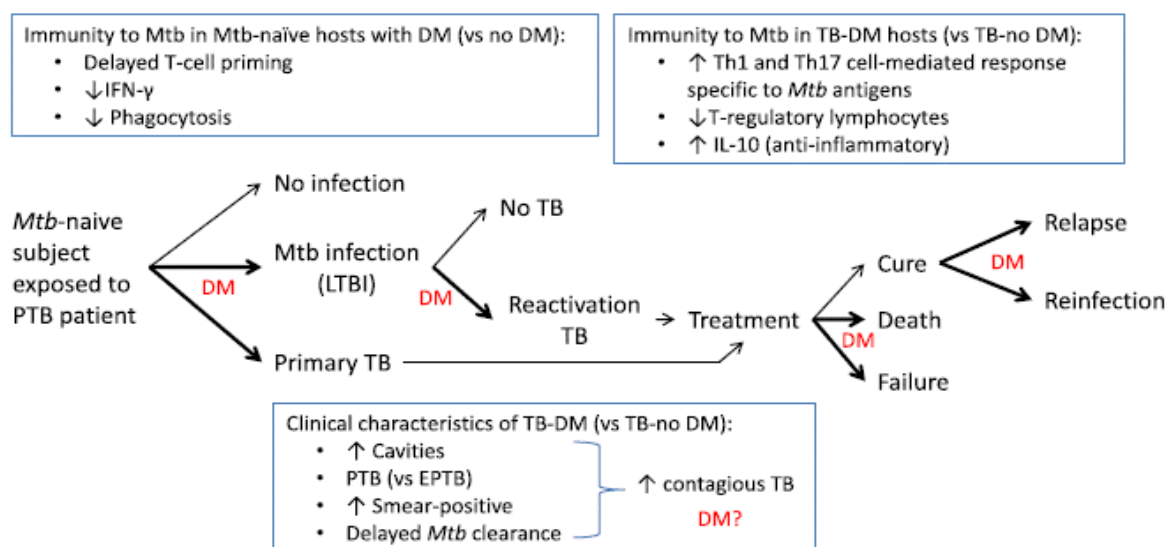
Figure 1.3 The influence of DM2 on TB outcome. DM2 appears to have influence TB pathogenesis as indicated by the bold arrows and "DM". Immune response to *M.tb* differs in DM2 and TB-DM2 individuals as indicated by the upper text boxes. TB-DM2 individuals are likely to exhibit clinical features linked to increased TB transmission however it is still unclear whether that influences disease spread to community (bottom text box). PTB, pulmonary TB; EPTB, extrapulmonary TB; LTBI, latent TB infection (53).

1.7.2 Poor glycaemic control due to TB treatment

Optimal control of blood glucose may improve TB treatment outcomes therefore prevent various diabetes complications. However, TB patients often experience reduced bodyweight, appetite and physical activity which all may have an influence on the homeostasis of glucose (54). In contrast, TB treatment may have opposite effects such as increased body weight, appetite and physical activity as well as decreased inflammation. However, the side effects of these anti-TB drugs in some patients

are increased inflammation or lack of appetite (54). In active TB patients chronic inflammation may result in insulin resistance, however, this is often reversed during TB treatment. Furthermore, good glucose control depends upon quality health care systems (54). A study conducted by Leung and colleagues indicated the association of DM2 with moderate increased risk to culture-confirmed, active pulmonary TB (55). This increased risk was detected mainly among participants with DM2 with a baseline glycated haemoglobin (HbA1c) > 7% as compared to those with a baseline HbA1c < 7%, who were not at increased risk. Poor glycaemic controls were observed to be the cause of the increased risk to TB. Participants with well-controlled glycaemia were shown to be at lower risk to develop TB despite the control of other confounding factors such as body mass index (BMI).

1.7.3 Drug to drug interactions in TB patients with DM2

DM2 is shown to have a negative effect on the prognosis as well as the treatment efficacy of TB. However, there are conflicting results and the effects of DM on the efficacy of TB treatment is not well understood. The pharmacokinetics of various anti-TB drugs are altered due to DM2 (56, 57). The effectiveness of anti-TB drugs is mostly dependant on their plasma concentration. Differences in distribution, excretion, absorption and metabolism in people with DM2 may cause altered plasma levels. Decreased anti-TB drug levels within plasma are associated with drug resistance which in turn complicates the course of TB treatment in people with the TB-DM2 comorbidity (56).

Rifampicin is a strong inducer of the hepatic microsomal enzyme system which often interacts with other drugs, reducing sulphonylureas and biguanides serum levels (58). Therefore, adjustments to oral anti-diabetic drugs should be made in individuals with co-existing DM2. Other anti-TB drugs rarely affect blood glucose levels (58). Several clinical challenges are present in the TB-DM2 comorbidity. Firstly, a severe infection caused by the comorbidity increases the catabolism and blood glucose levels, making diabetes difficult to treat (59). Secondly, rifampicin itself may have hyperglycaemic effects. Lastly, activation of CYP2C9 (a liver enzyme involved in metabolism of various sulphonylurea groups in oral hypoglycaemic agents) by rifampicin, decreases the drug efficacy of anti-diabetic drugs. In addition, INH predisposes one to peripheral neuropathy that may worsen or imitate diabetic peripheral neuropathy. Furthermore, PZA may interfer with urine ketone testing which should be kept in mind when assessing diabetic ketoacidosis. Theoretically, it would seem as if individuals with diabetes require an extended TB treatment regimen due to the increased failure to treatment (59). After correcting for body weight, which was found to be 20% higher in TB-DM2 individuals, reduced plasma rifampicin concentrations was associated with DM2 especially those with more profound hyperglycaemia. This indicates the influence of DM2 on the

pharmacokinetics of TB drugs. Furthermore, high bodyweight may reduce the rifampicin concentrations especially during the continuation phase of treatment (2-6 months). Though it might be difficult to resolve when fixed drug cocktails are used, TB drug dosage should be adjusted based on patient bodyweight (54).

Even though TB affects glucose tolerance, the TB treatment may also aggravate glycaemic control in patients with DM2 (60). The cytochrome P450 system found in the liver metabolises most of the oral hypoglycaemic agents (34). The main drug used for the management of TB, rifampicin, is a strong inducer of all the P450 systems, which includes CYP3A4, where drugs are generally metabolized. This inducer effect causes a reduced concentration of the hypoglycaemic drugs hence worsens glycaemic control which thus impairs the functions to challenge the pathogen which in this case is *M.tb*. Furthermore, individuals under rifampicin treatment need increased insulin dosages. Additionally, 53% of rifampicin concentrations are reduced when given together with oral hypoglycaemic drugs, which raises concerns for the development of drug resistance and occurrence the of treatment failure.

1.8 Aim

 The aim of the project is to assess soluble and cellular immunological markers of treatment response in TB patients, with and without DM2 and their association with glycaemic control.

1.9 Objectives

1. To establish whether DM2 alters the serum cytokine concentrations and to determine their association with clinical parameters associated with DM2.

2. To establish whether TB-DM2 comorbidity changes the frequencies of different T cell subsets (naïve, central memory, effector memory and terminally differentiated effector memory) compared to TB patients without DM2.

CHAPTER 2

Material and Methods

2.1 Study Participants

Forty TB patients, 23 TB patients with type 2 diabetes (TB-DM2), 14 patients with type 2 diabetes (DM2) and 11 healthy controls were recruited from the Western Cape, South Africa as part of an ongoing TANDEM study funded by European Union (EU). This study was approved by the Human Research Ethics Committee (HREC) of the Faculty of Medicine and Health Sciences in Stellenbosch University; Ethics reference number N13/05/064.

Pulmonary TB was diagnosed by a positive GeneXpert test or a positive sputum smear along with a positive Mycobacterial Growth Indicator Tube (MGIT) culture and abnormal chest X-ray. All individuals received the standard TB treatment (Directly Observed Treatment Short Course – DOTS which is rifampicin, ethambutol, isoniazid and pyrazinamide for 2 months (Intensive phase), followed by rifampicin and isoniazid for 4 months (Continuation phase)). Patients were eligible for this study if they were between the ages of 18 and 60, willing to give written informed consent to participate in this study and willing to be HIV tested or willing to have their HIV status disclosed from clinical records to study field worker. TB-DM2 and DM2 patients had to have a HbA1c $\geq$ 6.5% as well as a fasting glucose of $\geq$ 11.1 mmol/L (200 mg/dL). TB-DM2 patients received diabetes treatment (metformin, gliclazide, insulin, rapid acting insulin and glimepiride) at different doses and drug combination. Patients were excluded from the study if they were severely anaemic (HB < 10g/l), steroid or immunosuppressive drug users for the past 6 months, HIV positive, were currently pregnant and breastfeeding or were diagnosed with type 1 diabetes (DM1).

TB patients were followed up during TB treatment and blood and sputum were collected at TB diagnosis (prior to initiation of treatment (Baseline; BL), month 2 (M2) of treatment and month 6 (M6). Sample collection for DM2 patients and healthy controls were only done at a single time point. Serum samples and saliva aliquots, for all study participants, were stored at -80°C until downstream analysis. Subsets of the total number of study participants were included in different assays (Multiplex bead assay, HO-1 ELISA and Flow cytometry). Study participant number was steered by the matching of individuals, high costs of kits and number of study participants which can be included in the kits.

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2.2 Sample Collection and processing

2.2.1 Serum

Blood was collected into 4 ml plain BD vacutainer® tubes (BD Biosciences, Franklin lakes New Jersey, United States) and centrifuged at 2000 x g for 10 minutes. Serum was then collected, harvested, aliquoted and frozen at -80 °C until further use.

2.2.2 Sputum

Sputum samples collected from study patients were cultured by means of the MGIT protocol which was processed in the Biosafety Level Category 3 (BSL3) laboratory. Further sputum processing was carried out in the BSL3 laboratory.

2.2.2.1 The MGIT method

The MGIT method is utilized for the detection and recovery of the mycobacteria. The tubes contain Middlebrook 7h9 broth containing OADC enrichment (Sigma Aldrich, St Louis, USA) and PANTA (polymyxin B-amphotericin B-nalidixic acid-trimethoprim-azlocillin) antibiotic mixture (Sigma-Aldrich), one of the most commonly used liquid media for mycobacterium cultures. OADC enrichment contains oleic acid, a long-chain fatty acid utilized in the metabolism of the organism; albumin that protects the tubercle bacilli against toxic agents; dextrose an energy source; catalase that kills toxic peroxides in the medium; and sodium chloride to maintain osmotic equilibrium. An oxygen quenched fluorochrome is embedded in the silicone at the bottom of the tube. During bacterial growth within the tube, carbon dioxide replaces free oxygen. As the free oxygen reduces, the fluorochrome is no longer inhibited causing fluorescence within the MGIT tube when visualized under UV light. Fluorescence intensity is directly proportional to the degree of oxygen depletion. Bacterial growth therefore increases the fluorescence. The MGIT tubes are incubated in a BACTEC MGIT 960 instrument and are continuously monitored manually or in an automated fashion. The instrument declares a tube negative if no fluorescence is detected after for 42 days. Bacterial growth can be detected visually by observing the presence of non-homogeneous light turbidity or small granular appearance in the medium.

2.2.2.2 Microbiological sputum processing

Prior to the addition of Mycoprep solution (Sigma-Aldrich), 1 ml of sputum was transferred to a 50 ml tube (BD). For degradation to take place, samples were placed on a shaker for 15 minutes. Phosphate buffer (PO_4; Sigma-Aldrich) was then added to the solution neutralizing the reaction then

centrifuged at 4°C at 3000 x *g* for 15 minutes. Furthermore, 800 µl of PANTA solution was added into the MGIT tubes and mixed by inversion. After centrifugation of sputum mixture, supernatants were discarded and 1 ml of PO_4 was added to the pellet. Half a ml of solution was added to the MGIT tube and inserted into the BACTEC MGIT 960 instrument after which it was monitored for up to 42 days. The instrument records the time to positivity (TTP) where samples with a TTP less than 4 days were considered as contaminated. A MGIT tube containing only PO4 buffer was used as a negative control. If at any point in time the negative control had a positive result, another sputum aliquot (which was stored at -80 °C) was re-cultured.

2.2.2.3 Ziehl-Neelsen (ZN) staining

The ZN stain is a differential bacteriological stain that identifies acid-fast mycobacteria which are capable of retaining carbolfuchin when treated with an acid. Staining for acid fast bacteria was done on smear MGIT positive patients. The molecular weight of the mycobacterium lipid capsule inhibits penetration of aqueous based staining solution allowing the capsule to be waxy at room temperature therefore detectable under the microscope. One ml of positive culture was smeared onto a microscope slide and the bacteria heat-killed by incubating the slide on a heating block for 2 hours at 80°C. The slide was then placed on a staining rack where carbolfuchin (Sigma-Aldrich) was poured over slide. Using a Bunsen burner, slides were heated and left to stand for 5 minutes. Slides were then cleaned with water, then alcohol was poured over the slides and left to stand for 2 minutes. Slides were cleaned again with water, then methylene blue (BD) was poured over slides and left to stand for 2 minutes. Slides were cleaned, air dried and examined by means of a light microscope. Bacteria was counted and given a negative or positive score (scanty, +, ++ or +++). This is the grading system of mycobacterium where smears were graded positive if the following is observed: when $1-9$ acid fast bacilli were seen in 100 microscopic fields (scanty positive), when $10-99$ acid fast bacilli were seen in 100 microscopic fields (scored +), when $1-10$ acid fast bacilli were seen in at least 50 microscopic fields (scored ++), and when more than 10 acid fast bacilli were seen in at least 20 microscopic fields (scored +++).

2.2.3 Peripheral blood mononuclear cells (PBMCs) Isolations

PBMCs were isolated from whole blood by Ficoll-Paque PLUS (D > 1.077g/ml; GE Healthcare Life Sciences, Pittsburgh, PA) density gradient centrifugation. Briefly, blood was collected into $4\times$ 9 ml Sodium-Heparin (NaHep) tubes (BD Biosciences) and was then transferred to 50 ml tubes, diluted 1:1 with phosphate-buffered saline (PBS; Lonza BioWhittaker, Verviers, Belguim) and layered on to Ficoll-Paque (GE Healthcare Life sciences). The layered blood was then centrifuged at 400 x *g* for

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25 minutes in order to form a clear separation of cells. A buffy coat containing the PBMCs was transferred into a new 50 ml tube and washed twice with PBS (Lonza). After washing the cells, the PBMCs were diluted 1:10 in the viability stain Tryphan blue (Sigma Aldrich) and counted using the Countess automated cell counter (Life technologies, California, United States). The cells were then resuspended in cryomedia that consisted of 10 % Dimethyl sulfoxide (DMSO; GE Healthcare Life sciences) and Fetal bovine serum (FBS; GE Healthcare Life sciences) and aliquoted. Aliquots were stored into the Freezing container, Nalgene® Mr. Frosty (ThermoFisher Scientific, Massachusetts, USA) overnight at -80 °C and transferred to liquid nitrogen the following day until further use. The yield range per 1 ml peripheral blood of donors stored is between $0.8 - 3.0 \times 10^6$ cells/ml.

2.3 Quantification of serum cytokine concentrations using the Multiplex bead assay

2.3.1 Human Cytokine multiplex bead assay

Fourteen TB patients and 11 TB-DM2 patients were selected from the cohort above and included in the serum cytokine analysis. The patients were 1:1 age and gender matched however, 3 TB patients were added to fill the plate. Serum cytokines were measured at BL, M2 and M6 using the human cytokine, chemokine and growth factor panel (Bio-Rad Laboratories, Hercules, CA, USA). Concentrations of 29 host markers were measured: Interleukin (IL)-1β, IL-1ra, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, Eotaxin, Fibroblast growth factor (FGF), granulocyte colony stimulating factor (G-CSF), granulocyte monocyte stimulating factor (GM-CSF), interferon (IFN)-γ, IFN-α2, Interferon inducible protein (IP)-10, monocyte chemotactic protein (MCP)-1(MCAF), macrophage inflammatory protein (MIP)-1α, macrophage inflammatory protein (MIP)-1β, platelet-derived growth factor (PDGF), Rantes (CCL5), tumour necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF).

The assay was performed according to the manufacturer's instructions. Samples were diluted in a 1:4 dilution prior to experiment and run in singlet format due to the high cost of the kits. Diluted antibody-coupled beads were vortexed and added to each well. Diluted standards, controls and samples were added to the appropriate wells indicated on the plate template prepared prior to experiment. The plate was then sealed and incubated with agitation on a plate shaker for 45 minutes. Diluted detection antibodies and streptavidin-PE were then added to each well and incubated with agitation for 30 and 10 minutes, respectively. After each incubation period, plate was washed 3 times by means of the Bio Plex pro wash station (Bio-Rad laboratories). Sheath fluid was then added to each well and the plate placed on the plate shaker prior to data acquisition.

Concentrations of the quality controls of the kits were within the expected ranges. The standard curve for all analytes ranged from 3.2–10000 pg/ml. Bio-Plex Manager Software, version 6.1 was used to measure the median fluorescence intensity of each analyte.

2.3.2 Acute phase multiplex bead assay

Fourteen TB patients and 11 TB-DM2 patients were selected from the cohort above and included in the serum cytokine analysis. The patients were 1:1 age and gender matched however, 3 TB patients were added to fill the plate. Two sets of customized Bio Rad kits were used on the Bioplex platform (Bio Plex™, Bio Rad Laboratories). Serum cytokines were measured at BL, M2 and M6 using the acute phase 5 and 4 plex cytokine panel. Concentrations of 9 host markers were measured: Procalcitonin (PCT), ferritin, tissue-type plasminogen activator (Tpa), fibrinogen, Serum amyloid protein A (SAA), α-2-macroglogulin (A2M), haptoglobin, C-reactive protein (CRP), Serum amyloid protein P (SAP).

The assay was performed according to the manufacturer's instructions. Samples were diluted in a 1:100 dilution and 1:10 000 dilution for the 4 and 5 plex, respectively and run in singlet format due to high cost of kits. Plate was pre-wet prior to the addition of diluted antibody-coupled beads to each well after which it was washed twice by means of the Bio plex Pro wash station. Diluted standards, controls and samples were then added to the appropriate wells indicated on the plate template, prepared prior to experiment. The plate was then sealed and incubated with agitation on a plate shaker for an hour. Detection antibodies and streptavidin-PE were added to each well and the plate then incubated on a plate shaker for 30 and 10 minutes, respectively. After each incubation period, plates were washed 3 times. Sheath fluid was then added to each well and the plate placed on the plate shaker prior to data acquisition.

Each plate contained an internal control which was prepared prior to the study and is routinely used in the laboratory. The internal control had been prepared and stored at -20°C prior to usage. The internal control was prepared by the use of whole blood from healthy individuals that were stimulated with the mitogen Phytohaemagglutinin (PHA; Sigma-Aldrich) for 4 days to stimulate cytokine production. All analytes measured in the quality control reagents of the kits were within the estimated ranges. The Bio Plex Manager Software, version 6.1 was used for the analysis of the median fluorescence intensity. The beads were analysed on the Bio-plex array reader (Bio-rad, Hercules, CA, USA). A standard curve with a 5PL (Parameter Logistic) spline curve-fitting was used for calculating cytokine/chemokine concentrations in samples.

2.4 Quantification of Heme Oxygenase 1 (HO-1) in serum

For the HO-1 ELISA (Abcam, Cambridge, United Kingdom) serum samples from 40 TB and 20 TB-DM2 patients were used at BL, week 2 (W2), M2 and M6. The patients were matched by age and sex at a ratio of 1 TB-DM2 to 2 TB patients. The assay was performed according to the manufacturer's instructions. Neat samples were used and were run in duplicates. Standards and samples were added to the appropriate wells as indicated on the plate templates and the plate incubated with agitation for 30 minutes. The HO-1 polyclonal detection antibody and horseradish peroxidase (HRP) conjugated anti-IgG antibody were both added to each well and incubated for an hour each. Plates were washed three times manually after each incubation period. A substrate solution was added producing a blue colouration. The following step was to add a stop solution to each well prior to being read at 450 nm on an iMark microplate reader (Bio-rad).

2.5 Quantification and differentiation of cell subsets using flow cytometry

2.5.1 MultiTEST assay

The multiTEST 4 colour reagent uses a lyse/no-wash method for immunofluorescent staining of human blood specimens. The TruCOUNT tubes contain a lyophilized pellet with a known quantity of fluorescent beads. Whole blood was added to the TruCOUNT tubes containing the MultiTEST reagents and absolute counts as well as lymphocyte percentages of T cytotoxic ($CD3^+$ $CD8^+$), T helper ($CD3^+$ $CD4^+$) and total ($CD3^+$) cells enumerated using the FACS Canto flow cytometer. The second tube is used to enumerate natural killer ($CD3^-$ $CD16^+$/$CD56^+$), B ($CD3^-$ $CD19^+$) and mature T ($CD3^+$) subsets. Fourteen TB-DM2 patients and 36 TB patients were selected from the cohort above and included in the BD Multiset assay (Becton Dickinson (BD), San Jose, CA). Enumeration of cell subsets were done at BL and M6. Twenty µl of the MultiTEST reagent was pipetted into the bottom of the tube. Anti-coagulated whole blood was mixed and 50 µl was added into the bottom of the tube avoiding smearing blood down the side of the tube. The tube was then capped and vortexed gently and incubated for 15 minutes in the dark at room temperature (20 - 25°C). 450 µl of $1 \times$ FACS lysing solution was added to the tube. The tube was then capped and vortexed again gently and incubated for 15 minutes in the dark at room temperature. The samples were then analysed by means of the FACS Canto cytometer and using the FACS Diva 6.0 software (BD).

2.5.2 T cell phenotyping in TB, TB-DM2, DM and HC patients

T cell phenotyping was performed by the use of 8 colour Flow cytometric analysis on the FACS Canto cytometer and using the FACS Diva 6.0 software (BD). Twenty-three TB-DM2, 18 TB, 13 DM2 and 8 healthy controls were selected from the cohort above were included in this assay. Viable cryopreserved PBMCs (see section 2.2.3) were thawed using prepared thawing media (RPMI 1640; Biowest, Riverside, United States; 10 % FBS- GE Healthcare Life sciences; 5 % L-glutamin- Sigma) and washed in PBS (Lonza) once after which cells were incubated for 15 min in the presence of LIVE/DEAD Zombie aqua fixable viability dye (Biolegend, San Diego, California, US). After the incubation period, cells were washed twice initially with PBS (Lonza) and then with FACS Lysing solution (BD). To determine the proportion of different T cell subsets, cells were stained for an hour with the following antibodies: anti-CD3$_{PerCP-Cy5.5}$, anti-CD4$_{BV421}$, anti-CD38$_{APC/Cy7}$, anti-CD45RA$_{APC}$ (all from BD) and anti-CD197$_{PE}$ (Biolegend). After cell surface staining, cells were washed twice and resuspended with FACS lysing solution (BD). At least 100 000 cells were acquired for reproducibility of results. The FlowJo software version 10.1 (Treestar, San Carlos, CA) was used to analyse the data.

2.6 Statistical analysis

Statistical differences between two study groups were analyzed by the use of the unpaired Student's t test. This test was used to compare two sets of quantitative data of samples collected independently of one another. A value of $p < 0.05$ indicated significant difference. Analyses of data were done by means of the GraphPad prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA) and correlations with the spearman rank test which measures the strength and direction of association between two ranked variables. Further data analysis was done by means of Statistica version 11 (StatSoft, Tulsa, Oklahoma, USA). Results were transformed by means of logarithm base-10 to normalize the distributions. For the Luminex and ELISA analysis, study groups were also analysed by a mixed-model repeated-measures analysis of variance (ANOVA) with a Fisher LSD Post hoc test using the Statistica. Analysis of the T cell subsets was done by means of two-way ANOVA with a Bonferroni Post hoc test using GraphPad prism version 5.00. An ANOVA test was used to determine whether there are any statistically significant differences between the median of three or more independent (unrelated) groups. Demographic and biochemical parameters within the T cell subsets was done by means of the non-parametric Kruskal-Wallis test with a Dunns Post hoc test using GraphPad prism version 5.00.

CHAPTER 3

Elevated serum markers found in TB-DM2 patients verses TB patients may be characterized by dysregulated immune response to *M.tb*

3.1 Introduction

Effective immune responses to *M.tb* are critical for a successful TB treatment outcome and to minimise treatment failures as well as reactivation of the disease (61). The release of various cytokines is achieved by the complex interaction of *M.tb* with the immune system. A balance between pro- and anti-inflammatory immune responses mediated by cytokines is required and contributes to a favourable outcome of TB treatment (62). The aim of this study was to establish whether dysregulation of specific immunological pathways exist during the TB-DM2 comorbidity and whether such a dysregulation is associated with poor TB treatment response.

3.2 Methods

Serum samples were collected from 14 TB patients and 11 patients with the TB-DM2 comorbidity at BL (prior to TB treatment), M2 and M6 (end of treatment). The concentrations of 38 host markers were evaluated in the serum samples by means of the luminex technology as described in Chapter 2. The HO-1 concentrations were evaluated by means of a HO-1 ELISA using serum samples from 40 TB and 20 TB-DM2 patients at BL, W2, M2 and M6.

3.3 Results

3.3.1 Demographics and biochemical features of the study population

Given that among TB patients, DM2 is the most prevalent comorbidity and this comobidity increases by age (63), this study focused on patients from the ages of 18 to 60. Study patients were 1:1 age and sex matched for reproducibility of results, however, some patients were matched 2:1. An additional 3 TB patients were added to fill the plate of the assay. Therefore, no significant differences were found in the age between study patients ($p = 0.6609$) (Table 3.1). No statistical differences were found in the BMI between the two groups at all time points however, as expected; individuals with TB-DM2 comorbidity had a higher BMI (22.61 ± 5.248) at BL compared to TB patients (19.20 ± 2.599; Table 3.1). As expected BMI of all study patients gradually increased throughout treatment (Table 3.1).

With regards to the biochemical features between study patients, there was a trend that TB-DM2 individuals have higher random blood glucose compared to the TB individuals (Table 3.2). The haemoglobin concentrations did not differ between study patients. Glycated haemoglobin (HbA1c) measured at BL and M6 in study patients was higher in the TB-DM2 patients as compared to the TB patients with significant difference shown at BL ($p < 0001$; Table 3.2). In comparison to timepoints, in TB-DM2 group HbA1c levels decreased from BL (9.655 ± 2.994) to M6 (7.875 ± 3.701), however, did not differ in the TB group between time points. Figure 3.1 is a 2D categorized scatterplot that exhibits the different concentrations of HbA1c in both study patients individually. As shown by the scatterplot, individually, the TB-DM2 patients had higher HbA1c concentrations as compared to TB (Figure 3.1). The lipid profile (HDL cholesterol, LDL cholesterol, total cholesterol and triglycerides) and creatinine concentrations measured at W2 and M6 in the TB-DM2 group had no significant differences. However, HDL cholesterol, total cholesterol, triglycerides and creatinine concentrations were higher at M6 compared to W2 in this group. LDL cholesterol levels on the other hand displayed the opposite with higher levels at W2 compared to M6 (Table 3.2).

Table 3.1 Demographics of study patients

Study Demographics	TB (n=14)	TB-DM2 (n=11)	p-value
Age (years)*	42.71 ± 10.10	42.09 ± 9.159	P = 0.6609
Sex (F/M)	6/8	6/5	
BMI (kg/m²)* -Baseline -Month 2 -Month 6	19.20 ± 2.599 20.02 ± 2.145 20.24 ± 2.494	22.61 ± 5.248 23.19 ± 4.833 23.54 ± 5.762	P = 0.0667 P = 0.1063 P = 0.2180

*Results are shown as mean ± SD, F: female M: male, BMI: body mass index
Statistical diference is determined by the Mann Whitney t test. A $p < 0.05$ is considered significant.

Table 3.2 Biochemical parameters of study patients

Study Demographics	TB (n=14)	TB-DM2 (n=11)	p-value
Random blood glucose (mg/dL)	5.746 ± 1.321	8.750 ± 4.098	P = 0.0536
Haemoglobin	13.29 ± 1.821	13.08 ± 2.239	P = 0.9563
HbA1c* -Baseline -Month 6	5.793 ± 0.4779 5.582 ± 0.4094	9.655 ± 2.994 7.875 ± 3.701	P < 0.0001 [a] P = 0.4054
HDL* cholesterol (mg/dL) -Week 2 -Month 6	-	1.260 ± 0.5232 1.300 ± 0.3317	P = 0.4603
LDL* cholesterol (mg/dL) -Week 2 -Month 6	-	2.780 ± 0.9223 2.363 ± 0.4307	P = 0.3976
Total cholesterol (mg/dL) -Week 2 -Month 6	-	4.530 ± 1.189 4.689 ± 0.9765	P = 0.9346
Triglycerides (mg/dL) -Week 2 -Month 6	-	1.080 ± 0.4290 2.089 ± 1.668	P = 0.3047
Creatinine -Week 2 -Month 6	-	57.50 ± 16.26 61.56 ± 16.19	P = 0.6532

*Results are shown as mean ± SD, HbA1c: Glycated haemoglobin, HDL: High density lipoprotein, LDL: Low density lipoprotein
[a] Statistical difference as determined by the Mann Whitney t test. A $p < 0.05$ is considered significant.
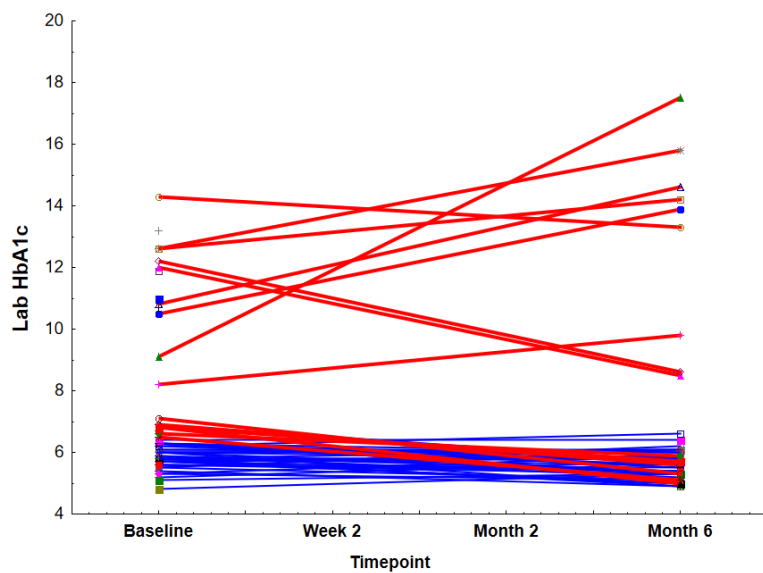
Figure 3.1 2D categorized scatterplot of HbA1c Concentrations. An individual scatterplot that exhibits different HbA1c concentrations in TB (n=14) and TB-DM2 (n=11) measured at BL and M6 end of treatment. Study groups were also analysed by a mixed-model repeated-measures analysis of variance (ANOVA) with a Fisher LSD Post hoc test using the Statistica program version 11. Red lines – TB-DM2; Blue lines – TB.

3.3.2 TB-DM2 comorbidity is associated with altered serum marker concentrations compared to TB without DM2

3.3.2.1 Increased Heme Oxygenase-1 (HO-1) levels exhibited in TB group towards end of treatment

Although the main mechanisms in the pathogenesis of TB-DM2 is still not well understood, oxidative stress induced by hyperglycaemia, free fatty acids (FFA) as well as glucose fluctuations was proposed to influence the development of DM2 (64). Also known as Hsp32, HO-1 is an inducible isoform of heme oxygenase that is highly responsible to various types of stimuli that cause oxidative stress and is up regulated in the presence of oxidants, UV irradiation as well cytokines (65, 66). Highly expressed in the lung tissue, HO-1 is a crucial enzyme in response to stress (67). Active TB patients have reduced systemic antioxidant levels and increased production of free radicals, demonstrating excessive oxidative stress associated with the disease. Enhanced plasma HO-1 expression has been found in patients with various pulmonary disorders such as asthma and chronic obstructive pulmonary disease. Of these disorders, the plasma HO-1 levels were associated with disease severity. Therefore, to compare the effects of altered HO-1 levels in TB management, HO-1 levels were measured in both groups at BL, W2, M2 and M6 from 40 TB and 20 TB-DM2 study participants. With the TB participants from BL to W2, HO-1 concentrations significantly decreased however increased from W2 and significantly from M2 to M6 (Figure 3.2). In the TB-DM2, HO-1 concentrations fluctuate, but no significant changes observed during treatment.
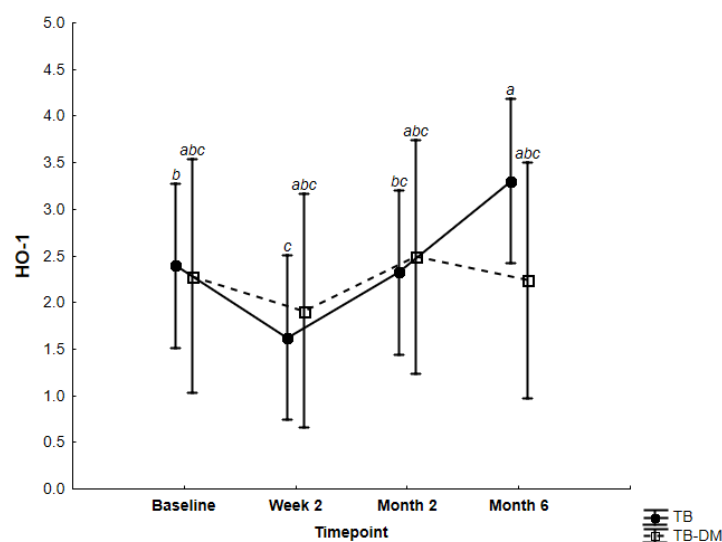
Figure 3.2 Increased Heme Oxygenase-1 (HO-1) levels exhibited in TB group towards end of treatment. HO-1 measured in TB (n=40) and TB-DM2 (n=20) patients at BL, W2, M2 and M6 following anti-tuberculosis treatment. Results were log-transformed. Study groups were analysed by a mixed-model repeated-measures analysis of variance (ANOVA) with a Fisher LSD Post hoc test. Letters indicate statistical differences. The same letter suggests no difference whereas different letters indicate a statistically significant difference.

3.3.2.2 FGF, G-CSF, GM-CSF, MIP-1β and IL-9 serum markers were significantly higher in TB-DM2 patients at baseline, month 2 and end of treatment

TB-DM2 patients had significantly higher concentrations of FGF, G-CSF, GM-CSF, MIP-1β and IL-9 as compared to the TB patients from baseline throughout the course of TB treatment (Figure 3.3 A-E). In both TB and TB-DM2 patients the serum concentrations of these markers decreased significantly from BL to M2, whereafter they remained unchanged, indicating a TB treatment specific effect. As DM2 is characterised by chronic inflammation, the increased concentrations of these serum markers is likely due to chronic inflammation driven by DM2.
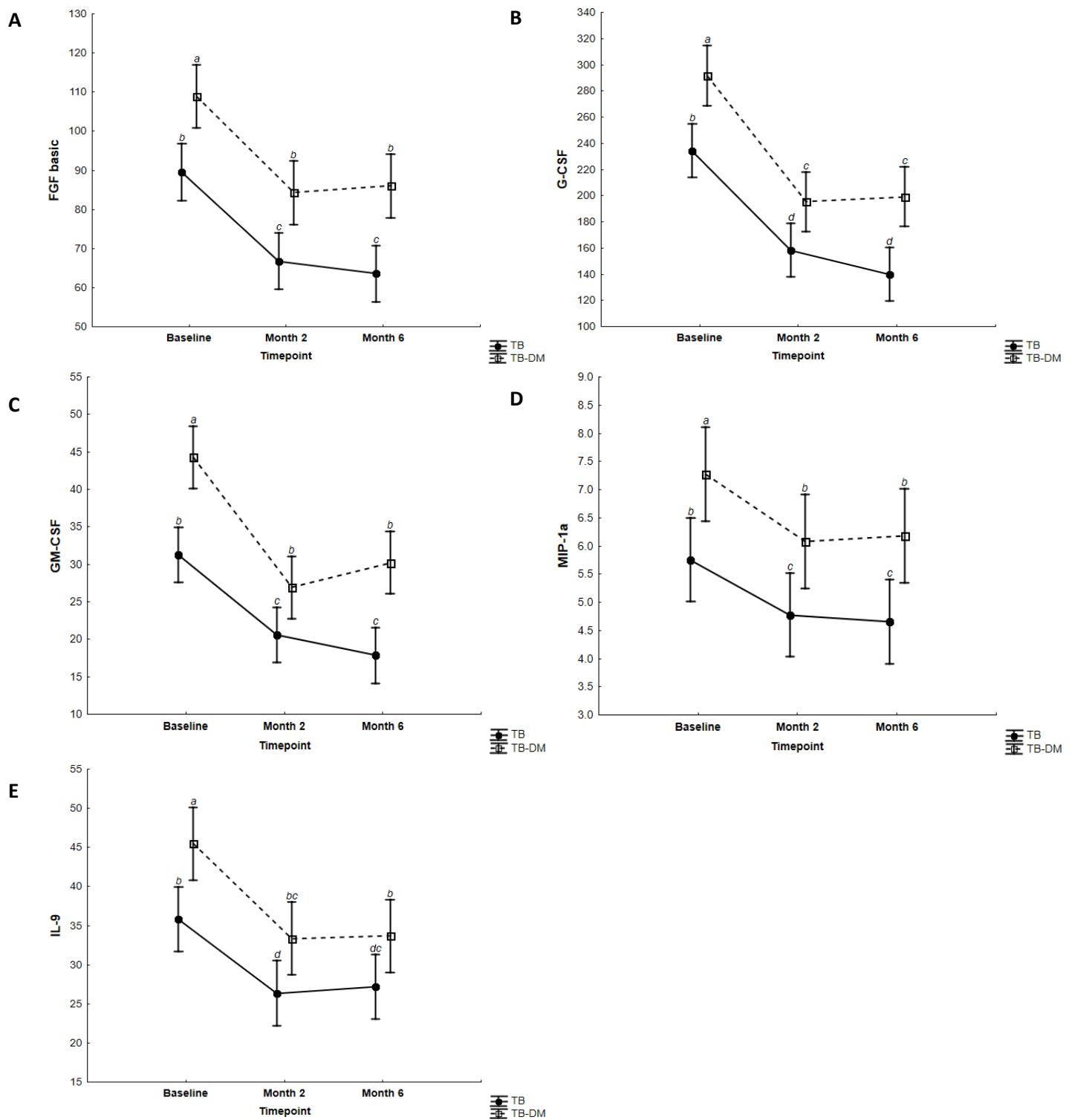
Figure 3.3 FGF, G-CSF, GM-CSF, MIP-1β and IL-9 serum markers were significantly higher in TB-DM2 patients at baseline, month 2 and end of treatment. Increased **A**. FGF, **B**. G-CSF, **C**. GM-CSF, **D**.MIP-1β and **E**. IL-9 concentrations found in TB-DM2 patients. Serum cytokine markers measured in TB (n=14) and TB-DM2 (n=11) patients at BL, M2 and M6 end of anti-tuberculosis treatment. Results were log-transformed. Study groups were analysed by a mixed-model repeated-measures analysis of variance (ANOVA) with a Fisher LSD Post hoc test. Letters indicate statistical differences. The same letter suggests no difference whereas different letters indicate a statistically significant difference.

3.3.2.3 VEGF concentrations are significantly higher in TB-DM2 patients at baseline and month 2 but not at the end of treatment

VEGF, a homodimetric molecule, is a heparin-binding glycoprotein of 34 to 42 kd that forms one of the crucial inducers of vascular permeability as well as angiogenesis (68). VEGF is a growth factor assumed to act mainly on vascular endothelial cells (69). Increasing evidence has suggested that VEGF plays a role in the pathophysiology of diabetes (69). Elevated VEGF concentrations are mostly found in individuals with high blood pressure and DM. As expected, the TB-DM2 patients had significantly higher VEGF concentrations at BL and M2 but not M6 when compared with the TB patients (Figure 3.4). In both respective groups, the VEGF concentrations significantly decreased with TB treatment from BL to M2, but remained unchanged between M2 and M6 (Figure 3.4).
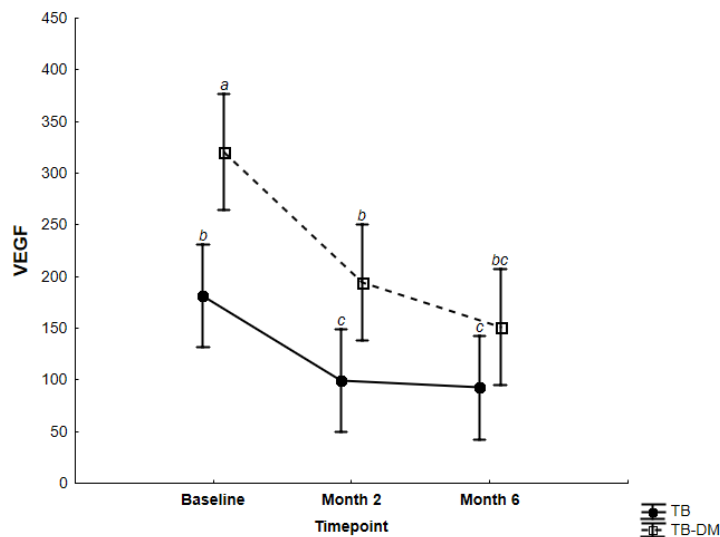
Figure 3.4 VEGF concentrations are significantly higher in TB-DM2 patients at baseline and month 2 but not at the end of treatment. VEGF concentrations measured in TB (n=14) and TB-DM2 (n=11) patients at BL, M2 and M6 end of anti-tuberculosis treatment. Results were log-transformed. Study groups were analysed by a mixed-model repeated-measures analysis of variance (ANOVA) with a Fisher LSD Post hoc test. Letters indicate statistical differences. The same letter suggests no difference whereas different letters indicate a statistically significant difference.

3.3.2.4 Significant differences in serum cytokine concentrations at baseline and end of treatment between TB and TB-DM2 groups

TB-DM2 patients had higher concentrations of IL-6, IL-7, IL-1β, IL-1ra, IL-4, IL-8, IL-13, IL-17, IFN-γ and TNF-α when compared to the TB group with significant differences found at BL and M6, but not M2 of treatment (Figure 3.5 A-J). From BL to M2 of treatment, serum cytokine concentrations mentioned above decreased in both groups. In the TB patients, IL-6, IL-7, IL-1rα, IL-4, IL-8, IL-13, IL-17, IFN-γ and TNF-α concentration further decreased from M2 to M6 of treatment, but did not reach statistical difference. In TB-DM2 patients, IL-1β, IL-ra, IL-4, IL-8, IL-13, IL-17, IFN-γ and TNF-α concentrations increased from M2 to M6 of treatment with only IL-1ra reaching significantly higher concentrations. It is likely that a decrease in bacterial load during the intensive phase of treatment (BL to M2) drives the decrease in these cytokines. However, during the continuation phase of treatment with only 2 drugs it is possible that mycobacterial replication increases in TB-DM2 patients and that the increase in these cytokines from M2 to M6 is an early indication thereof. However, IL-6 and IL-7 remained constant in the TB-DM2 group from M2 to M6 of treatment (Figure 3.5 A and B).
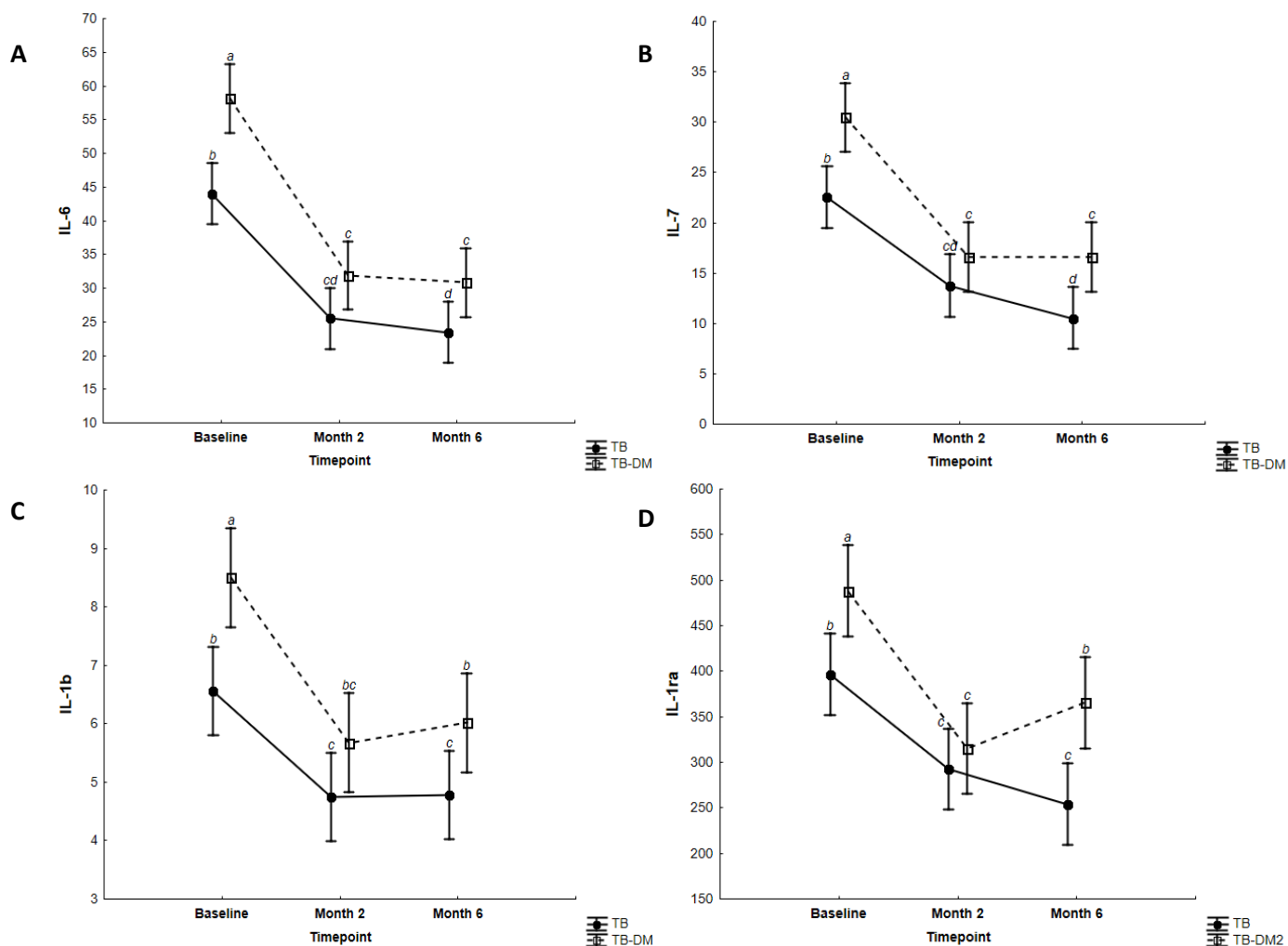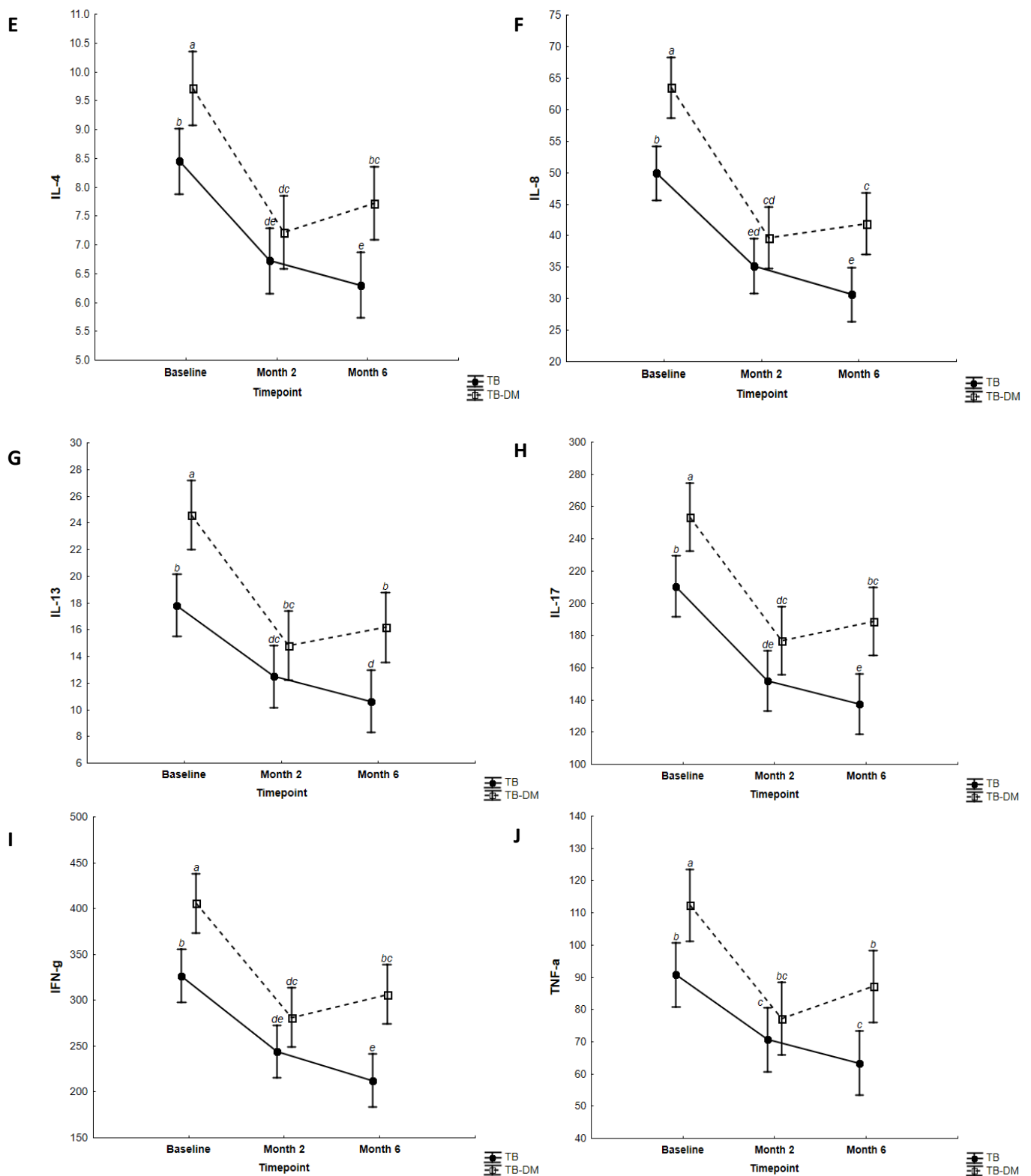
Figure 3.5 Significant differences in serum cytokine concentrations at baseline and end of treatment between TB and TB-DM2 groups. Increased **A.** IL-6, **B**. IL-7, **C**. IL-1β, **D.** IL-1ra, **E**. IL-4, **F.** IL-8, **G**. IL-13, **H**. IL-17, **I.** IFN-γ and **J.** TNF-α concentrations found in TB-DM2 patients. Serum cytokine markers measured in TB (n=14) and TB-DM2 (n=11) patients at BL, M2 and M6 end of anti-tuberculosis treatment. Results were log-transformed. Study groups were analysed by a mixed-model repeated-measures analysis of variance (ANOVA) with a Fisher LSD Post hoc test. Letters indicate statistical differences. The same letter suggests no difference whereas different letters indicate a statistically significant difference.

3.3.2.5 Significant differences in serum cytokine concentrations at the end of treatment between TB and TB-DM2 groups

A subgroup of cytokines showed significant differences between TB and TB-DM2 patients only at the end of treatment. This subgroup can be further divided into cytokines which were significantly higher in TB-DM2 compared to TB at month 6 (IL-5, IL-10, IFN-α2 and α2m) and those which were significantly lower in the TB-DM2 patients (Fibrinogen and PCT). Significant differences between respective groups were only observed at the end of treatment (M6) and these were IL-5, IL-10, IFN-α2, fibrinogen, α2m and PCT (Figure 3.6). In the TB-DM2 patients, IL-5, IL-10 and IFN-α2 concentrations were higher when compared with TB patients. From BL to M2 of treatment, serum cytokine concentrations mentioned above significantly decrease in both respective groups. However, from M2 and M6 end of treatment, IL-5, IL-10 and IFN-α2 concentrations increase in the TB-DM2 patients but gradually decrease in the TB patients (Figure 3.6 A-C). Interestingly with regards to the TB patients, fibrinogen and PCT concentrations were higher than the TB-DM2 patients (Figure 3.6 E and F). In both respective groups α2m concentrations decrease from BL to M6 of treatment with the TB group displaying significant change overtime. However, the cytokine concentration was higher in the TB-DM2 when compared to the TB group at M6 of treatment (Figure 3.6 D). Fibrinogen concentrations significantly decreased in TB patients from BL to M2. Interestingly, from M2 to M6 of treatment, fibrinogen concentrations increase in the TB patients while the opposite occurs with the TB-DM2 patients (Figure 3.6 E). In both respective groups from BL to M2 of treatment, PCT remained constant. Similar to fibrinogen, from M2 to M6 PCT concentrations increase in the TB patients but remained unchanged in the TB-DM2 patients.
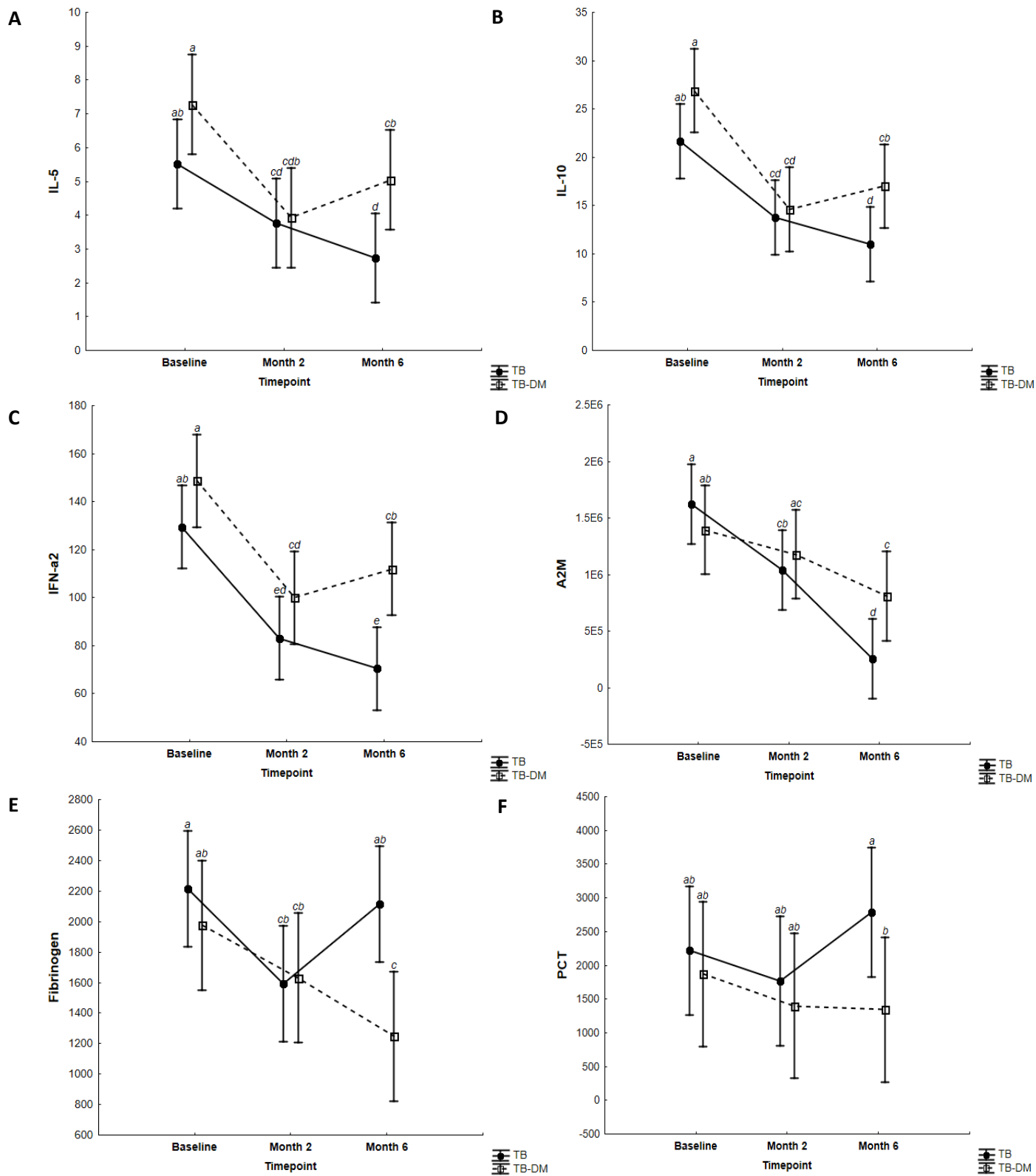
Figure 3.6 Significant differences in serum cytokine concentrations at the end of treatment between TB and TB-DM2 groups. Increased **A**. IL-5, **B.** IL-10, **C**. IFN-α2 and **D**. α2m concentrations found in TB-DM2 patients while **E**. Fibrinogen and **F**. PCT concentrations were found to be increased in TB patients. Serum cytokine markers measured in TB (n=14) and TB-DM2 (n=11) patients at BL, M2 and M6 end of anti-tuberculosis treatment. Results were log-transformed. Study groups were analysed by a mixed-model repeated-measures analysis of variance (ANOVA) with a Fisher LSD Post hoc test. Letters indicate statistical differences. The same letter suggests no difference whereas different letters indicate a statistically significant difference

3.3.2.6 Haptoglobin concentrations are significantly higher in TB-DM2 compared to TB at month 2 but not at baseline or end of treatment DM2

Haptoglobin is a haemoglobin (Hb)-binding protein which is also an acute-phase protein and its synthesis is elevated during inflammation (70). With immunomodulatory properties, haptoglobin stimulates or inhibits the immune response. Elevated levels of this acute-phase protein is mostly found during infectious inflammatory processes (71). As shown in figure 3.7 from BL to M2 of treatment, haptoglobin serum concentrations significantly decreased in TB patients, however, remained unchanged with the TB-DM2 patients. Haptoglobin concentrations were significantly higher in TB-DM2 patients compared to TB patients but only at M2.
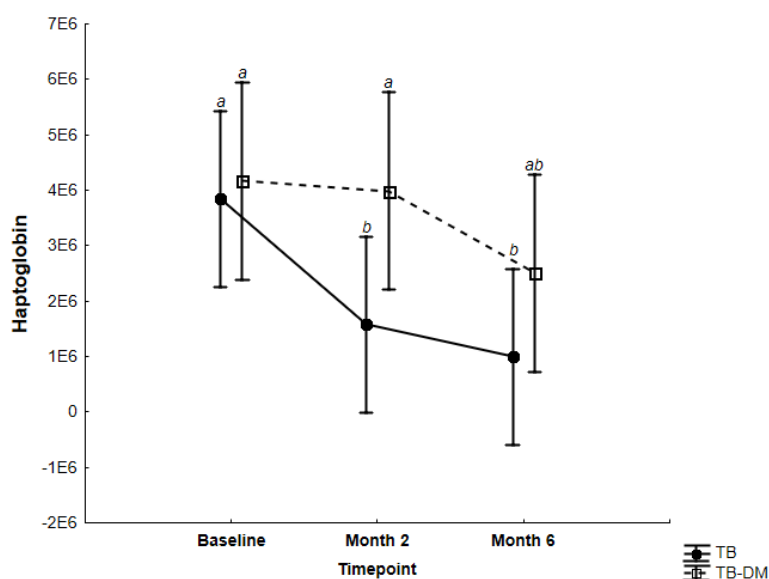
Figure 3.7 Haptoglobin concentrations are significantly higher in TB-DM2 compared to TB at month 2 but not at baseline or end of treatment DM2. Haptoglobin concentrations measured in TB (n=14) and TB-DM2 (n=11) patients at BL, M2 and M6 end of anti-tuberculosis treatment. Results were log-transformed. Study groups were analysed by a mixed-model repeated-measures analysis of variance (ANOVA) with a Fisher LSD Post hoc test. Letters indicate statistical differences. The same letter suggests no difference whereas different letters indicate a statistically significant difference.

3.3.2.7 MCP-1(MCAF) serum concentrations are significantly higher in TB-DM2 patients at diagnosis

MCP-1(MCAF) is a chemoattractant marker which Is produced by mononuclear cells and several non-leukocytic cells such as renal resident cells (72). It is upregulated during an inflammatory immune response (73). As the name indicates, MCP-1(MCAF) serves as a monocyte chemoattractant, additionally it is also crucial for memory T cells as well as natural killer cells. It is a crucial chemoattractant involved in several pathologies as it is expressed in most inflammatory disorders associated with the recruitment of monocytes, together with delayed hypersensitivity reactions and bacterial infections (74). Significantly high of MCP-1(MCAF) levels has been found to be circulating in patients with DM2 (74). Significant difference was seen at BL only with higher MCP-1 (MCAF) concentrations in the TB-DM2 patients when compared to the TB patients. From BL to M2 of treatment, MCP-1 (MCAF) concentrations significantly decreased in TB-DM2 patients whilst MCP-1 concentrations remained unchanged in TB patients (Figure 3.8).
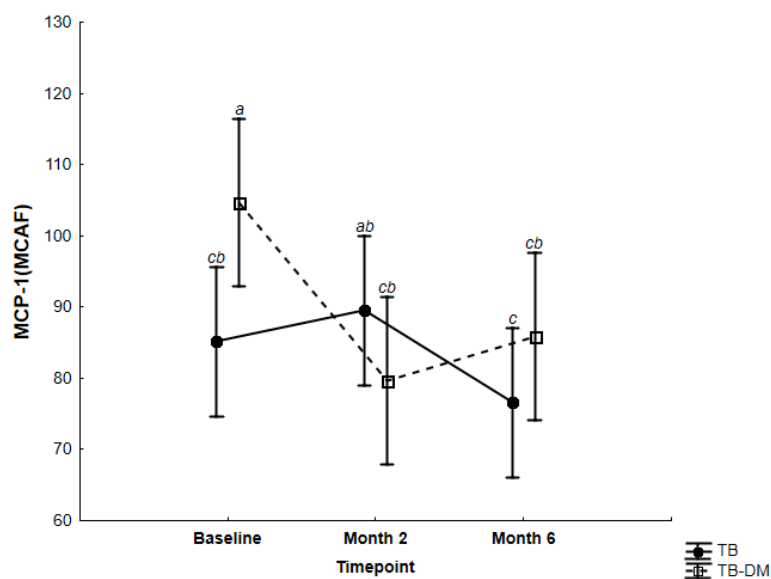
Figure 3.8 MCP-1(MCAF) serum concentrations are significantly higher in TB-DM2 patients at baseline. MCP-1 (MCAF) concentrations measured in TB (n=14) and TB-DM2 (n=11) patients at BL, M2 and M6 end of anti-tuberculosis treatment. Results were log-transformed. Study groups were analysed by a mixed-model repeated-measures analysis of variance (ANOVA) with a Fisher LSD Post hoc test. Letters indicate statistical differences. The same letter suggests no difference whereas different letters indicate a statistically significant difference.

3.3.2.8 No significant differences in serum cytokine concentrations throughout TB treatment in both groups

No significant differences in IL-2, IL-12(p70), IP-10, MIP-1β, Ferritin, Eotaxin, CRP, PDGF, RANTES, SAA, SAP and Tpa serum concentrations were found at any time points between the study groups. Data for IL-1α and IL-15 are not shown due to very low detection measured.

3.3.3 Correlation between body mass index (BMI) and serum cytokine concentrations found at month 2 and month 6

An impaired immune system can be due to nutrition disequilibrium, which might increase the risk of developing active TB. Additionally, the treatment outcome may also be affected in TB patients due to a declined immunity (75,76). Higher BMI increases the risk of DM2, however has been observed to be protective during TB (77). Therefore correlation graphs were plotted to determine the relationship between serum cytokines measured and BMI. BMI was calculated at BL, M2 and M6 in both TB and TB-DM2 groups. No significant correlation between BMI and measured serum cytokines was found at BL. In a study conducted by Suriyaprom *et al* it was found that the relationship between BMI and α2m concentration were inversely correlated, meaning the higher the BMI, the lower the serum α2m concentration (78). This confirms our observation where α2m was found to be negatively correlated with BMI at M2 (Figure 3.9 A). Pro-inflammatory cytokine TNF-α poses crucial weight regulating properties and has an influence on lipid metabolism (79). In agreement with the literature, TNF-α was found to be positively correlated with BMI (Figure 3.9 B). With regards to eotaxin and fibrinogen, both serum cytokine concentrations were negatively correlated with BMI at M6 (Figure 3.9 C and D).
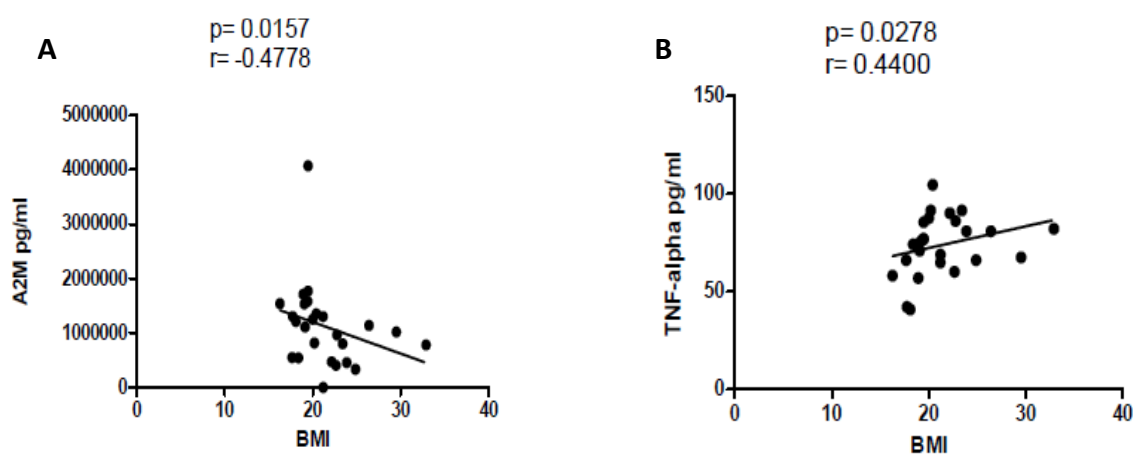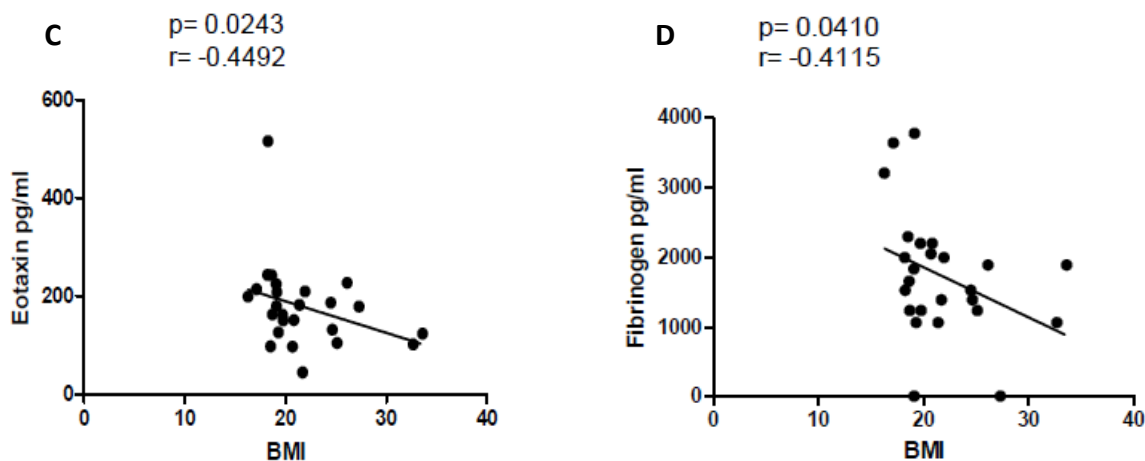
Figure 3.9 Correlation between body mass index (BMI) and serum cytokine concentrations found at month 2 and month 6. Correlation graphs that indicate the relationship between the clinical parameters and serum cytokine concentrations measured at BL, M2 and M6 from TB (n=14) and TB-DM2 (n=11) patients. Significant correlations were found at M2 between BMI and α2m **(A)** and BMI and TNF-α **(B)** as well as M6 with Eotaxin **(C)** and Fibrinogen **(D)**. Two-sided p-values with p < 0.05 were considered statistically significant. Correlations were calculated using the Spearman Rank correlation.

3.3.4 Correlation between blood glucose and serum cytokine concentrations found at baseline

In patients with DM2 there is an altered immune response that gives rise to increased susceptibility to infection or enhanced development of active TB disease. This may be due to changes such as altered random blood glucose or chronic hyperglycaemia, one of the major characteristics of DM2 (80). Therefore to understand the relationship between hyperglycaemia and the production of cytokines in TB, correlations were done between the measured serum cytokines and random plasma glucose and HbA1c concentrations in both TB and TB-DM2 patients. The HbA1c percentages from TB-DM2 patients were measured at BL as well as at the end of treatment. Random plasma glucose was measured in both groups at BL only. At BL, HbA1c correlated positively with IL-1β, IL-4, IL-6, IL-7, IL-8, IL-9, G-CSF, GM-CSF, IFN-γ and MCP-1(MCAF) (Figure 3.10 A-J). Although not significant IL-1β, IL-4, IL-6, IL-7, G-CSF, GM-CSF, IFN-γ and MCP-1(MCAF) maintained the positive relationship at M6 of treatment while IL-8 and IL-9 were found to be negatively correlated with HbA1c at M6 of treatment (Data not shown). Random plasma glucose positively correlated with PDGF-bb and VEGF at BL (Figure 3.10 K and L).

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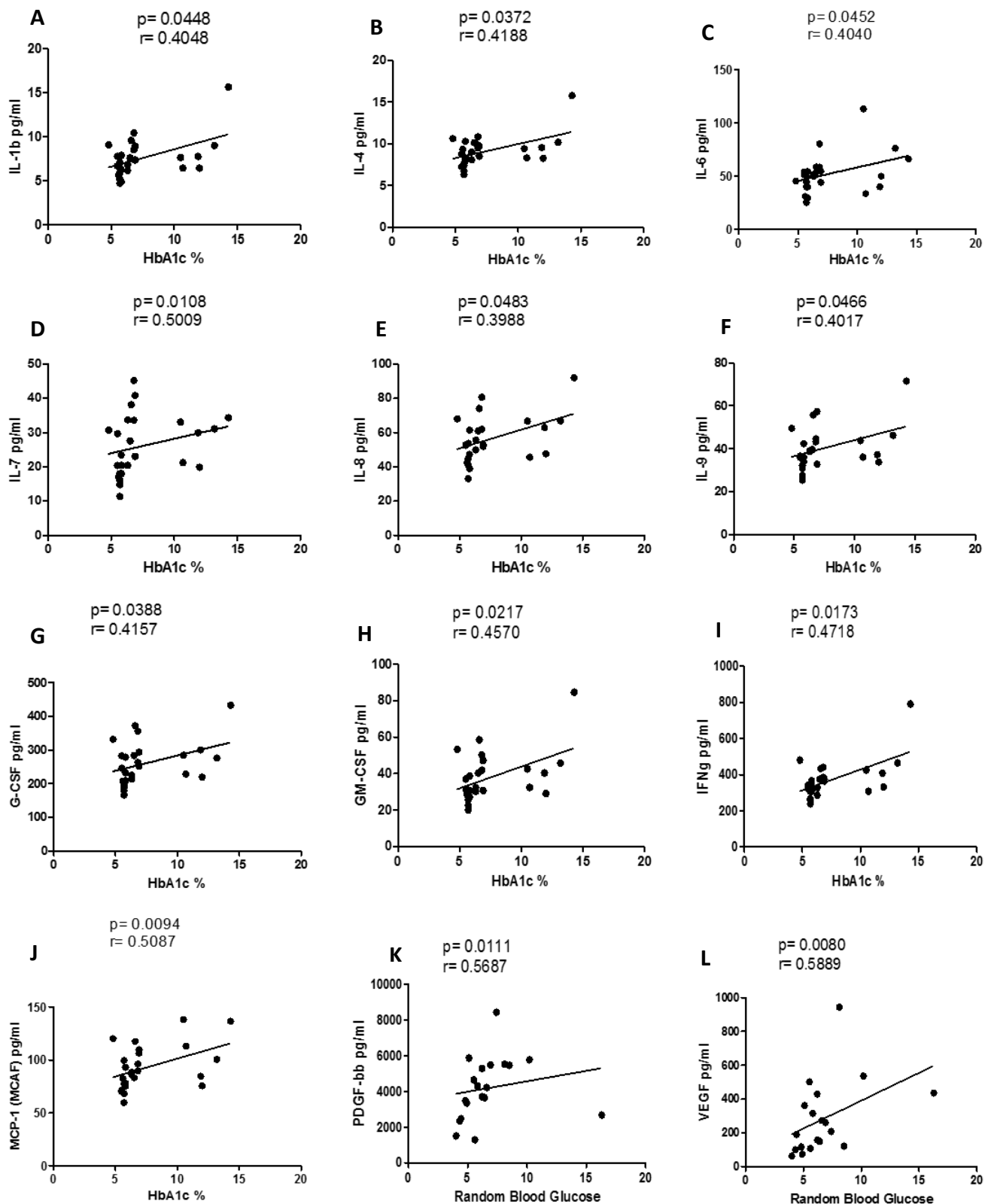
Figure 3.10 Correlation between blood glucose and serum cytokine concentrations found at baseline. Correlation graphs that indicate the relationship between the clinical parameters and serum cytokine concentrations measured at BL, M2 and M6 from TB (n=14) and TB-DM2 (n=11) patients. Significant correlations were found at BL between blood glucose and; IL-1β (**A**), IL-4 (**B**), IL-6 (**C**), IL-7 (**D**), IL-8 (**E**), IL-9 (**F**), G-CSF (**G**), GM-CSF (**H**), IFN-γ (**I**), MCP-1 (MCAF) (**J**), PDGF (**K**) and VEGF (**L**). Two-sided p-values with p < 0.05 were considered statistically significant. Correlations were calculated using the Spearman Rank correlation.

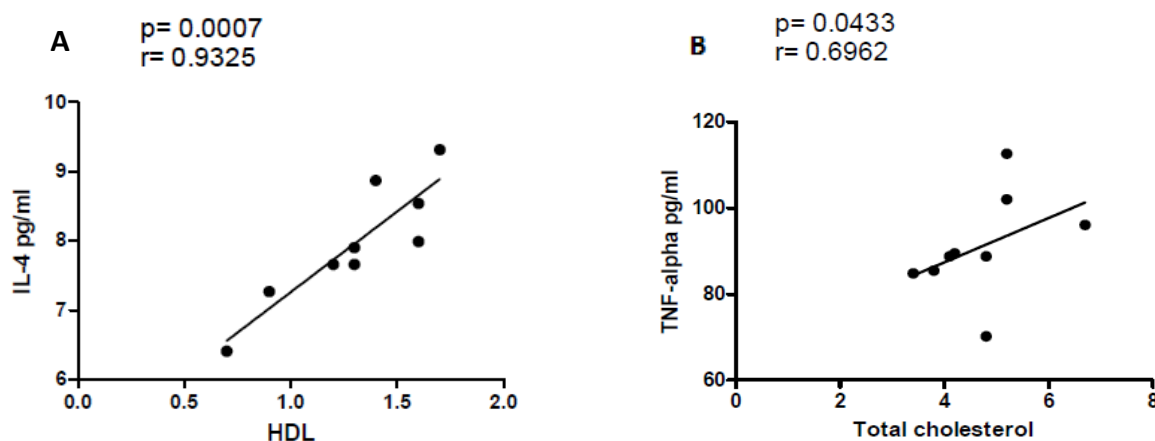### 3.3.5 Correlations between lipid profile and serum cytokine concentrations found at the end of treatment

During DM2 there are abnormalities in lipoproteins concentrations due to insulin deficiency or resistance affecting pathways involved in lipid metabolism (81, 82). This includes, decreased HDL cholesterol levels, increased number of small dense LDL particles and elevated triglycerides. Secreted from hepatocytes and adipocytes, IL-4 plays a role in moderating insulin sensitivity and local immune response (83). A study conducted by Chang-Hui Tsao *et al*, demonstrated that IL-4 is not only shown to improve glucose tolerance and insulin sensitivity but inhibits the increase of lipids in fat tissues leading to reduced weight gain and fat mass (83). This suggests that IL-4 plays a role in the susceptibility to DM2 and lipid metabolism. In the lipid profile measured only on the TB-DM2 patients, indeed IL-4 was shown to be positively correlated with HDL cholesterol at M6 displaying its role in lipid metabolism (Figure 3.11 A). Originally, TNF-α was defined as a cachectin, a protein involved in cachexia development (84). Later TNF-α was shown to be involved in lipid metabolism such as the increase of serum triglycerides which decreases the adipose tissue lipoprotein lipase activity. The latter increases free fatty acids as well as decreases HDL cholesterol levels (85). Few studies have shown the effect of TNF-α on total cholesterol, however, this study shows the positive correlation between the two parameters displaying its role in lipid metabolism (Figure 3.11 B).

Figure 3.11 Correlations between lipid profile and serum cytokine concentrations found at the end of treatment. Correlation graphs that indicate the relationship between lipid profile measured from TB-DM2 patients and serum cytokine concentrations measured at BL, M2 and M6 from TB (n=14) and TB-DM2 (n=11) patients. Significant correlations were found at M6 between HDL and IL-4 **(A)** as well as total cholesterol and TNF-α **(B)**. Two-sided p-values with p < 0.05 were considered statistically significant. Correlations were calculated using the Spearman Rank correlation

3.3.6 Treatment induced changes in T cell and B cell subset of TB-DM2 patients

To determine the percentage and absolute count of T lymphocytes, B lymphocytes and natural killer cells, whole blood was stained with anti-CD3, -CD8, -CD4, -CD45, -CD19, -CD16 and -CD56 antibodies and percentages of different immune cell subsets determined in both TB and TB-DM2 patients.

The percentage and absolute count of T lymphocytes, B lymphocytes and natural killer cells were further correlated with the clinical parameters and measured cytokines to evaluate their respective relationships (Table 3.3). At BL the percentage and absolute count of CD8 T cells exhibited a significant positive relationship with haemoglobin (CD8+ % Lymph: p = 0.01 r = 0.37; CD8+ Abs Cnt: p = 0.03 r = 0.31). However, when correlated with serum cytokines, SAA was negatively correlated with the absolute count of CD8 T cells (p = 0.05; r = -0.43) while α2m exhibited a positive relationship (p = 0.05; r = 0.42) with the percentage of these cells (Table 3.3). The percentage of CD4 T cells exhibited a significant negative correlation with both IL-6 and IL-10 (Table 3.3). HO-1 concentrations were also correlated with measured serum cytokines as well as multiset flow cytometry data to evaluate if a relationship exists between cytokines or T cell markers and HO-1 concentrations (Table 3.4). At BL there was a negative correlation between HO-1 and eotaxin (p = 0.05; r = -0.40) as well as HO-1 and the percentage of CD3+ lymphocytes (p = 0.04; r = -0.30; Table 3.4). The B lymphocytes were positively correlated with overall HO-1 (p = 0.04; r = 0.31) concentrations at BL. At the end of treatment, there was a negative correlation between MCP-1(MCAF) (p = 0.03; r = -0.43) and a positive relationship between Tpa and HO-1 concentrations (p = 0.02; r = 0.46) (Table 3.4).

Table 3.5 is a correlation table between T cell, B cell and natural killer cells markers and clinical parameters or serum cytokines at M6. BMI exhibited a positive correlation with the absolute count of CD19 expressing B cells (p = 0.04; r = 0.31). Both the percentage and absolute count of the CD3+ T cells and CD4+ T cells exhibited a positive correlation with both total cholesterol and triglycerides. When correlated with serum cytokines, IL-1ra exhibited a positive correlation with the percentage of CD8+ T cells. SAA and MCP-1(MCAF) were positively correlated with the T cells and B cells. The percentage of CD4+ T cells and absolute count of the CD45+ memory T cells exhibited a negative relationship with IL-10 and IL-12(p70), respectively. IL-17 exhibited a negative correlation with both the absolute count and percentage of the CD16+CD56+ natural killer cells. Figure 3.12 illustrates the scatterplot of the individual patients where cell subsets were measured at BL and M6.

Table 3.3 Percentages and absolute cell counts correlated with clinical parameters or measured serum cytokines at baseline

Variable 1	Variable 2	Spearman	p-value	# cases
Clinical Parameters:				
-Haemoglobin	CD3+CD8+% Lymph	0.37	0.01	47
-Haemoglobin	CD3+CD8+ Abs Cnt	0.31	0.03	47
Serum cytokines:				
-IL-6	CD3+CD4+% Lymph	-0.45	0.04	22
-IL-10	CD3+CD4+% Lymph	-0.54	<0.01	22
-SAA	CD3+CD8+ Abs Cnt	-0.43	0.05	22
-A2M	CD3+CD8+% Lymph	0.42	0.05	22

Table 3.4 Percentages and absolute cell counts correlated with clinical parameters or HO-1 at baseline and month 6

Variable 1	Variable 2	Spearman	p-value	# cases
Baseline:				
-Eotaxin	HO-1	-0.40	0.05	25
-CD3+ % Lymph	HO-1	-0.30	0.04	47
-CD19+ Abs Cnt	HO-1	0.31	0.04	47
Month 6:				
-MCP-1(MCAF)	HO-1	-0.43	0.03	25
-Tpa	HO-1	0.46	0.02	25

Table 3.5 Percentages and absolute cell counts correlated with clinical parameters or measured serum cytokines at month 6

variable 1	variable 2	Spearman	p-value	# cases
Clinical Parameters: -BMI	CD19+ Abs Cnt	0.31	0.04	47
-Total Cholesterol	CD3+ % Lymph	0.63	0.04	11
-Total Cholesterol	CD3+ Abs Cnt	0.59	0.05	11
-Total Cholesterol	CD3+CD4+% Lymph	0.60	0.05	11
-Total Cholesterol	CD3+CD4+ Abs Cnt	0.73	0.01	11
-Triglyceride	CD3+ % Lymph	0.67	0.02	11
-Triglyceride	CD3+ Abs Cnt	0.60	0.05	11
-Triglyceride	CD3+CD4+% Lymph	0.65	0.03	11
-Triglyceride	CD3+CD4+ Abs Cnt	0.74	<0.01	11
Serum Cytokines: -IL-1ra	CD3+CD8+% Lymph	0.42	0.05	23
-IL-10	CD3+CD4+% Lymph	-0.48	0.02	23
-IL-12(p70)	CD45+ Abs Cnt	-0.46	0.03	23
-IL-17	CD16+CD56+% Lymph	-0.42	0.05	23
-IL-17	CD16+CD56+ Abs Cnt	-0.55	<0.01	23
-MCP-1(MCAF)	CD3+ % Lymph	0.42	0.04	23
-SAA	CD19+ % Lymph	0.42	0.05	23
-SAA	CD19+ Abs Cnt	0.42	0.05	23
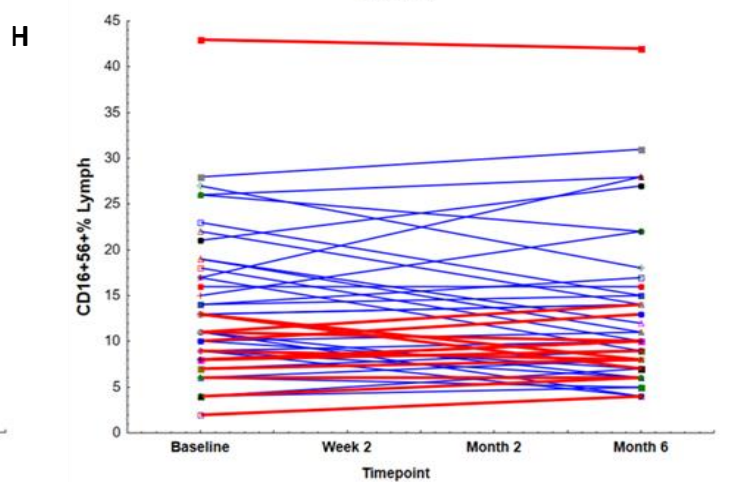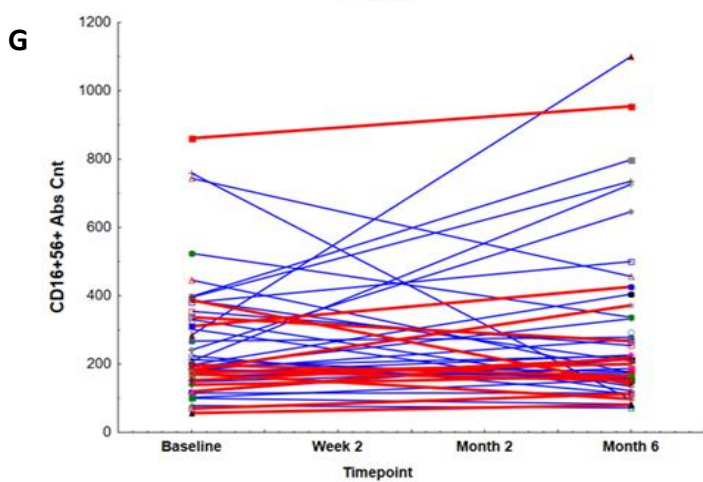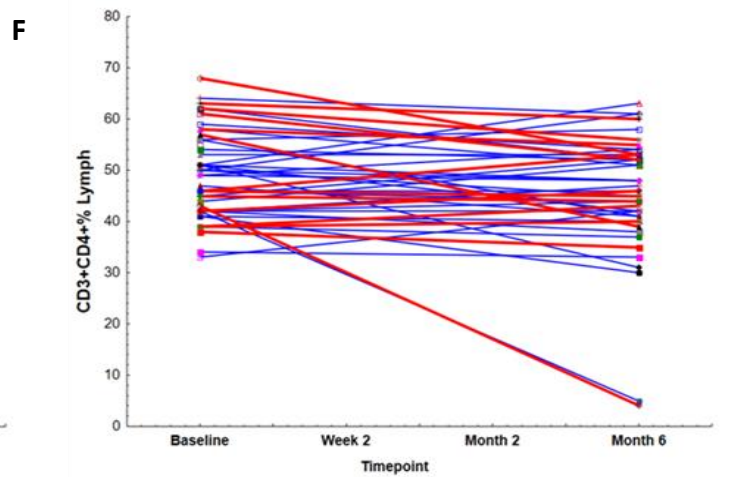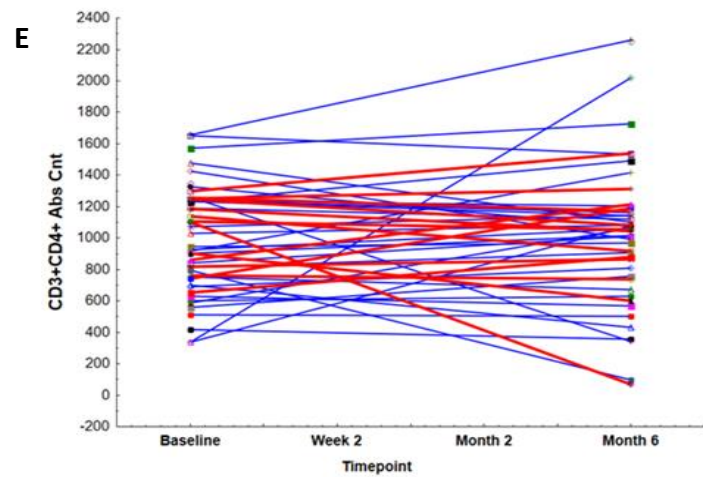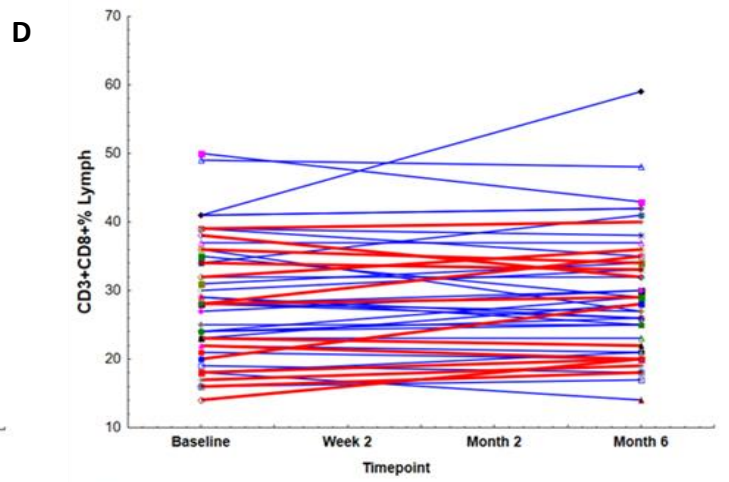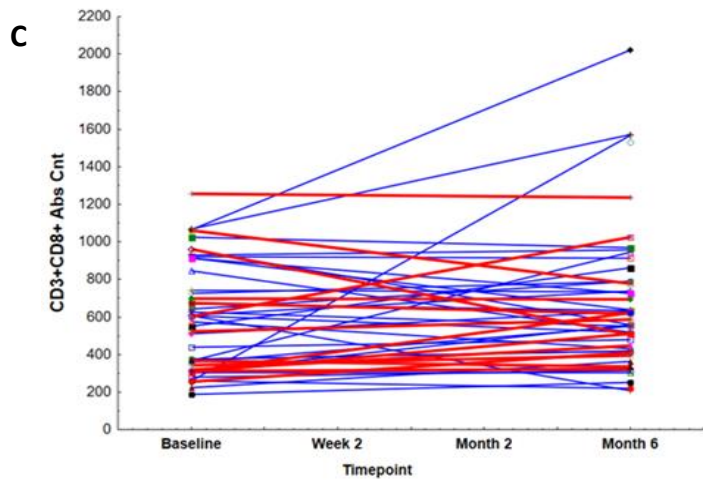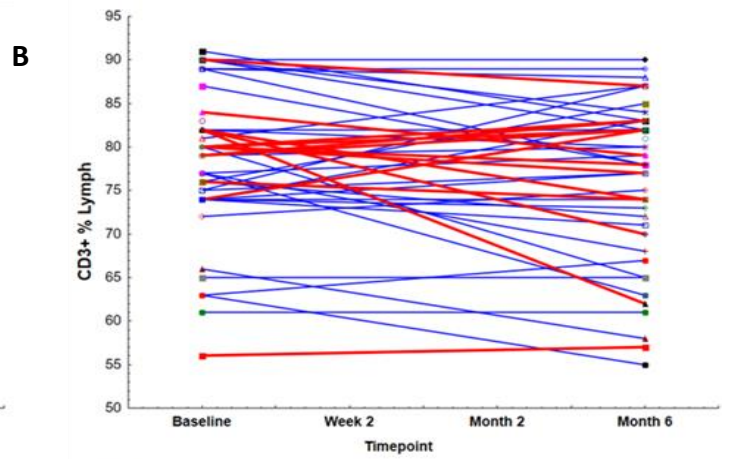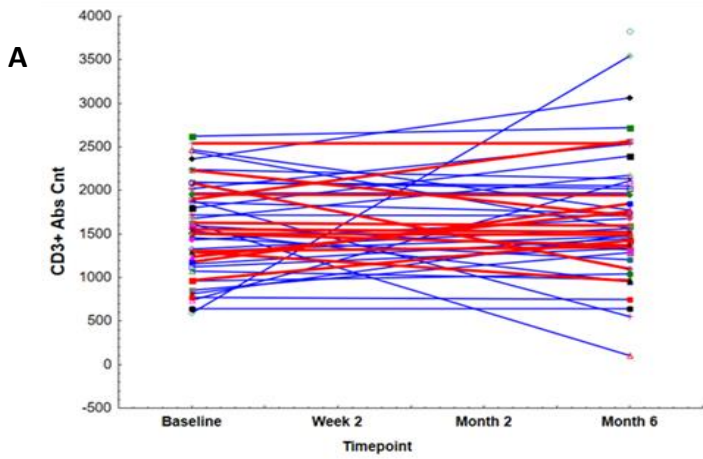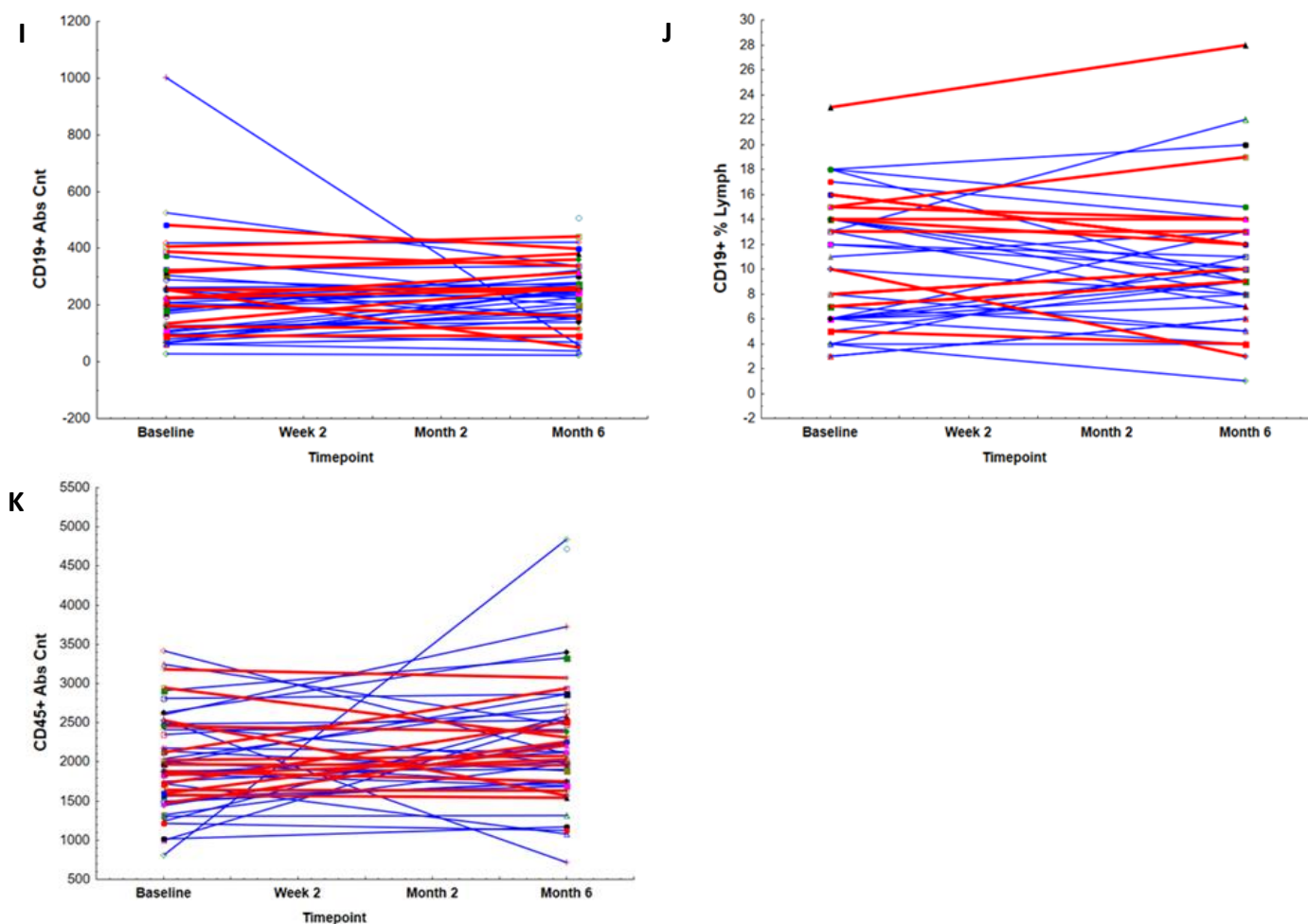
Figure 3.12 Treatment induced changes in T cell and B cell subset of TB-DM2 patients. A scatterplot of different cell subsets in TB (n=36) and TB-DM2 (n=14) measured at BL and M6 end of treatment. Study groups were also analysed by a mixed-model repeated-measures analysis of variance (ANOVA) with a Fisher LSD Post hoc test using the Statistica program version 11. Red lines – TB-DM2; Blue lines – TB. Red lines – TB-DM2; Blue lines – TB.

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CHAPTER 4

Altered T cell subsets in patients with TB-DM2 comorbidity and TB patients during TB treatment

4.1 Introduction

Cells of the adaptive immune response particularly T cells assist in the containment of the *M.tb* bacterium limiting its spread (86, 87). Studies in humans and animal models have demonstrated that $CD4^+$ and $CD8^+$ T cells are crucial in the protection against *M.tb* (88). In a study by Kumar *et al*, TB-DM2 patients were shown to have altered frequencies of $CD8^+$ and $CD4^+$ T cell subsets (89). This suggests that DM2 has an influence on the function and phenotype of T cells during TB disease. Therefore, the aim of this part of the study was to verify if TB-DM2 patients receiving TB treatment have altered frequencies of different $CD8^+$ and $CD4^+$ T cell subsets at BL and M6.

4.2 Methods

In this study different T cell subsets were examined from 21 TB-DM2, 18 TB, 13 DM2 and 8 healthy controls (HCs) at BL (prior to TB treatment), M2 and M6. Cryopreserved PBMCs were thawed and stained for surface markers to distinguish different T cell phenotypes. Analysis was performed by Flow cytometric analysis on the FACS Canto cytometer. Lymphocytes were identified by their light-scattering properties (Figure 4.1) after which doublets and dead cells were excluded. $CD4^+$ T cells were identified as $CD4^+CD3^+$, while $CD8^+$ T cells were identified as $CD4^-CD3^+$. In $CD4^+$ and $CD8^+$ cells, 4 sub-populations were defined as follows: central memory (CM) cells ($CD197^+CD45RA^-$), naïve cells ($CD197^+CD45RA^+$), terminally differentiated effector memory (TEMRA) cells ($CD197^-CD45RA^+$) and effector memory (EM) cells ($CD197^-CD45RA^-$). Activated $CD4^+$ T and $CD8^+$ cells, were characterized by $CD38^+CD4^+$ and $CD38^+CD8^+$, respectively, and the 4 sub-populations characterised as mentioned above. The gating strategy as shown in Figure 4.2 was used to define the $CD4^+$ and $CD8^+$ T cell subsets.
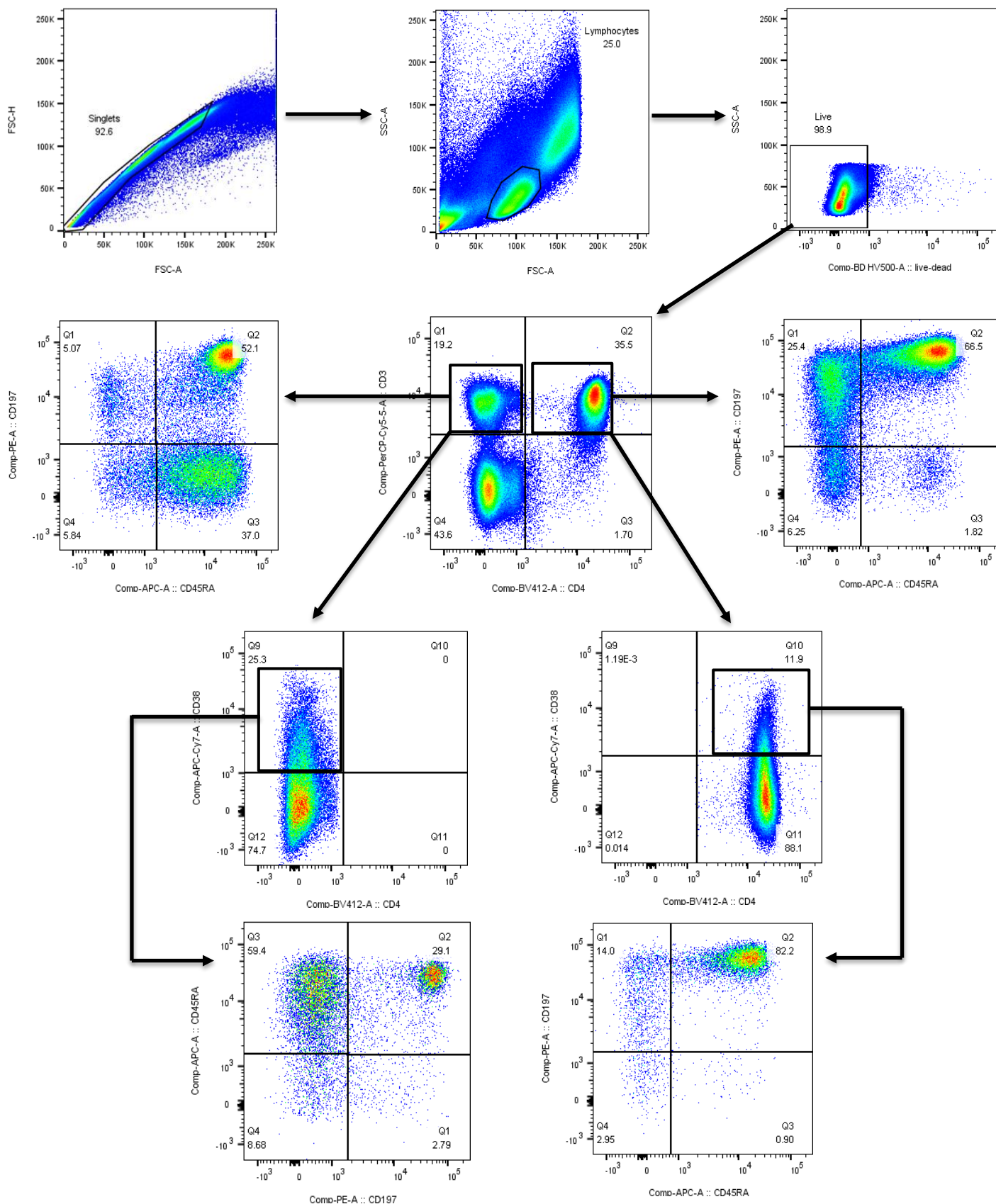
Figure 4.1 Gating strategy for CD4+ and CD8+ T cells and their subpopulations. Flow cytometric plots of CD4+ and CD8+ T cells with their subpopulations. Analysis was done by means of FlowJo software version 10.1. Viable CD4+ and CD8+ T cells were further subdivided into naïve and memory T cell subsets. Activated CD4+ and CD8+ T cells were characterized by the expression of CD38 activation marker. Naïve and memory T cell subsets were compared between activated and non-activated CD4+ and CD8+ T cells.

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4.3 Results

4.3.1 Demographics and biochemical features of the study population

For T cell phenotyping, samples from study patients aged 18 to 60 were age and sex matched, therefore there was no statistical difference in the age between all study patients with TB and TB-DM2 respectively (p = 0.7797) (Table 4.1). At BL, the BMI of DM2 patients was significantly higher compared to the TB patients as indicated by the different significant letters. No statistical difference was found in the BMI between TB and TB-DM2 patients at M2 and M6 end of treatment. As expected, BMI of both TB and TB-DM2 study patients gradually increased throughout treatment (Table 4.1).

Based on the biochemical features between study patients, the random blood glucose in DM2 patients was significantly higher followed by the TB-DM2 patients when compared to TB and HCs as indicated by the different significant letters (Table 4.2). As expected, DM2 patients had higher randon blood glucose compared to the other study patients. The haemoglobin concentrations did not differ among study participants. HbA1c measured at BL was statistically different between study patients a indicated by the different significant letters (Table 4.2). HbA1c levels measured in TB-DM2 and DM2 patients were found to be higher than the TB and HCs at BL. At M6 no statistical difference was found in HbA1c between TB-DM2 and TB patients. With regards to the TB group, the HbA1c levels did not differ throughout treatment. The lipid profile (HDL cholesterol, LDL cholesterol, total cholesterol and triglycerides) and creatinine concentrations measured in the TB-DM2 group increased during treatment, but no significant differences were observed (Table 4.2).

Table 4.1 Demographics of study patients

Study Demographics	TB-DM2 (n=21)	TB (n=18)	DM2 (n=13)	HC (n=8)	p-value
Age (years)*	46.19 ± 8.756	44.06 ± 9.396	45.46 ± 12.06	41.25 ± 11.52	
Sex (F/M)	12/9	9/9	6/7	5/3	
BMI (kg/m²)* -Baseline -Month 2 -Month 6	22.66 ± 5.449 [ab] 22.98 ± 5.232 23.10 ± 5.467	19.39 ± 2.598 [a] 20.11 ± 2.303 20.27 ± 2.565	27.86 ± 4.867 [b] - -	25.77 ± 7.495 [ab] - -	 P = 0.1468 P = 0.2204

*Results are shown as mean ± SD, F: female M: male, BMI: body mass index. Statistical difference is determined by the Mann Whitney t test and one-way analysis of variance (ANOVA). Statistical difference between study participants were indicated by means of different significant letters as determined the Kruskal Wallis test with Dunns Post hoc test. A p-value < 0.05 is considered significant.

Table 4.2 Biochemical parameters of study patients

Study Demographics	TB-DM2 (n=21)	TB (n=18)	DM2 (n=13)	HC (n=8)	p-value
Random Blood Glucose (mg/dL)	9.360 ± 5.027 [ab]	5.722 ± 1.224 [a]	15.59 ± 4.636 [b]	5.763 ± 1.084 [a]	
Haemoglobin	12.35 ± 2.307	13.34 ± 1.971	12.77 ± 1.592	-	P = 0.4216
HbA1c* -Baseline -Month 6	9.719 ± 2.656 [ac] 8.263 ± 3.818	5.794 ± 0.4544 [b] 5.567 ± 0.3581	10.38 ± 1.878 [c] -	5.388 ± 0.340 [b] -	 P = 0.0722
HDL* cholesterol (mg/dL) -Week 2 -Month 6	1.254 ± 0.4701 1.551 ± 0.6684	-	-	-	P = 0.1644
LDL* cholesterol (mg/dL) -Week 2 -Month 6	2.692 ± 0.8779 2.843 ± 1.092	-	-	-	P = 0.8702
Total cholesterol (mg/dL) -Week 2 -Month 6	4.431 ± 1.080 5.242 ± 1.518	-	-	-	P = 0.1898
Triglycerides (mg/dL) -Week 2 -Month 6	1.046 ± 0.3886 1.842 ± 1.450	-	-	-	P = 0.2062
Creatinine -Week 2 -Month 6	56.42 ± 14.98 66.62 ± 21.97	-	-	-	P = 0.1730

*Results are shown as mean ± SD, HbA1c: Glycated haemoglobin, HDL: High density lipoprotein, LDL: Low density lipoprotein. Statistical difference between study participants were indicated by means of different significant letters as determined the Kruskal Wallis test with Dunns Post hoc test. A p-value < 0.05 is considered significant.

4.3.2 T cell subsets in TB-DM2 patients versus TB patients throughout treatment

An appropriate T cell response is an essential factor in the control and host protection against intracellular bacterial infections (90). Increased Th1 cell-mediated immunity is a common finding in individuals with TB-DM2 comorbidity (90). Increased concentrations of inflammation markers, pro-inflammatory cytokines and increased frequencies of CD4$^+$ T cells, CD8$^+$ T cells as well as natural killer cells are associated with the TB and DM2 comorbidity (91). Increased expression of lymphocyte activation markers such as CD38 were shown to be associated with decreased CD4$^+$ T cell count (92). To establish the treatment induced changes on T cell subsets, T cell phenotyping in TB, TB-DM2, DM2 and HC was done. Frequencies of different T cell subsets (naïve, central memory, effector memory and terminally differentiated effector memory) were compared between study participants overtime.

We found that T naïve cells from activated CD4$^+$ T cells displayed a significant increase from BL to M2 of treatment in TB patients without DM2 (Figure 4.2 A). No significant difference was found in any of the other T cell subsets in both study groups (Figure 4.2 A and B). In the overall CD4$^+$ T cell population no differences in the subsets were seen in TB patients (figure 4.2 C). The CD4$^+$ central memory T cells increased throughout treatment in the TB-DM2 patients, however, the opposite occurred with the naïve T cells which displayed significant decrease over time in the same group (Figure 4.2 D). No significant difference was found in any of the other T cell subsets in this group (Figure 4.2 D). Interestingly, T naïve cells from activated CD8$^+$ T cells displayed a significant increase in both study groups throughout treatment (Figure 4.2 E and F). However, terminally differentiated effector memory T cells were found to be decreased in both study groups throughout treatment (Figure 4.2 E and F). No significant difference was found in any of the other T cell subsets from CD8$^+$ T cells in the TB group (Figure 4.2 G), however, in the TB-DM2 group, T naïve cells were found to be decreased throughout treatment (Figure 4.2 H).
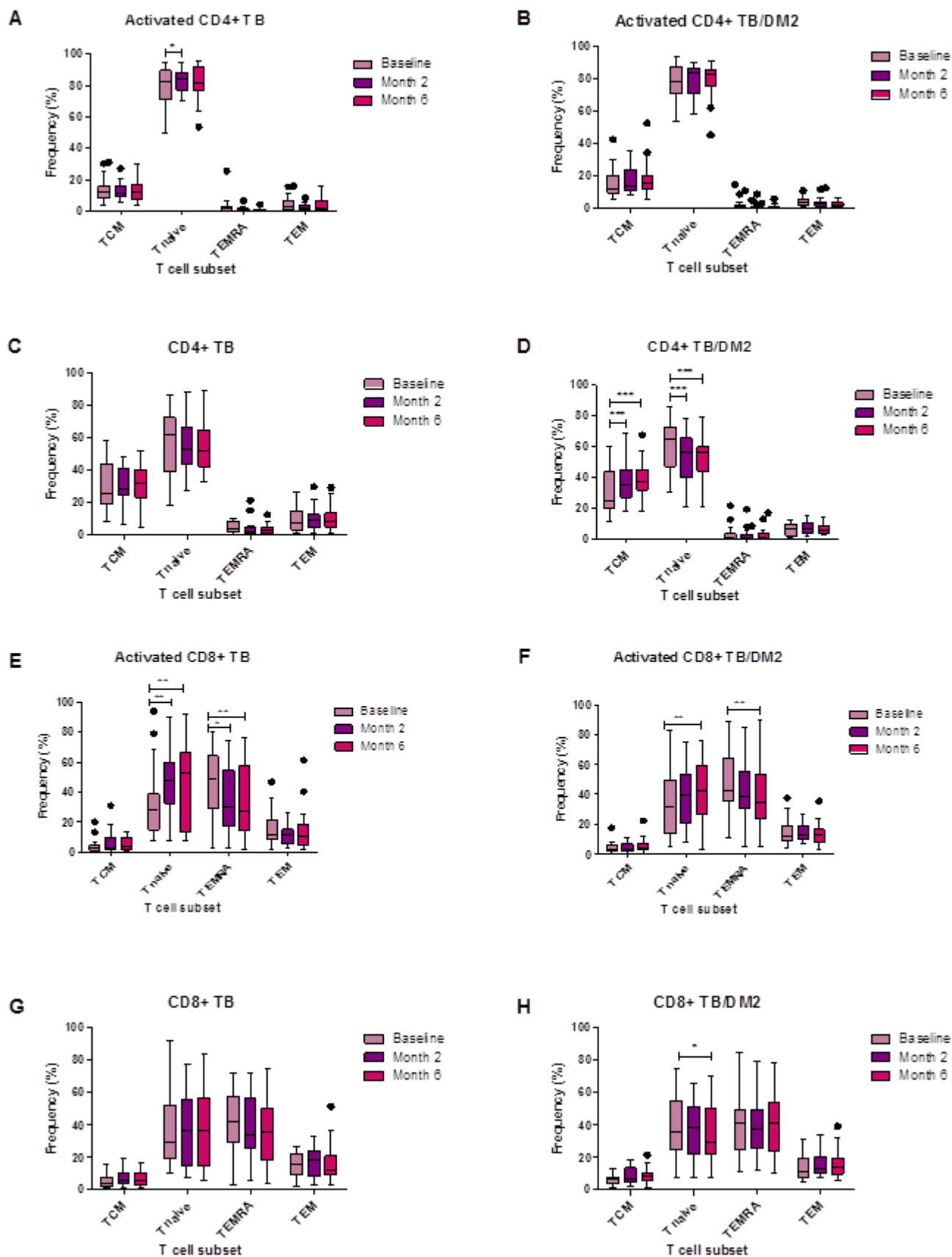
Figure 4.2 T cell subsets in patients with TB-DM2 comorbidity versus TB patients throughout treatment. T cell phenotyping from cryopreserved PBMCs of TB-DM2 (n=21), TB (n=18), DM2 (n=13) and HC (n=8) at BL, M2 and M6 end of treatment. Analysis of the T cell subsets was done by means of mixed-model repeated-measures two-way analysis of variance (ANOVA) with a Bonferroni Post hoc test using GraphPad prism version 5.00.

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4.3.3 T cell subsets observed at individual time points (BL, M2 and M6) in TB and TB-DM2 patients

Treatment induced changes of T cell subsets in TB-DM2 is still not well understood, however, DM2 seems to have an effect on the function and phenotype of T cells in TB disease (89). To determine the effect of DM2 on T cell subsets during treatment in our study cohort, different T cell subsets (as described above) were examined in TB-DM2, TB, DM2 and HCs at BL, M2 and M6. Based on individual time point plots, at BL, DM2 patients had significant increased central memory frequency when compared to TB and TB-DM2 patients from activated CD4$^+$ T cells (Figure 4.3 A). At M2 of treatment, the frequency of naïve T cells from the activated CD4$^+$ T cells were found to be higher in TB patients compared to the TB-DM2 patients (Figure 4.3 B). No significant difference was found in any of the other T cell subsets in both study groups at M6 (Figure 4.3 C). At BL, again DM2 patients had significant increased central memory frequency when compared to TB and TB-DM2 patients from total CD4$^+$ T cell population. HC displayed increased central memory frequency when compared to TB patients from total CD4$^+$ T cell population (Figure 4.3 D). The frequency of T naïve cells from total CD4$^+$ T cell population were found to be increased in TB-DM2 patients when compared with DM2 patients. Similar trend was seen where TB patients were found to have significant increased T naïve cells when compared to DM2 patients and HC (Figure 4.3 D). No significant differences were found at M2 and M6 in any of the other T cell subsets of the total CD4$^+$ T cell population (Figure 4.3 E and F). Interestingly, at BL, terminally differentiated effector memory T cells from activated CD8$^+$ T cell were found to be increased in HC group when compared to TB and TB-DM2 patients (Figure 4.3 G). However, at M2 and M6 no significant difference were found in any other T cell subset between study groups (Figure 4.3 H and I). No significant difference was found in any of the other T cell subsets from CD8$^+$ T cells between study groups throughout treatment (Figure 4.3 J-L)
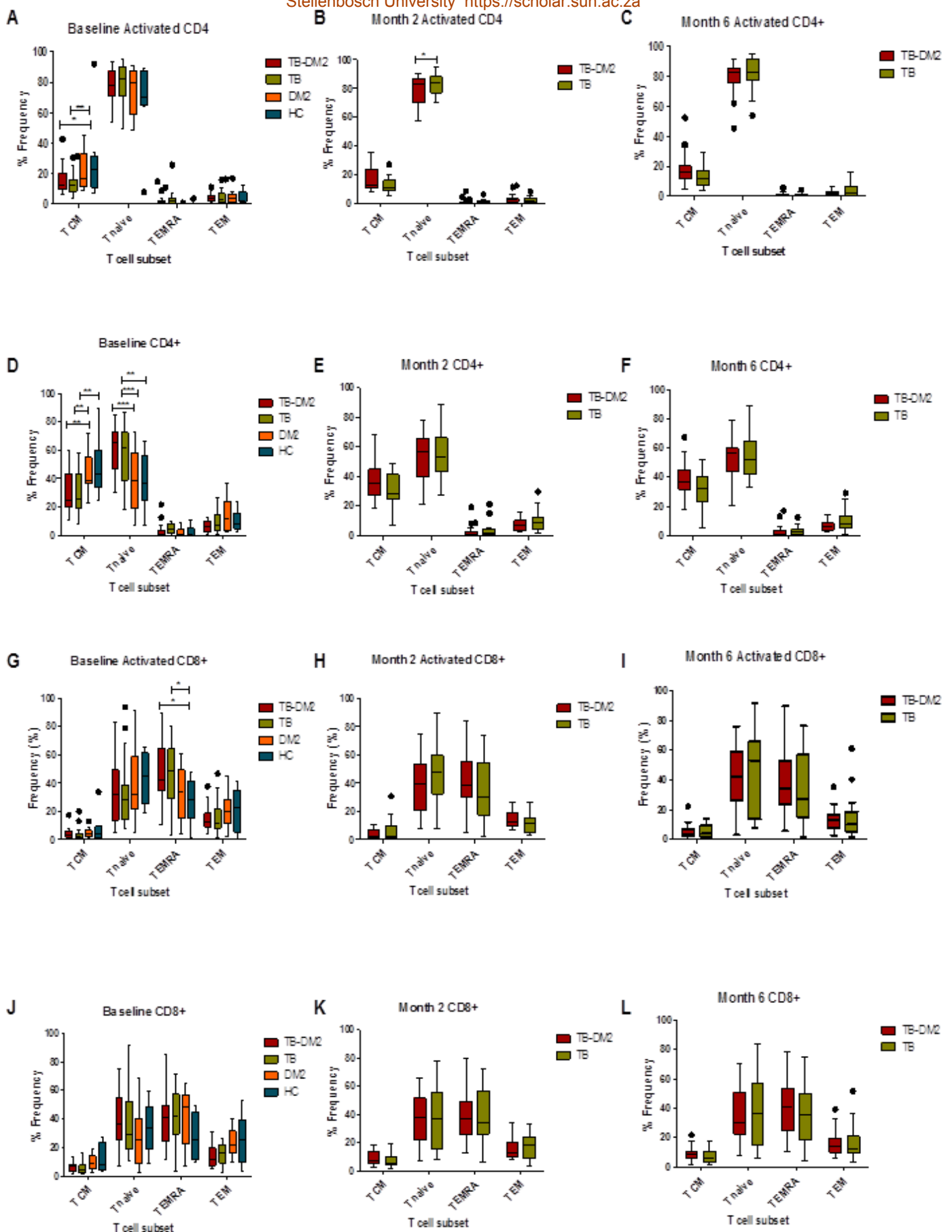
Figure 4.3 T cell subsets observed at individual time points (BL, M2 and M6) in TB and TB-DM2 patients. T cell phenotyping from cryopreserved PBMCs of TB-DM2 (n=21), TB (n=18), DM2 (n=13) and HC (n=8) at BL, M2 and M6 end of treatment. Analysis of the T cell subsets was done by means of mixed-model repeated-measures two-way analysis of variance (ANOVA) with a Bonferroni Post hoc test using GraphPad prism version 5.00.

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CHAPTER 5

Discussion

5.1 Demographics and clinical parameters of study patients

Various immunological factors such as chemokines, cytokines, antibodies, neutrophils, macrophages, T cells subsets as well as patterns specific for migration of T cells are crucial for immunity to TB (98). Increased low grade inflammation, which may be due to hyperglycaemia, is a crucial characteristic of DM2 and insulin resistance (99). Patients with DM2 experience a dysfunctional immunity with altered immunological components such as altered cytokine and chemokine levels, varied number and activation state of immune cell subsets and enhanced tissue fibrosis as well as apoptosis (35).

As one ages, the risk of non-communicable diseases, especially DM2, increases. Similar to a study conducted by Gil-Santana *et al*, TB-DM2 patients were found on average to be older than other study patients (100). The risk factors for DM2 are mainly obesity, increased age and female gender due to higher BMI (100). We showed in the present study that TB-DM2 patients had significantly higher BMI values compared to TB patients at BL. It is well known that TB patients have low BMI due to the cachexic state of the acute illness and TB was in the past referred to as "wasting". Increased TB risk has also been associated with low BMI and poor nutrition. Disease severity has also been reported to be higher in underweight patients (75) and during TB treatment BMI increases in TB patients. In a study conducted by Workneh *et al* during the course of treatment, TB-DM2 exhibited a significant increase in BMI compared to the TB patients (102). Although not significant, similar results were found in the present study. This highlights the significance of drug dosage based on the weight of patients presenting with both diseases.

To date, it remains unclear whether the improvement of glycaemic control in DM2 patients would decrease the risk of TB. However, good glycaemic control has been linked to improved clinical outcomes in certain infections as well as reduced the risk of infectious complications from surgery (103). As expected, DM2 patients had higher fasting blood glucose and HbA1c compared to TB patients at BL. Among study patients, significant differences were found in both fasting blood glucose and HbA1c at BL. Although not significant, TB-DM2 patients displayed higher HbA1c compared to the TB patients at the end of treatment. Few studies examined the link between the risk of TB and glycaemic control. A study conducted by Leung *et al* in Hong Kong, showed that DM2 patients with

a HbA1c $\geq$ 7% had increased susceptibility to TB compared to patients without DM2 (55). The lipid profile in TB patients may have an influence on drug susceptibility, clinical characteristics, pathophysiological aspects as well as the progression to disease (104). It has been shown that body fat is associated with increased susceptibility to DM2. Furthermore, DM2 was characterized as a multifactorial metabolic disorder comprising of resistance to insulin due to chronic hyperglycaemia, hypertension and hypercholesterolaemia (82). In this study, the lipid profile (HDL, LDL and Total cholesterol) measured only in TB-DM2 patients displayed a gradual increase from BL to M6 consistent with the increase in BMI. This may be due to resistance or deficiency to insulin which may influence the crucial enzymes and pathways in the lipid metabolism (82).

Patients with TB-DM2 comorbidity have increased serum concentrations of Th1 (IL-2, IFN-γ and TNF-α), Th17 (IL-17A) as well as various other pro-inflammatory cytokines (IL-1β, IL-6, IL-18). Furthermore, these patients have enhanced reactive T-helper cells and decreased regulatory T cells (Tregs) frequencies. It still remains unclear how TB immunopathology, susceptibility to TB and *M.tb* killing is influenced by DM2 associated inflammation. However, glucose intolerance has been found to be reduced following a successful TB treatment in studies investigating HbA1c levels in TB patients (105).

5.2 Relative increased serum cytokine concentrations in TB-DM2 patients compared to TB patients as an indication of chronic inflammation

An altered immune response has been hypothesised to increase the susceptibility to TB and accelerate the progression to active TB in patients with DM2, (56) yet the mechanism that underlies the association between TB and DM2 is not understood. Cytokines are crucial for host protection against *M.tb* of which IFN-γ and TNF-α are the best characterized (35). Although these pro-inflammatory cytokines are crucial for the containment of *M.tb*, the relative increase of these pro-inflammatory cytokines in TB-DM2 may contribute to increased immunopathology.

HO-1, produced in response to stress, is an antioxidant enzyme whose increased expression is found in human *M.tb* infection and serves as a biomarker for active disease (106). In this study, HO-1 levels significantly decreased from BL to W2 of treatment in TB patients. Interestingly, the HO-1 levels significantly increase towards the end of treatment which may indicate the persistence of the bacterium within the TB patients. With the TB-DM2 patients, HO-1 levels fluctuates throughout treatment, with no significance found. This may also indicate persistence of the bacterium within the TB-DM2 patients, however, further studies need to be done to evaluate this.

In our cohort of age and gender matched TB and TB-DM2 patients from Western Cape, serum levels of various chemokines, growth factors, pro and anti-inflammatory cytokines were measured and their alterations during the 6 months anti-tuberculosis drug therapy measured. The relative increase of serum cytokine concentration found in TB-DM2 may be an indication of stressed induced inflammation due to high glycaemic control. From diagnosis throughout the course of treatment, FGF, G-CSF, GM-CSF, MIP-1β and IL-9 were found to be higher in TB-DM2 patients when compared to TB patients. Interestingly in both groups, there was a decrease of these markers from BL to M2 where after remained constant. A similar TB treatment effect occurred with IL-6, IL-7, IL-1β, IL-1ra, IL-4, IL-8, IL-13, IL-17, IFN-γ and TNF-α as well as the growth factor, VEGF indicating a possible successful treatment based on the fact that it is found upregulated in response to hypoxia, inflammation as well as pro-inflammatory cytokines (107). Severity of inflammation have been associated with the levels of serum VEGF in patients with inflammatory induced lung disorders (107). A subgroup of cytokines (IL-5, IL-10 and IFN-α2), found to be significantly higher in the TB-DM2 patients at the end of treatment displayed an increase in TB-DM2 patients where the opposite was found with the TB patients.

Acute phase proteins are serum proteins that are found to be increased in TB patients (108). In contrast to the other markers measured, the measured acute phase proteins (α2m, Fibrinogen and PCT) were found to be higher in TB patients compared to TB-DM2 patients. The α2m is a carrier protein which interacts with various cytokines such as IL-6, TNF-α, IL-1β as well as growth factors. These factors play a critical role in host protection against *M.tb* (109). Interestingly, TB patients were found to have higher α2m levels at BL only which dramatically decreases throughout treatment. In both plasma and serum, α2m is shown to be used as a diagnostic and prognostic marker for various diseases including DM2 (110). In a study conducted by Annapooran *et al,* increased serum concentrations of α2m were found in DM2 patients suffering from either myocardial infarction or retinopathy (111). Alpha-2-M concentrations also positively correlated with the duration of DM2 (111). However, in contrast to findings, α2m levels displayed a gradual decrease throughout the course of treatment in the TB-DM2 patients. Fibrinogen is another acute phase protein where increased levels is associated with systemic inflammation during infection as well as other diseases and conditions such as aging, smoking, atherosclerosis and diabetes. In active TB patients, fibrinogen displayed higher sensitivity levels when compared to other inflammatory markers during the course of TB treatment (112). Heightened inflammation is crucial in the pathogenesis of DM2, however, in this study the TB patients displayed a sharp decrease of fibrinogen from BL to M2. In contrast to literature, from M2 to M6 (end of treatment), fibrinogen levels increased and were higher than in the TB-DM2 patients. A precursor of calcitonin, PCT, is induced by IL-2 and TNF-α during a bacterial infection. Therefore, serum PCT

serves as a marker for severe bacterial infection (113). Similar to a study conducted by Rohini *et al* where serum PCT levels were found to be increased in pulmonary TB patients (114), we found TB patients had higher PCT levels compared to TB-DM2 patients. In contrast to literature (114), at the end of treatment serum, PCT levels continued to increase in the TB patients. This may indicate the extent of bacterial infection present in the TB patients, however, further studies have to be done to investigate this. The opposite occurred for haptoglobin where TB-DM2 patients had higher levels compared to the TB patients suggesting the presence of chronic inflammation in the TB-DM2 patients. Higher serum levels of MCP-1(MCAF) was found in the TB-DM2 patients, however, a sharp decrease occurred from baseline to month 2 of treatment indicating the extent of disease in those patients as well as its associations with diabetes. Fluctuations of MCP-1(MCAF) levels did not reveal any significance in TB-infected patients without DM2.

5.3 Correlations between clinical parameters and serum cytokine concentrations

Generally a high BMI protects from TB disease. Latently TB-infected individuals with poor nutrition appear to exhibit disturbed systemic and TB-antigen specific cytokine response (115). In this present study, measured serum cytokine concentrations were correlated with patients' clinical parameters. In a study conducted by Zheng *et al* increased TNF-α was found higher in TB-DM2 patients when compared to TB patients (116). Its increased levels may explain the metabolic disturbances due to DM2. Common TB symptoms include weight loss and fever which are induced by TNF-α. The adverse effects in insulin resistance found on obese patients is due to the overexpression of TNF-α (117) which therefore explains its positive correlation ($p = 0.0278$; $r = 0.4400$) with BMI, especially at the continuation phase of treatment. Little is known about the influence of eotaxin levels on the BMI of TB patients, however, significantly increased levels were found at the end of treatment (118). This might explain its negative correlation with BMI at the end of treatment ($p = 0.0243$; $r = -0.4492$). Similar to the finding of Chung *et al* (110), the levels of α2m was negatively correlated with BMI at M2 of treatment as well as fibrinogen at the end of treatment.

By means of various metabolic disturbances of hyperglycaemia, DM2 may be able to alter immunity to *M.tb*. This allegation is supported by the association of blood glucose levels (or HbA1c levels) and the outcomes in immune response to TB (30). Despite the association between DM2 and risk to develop TB, it remains uncertain whether improvements in glucose control would alter the risk (103). HbA1c and random blood glucose levels indicate glycaemic levels where increased and revealed poor glucose control (119). Therefore the relationship between glycaemic control and serum cytokine markers were examined in TB-DM2 patients. HbA1c levels were found to be positively correlated with IL-1β, IL-4, IL-6, IL-7, IL-8, IL-9, G-CSF, GM-CSF, IFN-γ and MCP-1(MCAF) in TB-DM2

patients at baseline. Similar results occurred with measured growth factors (VEGF and PDGF) which were positively correlated with random blood glucose. DM2 is known to be a major risk factor for active TB development and its interaction is characterized by increased pro-inflammatory cytokines crucial in the pathogenesis of TB (120). This confirms the correlation found in TB-DM2 patients, indicating that the increased cytokine levels may be due to the high glucose levels. DM2 not only increases the susceptibility to TB, but their interaction has an influence of presentation and management of both diseases (46). TB treatment and the disease itself have a negative effect on hyperglycaemia, making DM2 challenging to treat. Individuals infected with HIV and having low CD4 counts, are associated with poor TB treatment outcomes. Similarly, chronic hyperglycaemia is linked to increased susceptibility to TB and poor TB treatment outcomes in individuals with TB-DM2 comorbidity. Chronic inflammation is due to chronic hyperglycaemia where both innate and adaptive immune response, although upregulated, are ineffective in the elimination of the bacilli. This therefore causes dysfunctional glucose regulation and worsens insulin resistance (46).

Increasing evidence has indicated the association between various human diseases such as TB and hypocholesterolemia. It has been shown that low blood cholesterol influences TB development which is in contrast to high blood cholesterol which aids in host protection against *M.tb*. Despite this known association of cholesterol and TB, little is known about to what extent treatment of the TB disease will influence the lipid levels in TB patients (121). The effects of IL-4 on obesity or development of insulin resistance is not well understood. It has been shown that IL-4 plays a crucial role in the metabolism of lipid/glucose by preventing lipid deposits as well as promote glucose tolerance (83). In the present study, similar to literature, IL-4 displayed a positive correlation with HDL cholesterol at month 6 in the TB-DM2 patients. This suggests the association of IL-4 and the homeostasis of energy. TNF-α, a pro-inflammatory cytokine, may have an influence on the pathogenesis of obesity and resistance to insulin. Animal models have shown that its production increases total cholesterol as well as LDL cholesterol levels by activating the synthesis of cholesterol (122). TNF-α was shown to be positively correlated with total cholesterol displaying its involvement with lipid metabolism. For appropriate function of immune system for host protection against TB, a sufficient cholesterol level is crucial (121).

5.4 Treatment induced changes of T cell subsets in TB and TB-DM2 patients

Risk factors such as malnutrition, poverty, immunodeficiencies and DM contribute to the increased risk to develop TB, therefore, an effective T cell response verifies either recovery from infection or development into disease. It is of interest to determine which T cell subset aid in anti-mycobacterial immunity. *M.tb*-specific CD4^{+} Th1 cell response is regarded as a crucial adaptive response due to its

protective abilities that produce certain cytokines, including IFN-γ and TNF-α, which recruit and activates innate immune cells. Although CD8^{+} T cells do not exhibit an immune response comparable to CD4^{+} T cells, they contribute to optimal host protection. CD8^{+} T cells have various anti-microbial functions not found in the CD4^{+} Th17 and Th1 T cells. Upon activation, their anti-microbial effects aid in controlling the *M.tb* infection by the release of cytotoxic molecules or cytokines which cause apoptosis of infected cell (123). Decreased CD4^{+} T cell numbers in the periphery of TB patients suggest that TB itself induces migration of this cell type from the periphery to the site of disease, the lung (93). Although it has been shown that *M.tb*-specific CD8^{+} T cells were mainly found in active TB patients (94), little is known about the regulation of CD8^{+} T cells on TB-DM2 comorbidity.

Both CD4^{+} and CD8^{+} T cells have further subpopulations based on the expression levels of chemokine receptors and activation markers. Four major T cell subsets can be differentiated: central memory, effector memory, effector and naïve T cells (89). In a study conducted by Pollock *et al*, active TB disease was found to be associated with increased frequency of central memory T cells (125). Furthermore, TB alters the memory T cell subsets of CD8^{+} T cells only, while DM2 alters the memory T cell subsets of CD4^{+} T cells (89). Kumar *et al*. found that CD4^{+} central memory T cells were significantly increased in TB-DM2 throughout treatment (89). Similar findings were observed in this study where central memory T cells exhibited an increase throughout treatment. However, in both activated and non-activated CD4^{+} T cells, TB and TB-DM2 patients had low frequency of central memory T cells when compared to the controls (Healthy controls and DM2 patients) at baseline. In the TB-DM2 patients, the frequency of naïve T cells displayed a decrease in both CD4^{+} and CD8^{+} T cells during treatment. This treatment effect occurring in the TB patients where naïve T cells from activated CD4^{+} T cells indicate an important treatment factor that alters the T cell composition.

The immune response to TB varies between patients of different ethnic origin which could possibly explain differences in cytokine expression levels (77). Majority of the study participants were of coloured origin therefore we did not correct for ethnicity. Despite this limitation, this study displays the need for personalized treatment due to different ethnic groups, environment, habit and most importantly co-morbid disorders. Future investigations should attempt in evaluating individual treatment medications in order to provide appropriate dosages based on disease severity. Finally, this study highlights the importance for screening for TB in patients with diabetes especially if patients originate from high TB burden regions.

5.5 Conclusion

Patients with the TB-DM2 comorbidity are characterised by a hyper-inflammatory state with increased serum cytokine concentrations and changes in the ratios of T cell subtypes, compared to TB patients without DM2. This imbalance of inflammatory cytokines may also affect treatment outcomes (62) which may possibly increase the susceptibility to treatment failures of reactivation of TB. With regards to the different T cell subsets in CD4$^+$ and CD8$^+$ T cells, the treatment was shown to have an influence overtime although not significant. These immune alterations occur both at BL as well as throughout TB treatment and may contribute to poor treatment outcomes in patients with TB-DM2 comorbidity. In the control of the bacterium, it is crucial that there is a balance of the immune response. It is important to better elucidate whether the host immune response is effective in controlling the infection. Consequently, a failed immune response will determine the transmission and dissemination of the bacilli to new host as well as better elucidate the treatment effect in TB patients.

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