



**Systemic lymphocyte trafficking markers in TB and
TB/HIV co-infections**

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PREFACE

The experimental work described in this thesis was conducted at the at the Centre for the AIDS Programme of Research in South Africa Laboratory, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa. This work was supervised by Dr Aida Sivo and Dr Kogielecum Naidoo.

This work has not been submitted in any form for any degree or diploma to any tertiary institution, where use has been made of the work of others, it is duly acknowledged in the text.

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Date 05.02.2019

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Date 01.02.2019

DECLARATION

I, Kimesha Pillay declare that:

(i) The research reported in this thesis, except where otherwise indicated, is my original work.

(ii) This thesis has not been submitted for any degree or examination at any other university.

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K. Pillay 

Date 05.02.2019

DEDICATION

For you,

Dad, Mum, Ma and Nadine

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LIST OF ABBREVIATIONS AND ACRONYMS

% – Percent

dH₂O – Deionized water

μl – Microliter

ACD – Acid citrate dextrose

AFB – Acid – fast bacilli

AIDS – Acquired Immunodeficiency Syndrome

APCs – Antigen presenting cells

ART – Antiretroviral Therapy

BAL – Bronchoalveolar lavage

BCG – Bacillus Calmette-Guérin

BMI – Body mass index

BV – Bacterial Vaginosis

CAPRISA – Centre of the AIDS Programme of Research in South Africa

CFP-10 – Culture filtrate antigen 10

CI – Confidence interval

CTL – Cytotoxic T lymphocytes

CV – Coefficient of variation

DCs – Dendritic cells

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-Linked Immunosorbent Assay

EMB – Ethambutol

ESAT-6 – Early secretory antigenic target 6

FTC – Emtricitabine

g – Grams

GALT – Gut Associated Lymphoid Tissue

GI – Gastrointestinal

GM-CSF – Granulocyte-macrophage colony-stimulating factor

HAART – Highly Active Antiretroviral Therapy

HCl – Hydrochloric acid

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV – Human Immunodeficiency Virus

HRZE – Isoniazid, Rifampicin, Pyrazinamide, Ethambutol

HRZM – Isoniazid, Rifampicin, Pyrazinamide, Moxifloxacin

IBD – Inflammatory bowel disease

IBS – Irritable bowel disease

ICAM-1 – Intercellular adhesion molecule 1

IFNs – Interferons

IFN- γ – Interferon gamma

IGRA – Interferon-gamma release assay

IL-1Ra – Interleukin-1 receptor antagonist

IMPRESS – Improving Retreatment Success

INH – Isoniazid

IP-10 – Interferon gamma-induced protein 10

IQR – Interquartile range

IRIS – Immune Reconstitution Inflammatory Syndrome

KZN – KwaZulu – Natal

LBP – Lipopolysaccharide Binding Protein

LFA-1 – Lymphocyte function-associated antigen 1

LPS – Lipopolysaccharide

LTBI – Latent TB Infection

M – Molar

M.tb – *Mycobacterium tuberculosis*

MAdCAM-1 – Mucosal addressin cell adhesion molecule 1

MCP-3 – Monocyte chemotactic protein-3

MD – Mean difference

MGIT – Mycobacteria growth indicator tube

MHC – Major Histocompatibility Complex

MIP-1 β – Macrophage inflammatory protein 1 β

ml – millilitre

MTCT – Mother to Child Transmission

N – Normality

NaOH – Sodium hydroxide

NK – Natural killer cells

OR – Odds ratio

PBMCs – Peripheral blood mononuclear cells

PBS – Phosphate buffer saline

PCR – Polymerase Chain Reaction

PCZCDC – Prince Cyril Zulu Communicable Disease Centre

PD-1 – Programmed cell death protein 1

PD-L1 – Programmed death-ligand 1

PET-CT – Positron emission tomography-computed tomography

PLHIV – People living with HIV

PPD – Purified protein derivative

PrEP – Pre-exposure prophylaxis

PRRs – Pattern recognition receptors

PZA – Pyrazinamide

RMP – Rifampicin

RNA – Ribonucleic Acid

RT – Room temperature

SA – South Africa

SAPiT – Starting Antiretroviral Therapy at Three Points in Tuberculosis

SD – Standard deviation

sICAM – Soluble Intercellular adhesion molecule

SIV – Simian Immunodeficiency Virus

sMAdCAM – Soluble Mucosal addressin cell adhesion molecule

sqrt – square root transformed

STIs – Sexually transmitted infections

sVCAM – Soluble Vascular cell adhesion molecule

TB – Tuberculosis

TB-IRIS – Tuberculosis associated Immune Reconstitution Inflammatory Syndrome

TDF – Tenofovir

TGF- β – Transforming growth factor β

Th – T helper

Th1 – T helper 1

Th17 – T helper 17

Th2 – T helper 2

TLR – Toll like receptor

TNF- α – Tumour necrosis factor- α

Treg – T regulatory

TRuTH – TB Recurrence upon Treatment with HAART

TST – Tuberculin Skin Test

VCAM-1 – Vascular cell adhesion molecule 1

VL – Viral Load

VLA – Very late antigen-4

WHO – World Health Organisation

α – alpha

β - beta

$\alpha 4\beta 1$ – alpha-4-beta-1

$\alpha 4\beta 7$ – alpha-4-beta-7

ABSTRACT

Background. Several studies demonstrate that immune inflammation and trafficking of immune cells to affected tissues plays a major role in the pathogenesis of tuberculosis (TB) and human immunodeficiency virus (HIV) infections; however, characterization of soluble markers of lymphocyte trafficking and inflammation in the context of TB and TB/HIV co-infection remains to be elucidated. Here we sought to evaluate the role of specific lymphocyte trafficking and inflammatory markers as predictors of TB disease.

Methods. The presented study was performed on stored plasma samples from TB Recurrence upon Treatment with HAART (TRuTH) and Improving Recurrence Success (IMPRESS) cohorts. TB recurrent cases (n = 37) were matched to controls (n = 103) on study arm in the original trial and antiretroviral therapy (ART) start date. A subset of cases was followed longitudinally at: preTB, active TB and postTB/cure timepoints. In IMPRESS a subset of HIV infected (n = 41) and HIV uninfected (n = 37) individuals were sampled at active TB disease and post TB/cure. Plasma concentrations of soluble mucosal addressin cell adhesion molecule (sMAdCAM), soluble intracellular adhesion molecule (sICAM), soluble vascular adhesion molecule (sVCAM), lipopolysaccharide binding protein (LBP) and transforming growth factor – beta (TGF- β) were measured using enzyme-linked immunosorbent assays (ELISAs) and Multiplex assays.

Results. Two analytes were associated with increased rate of TB recurrence in the univariate model: square root transformed (sqrt) sICAM (odds ratio [OR] 1.047, 95% confidence interval [CI] 1.014 – 1.081, p = 0.005) and sqrtLBP (OR 3.283, 95% CI 1.018 – 10.588, p = 0.047) and the multivariable model. Longitudinal analysis showed reduced levels of LBP,

sMAdCAM and sVCAM and an increase in levels of TGF- β 3 during the entire follow-up. In IMPRESS data, trends of increased plasma LBP from active TB to post TB/cure in HIV infected individuals and trends of reduced plasma LBP in HIV uninfected individuals post treatment were observed.

Conclusion. The TRuTH data demonstrates that plasma levels of sICAM and LBP can act as predictors of TB recurrence in HIV infected individuals receiving ART treatment. A decrease of plasma LBP levels from active TB to treatment completion in HIV uninfected individuals likely suggests that active TB and associated inflammatory changes are associated with gut inflammation and dysbiosis.

CHAPTER 1. Literature Review

1.1 TB Epidemic

Globally, the burden of Tuberculosis (TB) remains high even though the incidence rate is decreasing at 2% per annum (WHO, 2013). TB disease is established largely in the lungs (pulmonary), however the bacterium can spread to different organs (called extra pulmonary TB) (Harisinghani *et al.*, 2000, O'Garra *et al.*, 2013, WHO, 2016b). Despite being a preventable and treatable disease, TB is currently ranked as the ninth leading cause of mortality globally and ranks above human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) as the primary cause of mortality from an infectious agent, especially in low/middle income countries (Figure 1) (WHO, 2017). Globally in 2017, an estimated 1.3 million deaths occurred among HIV uninfected individuals. Additionally, 300 000 deaths occurred in HIV infected individuals who had TB. Among the estimated number of cases, 90% were adults and of this, 65% were male (WHO, 2017, Facts, 2017).

The highest rate of the TB burden is found in sub-Saharan Africa, where the HIV epidemic is the greatest (Zumla *et al.*, 2013, WHO, 2017). According to WHO, TB is responsible for a loss of 25,000 lives (excluding deaths due to co-infections) and an estimated 450,000 active cases a year in SA (Facts, 2017, WHO, 2016a). KwaZulu-Natal (KZN), one of the nine provinces in SA, has been burdened with the highest incidence of TB, followed by the Eastern Cape and Western Cape respectively. However, due to better screening and

treatment, KZN has shown a steady decline of TB incidence from 1185 to 685 per 100 000 population in the last five years (Facts, 2017).

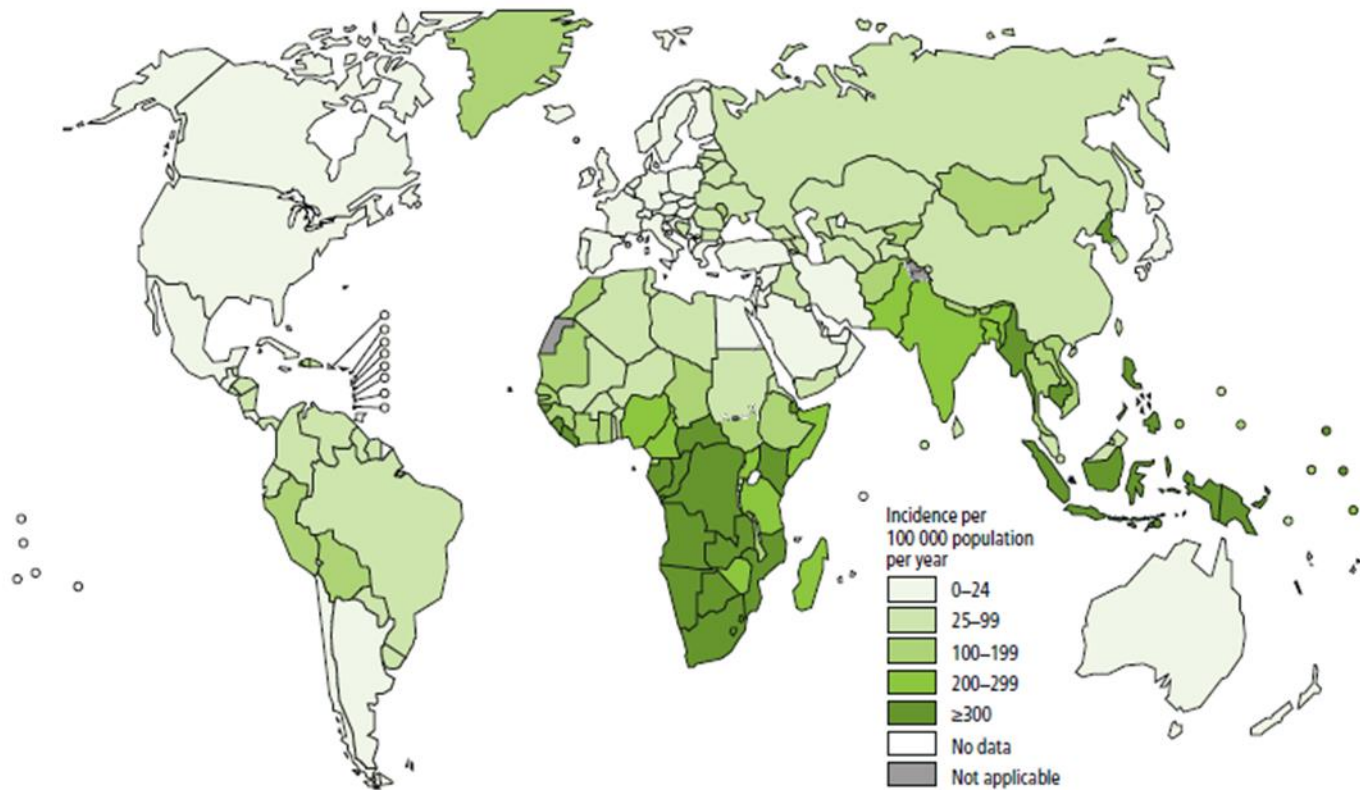


Figure 1. Global estimates of TB incidence in 2016 ¹

¹Adapted from (WHO, 2017), accessed on January 2018.

1.1.2. TB spectrum of disease

TB is caused by infection with *Mycobacterium tuberculosis* (*M.tb*), an acid – fast bacilli (AFB). The bacterium is spread predominantly through acts of coughing and sneezing and is a common cause of pulmonary TB (Algood *et al.*, 2003). *M.tb* is an effective intracellular pathogen (Van Crevel *et al.*, 2002) belonging to the *Mycobacteriaceae* family. It is an aerobic, non-motile organism, called a tubercle bacilli (rod shaped) (Knechel, 2009).

When an individual inhales the *M.tb* bacterium, the primary innate immune cells that encounter the infection are the alveolar macrophages (Behler *et al.*, 2012). Once bacterial dissemination occurs, T cell priming takes place and triggers the expansion of antigen specific T cells, which are later recruited to the lungs (Nunes-Alves *et al.*, 2014). The recruitment of T cells, B cells, macrophages and other leukocytes leads to the formation of granulomas, which can contain *M.tb*. However, in some individuals the granuloma is unsuccessful in enclosing the infection when the bacterial load is abundant. This allows for dissemination of the bacteria to other organs, where the *M.tb* can re-enter the respiratory tract or the bloodstream.

Although TB is an ancient disease, latent TB infection (LTBI) was only recognized at the end of the 19th century (Muñoz *et al.*, 2015). The asymptomatic individuals develop immune responses to *M.tb*, but do not present clinical evidence of active TB (Getahun *et al.*, 2015, Ernst, 2012). There are approximately 2 billion cases of LTBI, and 9 million cases of active TB (WHO, 2018a). LTBI has been known to transition into active disease (known as reactivation). Reactivation occurs in a 5-15% of latently infected individuals, predominantly in the first 5 years of active infection. Individuals who have LTBI are characterised as important reservoirs for new cases of active TB (Getahun *et al.*, 2015, Kiazzyk and Ball, 2017). Both bacterial and environmental factors have been found to play a role in LTBI reactivation, however, the exact mechanism is not fully understood. Active infection leads to clinical symptoms such as weight loss, fever and persistent coughing and is clinically known as active TB (Dheda *et al.*, 2010, O'Garra *et al.*, 2013, Harisinghani *et al.*, 2000). Active TB disease can arise after a new infection or after the reactivation of a latent form and it can be

diagnosed by the detection of the *M.tb* in tissue culture and sputum samples (O'Garra *et al.*, 2013, Lin and Flynn, 2010).

The spectrum of TB is diverse and recent research has shown that there is a continuous spectrum of metabolic bacterial activity and antagonistic immunological responses during transition from LTBI to active TB disease. Due to these findings, Drain *et al* has recently proposed the addition of two clinical states in the spectrum; (i) incipient TB and (ii) subclinical TB. Incipient TB infection is defined as an infection that has viable *M.tb* and is likely to advance to active TB disease without further intervention. These individuals do not have signs of active TB disease such as clinical symptoms and radiographic abnormalities. Subclinical TB disease is defined as disease caused by viable *M.tb* and does not cause clinical TB symptoms, however abnormalities related to active TB can be detected by radiologic and microbiologic assays (Achkar and Jenny-Avital, 2011, Drain *et al.*, 2018).

1.1.3. TB diagnosis, management and treatment

There are four methods by which active TB can be detected; these tests include chest X-ray/PET-CT, sputum smears (microscopy), molecular and culture based tests (Pai *et al.*, 2016). Microbiologic demonstration of AFB is the mainstay of diagnosis of *M.tb* infection. For more than a century, diagnosis of TB has been conducted by light microscopy on sputum smears (Lawn, 2015). Compared to other diagnostic tools, it is fairly quick and uses inexpensive materials (Lawn, 2015). However, there are limitations with this technique such as: (i) it has low sensitivity and requires a lot of manpower to examine and analyse resulting

in variability and (ii) the sputum sensitivity is limited to the amount of bacilli in the sample (Lawn, 2015, Gupta *et al.*, 2013).

The presence of *M.tb* can also be determined in clinical specimens using solid and liquid cultures. A number of culture media such as: (i) Lowenstein – Jensen (LJ) and (ii) Middlebrook 7H10/11 and (iii) Middlebrook 7H9 have been used to isolate and detect the presence of *M.tb* (SAITO, 1998). The disadvantage of using a culture based method is the turn over time; it takes 3-6 weeks for *M.tb* growth and isolation (Naveen and PeeraPur, 2012). Liquid media have been associated with shorter detection time and higher recovery of tubercle bacilli. Culture based assays such as the BD BACTEC™ – mycobacterium growth indicator tubes (MGIT) have improved the time to detection of *M.tb* (Naveen and PeeraPur, 2012).

Since 2010, a molecular assay based on the GeneXpert technology known as Xpert MTB/RIF has been used to diagnose TB and detect resistance to rifampicin (Albert *et al.*, 2016, WHO, 2014, Pai *et al.*, 2016). This molecular assay provides a greater precision than the sputum smear and therefore, the Xpert MTB/RIF is provisionally recommended as the first line diagnostic test used in presumed cases of active TB (children and adults) (Steingart *et al.*, 2013, Boehme *et al.*, 2010, Boehme *et al.*, 2011, Detjen *et al.*, 2015). Currently the WHO encourages the use of the Xpert MTB/RIF with patients who are HIV infected and who are suspected of having active TB, since sputum smears can only detect around 43% of these patients (Getahun *et al.*, 2007).

LTBI has no gold standard diagnostic test; however there are two tests to detect the presence of *M.tb*: the tuberculin skin test (TST) and the interferon gamma release assay (IGRA). The TST is based on a delayed hypersensitivity reaction which occurs when *M.tb* infected individuals are exposed to a mixture of antigens known as purified protein derivative (PPD). This results in a skin reaction that can be interpreted as a positive or a negative result (Huebner *et al.*, 1992). Although the TST is advantageous mostly in resource limited settings, it has major disadvantages such as: limited predictive value and the specificity of the test can be compromised by the Bacillus Calmette-Guérin (BCG) vaccination (Pai *et al.*, 2016). The IGRA test is a blood test, which measures the release of IFN γ from T cells following stimulation by early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) that are specific to the *M.tb* complex (Mahairas *et al.*, 1996, Sørensen *et al.*, 1995). However the IGRA test also provides poor predictive values (Rangaka *et al.*, 2012, Pai *et al.*, 2014, Pai *et al.*, 2016).

Treatment of active, drug sensitive TB in children (>8years and > 30kgs) and adults are separated into two phases. In the first phase (intensive phase) patients are given rifampicin (RMP), isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB) for 2 months. This helps to decrease the amount of infectious bacilli in the patients, which can be observed between 10-14 days from the start of treatment(Directorate, 2014, WHO, 2010). Thereafter, INH and RMP are administered for an additional 4 months to eliminate remaining bacilli, known as the continuation phase (second phase). These drugs are given in combination for no less than 6 months and no more than 9 months (Nahid *et al.*, 2016, WHO, 2010).

Currently the BCG vaccine is the only approved vaccine used to prevent active TB. The efficiency of the BCG vaccine against adult pulmonary cases conducted in clinical trials ranges between 0-80%, and it is currently not known why there is so much inconsistency in BCG vaccine efficacy, although phenotypic and genotypic differences between BCG strains are thought to be one of the main contributors (Mangtani *et al.*, 2013, Roy *et al.*, 2014, Pai *et al.*, 2016). Studies conducted on infants and children (<5 years) have shown that the effectiveness of the vaccine in the protection against TB ranges from 50-80%. In infants (<5 years), the vaccine is vital in preventing active TB and reducing morbidity and mortality rates (Barreto *et al.*, 2011, Pai *et al.*, 2016).

1.2. HIV Epidemic

Since the first documentation of the acquired immunodeficiency syndrome (AIDS) in the 1980's, HIV has claimed more than 35 million lives (UNAIDS, 2016). HIV infection is characterised by: (i) the progressive depletion of CD4+ T cells, (ii) chronic immune activation and immune exhaustion and (iii) subsequent opportunistic infections (Maartens *et al.*, 2014).

Approximately 36.9 million individuals of all ages have been reported to be living with HIV and almost 1.8 million individuals have acquired new HIV infections worldwide in 2017 (Figure 2) (UNAIDS, 2018). There are approximately 19 million HIV infected individuals in Africa, of which an estimated 7.2 million individuals are infected and living in SA (UNAIDS, 2018). Women between the ages of 15 and 24 compared to the opposite sex, have an eight time greater risk of HIV and acquire HIV at least 7 years earlier than men (Zuma *et al.*, 2016, Karim *et al.*, 2015). A study conducted in KZN looked at transmission networks and the risk of HIV infection; this study found that a key factor that influences HIV incidence is the age-disparate relationships between younger women and older men (De Oliveira *et al.*, 2017). Therefore, future prevention strategies in SA need to address sexual partnering in combination with treatment.

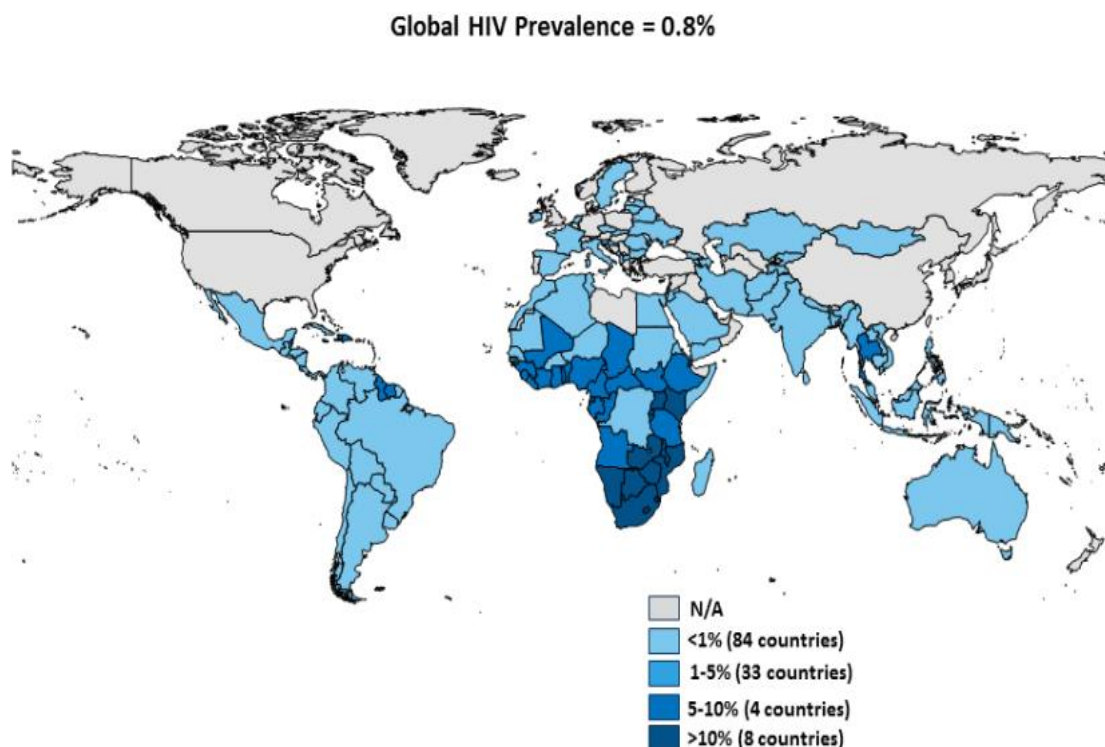


Figure 2. Global Prevalence of HIV in adults in 2016 ²

²Adapted from (UNAIDS, 2017)

1.2.1. HIV treatment and prevention

In the late 1990's, combination antiretroviral therapy (ART) regimens were developed, which transformed HIV from a progressive illness to a manageable chronic disease by suppressing viral replication (Maartens *et al.*, 2014). The development of ART has been a key accomplishment in the fight against HIV, however ART is not able to eliminate the infection (Volberding and Deeks, 2010, Simon *et al.*, 2006). With the current 'test and treat' strategy, ART is initiated after diagnosis is made irrespective of the CD4 cell count. In the hope of preventing HIV, oral pre-exposure prophylaxis (PrEP) is given to individuals who are at high risk for HIV infection. PrEP is a combination of tenofovir (TDF) and emtricitabine (FTC) and in clinical trials it has shown to reduce the risk of sexual acquisition in HIV-serodiscordant couples and in men who have sex with men (Eagle *et al.*, 2017, Grant *et al.*, 2010).

The quest for an effective HIV vaccine has been on-going for many years however, there has not been a clinical trial which has shown a vaccine that is adequately protective (Whaley and Mayer, 2014). The RV144 trial that was conducted between 2003 and 2006 on HIV uninfected Thai individuals, administered a vaccine containing a prime and boost regimen, which consisted of a mixture of the canarypox virus, ALVAC-HIV (prime) and AIDSVAX B/E gp120 (boost) (Bekker and Gray, 2017, Whaley and Mayer, 2014). The combined vaccine resulted in a 31.2% effectiveness in the modified intent to treat population, thus resulting in the first vaccine to show promising efficacy in decreasing HIV acquisition (Bekker and Gray, 2017, Whaley and Mayer, 2014). The HVTN 097 study in SA replicated the RV144 trial by looking at HIV subtype C. This study showed similar results to the RV144

study irrespective of the route of transmission, HIV subtype and ethnicity of the study population (Gray *et al.*, 2014, Bekker and Gray, 2017).

1.3. TB/HIV Co-infection

The leading cause of death in people infected with HIV is the co-infection with *M.tb* (Bell and Noursadeghi, 2018, Pawlowski et al., 2012, Sutherland *et al.*, 2014) and the risk of active TB increases substantially when HIV infection alters the course of *M.tb* infection (Bell and Noursadeghi, 2018). The epidemics of TB and HIV are closely related, such that the risk of acquiring TB in people living with HIV (PLHIV) is between 16 and 27 times greater than people living without HIV infection (WHO, 2018b). WHO reported that there had been 10.4 million cases of TB and from these estimates approximately 1.2 million cases were people who had HIV (WHO, 2017).

1.3.1. TB/HIV Epidemic in South Africa

The overlap between HIV and TB epidemics is greatest in sub-Saharan Africa, with more than 50% of TB individuals being co-infected with HIV (WHO, 2007). SA is ranked in the top five countries globally with respect to TB incidence and it ranks first in the number of cases of TB/HIV co-infection, with approximately 65% of TB individuals dually co-infected with HIV (WHO, 2013).

1.3.2. Difficulty in diagnosing TB in HIV infected individuals

There has been a dire need to improve TB diagnoses in sub-Saharan Africa (Kaforou *et al.*, 2013). Diagnostic tests for active TB have been challenging in co-infected individuals since sputum of HIV infected individuals have less bacilli than that of HIV uninfected individuals (Getahun *et al.*, 2010). HIV infection is known to compromise the TB diagnostic test strength and efficacy (Getahun *et al.*, 2011). Presently, there is no internationally recognized tool to screen for TB in individuals who are living with HIV (Getahun *et al.*, 2011). TB diagnostic tests such as smear microscopy and the chest radiography yield poor results in PLHIV. The recommended test for TB in individuals who are infected with HIV is the GeneXpert, however mycobacterial cultures remain the gold standard for diagnosis (Getahun *et al.*, 2010, WHO, 2018a). The complexities of accurately characterizing TB in HIV infected individuals highlights the need for research to identify novel biomarkers that can accurately diagnose or predict TB in HIV infected individuals, as this is crucial for timely management of co-infection.

1.3.3. Effect of ART on TB

Mortality remains high in individuals that have severe immunosuppression, regardless of effective TB treatment (Kyeyune *et al.*, 2010, Koenig *et al.*, 2009). The mortality rate of HIV infected individuals is reported to be around 30% in the first 2 months of TB treatment if ART is held back (Blanc *et al.*, 2011). Previous studies have argued both for and against delaying ART initiation (Blanc *et al.*, 2011). The “Starting Antiretroviral Therapy at Three Points in Tuberculosis” (SAPiT) study, showed that the initiation of ART during TB

treatment improved survival by 56% in HIV-TB co-infected individuals with < 500 per cubic millimeter CD4+ T cell count (Abdool Karim *et al.*, 2010).

Another complication of ART in TB patients, in resource limited settings is the immune reconstitution inflammatory syndrome (IRIS). IRIS is a highly recognized phenomenon, through which ART is hindered (French *et al.*, 2004, Hamill, 2003). It is as a result of rapid restoration of pathogen specific immune responses to an opportunistic infection and as a result, causes the deterioration of a treated infection or a new presentation of an existing subclinical infection. Typically IRIS occurs during the start of ART and has been associated with a wide range of pathogens, including mycobacteria (Hamill, 2003, French *et al.*, 2004, Lawn *et al.*, 2005a).

The burden of TB/HIV co-infection is high in low income and middle income settings. Therefore, patients that enter ART programmes are currently diagnosed with TB or later develop TB after the initiation of ART (Corbett *et al.*, 2003). A study conducted in SA showed that 25% of patients who attended a community based ART programme, received TB treatment at ART initiation. In the first year of ART treatment, the incidence of TB was 13.4 cases per 100 persons per year (Lawn *et al.*, 2006). Approximately one third of patients with TB/HIV co-infection that initiate ART in these settings are more likely to develop TB-associated IRIS (TB-IRIS) (Lawn *et al.*, 2005a), which is considered a major clinical challenge in countries that are resource limited (Colebunders *et al.*, 2006, Kumarasamy *et al.*, 2004, Manosuthi *et al.*, 2006, Lawn *et al.*, 2007). TB-IRIS is defined as an excessive immune response against *M.tb* that can occur in both HIV infected or HIV uninfected patients, who

are on or have completed TB treatment. In patients who are HIV infected, TB-IRIS occurs after the initiation of ART, independent of HIV viremia suppression. There are two forms of IRIS: (i) paradoxical IRIS, where a treated patient has a new or worsening symptom or has a recurrent episode, and (ii) unmasking IRIS, is an ART associated inflammatory manifestation of a subclinical infection (Meintjes *et al.*, 2008, Lanzafame and Vento, 2016, Michailidis *et al.*, 2005). Currently, the immunopathogenesis remains largely unknown. There is no diagnostic test for IRIS or TB-IRIS, therefore confirmation relies on case definition that include laboratory and clinical data (Lanzafame and Vento, 2016).

1.4. Brief overview of the immune response

1.4.1 Innate Immune Response

The innate immune response is nonspecific and is made up of a number of cells such as: macrophages, monocytes, neutrophils, natural killer (NK) cells and dendritic cells (DCs).

Phagocytic cells such as macrophages, monocytes, neutrophils and DCs are important in engulfing and removing the infected cells and pathogens. Macrophages are the first cells to recognize a pathogen; these cells then engulf the pathogen and initiate an inflammatory response. This results in the expression of the major histocompatibility complex (MHC) class II molecule on the surface of the cell, which allows the macrophage to present peptides of the processed pathogen to T lymphocytes (Fraser *et al.*, 1998, Aderem and Underhill, 1999).

Clearing of microbial components is accomplished by neutrophils which also have the ability to repair tissue. DCs are known as highly specialised phagocytes and have the ability to present engulfed antigens on their cell surface through the MHC molecules. These are then recognized by T cells inducing an adaptive immune response. T cells, when activated are able to produce cytokines that can activate inflammatory responses, such as type I interferons (IFNs) (Bell *et al.*, 1999, Altfeld *et al.*, 2011). NK cells play a key role in an initial immune response to infections and are activated by IFNs and other cytokines derived from macrophages. These cells interact with MHC class I molecules, through inhibitory and activating receptors. NK cells are able to differentiate between cells that are infected and uninfected by inhibitory receptors, thus conferring self-tolerance and permitting toxicity towards impaired cells (Caligiuri, 2008, Vivier *et al.*, 2008, Campbell and Hasegawa, 2013).

1.4.2 Adaptive Immune response

The adaptive immune response is mediated by T and B cell responses. In the lymphoid tissues the antigens are presented to the T cells by the MHC molecules. Activation of T cells initiates production of interleukin (IL)-2, which mediates T cell proliferation and differentiation into effector T cells.

MHC class I molecules carry antigens which are presented to the CD8⁺ T cells, classified as cytotoxic T lymphocytes (CTL) able to eliminate infected cells (Janeway Jr and Medzhitov, 2002, Santana and Esquivel-Guadarrama, 2006, Paranjape, 2005). MHC class II molecules present antigens to CD4⁺ T cells. CD4⁺ T cells can differentiate into T helper cell subsets (Th), such as: (i) T helper 1 (Th1), (ii) T helper 2 (Th2), (iii) T helper 17 (Th17) and (iv) T

regulatory (Treg) cells. IFN- γ and tumour necrosis factor- α (TNF- α) cytokines, which aid the protection against intracellular pathogens are mainly produced by Th1 cells (Chaplin, 2010). On the other hand, Th2 cells are able to stimulate the production of antibodies and cytokines such as IL-4, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are vital in producing an immune response to extracellular infections (Zheng and Flavell, 1997). The Th17 cells produce pro-inflammatory IL-17 and IL-22 cytokines, while tolerance for self-antigens is maintained by Treg cells (Weaver *et al.*, 2006, Sehrawat and Rouse, 2017).

Antigen specific antibodies are secreted by B cells against antigens which are presented to lymphocytes by APCs. Activation and differentiation of B cells is supported through Th2 helper cells. In adaptive immunity, antibodies contribute in many different ways: i) neutralising antibodies are able to prevent pathogens from entering the cell by binding to the pathogen, ii) phagocytosis is also enhanced by the coating of antibodies to the surface of the pathogen, which is recognized by the Fc receptors on the phagocytic cells or iii) by activating the complement system (Moir and Fauci, 2009, Haynes *et al.*, 2011).

1.5 Lymphocyte trafficking

Recruitment of leukocytes plays a major role during the host immune response to an infection. The process of lymphocyte trafficking comprises of a complex cascade from the blood into inflamed tissues, where each stage of the cascade is controlled by molecules such as chemokines and adhesion molecules (Bromley *et al.*, 2008, Abram and Lowell, 2009). This complex cascade occurs between leukocytes and the endothelium and includes

leukocyte rolling, tethering, firm adhesion, crawling and transendothelial migration. During lymphocyte trafficking there are two classes of molecules known as integrins and selectins with their respective ligands, that are known to aid in the adhesion of vascular endothelial cells and the circulation of leukocytes (Kinashi, 2005, Bromley *et al.*, 2008).

Integrins are cell surface receptors composed of alpha (α) and beta (β) chain heterodimers (Kinashi, 2005, Hynes, 1992). The heterodimer chain can be divided into integrin subfamilies based on their β chain such as: β 1, β 2, and β 4 (Imhof and Dunon, 1995). Integrins have essential roles in the host immune system, which include: (i) attachment of leukocyte to endothelial cells and APCs (ii) cytotoxic killing and (iii) extravasation of cells into tissues.

Trafficking of lymphocyte to tissues during inflammatory responses involves a sequence of complexed mechanisms which include: adhesion molecules and tissue based chemokine production. In this study we will assess the role of three adhesion molecules in TB disease: intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and mucosal addressin cell adhesion molecule 1 (MAdCAM-1).

ICAM-1 binds lymphocyte function-associated antigen 1 (LFA-1) expressed on neutrophils and is constitutively conveyed on certain cell types such as: endothelial cells, epithelial cells and DCs (Dustin *et al.*, 1986). ICAM-1 was found to be significant in leukocyte-endothelial interaction as well as in the interaction of T cells with APCs (Abram and Lowell, 2009). VCAM-1 is expressed in the central nervous system, bone marrow and skin where it binds to very late antigen-4 (VLA-4), a member of alpha-4-beta-1 (α 4 β 1) integrin family, expressed

on most leukocytes except neutrophils. VCAM-1 can be expressed by endothelial cells and its expression can be induced by HIV (Osborn *et al.*, 1989). Upregulation of VCAM-1 has been associated with immunity to pulmonary *M.tb* infection (Feng *et al.*, 2000). MAdCAM-1 is expressed largely in the gastrointestinal (GI) tract where it binds to alpha-4-beta-7 ($\alpha4\beta7$) and specifically recruits leukocytes to the intestinal mucosa (Streeter *et al.*, 1988).

1.6 Immune response to TB

1.6.1 Innate immune response to TB

The innate cells that play a major role in the primary response to TB include macrophages, neutrophils, DCs and NK cells. Alveolar macrophages initiate phagocytosis through pattern recognition receptors (PRRs). By initiating phagocytosis, the innate effector function begins and other immune cells are attracted by the production of inflammatory cytokines. IL-8 promotes the recruitment of neutrophils, thereafter NK cells and T cells produce IFN- γ upon stimulation with IL-12 (Sia *et al.*, 2015). In response to *M.tb*, IL-12 responses have been shown to play an important role since individuals who do not produce this cytokine were shown to be more susceptible to the infection (Casanova and Abel, 2002).

In sputum and bronchoalveolar lavage (BAL) of active TB patients, neutrophils are a major subclass of the innate immune cells and arrive within hours of infection. Neutrophils are able to produce and secrete a number of antimicrobial enzymes during infection, thus restricting growth in the infected macrophage (Eum *et al.*, 2010, Sia *et al.*, 2015). Apoptosis of infected

macrophages is promoted by neutrophil action, limiting the existence of *M.tb* in the infected host cells (Tan *et al.*, 2006, Sia *et al.*, 2015).

During mycobacterial infection, DCs are important for establishing an adaptive immune response. In response to *M.tb*, DCs are considered one of the main cells in presenting antigens to T cells. Upon stimulation, DCs are able to produce inflammatory cytokines which direct T cell response (TNF and IFN- γ) (Flynn and Chan, 2001) and recruit cells to the site of infection (IL-1 and IL-6) (Giacomini *et al.*, 2001). Previous studies have found that DCs have the ability to strengthen the cellular immune response against *M.tb* infection (Henderson *et al.*, 1997, Förtsch *et al.*, 2000, Tascon *et al.*, 2000). DCs are highly represented at sites of *M.tb* infection at the onset of inflammatory responses (Sertl *et al.*, 1986, Van Haarst *et al.*, 1994). Immature DCs are present in the mucosa of the lung and are highly specialized for antigen uptake as well as processing. Maturation occurs after DCs interact with *M.tb*. Thereafter DCs migrate to lymphoid organs, where they prime T cells through cell surface expression of MHC and costimulatory molecules and through the secretion of immunoregulatory cytokines such as: IL-12 (Banchereau and Steinman, 1998).

NK cells are granular lymphocytes characterized by a strong cytolytic capacity. NK cells were found to limit the replication of *M.tb* by producing specific soluble mediators (GM-CSF, IL-12, TNF- α , IL-22, and IFN- γ) (Zhang *et al.*, 2006). Granulysin and perforin are derived antimicrobial factors of NK cells, and are able to indirectly limit the progression of *M.tb* through the lysis of infected host cells (Sia *et al.*, 2015).

1.6.2 Adaptive immune response to TB

The protective response against *M.tb* is dependent on the dominant role of CD4⁺ T cells (Kaufmann *et al.*, 2005). Once CD4⁺ T cells are activated, cytokines such as IFN- γ , IL-2 and TNF are secreted resulting in the activation of macrophages (Flynn and Chan, 2001). The importance of CD4⁺ T cells in control of *M.tb* infection in humans is revealed by the steady increase in the risk of active TB in HIV infected individuals who have low CD4⁺ T cell counts (Selwyn *et al.*, 1989). Additionally, CD4⁺ T cells are essential in increasing APC function through the interaction of CD40-CD40L between CD4⁺ T cells and the APC.

Although the principle mechanisms of CD8⁺ T cell stimulation is not completely understood, it is known that CD8⁺ T cells add to a protective immunity against TB (Kaufmann, 2006, Cooper, 2009). Mycobacterial peptides presented by MHC I are needed for the stimulation of CD8⁺ T cells. The presentation usually takes place in the cytosol which is not freely accessed by *M.tb*. Therefore, two pathways have been suggested: (i) *M.tb* enters the cytosol of the infected DCs which results in direct loading of the MHC class I molecules or (van der Wel *et al.*, 2007) (ii) apoptosis of *M.tb* infected macrophages which releases the vesicle containing the mycobacterial antigen, leading to cross priming (Winau *et al.*, 2006). CD8⁺ T cells which are specific to mycobacterial antigens are able to produce IFN- γ and secrete cytolytic proteins (perforin and granulysin), thereby lysing the host cells and directly attacking *M.tb* (Stenger *et al.*, 1997).

Cytokines produced in response to *M.tb* infection are not always favorable to host protection and in turn have the ability to favor *M.tb* replication and persistence. A number of studies

both in humans and animals have found that cytokines play a significant role in defining the outcome of *M.tb* infection. Transforming growth factor β (TGF- β), an anti-inflammatory cytokine, has the ability to counteract the protective immunity against TB by inhibiting IFN- γ production and T cell proliferation, resulting in the suppression of the adaptive immune response (Toossi *et al.*, 1995). It has been previously shown that soluble adhesion molecules such as: sICAM and sVCAM correlate with TB disease severity (Mukae *et al.*, 2003). A study conducted by Shi *et al* found increased levels of sICAM-1 in millitary and advanced TB disease whereas, a study by Lai *et al* found that sICAM-1 levels where increased in individuals who had active TB compared to controls (SHI et al., 1993, Lai et al., 1993). Mukae *et al* found significantly higher sICAM-1 in individuals with active TB and found it to be the most sensitive biomarker of TB severity (Mukae *et al.*, 2003). Additionally, Feng *et al* showed in mice that the upregulation of sVCAM-1 was associated with immunity to pulmonary *M.tb* (Feng *et al.*, 1999).

1.7. Immune response to HIV

1.7.1. HIV transmission and mucosal immune responses

Transmission of HIV can occur through various routes such as: sexual transmission (vaginal, anal or oral), perinatal transmission (child birth) and percutaneous transmission (needle stick). At least 80% of all HIV infections occur through sexual contact via the mucosal surface and the other 20% occur mainly through percutaneous methods (Cohen *et al.*, 2011, Sharp and Hahn, 2011). While significantly reduced a small proportion of HIV transmission still occurs through mother to child transmission (MTCT) during birth and through

breastfeeding, especially in the resource limited settings. Majority of the individuals who are infected, acquire the virus via sexual contact through mucosal tissue (genital and rectal) and between these tissues the probability of transmission varies. During vaginal intercourse the probability of transmission ranges from 1/200 to 1/2000 whereas in anal intercourse the probability of transmission ranges from 1/20 to 1/300 (Kaul *et al.*, 2015).

Prolonged/chronic inflammation impairs mucosal barriers, permits HIV to enter target cells in order to establish infection (Carias *et al.*, 2013, Nazli *et al.*, 2010). Inflammation of the genital tract can be caused by sexually transmitted infections (STIs), bacterial vaginosis (BV) and genital micro-abrasions, which lead to an upregulation of soluble immune proteins and activation of susceptible cells in the vaginal mucosa (Fichorova *et al.*, 2001, Guimarães *et al.*, 1997).

1.7.2 HIV disease progression

HIV disease progression is marked by a destruction of CD4+ T cells compromising the host immune response (Okoye and Picker, 2013). There are varied rates at which HIV progresses to AIDS. These are classified as: (i) Elite controllers, living with HIV and able to maintain suppressed viral loads without ART (ii) Rapid progressors, which advance to AIDS within 3 years following infection, (iii) Intermediate, which assumes a time of 3-10 years since infection and (iv) Long term, where CD4 count is preserved for a great length of time without ART therapy (Ganesan *et al.*, 2010, Zeller *et al.*, 1996). CD4+ T cell counts, viral load (VL) and immune activation are classified as predictors of disease progression (Goujard *et al.*, 2006). One of the main causes of a gradual loss of CD4+ T cells is immune activation,

regardless of VL (Deeks *et al.*, 2004). Mucosal integrity is sustained by CD4⁺ T cells, and Th17 cells in particular, and their destruction causes damage to mucosal barriers and systemic translocation of microbes (Brenchley *et al.*, 2006b, Mogensen *et al.*, 2010). The translocation of bacterial products ('microbial translocation') results in the production of high amounts of inflammatory cytokines. These include TNF, IFN- α , IFN- β and IL-1 β which contribute to immune activation in chronically infected individuals (Sandler *et al.*, 2011, Miyake, 2006).

High level of viral replication in the gut associated lymphoid tissue (GALT) is the defining feature of acute HIV infection. Early studies conducted on rhesus macaques and simian immunodeficiency virus (SIV) models established that a profound depletion of gut CD4⁺ T cells was accompanied by increased viral replication in the GALT (Heise *et al.*, 1994, Veazey *et al.*, 1998). In human studies, it was shown that a similar depletion of CD4⁺ T cells was observed in the initial stages of HIV infection (Guadalupe *et al.*, 2003, Mehandru and Dandekar, 2008, Mehandru *et al.*, 2004). The rapid loss of CD4⁺ T cells results in damage to the physical integrity of the gut, which has been linked to chronic systemic immune activation (Brenchley and Douek, 2008a, Brenchley and Douek, 2008b). Therefore, there is substantial evidence to propose that the events which occur in the tissues of the gut during the initial infection play a significant role in the pathogenesis of AIDS (Breed *et al.*, 2015).

1.7.3 Systemic immune activation

During HIV infection the cytokines produced contribute directly to chronic immune activation, which begins during acute HIV infection. This in turn has an effect on lymphocytes in circulation and in the tissues. Previous studies have shown that HIV

replication in the GALT causes drastic CD4⁺ T cell depletion in HIV/SIV infection (Février *et al.*, 2011, Veazey *et al.*, 1998). The depletion is likely due to the infected CD4⁺ T cells being directly killed, however this is exacerbated by pro-inflammatory cytokine (IFN- α and TNF- α) production, which induce activation and death of uninfected CD4⁺ T cells (Groux *et al.*, 1992). In addition, the depletion of Th17 cells results in the structural damage of the GALT tissues as well as the translocation of microbial products from the gut into systemic circulation (Brenchley *et al.*, 2008, Brenchley *et al.*, 2006b, Marchetti *et al.*, 2011, Kamat *et al.*, 2010, Moir *et al.*, 2011).

In pathogenic SIV/HIV infections, the translocation of microbial products from the GI tract and the associated systemic inflammation have been suggested to be a major driver of chronic immune activation (Marchetti *et al.*, 2011, Zevin *et al.*, 2016). A major marker of microbial translocation is lipopolysaccharide (LPS) which is a component of the gram-negative bacterial cell wall and a recognized agonist of toll-like receptor (TLR) 4 (Beutler, 2000, Caradonna *et al.*, 2000, Cooke *et al.*, 2001). It was originally proposed by Brenchley *et al* that microbial translocation is a key mechanism of mucosal immune dysfunction, chronic systemic immune activation and HIV disease progression (Brenchley *et al.*, 2006a). A subsequent study by Brenchley *et al* showed significant increases of LPS levels in HIV infected individuals and SIV infected rhesus macaques, which correlated positively with innate and adaptive immune activation measures (Brenchley *et al.*, 2006b). Since Brenchley *et al* initial finding, a number of studies have found an association between HIV infection and a substantial loss of CD4⁺ T cells in the gut, thereby resulting in microbial translocation (Hunt *et al.*, 2014, Marchetti *et al.*, 2008).

1.8. Immune responses in HIV/TB infection

1.8.1 Immune complexities of HIV and TB co-infection

The progressive reduction of absolute CD4⁺ T cell numbers is the most obvious defect caused by HIV that strongly correlates with an increased risk of TB (Sonnenberg *et al.*, 2005). Due to the increase in TB risk just after HIV acquisition, there has been a growing interest in establishing if early HIV infection eliminates *M.tb* specific CD4⁺ T cells and whether it impairs their functional potential (Sonnenberg *et al.*, 2005, Lawn *et al.*, 2005b).

A number of cross sectional studies have shown that in individuals who have latent TB, the frequency of *M.tb* specific CD4⁺ T cells is decreased by HIV-1 in the periphery and at the site of disease (Jambo *et al.*, 2014, Serbina *et al.*, 2001, Rangaka *et al.*, 2007). A study conducted by Geldmacher *et al* looked at a small group of participants and after seroconversion found that majority of the participants had a fast decline in the rate of ESAT-6/CFP-10 and PPD specific CD4⁺ T cells, attaining an undetectable level a year after infection (Geldmacher *et al.*, 2008). Additional studies have shown that in HIV-1 co-infected individuals with CD4 counts < 200 cells/mm³, there was an increased frequency of ESAT-6/CFP-10 specific CD4⁺ T cells in active and latent TB (Hammond *et al.*, 2008, Rangaka *et al.*, 2007). From a mechanistic point of view, the susceptibility of *M.tb* specific CD4⁺ T cells to HIV-1 has been associated with a poor secretion of macrophage inflammatory protein 1 β (MIP-1 β). MIP-1 β is a ligand of CCR5 and acts as an antagonist for HIV entry (Geldmacher *et al.*, 2010). Active TB is linked with elevated expression of CCR5 on CD4⁺ T cells, making

M.tb specific cells better targets for HIV-1 (Juffermans *et al.*, 2001, Rosas-Taraco *et al.*, 2006). Co-infection of *M.tb* and HIV-1 generally provides a microenvironment that has the ability to assist in the replication of HIV-1 and *M.tb* specific CD4+ T cell depletion. The remaining *M.tb* specific CD4+ T cells are not able to contain *M.tb* in co-infected individuals, suggesting that the functional properties of the remaining *M.tb* specific CD4+ T cells are impaired by HIV-1.

A number of studies have looked at the qualitative profile of *M.tb* specific CD4+ cells in the context of HIV-1 co-infection (Canaday *et al.*, 2015, Pollock *et al.*, 2016). A reduction in the polyfunctional potential of PPD and BCG specific Th1 CD4+ T cells in both the periphery and BAL has been associated with individuals who are HIV-1 infected and have latent TB; thereby skewing the cell functional profile toward a monofunctional TNF- α response (Kalsdorf *et al.*, 2009). Moreover, the proportion of CFP-10 specific CD4+ T cells that produce IL-2 is indirectly related to HIV-1 viremia in individuals who are HIV-1 infected and have latent TB. This limits the expansion of *M.tb* specific T cell upon antigen recall (Day *et al.*, 2008).

A systemic and chronic state of immune activation induced by HIV infection affects the innate and adaptive immune systems (Moir *et al.*, 2011). T cell phenotype is altered by HIV-1 related chronic immune activation, resulting in increased expression of activation markers and a shift in the memory profile of T cells toward the effector phenotype and increased expression of exhaustion markers on effector memory subsets (Liu *et al.*, 1997, Papagno *et al.*, 2004). In individuals who are co-infected with HIV-1 and latent TB, the expression of

HLA-DR in *M.tb* specific CD4+ T cells is significantly increased compared to individuals who are HIV-1 uninfected (Wilkinson *et al.*, 2016). In BCG vaccinated infants, there was an association between the frequency of circulating activated HLA-DR+ CD4+ T cells and elevated risk of active TB (Fletcher *et al.*, 2016). Therefore, it is plausible that HIV-induced T cell activation plays a role in increasing the risk of TB in HIV-1 infected individuals. Additionally, individuals who had latent TB expressed <10% of programmed cell death protein 1 (PD-1) on PPD specific CD4+ T cells irrespective of HIV-1 infection (Pollock *et al.*, 2016). Conversely the expression of PD-1 on *M.tb* specific CD4+ T cells was significantly elevated in HIV-1 co-infected individuals compared to HIV-1 uninfected individuals (Pollock *et al.*, 2016). The proliferation potential of PPD specific CD4+ T cells was marginally improved by blocking of PD-1/ programmed death-ligand 1 (PD-L1) pathways (Shen *et al.*, 2016), therefore suggesting that in HIV-1 co-infected individuals the overexpression of PD-1 encourages T cell exhaustion and contributes to a suboptimal *M.tb* response.

Furthermore, HIV-1 infection disrupts the balance in *M.tb* specific Th subsets, thus suggesting that *M.tb* specific responses are shifted toward a more pathogenic or inflammatory profile due to HIV-1 (Riou *et al.*, 2016). A study conducted by Bell *et al* looked at the Th responses in a small number of *M.tb*/HIV co-infected individuals by measuring transcriptional profiles of punch biopsies taken at the site of mycobacterial challenge (Bell *et al.*, 2016). Individuals, who were co-infected with a positive TST and a stable CD4 count, displayed conserved Th responses and poor IL-10 inducible responses, thus proposing that impairment of regulatory pathways could be harmful for *M.tb* control. This supports the idea that a stable immune response is vital for *M.tb* containment. In

comparison, individuals who were TB/HIV co-infected with clinically anergic TSTs showed a significant decrease in circulating CD4 counts. Additionally, the TST transcriptome of these individuals showed immunological anergy with a decrease in cellular recruitment, cytokine activity as well as in the innate immune responses (Bell *et al.*, 2016).

Animal studies have shown that CD8⁺ T cells specific to *M.tb* play a protective role in controlling *M.tb* infection during latency (Woodworth and Behar, 2006). A study conducted on macaques found that protection from TB reactivation was related to an increase in memory CD8⁺ T cell proliferation and high granzyme B production in the lung (Foreman *et al.*, 2016). Not many studies have looked at the impact of HIV-1 infection on *M.tb* specific CD8⁺ T response. However, a study conducted by Kalokhe *et al* in individuals with LTBI found that CD8⁺ T cells from individuals who are HIV-1 co-infected showed a decrease in the expression of CD107a and this likely suggests that HIV co-infection compromises the function of CD8⁺ T cells in LTBI (Kalokhe *et al.*, 2014). It is well established that the hallmark of HIV-1 mediated chronic activation has the ability to severely affect the CD8 compartment (Breton *et al.*, 2013). It is also possible that premature exhaustion of *M.tb* specific CD8⁺ T cells is promoted by HIV, which affects their functional capacity and potential to survive. Therefore, more studies need to be conducted in order to fully describe the effect of HIV-1 on *M.tb* specific CD8⁺ responses.

The immunological environment during *M.tb* infection is distinguished by irregularities in the production of cytokines and chemokines which are believed to (i) increase T cell activation, (ii) increase HIV replication and (iii) result in a dysfunctional immune response (Toossi *et*

al., 2001a, Goletti *et al.*, 1996, Toossi *et al.*, 2001b, Hertoghe *et al.*, 2000, Vanham *et al.*, 1996). The effect of cytokines and chemokines in TB/HIV infected individuals remains largely unexplored. There have been contradictory studies on the effect of TB treatment on VL and plasma cytokine response. Elevated pro-inflammatory immune response and a reduced VL in response to TB therapy were observed in some studies (Goletti *et al.*, 1996, Toossi *et al.*, 2001b, Hertoghe *et al.*, 2000, Vanham *et al.*, 1996, Dean *et al.*, 2002), whereas other studies have not seen this difference (Kalou *et al.*, 2005, Morris *et al.*, 2003, Lawn *et al.*, 1999). A study conducted by Mihret *et al* looked at the difference in the immune response before and after TB treatment in TB/HIV co-infected individuals compared to individuals who had TB and were HIV uninfected. The study found that there were statistical differences in the plasma levels of IFN- γ , IL-4, monocyte chemoattractant protein-3 (MCP-3), MIP-1 β and interferon gamma-induced protein 10 (IP-10) prior to and after TB treatment in HIV uninfected participants (Mihret *et al.*, 2014).

Previous studies conducted on TB/HIV co-infected individuals in KZN have shown differences in host responses to TB. A study conducted by Thobakgale *et al* found an association between the production of interleukin 1-beta (IL-1 β) by innate cells (monocytes and myeloid dendritic cells) following BCG and TLR 2,4,7/8 stimulation with differences in TB recurrence outcomes. They found that the production of IL-1 β in response to BCG stimulation was associated with increased TB risk in a SA population (Thobakgale *et al.*, 2017). Sivro *et al* measured cytokines in plasma samples from the TB Recurrence upon Treatment with HAART (TRuTH) cohort in SA and found that inflammatory cytokines such as: IL-1 β , IL-6 and interleukin-1 receptor antagonist (IL1-Ra) were associated with increased risk of TB reactivation (Sivro *et al.*, 2017) thus indicating that systemic

inflammation plays a role in TB disease. These studies provide evidence that the inflammatory cytokines have an impact on reactivation of TB and could be used as biomarkers for effective TB management.

1.8.2 Gut-Lung axis

The human microbiota is composed of microbial communities which reside at different sites such as the: gut, airways, vagina, skin and mouth (Cho and Blaser, 2012). Dysbiosis of the gut microbiota has been associated with respiratory infections and lung disorders (Trompette *et al.*, 2014, Shukla *et al.*, 2017). Studies previously conducted on mice have shown that due to antibiotic intake, certain species (such as *Clostridium* species) within the gut microbiota get depleted and this was shown to influence allergic inflammation and lung diseases (Russell *et al.*, 2013, Dharmage *et al.*, 2015, Metsälä *et al.*, 2015). Chronic gut diseases such as: inflammatory bowel disease (IBD) and irritable bowel disease (IBS) were shown to impact lung health (Roussos *et al.*, 2003, Rutten *et al.*, 2014). Pulmonary involvement such as: inflammation and impaired lung function is found in up to 50% of adults with IBD and 33% of patients with IBS (Yazar *et al.*, 2001, Keely *et al.*, 2012). Additionally, mouse studies have previously shown that after the administration of neomycin to remove sensitive gut bacteria, the lungs become increasingly susceptible to the influenza virus infection (Ichinohe *et al.*, 2011, Looft and Allen, 2012).

Opposite effect has been shown as well, where changes in the lung microbial community can influence the gut microbiota composition. In mice models, influenza infection in the respiratory tract increased the amount of *Enterobacteriaceae* and led to a reduction in

Lactobacilli and *Lactococci* species in the microbiota of the intestines (Looft and Allen, 2012). Sze *et al* showed in mice that the dysbiosis in the microbiota of the lungs after LPS administration was accompanied by the disturbance in their gut microbiota, which is due to bacteria moving from the lung and into the bloodstream (Sze *et al.*, 2014). Another important and currently unexplored research area is the impact of long-term TB treatment on bacterial communities of the vaginal tract and how these changes could be impacting HIV susceptibility.

Overall these findings indicate an inflammatory “cross talk” between different mucosal tissues. HIV-mediated destruction of the gut mucosal tissues and the resulting microbial translocation could have profound impacts on TB susceptibility and disease in co-infected individuals.

Rationale

Globally the burden of TB and HIV infections is highest in SA and often concomitant (Jacobson *et al.*, 2015). Therefore, there is a dire need to improve the diagnosis and management of TB/HIV co-infected individuals.

TB and HIV infections have been related to changes in a number of host protein expression (Sivro *et al.*, 2017, Achkar *et al.*, 2015). Inflammatory responses of both HIV and TB act similarly, whereby the initial response to infection is needed to prevent the infection however, if left uncontrolled it can lead to immune mediated pathology (Guo *et al.*, 2015, Sandler *et al.*, 2014, Veazey *et al.*, 2016). Trafficking of immune cells from the periphery to the affected tissues plays an important role in the pathogenesis of both TB and HIV (Wilson and Sereti, 2013). The migration of leukocytes from circulation to tissues is a multistep process, which is mediated by the interaction of cell surface glycoproteins (“homing receptors”) with endothelial molecules expressed in a tissue-restricted manner (“vascular addressins”) (Butcher *et al.*, 1999, Butcher and Picker, 1996). This study will look at three adhesion molecules: sICAM, sVCAM and sMAdCAM that are known to play an important role in cell recruitment to the mucosal tissues including lung and the GI tract as well as TGF- β and LBP, which are known for their role in inflammation/wound healing and microbial translocation respectively. Although several chemokine receptors have been implicated in T cell trafficking to the lung during disease progression, there continues to be a lack of data regarding lymphocyte trafficking markers and their role in TB. Therefore given the role of lymphocyte trafficking in HIV, it is important to assess the role of soluble lymphocyte trafficking markers in TB and TB/HIV co-infection. Susceptibility to both HIV and TB infection as well as the course of the disease are likely to be affected by the inflammation induced changes in cytokine/chemokine expression.

Here we hypothesize that concentrations of soluble lymphocyte markers will increase during active TB disease and will contribute to inflammation in TB and TB/HIV co-infection.

Aim

Characterize soluble markers of lymphocyte trafficking and inflammation in the context of TB and TB/HIV co-infection

Hypothesis

Increased plasma expression of lymphocyte trafficking markers (ICAM, VCAM and MAdCAM) as well as inflammatory LBP and TGF- β will positively associate with onset of TB disease and can be used to predict TB recurrence.

Specific objectives

1. To evaluate the role of ICAM, VCAM, MAdCAM, TGF- β and LBP plasma expression as predictors of TB recurrence (TRuTH) by standard and multiplex ELISAs
2. To evaluate the effect of active TB disease and TB treatment completion (IMPRESS) on the plasma expression of soluble ICAM, VCAM, MAdCAM, TGF- β and LBP by standard and multiplex ELISAs

CHAPTER 2. Materials and Methods

2.1. Ethics

Informed written consent was obtained from all study participants prior to enrolment. The University of KwaZulu-Natal's Biomedical Research Ethics Committee (BE659/17) approved this study. Ethical approval received covered the use of stored samples in this study.

2.2. Study participants

The presented study was performed on stored plasma specimens from the CAPRISA 005 TRuTH and the CAPRISA 011 Improving Retreatment Success (IMPRESS) cohorts. Patients in both studies were recruited and treated at an urban clinic (CAPRISA eThekweni Research Clinic) in KZN, South Africa.

2.2.1. TRuTH cohort

TRuTH was a cohort observational study that investigated the rate of TB recurrence in adults who completed therapy for pulmonary TB and were on highly active antiretroviral therapy (HAART) (Clinical trial NCT 01539005). Participants from this cohort were previously enrolled in the CAPRISA 003 SAPiT trial, which investigated the timing of ART initiation during pulmonary TB treatment. Participants that entered TRuTH had a confirmatory negative status for TB upon completion of TB treatment. While retained on ART, participants were screened four times a year for a maximum of 4 years for recurrence of TB.

A nested case: control study was conducted for TRuTH samples, where cases were defined by the recurrence of TB with the availability of a pre-recurrence sample, which was matched to controls who were participants that had no indication of recurrence during follow up. To analyse the effect of measured analytes on TB recurrence, case:control matching (1:3) was carried out based on the ART start date. Cases were sampled at a minimum of 3 and maximum of 9 months prior to TB recurrence and controls at comparable time points to minimize the difference in sample cryopreservation length between groups. A subset of cases was followed longitudinally at additional time points: Recurrence/TB (2-month window before or after TB recurrence) and Cure/Post TB (capturing a 2 month-window before and 3 month- window after recurrent TB treatment completion date).

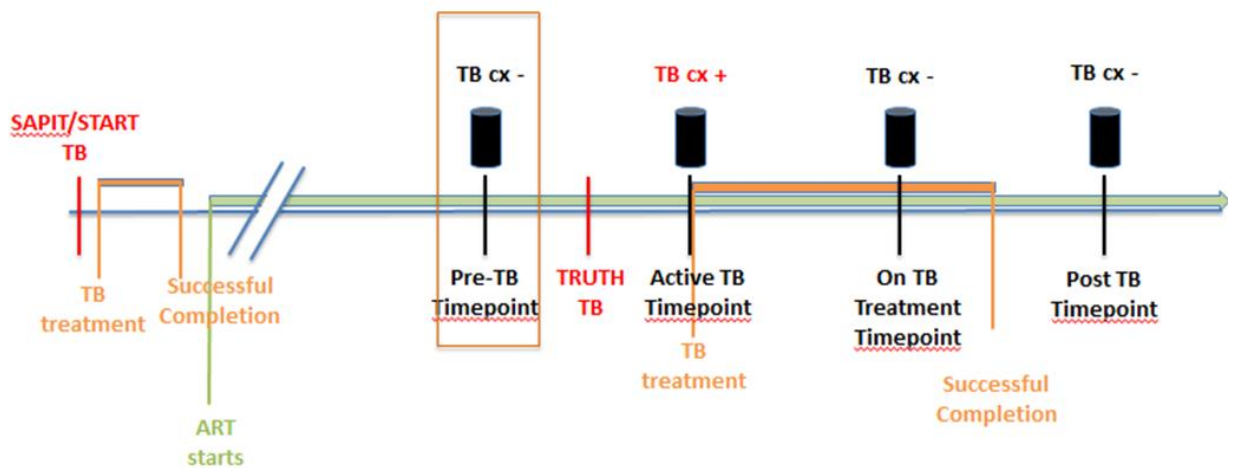


Figure 3. Pictorial timeline illustrating sample collection in TRuTH

2.2.2. IMPRESS cohort

IMPRESS was an open labelled phase 1 and 2 randomized control study (Clinical trials NCT 02114684), which compared previously treated TB patients with two regimens for treatment of smear positive pulmonary TB. The first was a 24 week regimen consisting of Moxifloxacin, INH, RMP and PZA taken orally and the second was a standard drug regimen (INH, RMP, PZA and EMB). Patients were stratified by HIV status and randomized into the intervention and control arm, where TB therapy was administered for 6 months. Participants included in this study had to be >18 years of age, have had a documented history of confirmed TB in the last 3 years and have had a positive sputum smear of *M.tb*. This study consisted of men and women who were HIV infected and uninfected.

To analyse the effect of active disease and TB treatment completion on the expression of the here measured analytes (MAdCAM, ICAM, VCAM, LBP, TGF- β 1, TGF- β 2 and TGF- β 3), a subset of HIV infected and HIV uninfected men and women were sampled at two time-points: active disease and post TB treatment completion. No matching was performed.

2.3. Collection and Processing of Samples

Acid citrate dextrose (ACD) and Ethylenediaminetetraacetic acid (EDTA) tubes were used to collect peripheral blood. The blood was centrifuged for 7 minutes at 1400rpm, the plasma was retrieved and cryopreserved for future analysis at -80°C.

2.3.1. Chest Radiograph

Participants underwent a digital chest x-ray every 6 months at the Prince Cyril Zulu Communicable Disease Centre (PCZCDC).

2.3.2. HIV testing

Uni-Gold (Trinity Biotech, Ireland) and Alere Determine (Alere Scarborough, Inc) rapid HIV tests were used to test the samples for HIV. Results positive for HIV were confirmed by a second licenced rapid HIV test and the discordant results were confirmed by an Enzyme-Linked Immunosorbent Assay (ELISA).

2.3.3. Analysis of CD4 count

All participants who were HIV positive had their CD4 counts measured by collecting whole blood in EDTA tubes and analysed with the TruCOUNT method (BD Biosciences, San Jose, US).

2.3.4. Analysis of viral load

A nested PCR with Expand Long Template PCR system (Roche Diagnostics) was used to determine viral loads in HIV positive samples. The assay yields a lower detection limit of 40 HIV RNA copies/ml with a range of 40-10⁷ HIV RNA copies/ml.

2.4. Multiplex Assays

2.4.1. Millipore Milliplex MAP Human Sepsis assay

The Milliplex MAP Human Sepsis kits were used to simultaneously quantify ICAM and VCAM, by using small volumes of plasma. Prior to the start of the assay, all reagents were brought to room temperature (RT). The reagents were prepared as per manufacturer's instructions, with minor changes. A fourfold dilution series was made for the standard (Figure 3). The serum matrix, once reconstituted with deionized water (dH₂O) was further diluted in sample diluent. The patient plasma samples (5 µl) were diluted in 195 µl of serum matrix to get a final 1:40 dilution.

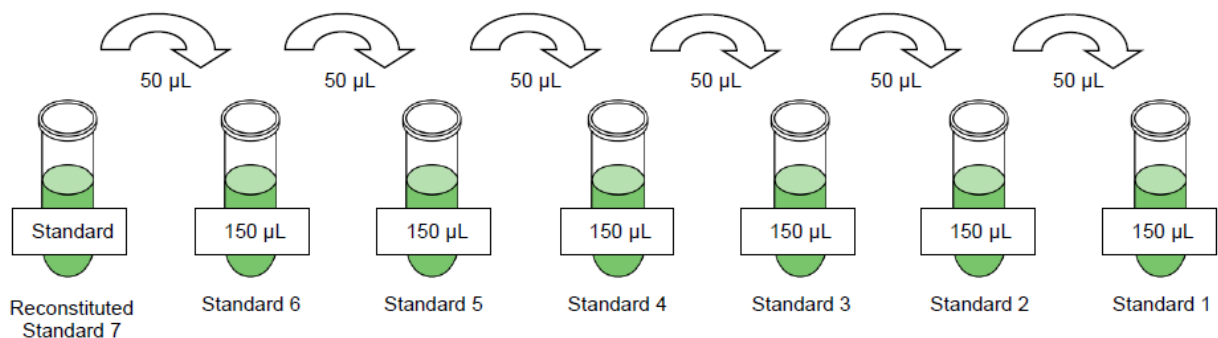


Figure 4. Illustration of the fourfold Milliplex MAP Human Sepsis standard serial dilution ³

³Adapted from the Millipore Milliplex instruction manual

Plates were pre-wet with 200 µl/well of assay buffer and incubated on a shaker for 10 minutes at RT. Following the removal of assay buffer, 25 µl of standards and controls was added to the respective wells and 25 µl of assay buffer was added to all the sample wells. Matrix solution (25 µl/well) was added to the wells containing the background, standards and controls. Diluted samples (25 µl) were added to the appropriate wells and 25 µl of magnetic

beads were added to each well in the plate. The plate was then sealed, covered with foil and incubated on a shaker at 250 ± 50 rpm overnight at 4°C .

The plate was washed twice with $100\ \mu\text{l}$ wash buffer on the Bio-Plex Wash Station. $25\ \mu\text{l}$ of detection antibodies/well were added to the plate and incubated for 1 hour at RT. After incubation, $25\ \mu\text{l}$ of Streptavidin-Phycoerythrin was added and the plate was incubated for 30 minutes at RT. The plate was then washed three times with $100\ \mu\text{l}$ of wash buffer and $100\ \mu\text{l}$ of sheath fluid was added to each well. The beads were re-suspended on a shaker for 5 minutes at 250 ± 50 rpm. The plate was analysed on the Bio-Plex™ 200 plate reader.

Commercial Kits used:

Customized Milliplex MAP Human Sepsis Mag Bead Panel 1 (ICAM and VCAM) (Cat# HSP1MAG-63K-02)

Equipment Used:

Bio-Plex Pro™ II Wash Station

Bio-Plex™ 200 System

Stuart Orbital Shaker

Vortex

2.4.2. Transforming growth factor – beta (TGF-β) assay

The Transforming growth factor-beta (TGF-β) kit (Bio-Rad, USA) was used to determine the plasma levels of TGF-β1, TGF-β2 and TGF-β3. Prior to the start of the assay, all reagents, samples and controls were brought to RT and were prepared as per the manufacturer’s instructions. A fourfold serial dilution was made of the standard (Figure 4).

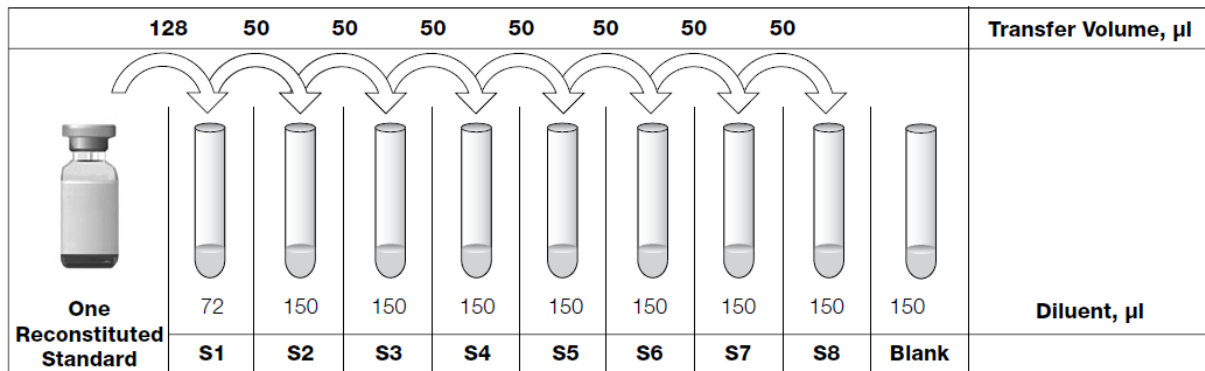


Figure 5. Illustration of the fourfold TGF-β standard serial dilution⁴

⁴Adapted from the Bio-Plex Pro instruction manual

Once thawed, the samples were activated by adding 2.5 μl of 1 N hydrochloric acid (HCl) to 12.5 μl of sample. The samples were then vortexed and incubated at RT for 10 minutes. Thereafter, the samples were neutralized by adding 2.5 μl of 1.2 N Sodium hydroxide (NaOH)/ 0.5 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). To make 100 ml of 1.2 N NaOH/ 0.5 M HEPES, 20 g of NaOH was added to 20 ml of dH₂O and topped up to 50 ml with dH₂O to make 10 N NaOH . Thereafter, 12 ml of 10N NaOH was added to 75 ml of dH₂O. Once mixed well, 11.9 g of HEPES was added and the volume was adjusted to 100 ml with dH₂O.

To run the assay, the magnetic beads were vortexed and added (50 μ l/well) to the plate. The plate was washed twice (100 μ l) on the Bio-Plex wash station. The diluted standards, samples, controls and blanks were vortexed for 5 seconds and 50 μ l was added to the respective wells. The plate was covered with sealing tape and incubated on a shaker at 250 ± 50 rpm at RT for 2 hours. The plate was washed three times with 100 μ l of wash buffer. Detection antibodies were vortexed and 25 μ l was added to each well of the plate. The plate was covered with aluminium foil and incubated at room temperature for 1 hour on a shaker at 250 ± 50 rpm. The plate was washed three times with 100 μ l of wash buffer and 50 μ l per well of SA-PE was added to the plate. The plate was covered with aluminium foil and incubated on a shaker at 250 ± 50 rpm for 30 minutes at RT. The plate was then washed three times, and the beads were re-suspended with 125 μ l of assay buffer. The plate was analysed on the Bio-Plex™ 200 system.

Commercial Kits and reagents used:

1N Hydrochloric Acid (Cat# KC-HCL-1N)

NaOH pellets (Cat# S8045-500G)

HEPES Free Acid Ultrol Grade (Cat#391338-100GM)

Bio-Plex Pro TGF- β Panel (Cat#171W4001M)

Equipment used:

Bio-Plex Pro™ II Wash Station

Bio-Plex™ 200 System

Stuart Orbital Shaker

Vortex

2.5. Standard Sandwich ELISA Assays

2.5.1. Mucosal Addressin Cell Adhesion Molecule 1 (MAdCAM-1) assay

The Hycult Biotech HK337 Human sMAdCAM-1 ELISA Kits (Hycult, USA) were used to determine the level of sMAdCAM-1 in plasma samples. Prior to the start of the assay, all the reagents were brought to RT. The reagents were prepared as per manufacturer's instructions. A serial dilution was made for the standards (Figure 5). The samples (20 μ l) were diluted in 180 μ l of dilution buffer for a final 1:10 dilution.

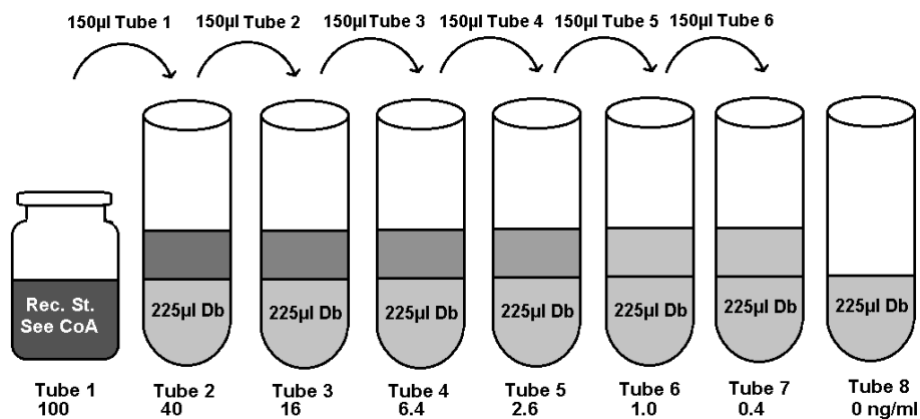


Figure 6. Illustration of the sMAdCAM-1 standard serial dilution⁵

⁵Adapted from the Hycult Biotech HK337 instruction manual

In a pre-coated plate 100 µl of standards, samples and controls were added to their respective wells and incubated on a plate shaker for 1 hour at 250± rpm. The plate was washed manually three times with 200 µl of wash buffer. The plate was then coated with 100 µl of diluted tracer and incubated for 1 hour at RT. After incubation, the wash step was repeated and thereafter diluted streptavidin-peroxidase substrate (100 µl/well) was added and the plate was incubated for 1 hour at RT. The wash step was repeated and 100 µl of TMB substrate was added to each well of the plate and incubated for 30 minutes at RT. The reaction was stopped by adding stop solution (2% oxalic acid) (100 µl /well) to the plate. The plate was analysed at 450nm on the ELISA plate reader.

Commercial Kits used:

Human sMAdCAM-1 ELISA kit (Cat#HK337-02)

Equipment used:

Versamax ELISA Plate Reader

Stuart Orbital Plate Shaker

Vortex

2.5.2. Human Lipopolysaccharide – Binding Protein (LBP) Assay

The Human Lipopolysaccharide-Binding Protein (LBP) kit (R&D systems, USA) was used to measure the human LBP plasma levels. Prior to the start of the assay, all reagents were brought to RT. A two fold serial dilution of the standard was made (Figure 6). Capture antibody was diluted in phosphate buffer saline (PBS) without a carrier protein. The plate was coated with the diluted capture antibody; it was then sealed and incubated overnight at RT. On the following day, the plate was manually washed three times with wash buffer (200 μ l/well). The plate was blocked with 300 μ l of reagent diluent and incubated for 1 hour at RT. Wash step was repeated after incubation.

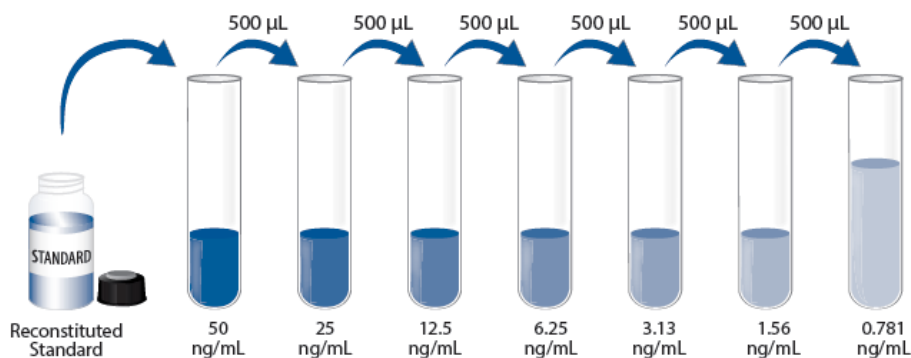


Figure 7. Illustration of the twofold LBP standard serial dilution⁶

⁶Adapted from the DuoSet ELISA instruction manual

To a pre-coated plate 100 μ l of sample and standards were added to their respective wells; the plate was then covered and incubated at RT for 2 hours. The plate was manually washed three times with wash buffer (200 μ l/ well). Streptavidin-HRP (100 μ l/well) was added to the plate, covered with foil and incubated at RT for 20 minutes. The wash step was repeated and

substrate solution (100 μ l/well) was added to the plate and then incubated at RT for 20 minutes. Stop solution (2N H₂SO₄) (50 μ l/well) was added to the plate. The plate was read immediately at 450nm with a wavelength correction of 540nm on an ELISA microplate reader.

Commercial Kits and Reagents Used:

Human LBP DuoSet, 5 Plate (Cat# DY870-05)

DuoSet Ancillary Reagent Kit 2, 5 Plate (Cat# DY008)

Equipment used:

Versamax ELISA Plate Reader

Stuart Orbital Shaker

Vortex

2.6. Statistical analysis

All specimens were analyzed, blinded to the clinical status, with longitudinal samples analyzed on the same plate. GraphPad Prism version 7.05 (GraphPad software, La Jolla, CA) and SPSS version 24 were used to perform the statistical analysis.

For comparison of baseline characteristics of TRuTH samples, the Wilcoxon signed ranks and the McNemar tests were used. TRuTH data were modeled using a univariate and

multivariable conditional logistic regression to account for matching. Data was square root transformed in order to achieve normality in distribution. Square root transformation was chosen due to the slightly right-skewed structure of the original dataset. TB recurrence status was used as a dependent variable with each analyte modeled independently as an independent variable. Multivariable analysis adjusted for a number of potential confounders including: age, body mass index (BMI), CD4 count, VL, presence of lung cavities, previous history of TB, and WHO disease stage (4 vs 3). Longitudinal analysis was performed by a paired t-test.

For comparison of baseline characteristics in IMPRESS, the Mann-Whitney and the Fishers exact tests were used. D'Agostino-Pearson omnibus normality test was used to determine normality, if the data was normally distributed a paired t-test was conducted and a Wilcoxon signed-rank test was conducted for non-normally distributed data. Individuals that failed treatment were excluded from the analysis.

CHAPTER 3. Results

3.1. Study Participants Characteristics and Demographics

3.1.1. TRuTH cohort

The final analysis included 139 participants, of which 37 were cases and 102 were controls. For cases there were 18 males and 19 females and for controls there were 45 males and 57 females. During sample collection the median age was 36 [interquartile range (IQR) 33-41] years for cases and 39 (IQR 33-46) years for controls ($p=0.0179$). The median CD4 count for cases was 479 cell/mm³ (IQR 339-834) and for controls 458.5 cells/mm³ (IQR 358.8 – 635.3) ($p=0.596$). Despite ART some participants had detectable viral loads: the mean VL for cases was 1.61 log copies/ml and 1.52 log copies/ml for controls ($p=0.376$). A detailed list of the cohort characteristics can be seen in Table 1.

Table 1. Cohort characteristics of study participants from TRuTH

Variables	Recurrence of TB (N = 37)	No Recurrence of TB (N = 102)	p-value
SAPiT enrollment			
Randomization arm, n (%)			
Early Arm	12 (32.4)	11 (10.8)	-
Post-Intensive Phase	15 (40.5)	39 (38.2)	
Post-Continuation Phase	10 (27)	52 (51)	
Age (y), [median(IQR)]	32 (27 - 37)	34 (28 - 40)	0.037
Body mass index (kg/m ²)	21.51 (19.57 - 23.20)	21.84 (19.86 - 25.70)	0.055
CD4 count (cells/mm ³), median (IQR)	146 (72.25 - 236)	144.5 (81 - 256)	0.682
Viral load (log copies/mL), mean (SD)	5.05 (0.80)	5.15 (0.76)	0.36
Gender, n (%)			-
Male	18 (48.6)	45 (44.1)	
Female	19 (51.4)	57 (55.9)	
WHO Stage, n (%)			0.763
3	35 (94.6)	95 (93.1)	
4	2 (5.4)	7 (6.9)	
Previous TB, n (%)			0.3545
Yes	13 (35.1)	32 (31.4)	
No	24 (64.9)	70 (68.6)	
Lung cavities, n (%)			0.245
No	19 (51.4)	53 (52.0)	
One	9 (24.3)	33 (32.4)	
Both	5 (13.5)	5 (4.9)	
SAPiT ART Initiation			
CD4 count (cells/mm ³), median (IQR)	144 (90.50 - 266)	155 (93 - 259.30)	0.6793
Viral load (log copies/ml), mean (SD)	4.94 (0.82)	5.05 (0.84)	0.466
End of TB treatment in SAPiT			
CD4 count (cells/mm ³), median (IQR)	223 (144 - 447)	191 (101.8 - 328.3)	0.0008
Viral load (log copies/ml), mean (SD)	2.98 (1.09)	3.96 (1.41)	<0.0001
Variables at sample collection			
Age (y), median (IQR)	36 (33 - 41)	39 (33 - 46)	0.0179
CD4 count (cells/mm ³), median (IQR)	479 (339 - 834)	458.5 (358.8 - 635.3)	0.596
Viral load (log copies/ml), mean (SD)	1.61 (0.70)	1.52 (0.60)	0.376

Abbreviation: ART, antiretroviral therapy; IQR, interquartile range; SD, standard deviation; TB, tuberculosis; WHO, World Health Organization

3.1.2. IMPRESS Cohort

The IMPRESS study was an open labelled randomized control trial which included both HIV infected and HIV uninfected participants who had active TB. The original study design included 41 HIV infected and 37 HIV uninfected participants, however due to sample availability the main analysis included 41 HIV infected and 33 HIV uninfected participants. Of the 78 participants, there were 24 and 31 HIV infected and uninfected male and 17 and 6 HIV infected and uninfected female participants respectively ($p=0.024$). The median age was 35 (IQR 31.75-42.5) years for HIV infected participants and 33 (IQR 24-45) years for HIV uninfected participants ($p=0.490$). As expected, HIV infected participants had a higher number of previous TB episodes compared to HIV uninfected participants and 3 HIV infected participants failed TB treatment (Mahtab and Coetzee, 2017). A detailed list of the cohort characteristics can be seen in Table 2.

Table 2. Cohort characteristics of study participants from IMPRESS

Variables	HIV Infected (N=41)	HIV Uninfected (N=37)	p-value
IMPRESS Enrolment			
Randomization arm, n (%)			1
HRZE – Control	21 (51.2)	19 (51.4)	
HRZM – Active	20 (48.8)	18 (48.6)	
Age (y), [median (IQR)]	35 (31.5-42.5)	33 (24 – 45)	0.490
Gender, n (%)			0.024
Male	24 (58.5)	31 (83.8)	
Female	17 (41.5)	6 (16.2)	
Body mass index (kg/m ²)	19.7 (17.75 – 22.81)	19.6 (18.28 – 21.51)	0.732
CD4 count (cells/mm ³), median (IQR) ^a	243.5 (124.8 – 396)	-	
Viral load (log copies/mL), mean (SD) ^b	3.60 (1.75)	-	
Chest Findings, n (%) ^c			-
Abnormal	40 (100)	30 (100)	
Normal	0	0	
Cavities Right, n (%) ^d			0.237
Yes	18 (45.0)	18 (60.0)	
No	22 (55.0)	12 (40.0)	
Cavities Left, n (%) ^e			0.335
Yes	16 (40.0)	16 (53.3)	
No	24 (60.0)	14 (46.7)	
IMPRESS Follow Up			
Body mass index (kg/m ²)	21.17 (19.45 – 24.33)	21.67 (19.74 – 22.28)	0.515
CD4 Count, n (%)			-
Detectable	4 (9.8)	-	
Undetectable	37 (90.2)	-	
Viral load, n (%)			
Detectable	16 (39)	-	
Undetectable	25 (61)	-	
Previous TB, n (%) ^f			0.703
1	35 (85.4)	32 (86.5)	
2	4 (9.8)	3 (8.1)	
3	-	1 (2.7)	
4	-	1 (2.7)	
WHO staging, n (%)			
3	37 (90.2)	37 (100)	
4	4 (9.8)	-	
Chest Findings, n (%) ^g			0.660
Normal	2 (5.7)	3 (9.7)	
Abnormal	33 (94.3)	28 (90.3)	
Cavities Right, n (%) ^h			0.561
Yes	7 (21.2)	8 (28.6)	
No	26 (78.8)	20 (71.4)	
Cavities Left, n (%) ⁱ			0.375
Yes	6 (18.2)	8 (28.6)	
No	27 (81.8)	20 (71.4)	
Failed TB Treatment, n (%)	2 (4.9)	0	
Recurrent TB, n (%)	1 (2.4)	0	
Missing data: a ² , b ¹ , c ¹⁸ , d ⁸ , e ⁸ , f ² , g ¹² , h ¹⁷ , i ¹⁷			
Abbreviations: IQR, interquartile range; HRZE, Isoniazid, Rifampicin, Pyrazinamide, Ethambutol; HRZM, Isoniazid, Rifampicin, Pyrazinamide, Moxifloxacin; SD, standard deviation; TB, tuberculosis; WHO, World Health Organization.			

3.2. Quality Control of Assays

3.2.1. Detectability and inter- and intra-plate variability of measured analytes for TRuTH and IMPRESS

Detection levels of the measured analytes [sMAdCAM, sICAM, sVCAM, LBP and TGF- β (TGF- β 1, TGF- β 2 and TGF- β 3)] was assessed for study samples. Figures 7A and 7B depicts the percent (%) detectability of the measured analytes in TRuTH and IMPRESS samples respectively. All TRuTH samples measured for sMAdCAM, sICAM, sVCAM and LBP produced a value within the range of the standard curve (100% detectability, Figure 7A). TRuTH samples measured for TGF- β 1, TGF- β 2 and TGF- β 3 had high levels of detectability, where TGF- β 1 was 97.92% and TGF- β 2 and TGF- β 3 were 89.06% respectively (Figure 7A). All IMPRESS samples measured for sICAM, sVCAM, LBP, TGF- β 1, TGF- β 2 and TGF- β 3 had values inside the range of the standard cure, whereas sMAdCAM had % detectability of 99.34% (Figure 7B).

To check for discrepancies and inaccuracies in the way the assays were performed, the intra-plate and inter-plate % coefficient of variation (CV) for each soluble marker was calculated (Table 3). For sMAdCAM, sICAM, sVCAM and LBP, 7 standards were run in duplicate whereas for the TGF- β (TGF- β 1, TGF- β 2 and TGF- β 3) 8 standards were run in duplicates to determine the intra-plate variability of the assays. It should be noted from Table 3 that the intra-plate variability was relatively low for all the soluble markers measured [Median CV = 3.20 for sMAdCAM, 4.40 for sICAM, 3.91 for sVCAM, 3.50 for LBP, and 6.30 for TGF- β].

For the inter-plate variability analysis, 4 control plasma samples were included on all plates. Inter-plate variability of sMAdCAM, sICAM, sVCAM and LBP for both TRuTH and IMPRESS were combined since the controls used for these assays were the same. It can be noted that the inter-plate variability of sICAM, sVCAM, LBP and TGF- β were relatively low [Median Coefficient of variation (CV) 12.27 for sICAM, 11.65 for sVCAM, 11.62 for LBP and 17.43] indicating consistency between the plates (Table 3). sMAdCAM % CV was slightly higher [Median Coefficient of variation (CV) 29.80], potentially due to differences in the color reagent development at the final step of the assay (Table 3). To minimize the effects of inter-plate variability on data analysis, longitudinal samples from the same individuals were run on the same plate.

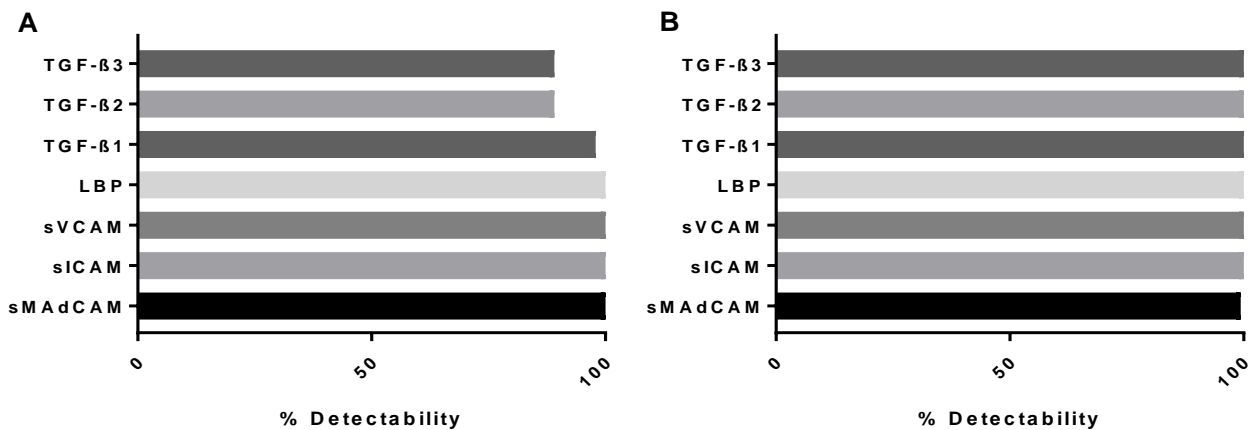


Figure 8. Percent (%) Detectability of measured analytes [sMAdCAM, sICAM, sVCAM, LBP and TGF- β (TGF- β 1, TGF- β 2 and TGF- β 3)] in A) TRuTH and B) IMPRESS plasma samples.

Table 3. Intra- and Inter-plate percent (%) coefficient of variation (CV) for plasma sMAdCAM, sICAM, sVCAM, LBP and TGF- β measured for TRuTH and IMPRESS samples.

Soluble Marker	Intra-plate %CV median (IQR)	Inter-plate %CV median (IQR)
sMAdCAM	3.20 (0.80 - 8.60)	29.80 (28.11 - 33.27)
sICAM	4.40 (1.19 - 9.43)	12.27 (11.18 - 13.35)
sVCAM	3.91 (2.18 - 10.56)	11.65 (9.72 - 13.58)
LBP	3.50 (1.60 - 7.50)	11.62 (10.49 - 15.94)
TGF- β	6.30 (0.90 – 14.63)	17.43 (10.17 – 26.69)

3.3 Measured analytes as predictors of TB recurrence

To determine if measured plasma analytes had an effect on the rate of TB recurrence, we conducted a univariate and multivariable conditional logistic regression analysis on the cases (Recurrence of TB) and controls (No recurrence of TB). In the univariate analysis, two cytokines were associated with increased rates of TB recurrence: sICAM (OR 1.047, 95% CI 1.014 – 1.081, $p = 0.005$) and LBP (OR 3.283, 95% CI 1.018 – 10.588, $p = 0.047$) (Table 4, Figure 8). This stayed true in multivariable analysis after correcting for WHO stage of the disease, BMI, lung cavities, age, CD4 count, VL, gender and previous history of TB: sICAM (OR 1.052, 95% CI 1.012 – 1.095, $p = 0.011$) and LBP (OR 5.133, 95% CI 1.309 – 20.127, p

= 0.019). Additionally, TGF- β 3 (OR 1.445, 95% CI 1.023 – 2.042, $p = 0.037$) was associated with increased risk of TB recurrence in the multivariable model and a statistical trend was observed for TGF- β 1 (OR 1.071, 95% CI 0.988 – 1.161, $p = 0.097$) and TGF- β 2 (OR 1.210, 95% CI 0.980 – 1.494, $p = 0.076$) (Table 4). Lower BMI was a significant predictor of TB recurrence in all the multivariable models ran. When analysed separately lower BMI was associated with increased risk of TB recurrence (OR 0.931, 95% CI 0.868 – 0.998, $p = 0.043$). Increased risk of TB recurrence in individuals with elevated sICAM and LBP plasma levels likely reflect the effects of systemic inflammation and lung-gut inflammatory cross talk respectively.

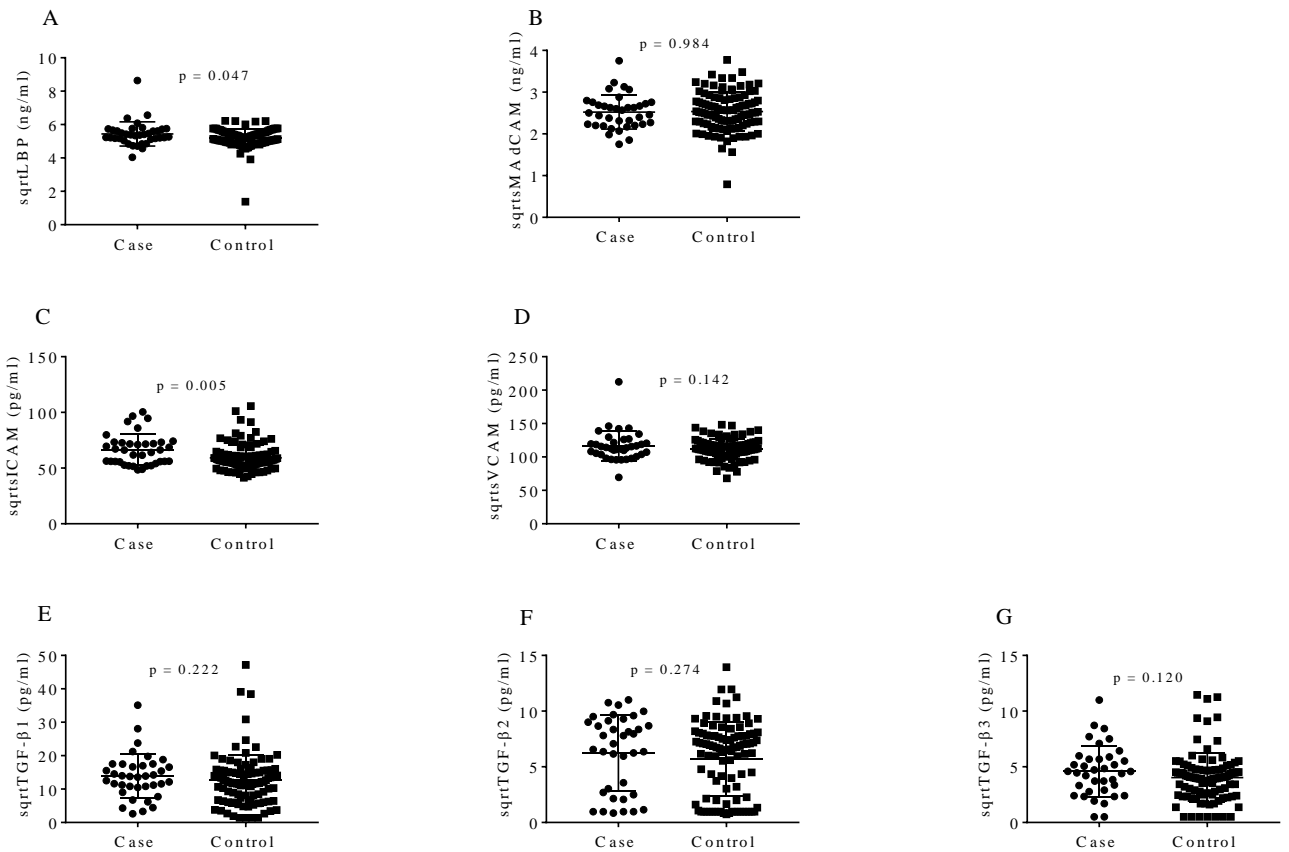


Figure 9. Plasma levels of A) LBP, B) sMAdCAM, C) sICAM, D) sVCAM, E) TGF- β 1, F) TGF- β 2 and G) TGF- β 3 differentially expressed between controls ($n = 103$) and cases ($n = 37$) in TRuTH. P-values indicated in the figures are the result of univariate conditional logistic regression.

Table 4. Univariate and multivariable analysis of TRuTH plasma analytes (sMAdCAM, sICAM, sVCAM, LBP, TGF- β 1, TGF- β 2 and TGF- β 3) as predictors of TB recurrence.

Cytokine	Univariate		Multivariable ¹	
	OR (95% CI)	p-value	OR (95% CI)	p-value
sMAdCAM	0.991 (0.390 – 2.520)	0.984	0.671 (0.196 – 2.305)	0.527
sICAM	1.047 (1.014 – 1.081)	0.005	1.052 (1.012 – 1.095)	0.011
sVCAM	1.017 (0.994 – 1.041)	0.142	1.014 (0.985 – 1.045)	0.351
LBP	3.283 (1.018 – 10.588)	0.047	5.133 (1.309 – 20.127)	0.019
TGF- β 1	1.046 (0.973 – 1.123)	0.222	1.071 (0.988 – 1.161)	0.097
TGF- β 2	1.095 (0.930 – 1.289)	0.274	1.210 (0.980 – 1.494)	0.076
TGF- β 3	1.214 (0.951 – 1.549)	0.120	1.445 (1.023 – 2.042)	0.037

¹Multivariable analyses adjusted for WHO stage of the disease, BMI, lung cavities, age, CD4 count, VL, gender and previous history of TB.

3.4 Longitudinal changes in plasma expression of measured analytes

A small subset of cases was followed longitudinally to observe the changes in plasma expression of measured analytes (sMAdCAM, sICAM, sVCAM, LBP, TGF- β 1, TGF- β 2 and TGF- β 3) during active TB disease and in response to TB treatment completion. We compared changes in the plasma expression of measured analytes between the following time-points: pre-TB and TB recurrence (n = 30), TB recurrence to post TB treatment/Cure (n = 14) and pre-TB to post TB treatment/Cure (n = 13). Expression levels of 2 cytokines decreased from pre-TB time-point to active TB disease: LBP (mean difference [MD] 4.831, 95% CI 2.693 – 6.969, p<0.0001) and sMAdCAM (MD 1.535, 95% CI 0.8862 – 2.183,

p<0.0001) (Figure 9A, Figure 9B respectively, Table 5), whereas TGF- β 3 (MD -75.69, 95% CI -100.4 – 50.96, p<0.0001) levels increased (Figure 10C, Table 5). No significant differences were observed in measured analyte expression between active TB and post TB treatment/Cure time-point. From pre-TB time-point to post TB treatment (or during the entire follow-up), expression of 3 analytes decreased: LBP (MD 3.855, 95% CI 0.5703 – 7.139, p = 0.0251), sMAdCAM (MD 1.153, 95% CI 0.2542 – 2.052, p = 0.0162) and sVCAM (MD 2373, 95% CI 945.8 – 3800, p = 0.0162) (Figure 9A, Figure 9B, Figure 9D respectively, Table 5), whereas TGF- β 3 (MD -100.4, 95% CI -128 – 72.88, p<0.0001) increased (Figure 10C, Table 5). Decrease in LBP, sMAdCAM and sVCAM and an increase in TGF- β 3 during the entire longitudinal follow-up likely reflects the increased duration of ART use and the associated immune reconstitution.

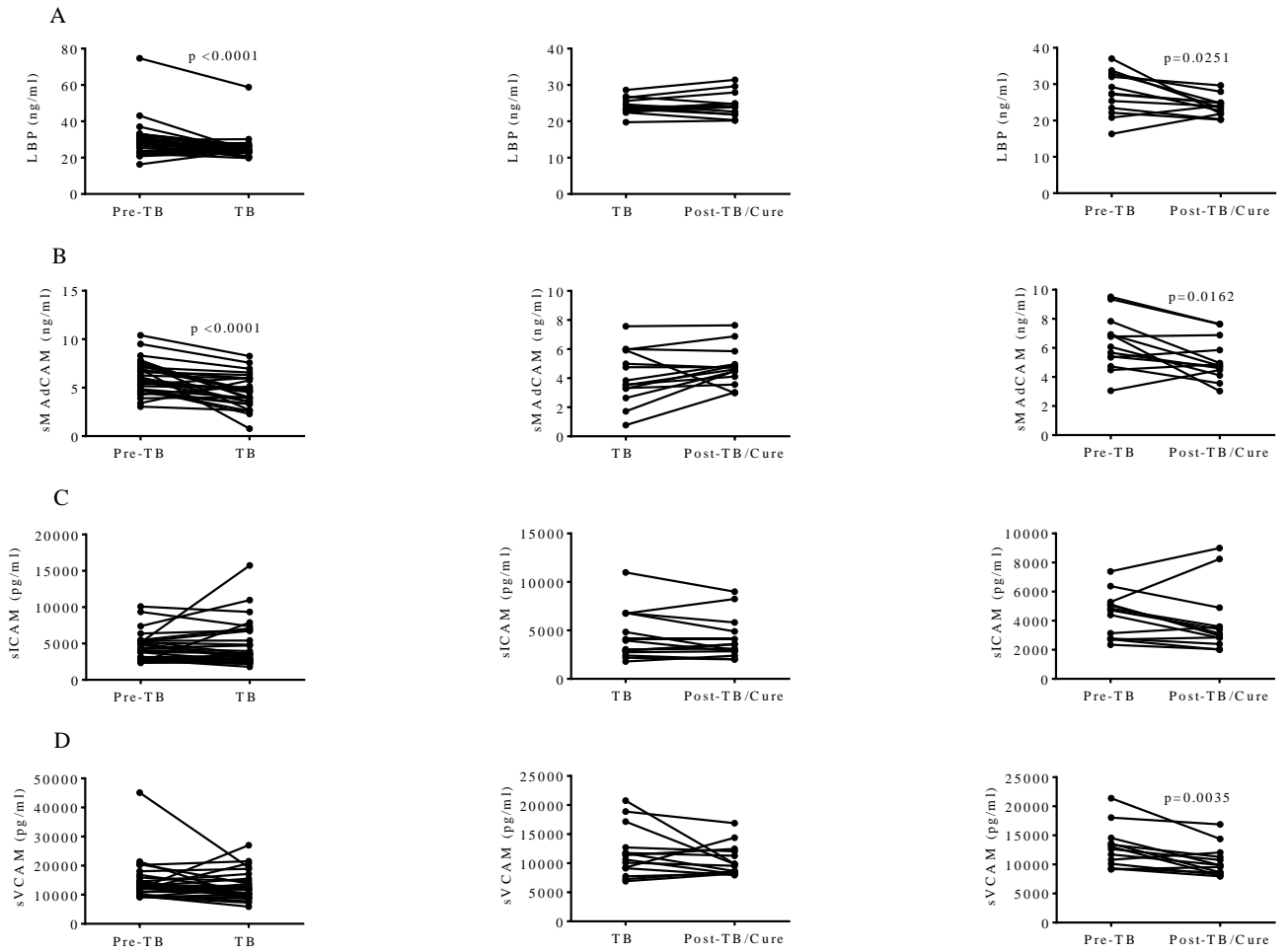


Figure 10. Longitudinal paired analysis of plasma A) LBP, B) sMAdCAM, C) sICAM and D) sVCAM expression at following time points: PreTB to TB (n = 30), TB to PostTB/Cure (n = 14) and PreTB to PostTB/Cure (n = 13).

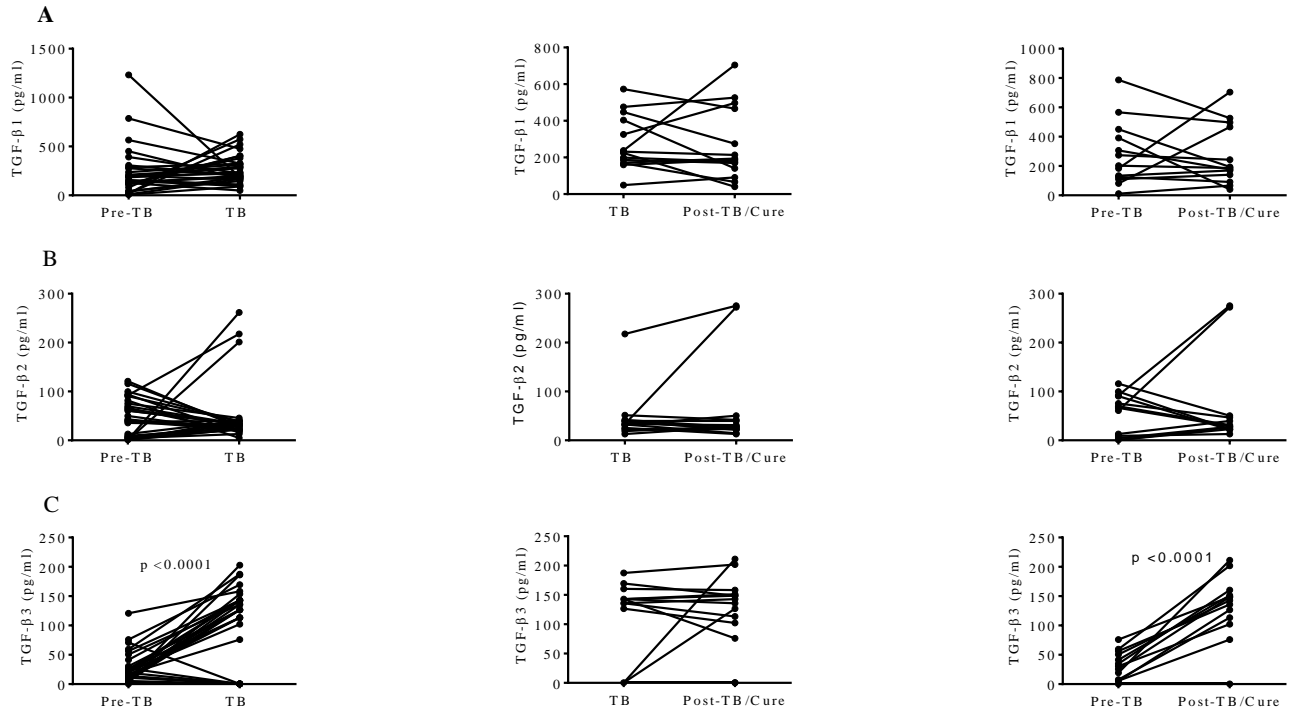


Figure 11. Longitudinal paired analysis of plasma A) TGF- β 1, B) TGF- β 2 and C) TGF- β 3 expression at following time points: PreTB to TB (n = 30), TB to PostTB/Cure (n = 14) and PreTB to PostTB/Cure (n = 13).

Table 5. Longitudinal paired analysis of TRuTH plasma cytokine (sMAdCAM, sICAM, sVCAM, LBP, TGF- β 1, TGF- β 2 and TGF- β 3) expression at following time-points: PreTB to TB (n = 30), TB to PostTB (n = 14) and PreTB to PostTB (n = 13). (Mean difference refers to difference in means between two timepoints)

Variable	PreTB - TB			TB – PostTB/Cure			PreTB – PostTB/Cure		
	N	Mean difference (95% CI)	p-value	N	Mean difference (95% CI)	p-value	N	Mean difference (95% CI)	p-value
sMAdCAM	30	1.535 (0.8862 to 2.183)	<0.0001	14	-0.6479 (-1.452 to 0.156)	0.1052	13	1.153 (0.2542 to 2.052)	0.0162
sICAM	30	-296.2 (-1225 to 632.5)	0.5193	14	342.5 (-284.4 to 969.4)	0.2590	13	459.2 (-428.6 to 1347)	0.2818
sVCAM	30	1440 (-1041 to 3920)	0.2448	14	1375 (-881.3 to 3632)	0.2107	13	2373 (945.8 to 3800)	0.0035
LBP	30	4.831 (2.693 to 6.969)	<0.0001	14	-0.2771 (-1.416 to 0.8621)	0.6081	13	3.855 (0.5703 to 7.139)	0.0251
TGF-β1	30	-35.94 (-146.8 to 74.87)	0.5123	14	7.641 (-94.58 to 109.9)	0.8742	13	9.262 (-137.1 to 155.7)	0.8927
TGF-β2	30	-2.673 (-31.5 to 26.15)	0.8509	14	-17.88 (-56.44 to 20.68)	0.3348	13	-13.89 (-68.17 to 40.38)	0.5872
TGF-β3	30	-75.69 (-100.4 to -50.96)	<0.0001	14	-16.4 (-56.62 to 23.82)	0.3943	13	-100.4 (-128 to -72.88)	<0.0001

3.5 Correlation with previously measured plasma cytokines/chemokines

Previously, Sivro *et al* measured the plasma expression of 23 different cytokines/chemokines to look at the influence they have on the risk of TB recurrence (Sivro *et al.*, 2017). Here we examined if there was a correlation between the cytokines measured by Sivro *et al* and the plasma expression of sMAdCAM, sICAM, sVCAM, LBP, TGF- β 1, TGF- β 2 and TGF- β 3 (Sivro *et al.*, 2017). Expressions of 23 cytokines/chemokines were correlated with sMAdCAM, sICAM, sVCAM, LBP, TGF- β 1, TGF- β 2 and TGF- β 3 in 21 overlapping plasma samples. We observed a statistically significant correlation between IL-27 and sVCAM ($r=0.438$, $p=0.047$), IL-7 and TGF- β 2 ($r=0.481$, $p=0.027$), IL-6 and LBP ($r=0.623$, $p=0.003$), IL-6 and TGF- β 2 ($r=0.438$, $p=0.047$), sCD14 and TGF- β 1 ($r=0.456$ $p=0.038$) and sCD14 and TGF- β 1 ($r=0.456$, $p=0.038$) (Table 6).

Table 6. Correlation of plasma cytokines/chemokines by Sivro *et al.* with the measured analytes (sMAdCAM, sICAM, sVCAM, LBP, TGF- β 1, TGF- β 2 and TGF- β 3).

Cytokines/chemokines (N=23)	sMAdCAM	sICAM	sVCAM	LBP	TGF- β 1	TGF- β 2	TGF- β 3
IL1 β	r=-0.316 p=0.162	r= -0.299 p= 0.188	r=-0.057 p=0.806	r=0.337 p=0.135	r=0.123 p=0.594	r=0.194 p=0.400	r=0.305 p=0.179
IL1Ra	r=-0.250 p=0.274	r= -0.346 p= 0.124	r=-0.049 p=0.832	r=0.335 p=0.137	r=0.068 p=0.769	r=0.108 p=0.641	r=0.220 p=0.338
IL2	r=0.234 p=0.308	r= -0.348 p= 0.122	r=0.216 p=0.346	r=0.102 p=0.661	r=-0.098 p=0.672	r=-0.151 p=0.514	r=-0.077 p=0.739
IL-6	r=-0.031 p=0.894	r= 0.114 p=0.624	r=0.264 p=0.248	r=0.623 p= 0.003*	r=0.337 p=0.136	r=0.438 p= 0.047	r=0.321 p=0.156
IL-7	r=-0.134 p=0.563	r=-0.342 p=0.130	r=0.242 p=0.291	r=0.273 p=0.232	r=0.345 p=0.125	r=0.481 p= 0.027	r=0.231 p=0.314
IL-10	r=-0.227 p=0.322	r=-0.252 p=0.271	r=0.161 p=0.486	r=0.387 p=0.083	r=0.194 p=0.401	r=0.286 p=0.209	r=0.174 p=0.452
IL-15	r=0.369 p=0.099	r=0.369 p=0.099	r=0.258 p=0.258	r=-0.148 p=0.523	r=0.074 p=0.750	r=0.000 p=1.000	r=0.074 p=0.750
IP10	r=0.051 p=0.827	r= -0.273 p= 0.232	r=0.104 p=0.654	r=0.264 p=0.248	r=0.303 p=0.182	r=0.353 p=0.117	r=0.296 p=0.192
TNF- α	r=-0.020 p=0.931	r= 0.066 p= 0.775	r=0.360 p=0.109	r=0.338 p=0.134	r=0.251 p=0.272	r=0.254 p=0.266	r=0.417 p=0.060
IL1 α	r=-0.057 p=0.807	r= 0.049 p= 0.831	r=0.105 p=0.651	r=0.105 p=0.651	r=-0.149 p=0.519	r=-0.128 p=0.579	r=-0.096 p=0.680
IL-18	r=-0.334 p=0.139	r=-0.255 p=0.265	r=-0.219 p=0.339	r=0.117 p=0.614	r=-0.010 p=0.964	r=0.067 p=0.773	r=0.141 p=0.542
MIG	r=0.019 p=0.933	r= 0.062 p=0.788	r=-0.019 p=0.933	r=0.131 p=0.571	r=-0.145 p=0.529	r=-0.012 p=0.960	r=-0.180 p=0.435

IFN- β	r=0.035 p=0.880	r= -0.366 p=0.103	r=-0.319 p=0.158	r=-0.313 p=0.168	r=-0.149 p=0.520	r=-0.120 p=0.605	r=-0.139 p=0.548
IFN- λ 2	r=-0.316 p=0.162	r=-0.035 p=0.881	r=-0.280 p=0.219	r=0.106 p=0.647	r=-0.063 p=0.788	r=0.052 p=0.822	r=-0.109 p=0.638
IFN- λ 1	r=0.148 p=0.523	r=-0.074 p=0.750	r=0.222 p=0.334	r=-0.222 p=0.334	r=0.111 p=0.633	r=0.037 p=0.874	r=0.037 p=0.874
IFN- α 2	r=0.064 p=0.782	r=0.118 p=0.611	r=0.301 p=0.184	r=0.023 p=0.921	r=0.161 p=0.487	r=0.231 p=0.313	r=0.182 p=0.429
IFN- γ	r=0.054 p=0.816	r=0.412 p=0.064	r=0.338 p=0.133	r=-0.184 p=0.426	r=-0.049 p=0.833	r=-0.068 p=0.768	r=0.047 p=0.840
IL-8	r=0.045 p=0.847	r=-0.002 p=0.995	r=0.007 p=0.976	r=0.052 p=0.823	r=-0.152 p=0.509	r=-0.170 p=0.461	r=-0.148 p=0.521
IL12p40	r=-0.265 p=0.245	r=-0.356 p=0.113	r=-0.248 p=0.278	r=-0.092 p=0.691	r=0.141 p=0.542	r=0.162 p=0.483	r=0.063 p=0.785
IL12p70	r=-0.243 p=0.289	r=-0.071 p=0.760	r=-0.156 p=0.500	r=0.000 p=1.000	r=-0.308 p=0.174	r=-0.210 p=0.360	r=-0.410 p=0.065
IL-27	r=0.258 p=0.258	r=0.167 p=0.470	r=0.438 p=0.047	r=0.141 p=0.542	r=0.088 p=0.706	r=-0.035 p=0.881	r=0.284 p=0.213
I-FAB	r=-0.013 p=0.956	r=-0.065 p=0.778	r=0.089 p=0.703	r=-0.197 p=0.392	r=0.275 p=0.228	r=0.191 p=0.406	r=0.200 p=0.385
sCD14	r=-0.212 p=0.357	r=0.121 p=0.602	r=0.179 p=0.437	r=-0.25 p=0.915	r=0.513 p=0.017	r=0.456 p=0.038	r=0.418 p=0.059

* P values that pass multiple comparisons are indicated with a star (*)

3.6 Changes in the expression of measured analytes from active TB disease to TB treatment completion in IMPRESS

Next we followed the HIV infected (n= 38) and HIV uninfected (n = 33) participants longitudinally to observe the changes in plasma expression of measured analytes (LBP, sMAdCAM, sICAM, sVCAM, TGF- β 1, TGF- β 2 and TGF- β 3) from active TB to post TB/cure. We observed no significant differences in the expression of sMAdCAM, sICAM, sVCAM, TGF- β 1, TGF- β 2 and TGF- β 3 between active TB and post TB cure in HIV infected and HIV uninfected participants (Figure 11 B-D, Figure 12 E-G, and Table 7). A trend was observed towards decreased LBP in HIV uninfected participants following TB treatment (MD 2.026, 95% CI -0.122 to 4.174, $p = 0.0638$). However in HIV infected participants there was a trend towards higher LBP post TB cure (MD -2.081, 95% CI -4.364 to 0.2021, $p = 0.0726$) (Figure 11A Table 7). The observed increase in LBP levels in HIV infected participants during follow-up is likely a result of HIV disease progression, whereas a decrease in LBP levels post TB cure in HIV uninfected participants could suggest that active TB is associated with gut inflammation and dysbiosis.

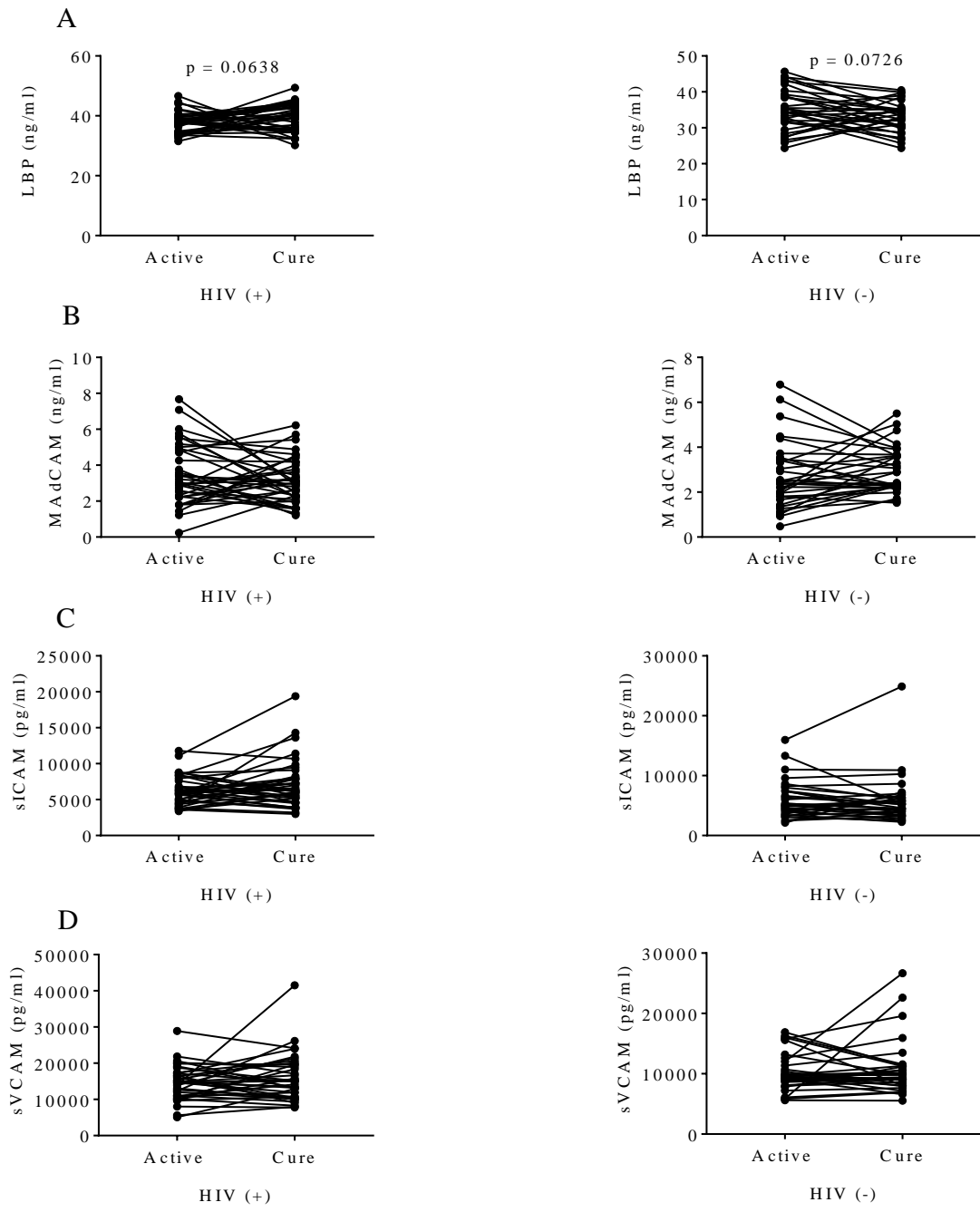


Figure 12. Longitudinal paired analysis of plasma A) LBP, B) sMAdCAM, C) sICAM and D) sVCAM expression at active TB to Post TB/Cure in HIV infected (n = 38) and HIV uninfected (n = 33) participants.

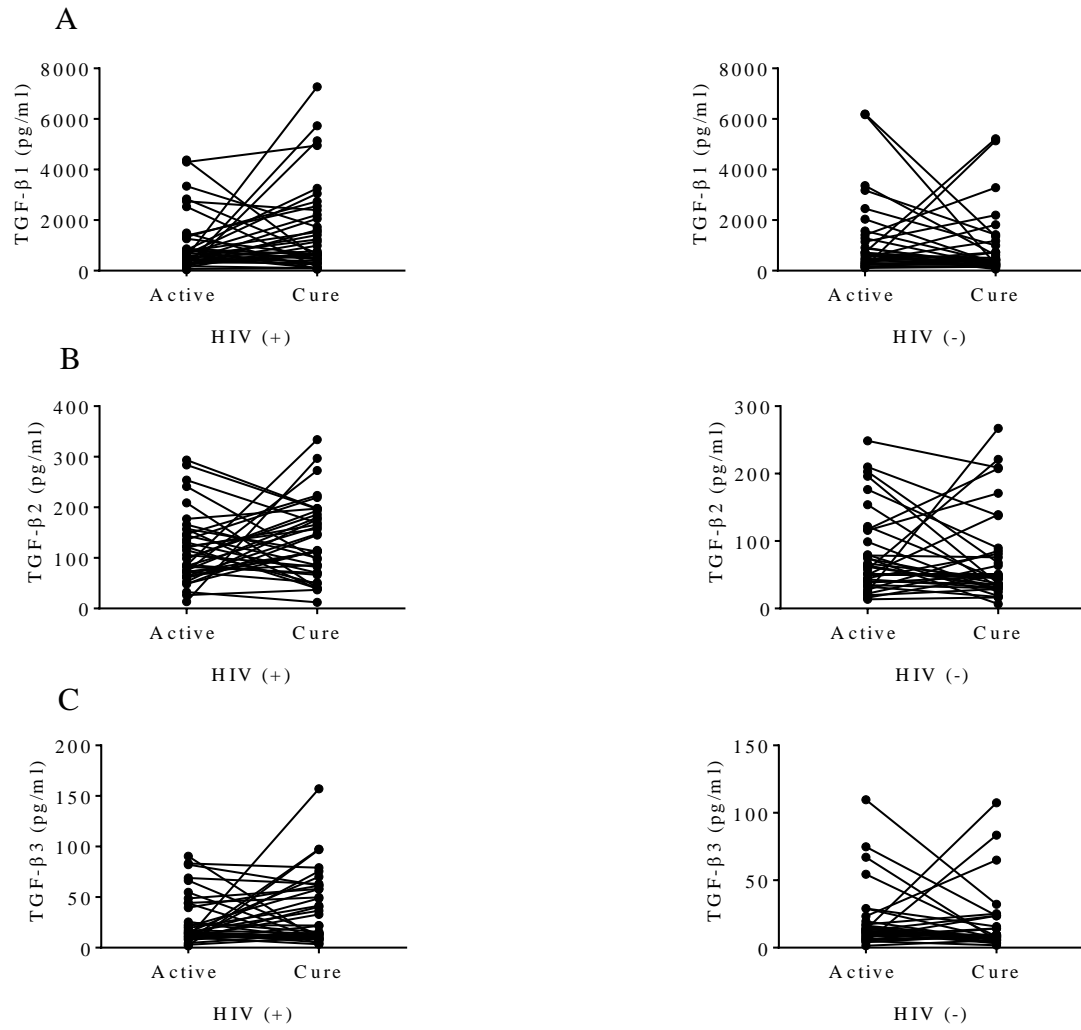


Figure 13. Longitudinal paired analysis of plasma A) TGF-β1, B) TGF-β2 and C) TGF-β3 expression at active TB to Post TB/Cure in HIV infected (n = 38) and HIV uninfected (n = 33) participants.

Table 7. Longitudinal paired analysis of measured analytes from active TB to post TB/cure in HIV infected (n = 38) and HIV uninfected (n = 33) participants.

Variable	HIV Infected Active TB – Post TB/Cure			HIV Uninfected Active TB – Post TB/Cure		
	N	Mean Difference (95% CI)	p-value	N	Mean Difference (95% CI)	p-value
sMAdCAM	38	-0.3563 (-0.9752 to 0.2626)	0.2509 ^a	33	0.2658 (-0.22 to 0.7515)	0.2980 ^b
sICAM	38	905.4 (-115.6 to 1926)	0.1941 ^b	33	-206.6 (-1201 to 788.2)	0.2798 ^b
sVCAM	38	1200 (-991.7 to 3392)	0.6360 ^b	33	485.9 (-1187 to 2158)	0.7914 ^b
LBP	38	2.026 (-0.122 to 4.174)	0.0638 ^a	33	-2.081 (-4.364 to 0.2021)	0.0726 ^a
TGF-β1	38	529.7 (-131.5 to 1191)	0.1365 ^b	33	-246.5 (-959.9 to 467)	0.3483 ^b
TGF-β2	38	18.83 (-14.25 to 51.92)	0.3965 ^b	33	-7.006 (-35.56 to 21.55)	0.3403 ^b
TGF-β3	38	10.19 (-3.399 to 23.78)	0.1612 ^b	33	-2.42 (-13.86 to 9.019)	0.2509 ^b

^a p-value is represented from the paired t-test

^b p-value is represented from the Wilcoxon matched-pairs signed rank test

3.7 Changes in the expression of measured analytes between HIV infected and HIV uninfected individuals at active TB and following completion of TB treatment

Finally, we wanted to characterize the effect of HIV infection on the measured analytes in active TB and post TB treatment subgroups. The univariate analysis revealed that the plasma expression of most analytes was significantly higher in HIV infected participants compared to HIV uninfected participants. This is expected and is likely a result of HIV induced inflammatory changes. At active TB time-point, it was observed that four analytes had significantly higher levels in HIV infected participants compared to HIV uninfected participants: LBP (Median 37.73, IQR 34.29 – 39.32, $p = 0.04$), sMAdCAM (Median 3.01, IQR 2.243 – 4.87, $p = 0.03$), sVCAM (Median 14635, IQR 10893 - 17121, $p < 0.0001$) and TGF- β 2 (Median 98.96, IQR 70 – 149.5, $p = 0.01$) (Figure 13 A, B, D, F). At cure time-point, it was observed that six analytes had significantly higher levels in participants who were HIV infected compared to HIV uninfected participants: LBP (Median 40.28, IQR 35.38 – 43.66, $p < 0.0001$), sICAM (Median 6245, IQR 5029 – 8046, $p = 0.0084$), sVCAM (Median 14530, IQR 10543 – 19596, $p < 0.0001$), TGF- β 1 (Median 736.9, IQR 371.7 - 2257, $p = 0.05$), TGF- β 2 (Median 114.3, IQR 70.31 – 187.8, $p = 0.0001$) and TGF- β 3 (Median 18.56, IQR 9.763 – 58.93, $p = 0.0017$) (Figure 14 A, C, D, E, F, G).

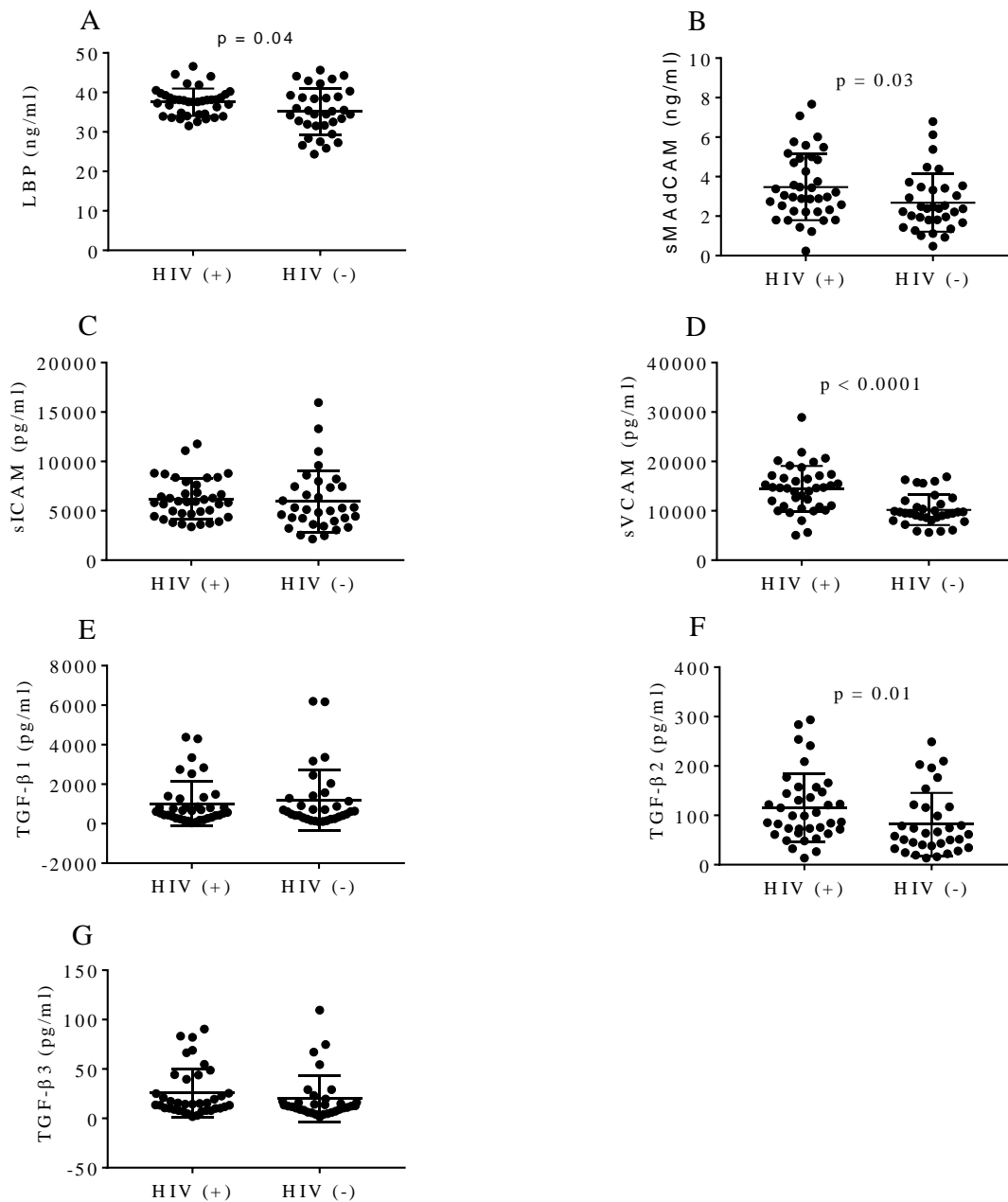


Figure 14. Changes in plasma expression of A) LBP, B) sMAdCAM, C) sICAM, D) sVCAM, E) TGF-β1, F) TGF-β2 and G) TGF-β3 between HIV infected (n = 38) and HIV uninfected (n = 33) individuals at active TB.

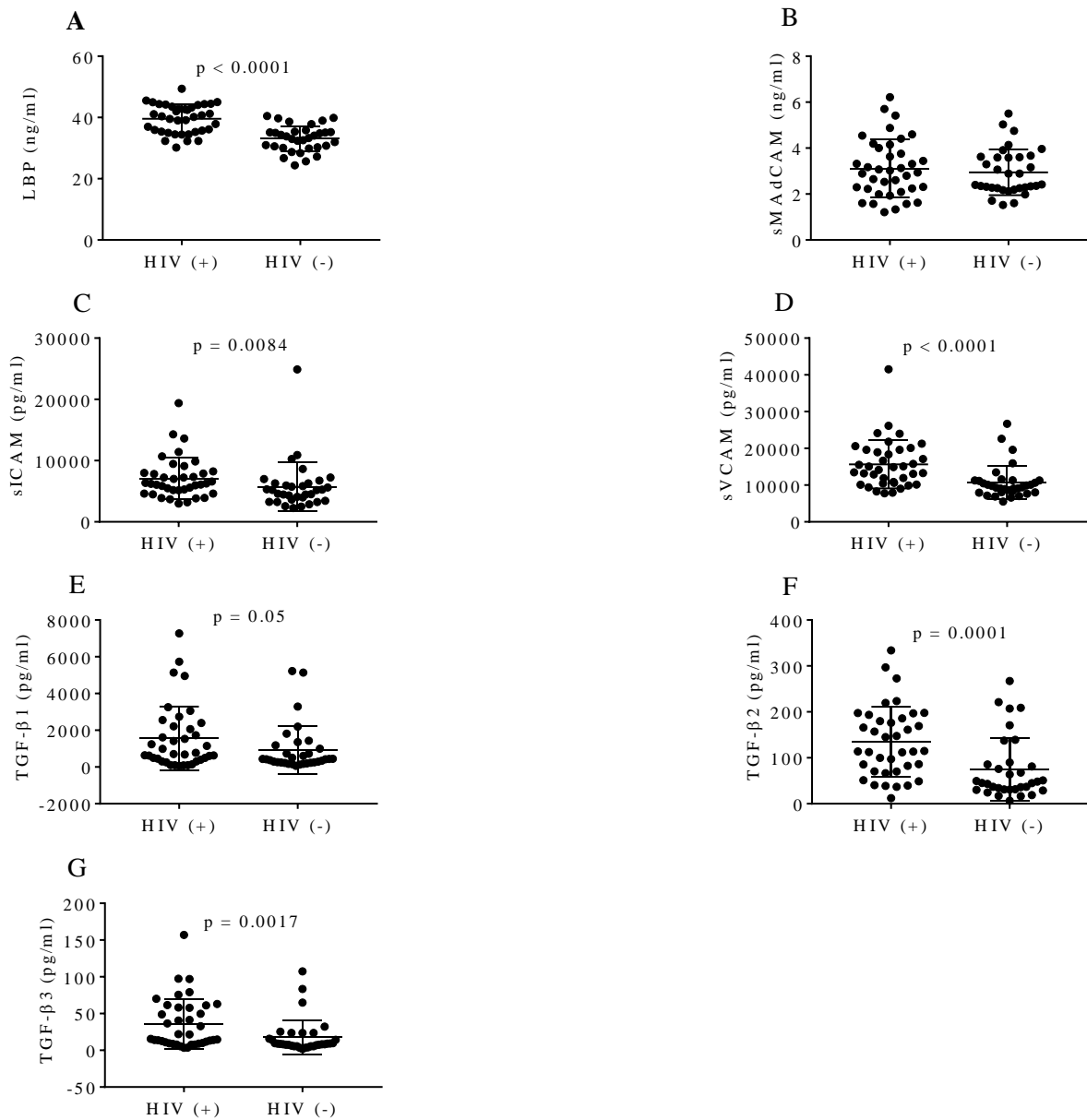


Figure 15. Changes in plasma expression of A) LBP, B) sMAdCAM, C) sICAM, D) sVCAM, E) TGF- β 1, F) TGF- β 2 and G) TGF- β 3 between HIV infected (n = 38) and HIV uninfected (n = 33) individuals following the completion of TB treatment.

CHAPTER 4: Discussion

TB/HIV co-infection is one of the major health burdens affecting SA (Jacobson *et al.*, 2015, Churchyard *et al.*, 2014). The host inflammatory response to TB and HIV infections acts similarly; the initial response is needed to prevent and contain the infection, however if left untreated the inflammation can lead to immune mediated pathology. Previous studies have looked at the role of lymphocyte trafficking markers in TB and HIV respectively (Byrareddy *et al.*, 2014, Byrareddy *et al.*, 2016, Bai *et al.*, 2018), however there continues to be a lack of data regarding lymphocyte trafficking markers in TB and TB/HIV co-infection.

4.1 Measured analytes as predictors of TB recurrence risk in TRuTH cohort

Here we examined if the expression of sMAdCAM, sICAM, sVCAM, LBP, TGF- β 1, TGF- β 2 and TGF- β 3 had an effect on the rate of TB recurrence. In the univariate and multivariable logistic model, the risk of TB recurrence was significantly associated with increases in the plasma expression of sICAM and LBP, known to play a role in inflammation and microbial translocation respectively. ICAM is present on endothelial cells and its expression is increased by pro-inflammatory cytokines (Wolf, 2009). ICAM is involved in the firm arrest and transmigration of leukocytes from blood vessels to tissues (Lawson and Wolf, 2009). Previous studies have shown that the concentration of sICAM is elevated in patients with active TB disease (Lai *et al.*, 1993). A decrease in ICAM levels was observed in response to anti-TB treatment suggesting that the serum concentration of sICAM is linked to bacterial activity (Demir *et al.*, 2002, Ramirez *et al.*, 1994, Lai *et al.*, 1993, Mukae *et al.*, 2003). Our data supports these findings. Increase in sICAM prior to TB disease observed in our study

likely reflects increased systemic inflammation as a result of increased bacterial replication and activation of latent disease.

In serum, LBP is present as a soluble acute-phase protein which binds to LPS and stimulates an immune response by presenting the LPS to important cell surface PRRs such as CD14 and TLR4. Increased concentrations of LBP have been observed in patients with sepsis and in healthy individuals injected with LPS (Froon *et al.*, 1995, van der Poll *et al.*, 1997). A study conducted by Juffermans *et al.* showed that levels of LBP was elevated in individuals with active TB and declined during treatment, suggesting that LBP may have importance in the host reaction to TB (Juffermans *et al.*, 1998). Increased levels of plasma LBP were associated with an increased risk of TB recurrence in this study. Since the TRuTH cohort only includes HIV co-infected individuals, increased LBP levels likely reflect the HIV induced microbial translocation as well as TB bacterial activity. There was a strong positive correlation between LBP and IL-6 measured by Sivro *et al.* IL-6 was one of the main pro-inflammatory cytokines associated with TB recurrence in the previous study (Sivro *et al.*, 2017). Interestingly, LPS was shown to be a strong inducer of IL-6 and ICAM-1 suggesting that HIV associated microbial translocation could be the main driver of inflammation, driving the increased risk of TB recurrence (Sawa *et al.*, 2008). Dysbiosis of the gut microbiota due to HIV is known to cause systemic inflammation and as a result can lead to opportunistic infections including TB. Furthermore gut inflammation can be linked to lung inflammation, since 50% of adults who suffer with IBD and 33% of individuals who suffer with IBS have pulmonary involvement (Yazar *et al.*, 2001, Keely *et al.*, 2012). This likely indicates that there is an inflammatory cross talk between different mucosal sites and that inflammation and dysbiosis in the gut can lead to inflammatory changes in the lungs (Budden *et al.*, 2017, Brenchley *et al.*, 2006b).

After adjusting for covariates, the risk of TB recurrence was associated with increased concentrations of TGF- β 3 and although not statistically significant, there was a statistical trend toward increased concentration of TGF- β 1. TGF- β belongs to a superfamily of cytokines which include three isoforms; TGF- β 1, TGF- β 2, and TGF- β 3 (Morikawa *et al.*, 2016). TGF- β is involved in enhancing regulatory T cell responses and having stimulatory and inhibitory effects on various cell types (Nathan and Sporn, 1991, Yano *et al.*, 2012). Previous studies have reported that the production of TGF- β by monocytes and DCs is induced by mycobacterial products and as a result TGF- β is produced in excess during active TB and at the site of infection (Toossi *et al.*, 1995, Condos *et al.*, 1998). Excess TGF- β was shown to suppress T cell responses to *M.tb* antigens (Hirsch *et al.*, 1997). These studies corroborate our findings, in that expression of TGF- β was elevated prior to active TB disease and likely reflects increased bacterial activity. Additionally, TGF- β 1 and TGF- β 2 expression was shown to positively correlate with sCD14 expression. It has previously been shown that active TB is associated with plasma sCD14, a known marker of monocyte activation (Sandler *et al.*, 2011).

Additionally, the risk of TB recurrence was significantly associated with a low BMI in our study. The effect of BMI on the risk of TB has been examined previously (Tverdal, 1986, Leung *et al.*, 2007, Lönnroth *et al.*, 2009b). There has been a consistent and strong log-linear relationship between the incidence of TB and BMI in different settings (Lönnroth *et al.*, 2009b). In a South African HIV infected cohort, HIV infected individuals with high BMI had a significantly reduced risk of mortality and TB after adjusting for HAART and CD4 count (Hanrahan *et al.*, 2010). Our data agrees with these findings showing that low BMI is associated with increased risk of TB likely due to impairment of the immune system, as a result of malnutrition.

4.2 Effect of active TB and treatment completion on analyte expression in TRuTH

We looked at the longitudinal changes in plasma expression of the measured analytes from prior to TB reactivation (pre-TB), during active TB disease and post TB treatment/cure. It was found that three analytes (LBP, sMAdCAM and TGF- β 3) had significant differences between pre-TB and active TB disease time-points. Between pre-TB and active TB disease there was a decreased concentration of LBP and sMAdCAM and an increased concentration of TGF- β 3. No differences were observed in the measured analytes between active TB disease and post TB treatment/cure time-points. Moreover, there was a significant decrease in the expression levels of LBP, sMAdCAM and sVCAM and a significant increase in the expression level of TGF- β 3 between pre-TB and post TB/cure time-points (or during the whole follow-up). The lack of differences between active TB disease and post treatment completion time-points suggest that the observed changes in the analyte expression during follow-up are likely a result of increased ART duration and HIV disease progression rather than a direct effect of TB disease activity (Papasavvas *et al.*, 2008).

A study by Feng *et al.* showed that *M.tb* infection induces the upregulation of VCAM-1 on pulmonary endothelium in mice with no changes in sMAdCAM (Feng *et al.*, 2000). sVCAM is a marker of vascular inflammation and plays a major role in leukocyte recruitment (Carlos and Harlan, 1994). A study by Lai *et al.* found increased expression of circulating VCAM-1 during active TB disease and this could be a mechanism in which the inflammatory leukocytes are attracted to the site of the lesion leading to the development of the disease (Lai *et al.*, 1993). A study conducted by Sharma *et al.* found increased concentrations of VCAM-1 at active TB disease, as well as after completing TB treatment, proposing that VCAM-1 is important in maintaining the lymphocytic inflammation in the lungs (Sharma *et al.*, 1992, Lai *et al.*, 1993). We did not observe any changes in sVCAM expression with active TB disease

with an overall decrease in concentrations of sVCAM and sMAdCAM during the entire study follow-up likely reflecting the effect of ART duration and decreased inflammation (Papasavvas *et al.*, 2008).

As with sVCAM and sMAdCAM we observed an overall decrease in plasma LBP expression during the study follow-up. ART was previously shown to lead to decreased plasma LBP levels (Nyström *et al.*, 2015) and this is likely reflected by our data.

Previous studies have reported increased concentrations of TGF- β in individuals with pulmonary (Dlugovitzky *et al.*, 2000) and severe TB (Fiorenza *et al.*, 2005). Here we observed an increase in TGF- β 3 between pre-TB and active TB time-point as well as an overall increase during the entire follow-up. While the initial increase might, to some degree, reflect the increased bacterial activity and active TB disease, TGF- β levels are known to increase steadily during HIV infection irrespective of ART (Wiercińska-Drapalo *et al.*, 2004). This sustained elevation in circulating TGF- β despite ART treatment is thought to contribute to immunosuppression and progression to AIDS, even in virally suppressed individuals (Theron *et al.*, 2017).

4.3 Effect of active TB and treatment completion on analyte expression in IMPRESS

Next, we examined the changes in the expression of LBP, sMAdCAM, sICAM, sVCAM, TGF- β 1, TGF- β 2 and TGF- β 3 from active TB disease to TB treatment completion in HIV infected and HIV uninfected individuals from IMPRESS. No significant differences were observed in the expression of sMAdCAM, sICAM, sVCAM, TGF- β 1, TGF- β 2 and TGF- β 3 between active TB disease and post TB treatment/cure in HIV infected and HIV uninfected individuals. Although not statistically significant, there was a trend towards decreased

concentrations of plasma LBP in HIV uninfected individuals following TB treatment. This potentially suggests that active TB is associated with inflammation of the gut that is resolved by successful treatment completion. A trend towards increased concentrations of plasma LBP was observed in HIV infected individuals following TB treatment, likely reflecting a decrease in the gastrointestinal integrity caused by progressing HIV infection (Funderburg *et al.*, 2013, Brenchley *et al.*, 2006b).

As expected plasma expression of majority of the measured analytes was increased in HIV infected individuals at active TB disease and post TB treatment time-points. HIV infection is known to increase the plasma expression of LBP (Ancuta *et al.*, 2008), sMAdCAM (Miao *et al.*, 2002), sICAM (Mastroianni *et al.*, 2000), sVCAM (Graham *et al.*, 2013) and TGF- β (Tudela *et al.*, 2014) contributing the overall chronic immune activation and disease progression.

Interestingly, we did not observe any differences in the measured analyte expression between males and females. Previous research has shown that males are more affected by TB compared to females, with a case notification of 2:1 (Neyrolles and Quintana-Murci, 2009). These gender differences can be due to both biological (such as hormone and immune differences) (Borgdorff *et al.*, 2000, Blaak, 2001, Salim *et al.*, 2004, Kolappan *et al.*, 2007, Boelaert *et al.*, 2007) as well as social (such as access to care and work environment)(Lönnroth *et al.*, 2009a, Lönnroth *et al.*, 2010, Hargreaves *et al.*, 2011) aspects. The process of case-notification is complicated and ultimately combines a number of factors such as: (i) difference in susceptibility and exposure, (ii) access to healthcare services and (iii) help seeking behaviour (Neyrolles and Quintana-Murci, 2009). Although some reviews

have suggested that under-notification of women in developing countries could be due to access to clinics, timely diagnosis or treatment and sex bias could be major influences on the rate of TB (Gordon and Rylance, 2009, Lönnroth *et al.*, 2008, Lienhardt *et al.*, 2005, Khan *et al.*, 2007). Therefore, it is likely that case notifications may not reveal the number of aspects between sexes and TB susceptibility (Neyrolles and Quintana-Murci, 2009). A case-control study conducted by Lienhardt *et al* found that male sex was a TB risk factor and was independent of other behavioural factors studied (Lienhardt *et al.*, 2005). Even with income, awareness and stigma accounted for, a survey conducted revealed that confirmed TB was seen 3 times more in males as compared to females (Borgdorff *et al.*, 2000, Salim *et al.*, 2004). Men are more susceptible to pulmonary TB based on specific biological sex factors such as: sex hormones, sex chromosomes and sex specific metabolic features (Neyrolles and Quintana-Murci, 2009, Klein and Flanagan, 2016). It can be seen from the IMPRESS cohort characteristics that active TB individuals that are HIV uninfected are predominantly males (31 males vs 6 females). The ratio of males to females with active TB changes in HIV infected IMPRESS and TRuTH cohort, and this is likely due to HIV infection. Young women in SA are at high risk of HIV infection, and once infected become at high risk of TB. Additional studies are needed to fully understand the immunological aspects of gender differences in TB susceptibility.

Study limitations and future directions

Our study could be improved in several ways. To expand this study and increase our understanding of lymphocyte trafficking in TB and TB/HIV co-infection, cell phenotyping looking at the cell surface integrins should be conducted. Ideally this should be done in mucosal samples including gut and lung tissues. Unfortunately at the time of the study we did

not have access to additional sample types such as matching PBMCs and mucosal samples. Additionally the study design did not include non-HIV infected and non-TB infected samples for comparison to “normal” plasma analyte levels. Microbiome analysis of the gut and lung mucosa should also be performed to look at the dysbiosis and gut-lung axis in TB/HIV co-infected individuals.

Future studies could also examine matched BAL and plasma samples in different and larger cohorts in order to identify and validate immune activation markers that could be used for development of a rapid point of care device to predict active TB.

Conclusion

Here we identified increased expression of plasma LBP and sICAM as predictors of TB recurrence in individuals who were receiving ART treatment in the TRuTH cohort. A trend in decreased levels of plasma LBP from active TB disease to treatment completion in HIV uninfected individuals from IMPRESS, could provide evidence that active TB and the associated inflammatory changes could lead to gut inflammation and dysbiosis. Increased plasma LBP in HIV infected individuals following the completion of TB treatment in IMPRESS cohort likely reflects gut damage and microbial translocation, as a result of HIV disease progression. This study has important implications in providing insight into the role of lymphocyte trafficking and inflammation markers in TB and TB/HIV co-infection.

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