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PLASMA INDOLEAMINE 2, 3 DIOXYGENASE ACTIVITY, A POTENTIAL BIOMARKER FOR TUBERCULOSIS

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A dissertation submitted to the Faculty of Health Sciences, University of The Witwatersrand, in fulfilment of the requirements for the degree of Master of Science Medicine

Johannesburg, 2016

Declaration

I Clement Adu-Gyamfi of Department of Molecular Medicine & Haematology declare that;

a) This dissertation is my own work.

b) This dissertation has not been submitted for any degree or examination to any other University.

c) This thesis does not contain other people's data, pictures, graphs or information, unless specifically acknowledged, and sources are listed in the referenced section.

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Abstract

Tuberculosis (TB) is a major global health challenge, especially in high HIV prevalence settings. To date, however, there is no validated biomarker for diagnosing TB in HIV infected patients. Indoleamine 2, 3 dioxygenase (IDO) is an immunoregulatory enzyme capable of modulating cell mediated immunity (CMI). IDO catalyses the breakdown of tryptophan (Trp) to its toxic metabolites collectively known as kynurenines (Kyn). Elevated IDO activity has been proposed as a prognostic biomarker for TB, however, there is no longitudinal data to assess the clinical significance of elevated IDO activity in HIV-TB co-infection. We investigated whether IDO activity, as measured by Kyn-to-Trp ratio, using ultra-performance liquid chromatography mass spectrometry (UPLC-MS/MS) can act as a biomarker for diagnosing TB in HIV infected patients who develop active TB disease.

Methodology

Kyn and Trp concentrations were measured simultaneously using UPLC-MS/MS in the plasma of 32 HIV infected patients who developed active TB during a longitudinal study and compared with 70 control subjects, age and CD4 cell count matched, in the same HIV infected cohort who did not develop TB.

Results

IDO activity was significantly higher in TB patients than controls at the time of TB diagnosis ($P = 0.0001$). At 6 months prior to TB diagnosis, IDO activity was significantly higher in those who developed TB than controls ($P = 0.0001$). Within 6 months of anti-TB treatment, IDO activity in TB patients declined to almost same levels as that of the controls. To evaluate diagnostic significance of IDO activity using a receiver operating characteristic (ROC) curve, we selected 0.70 as the optimal cut-off. At time of TB diagnosis using both laboratory confirmed and clinical TB as gold standard, IDO activity gave a diagnostic sensitivity of

100% and a specificity of 98.5% with Positive and Negative predictive values of 96.9% and 100% for detecting active TB cases.

Conclusion

Our results, demonstrate the plausibility of increased IDO activity as a biomarker of active TB in HIV positive patients. Further, IDO activity may be a useful biomarker for predicting progress to active TB disease within 6 months or monitoring response to TB treatment. Strengths of the study include inclusion of an HIV infected control group and a longitudinal study design.

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Dedication

To my parents
Your prayer works!

Table of Contents

Declaration ii
Abstract iii
Acknowledgements v
Dedication vi
Table of Contents vii
List of Figures and List of Tables x
Abbreviations xii
CHAPTER 1 1
Introduction 1
1.1 Study hypothesis 2
1.2 Aims and Objectives 3
CHAPTER 2 4
History and epidemiology of Tuberculosis 4
2.1 History of Tuberculosis 4
2.2 Epidemiology of Tuberculosis 4
2.3 Tuberculosis-HIV mortality 6
CHAPTER 3 8
Pathophysiology of Tuberculosis 8
3.1 Aetiological agent of Tuberculosis 8
3.2 Transmission of *M. tuberculosis* 9
3.3 Clinical presentation of Tuberculosis 12
3.4 Risk of Developing Tuberculosis over a lifetime 13
CHAPTER 4 14
Immunology of Tuberculosis 14
4.1 Innate immune responses to Tuberculosis 14
4.2 Adaptive immune response to *M. tuberculosis* 18
4.3 Cytokine and chemokines in immune response to *M. tuberculosis* 23
4.4 Macrophage activation in *M. tuberculosis* infection 24
4.5 The granuloma and latency in Tuberculosis 25
4.6 Dysfunctions in adaptive immune response during *M. tuberculosis* Infection 28
4.7 Tuberculosis - HIV immunopathology 29
4.8 Effect of HIV on the Tuberculosis granuloma 31
CHAPTER 5 33
Diagnosis and Treatment of Tuberculosis 33
5.1 Microbiological diagnosis of *M. tuberculosis* infection 33
5.2 Molecular diagnosis of *M. tuberculosis* 35

5.3 Immunological methods 38
5.4 Treatment of Tuberculosis 41
CHAPTER 6 44
Biomarkers in Tuberculosis 44
6.1 What is a biomarker/biological marker? 44
6.2 Clinical application of Tuberculosis biomarker(s) 45
6.3 Candidate biomarkers in Tuberculosis 46
6.7 Challenges of Tuberculosis biomarker search 52
6.8 Tuberculosis biomarker and other diseases 53
6.9 A plausible biomarker for Tuberculosis 53
CHAPTER 7 55
Indoleamine 2, 3 dioxygenase 55
7.1 IDO and immune suppression 56
7.2 IDO suppression of adaptive immune responses 57
7.3 "IDO-competent" cells and induction in tissues 58
7.4 IDO and human pathologies 58
7.5 The Kynurenine pathway of Tryptophan metabolism 61
7.6 IDO biochemistry and gene regulation 62
7.7 Regulation of functional IDO activity in tissue 63
CHAPTER 8 65
Role of IDO in Tuberculosis and HIV infections 65
8.1 Diagnostic significance of IDO activity in Tuberculosis 66
CHAPTER 9 68
Analytical method selection 68
9.0 Introduction 68
9.1 Method and instrument selection 68
9.4 Instrumentation 71
9.5 Operational principle of the triple quadrupole mass spectrometry (QqQ MS) 73
9.6 Chemicals and reagents 74
9.7 Analytical method optimization 76
9.8 Optimization of chromatography 77
9.9 Optimization of mass spectrometry 78
CHAPTER 10 79
Results of analytical method validation 79
10.1 Preparing calibration curves for Tryptophan and Kynurenine 80
10.2 Extraction efficiency or recovery 80
10.3 Matrix effect 81

10.4 Analytical specificity or selectivity....82
10.5 Linearity and analytical sensitivity....83
10.8 Lower Limit of Detection and Lowest limit of Quantitation (LOD & LOQ)....85
10.9 Assay accuracy....86
10.10 Precision....86
CHAPTER 11....92
Study design....92
11.1 Description of sample cohort....92
11.2 Ethical approval....92
11.3 Inclusion criteria....93
11.4 Exclusion criteria....93
11.5 Study subjects....93
CHAPTER 12....96
Study results....96
12.1 Demographic characteristics of Tuberculosis cases and controls....96
12.2 Clinical parameters in TB cases and controls....98
12.3 Plasma IDO activity in HIV infected TB cases and controls....99
12.4 Plasma IDO activity in laboratory confirmed TB and clinical TB....101
12.5 Plasma concentration of Kynurenine in HIV infected TB cases and controls....101
12.6 Plasma Tryptophan concentration in HIV infected TB cases and controls....103
12.7 Plasma Kynurenine or Tryptophan concentrations in laboratory confirmed TB and clinical TB....104
12.8 Plasma IDO activity fold change in HIV infected TB cases and controls....105
12.9 Diagnostic significance of plasma IDO activity....106
12.11 Plasma IDO activity and other clinical parameters....112
12.12 Plasma IDO activity in patients with pulmonary TB and extra-pulmonary TB....113
CHAPTER 13....115
Discussion and conclusion....115
Conclusion....122
References....124
Appendix A....134
Ethics clearance certificate....134
Appendix B....135
Study cohort....135
Appendix C....139
Study results....139
Appendix D....146

List of Figures and List of Tables

List of Figures

Figure 2.1: 2012 Worldwide distribution of Tuberculosis cases. 5
Figure 3.1: Structure of *M. tuberculosis* cell wall 9
Figure 4.1: Helper T cell subsets 20
Figure 7.1: A simplified diagram of the kynurenine pathway 62
Figure 9.1: UPLC-MS/MS system.....72
Figure 9.2: Arrangement of the triple quadrupoles rods in the Triple-quadrupole mass spectrometer.....74
Figure 10.1: UPLC-MS/MS chromatogram for analysis of Tryptophan and Kynurenine and Trp-d5 in plasma 83
Figure 10.2: Linearity of Tryptophan 84
Figure 10.3: Linearity of Kynurenine 85
Figure 12.1: (A & B) Plasma IDO variability in HIV infected patients who developed TB and controls over 5 time points.....100
Figure 12.1: Plasma IDO activity in HIV infected TB cases compared to HIV infected controls 100
Figure 12.2: Plasma IDO activity in Laboratory confirmed TB and clinical TB 101
Figure 12.3: Plasma Kynurenine levels of HIV infected patients who developed TB and HIV infected controls over 5 time points 103
Figure 12.4: Plasma Tryptophan level in HIV infected who developed TB and HIV infected control over 5 time points 104
Figure 12.5: Plasma Kynurenine or Tryptophan in confirmed TB cases and clinical TB 105
Figure 12.6: Plasma IDO activity fold change in controls and TB cases 106
Figure 12.7: ROC curve: using plasma IDO activity results at TBdx time point 108
Figure 12.8: ROC curve: Using plasma IDO activity results at TBdx time point excluding -6m results 109
Figure 12.9: ROC curve: Using plasma IDO activity at -6m and TBdx as gold standard positives 110
Figure 12.10: ROC curve: Using plasma IDO activity at -6m 112
Figure 12.11: Plasma IDO correlation with CD4, HIV viral load & CRP 113

List of Tables

Table 10.1: Extraction efficeiencies of Trp-d5 and Kyn-d4 in extracted plasma and non-extracted plasma.....81
Table 10.2: Matrix ionization or suppression of Trp-d5 and Kyn-d4 in plasma and distilled water.....82
Table 10.3: Limit of detection and Limit of quantitation 86
Table 10.4: Intra-day precision of Tryptophan in spiked plasma and pure standard solutions ..87
Table 10.5: Tryptophan inter day precision.....88
Table 10.6: Kynurenine intra-day precision assay.....89
Table 10.7: Kynurenine inter-day assay precision.....90
Table 12.8: Clinical and demographic characteristics of TB cases and control 96
Table 12.9: Diagnostic significance of IDO activity at TB diagnosis time point 107
Table 12.10: Diagnostic significance of IDO activity at TB diagnosis time point 109

Table 12.11: Diagnostic significance of IDO activity using -6m and TBdx time point as gold standard positives 110
Table 12.12: Diagnosing TB using IDO activity at -6m time point 111
Table 12.13: Correlation of plasma IDO activity with CD4 cell count, HIV viral load & CRP 112

Abbreviations

ADCC – Antibody dependent cellular cytotoxicity
AFB – Acid fast bacillus
AIDS – Acquired immunodeficiency syndrome
BCG – Bovis Calmette-Guerin
CD – Cluster of differentiation
CDC – Centre for disease control & prevention
CMI – Cell mediated immunity
CR – Complement receptors
CRP – C- reactive protein
CTL – cytotoxic T cell
DC – Dendritic cell
DOTS – Directly Observed treatment, short course
DST – Drug susceptibility testing
EPTB – Extra-pulmonary Tuberculosis
ESAT-6 – Early secretory antigen- 6
ETH – Ethambutol
Foxp3 – Fox-headbox p3
HAART – Highly active antiretroviral therapy
HIV – Human immunodeficiency virus
IDO – Indoleamine 2, 3 dioxygenase
IL – Interleukins
INH – Isoniazid
Kyn – Kynurenine
LAM – Lipoarabinomanann
LOD – Limit of detection
LOQ – Limit of quantitation
LTBI – Latent Tuberculosis infection
MCP – Monocyte chemotactic protein
MDR – Multi-drug resistant
MGIT – Mycobacteria growth in-tube
MHC – major histocompatibility complex
MR – Mannose receptors
MRM – Multiple reaction mode
mRNA – Messenger ribonucleic acid
NAAT – Nucleic acid amplification test
NADP – Nicotinamide adenine dinucleotide phosphate
NK cells – Natural killer cells
Nramp – Natural-resistance-associated macrophage protein
NTM – Non tuberculous mycobacterium
PAMP – Pathogen associated molecular patterns
PRR – Pathogen recognition receptors
Psig – Pounds per square inch gauge

PTB – Pulmonary Tuberculosis
PZA – Pyrazinamide
RCF – Relative centrifugal force
RNI – Reactive nitrogen species
ROS – Reactive oxygen species
RPF – Rifampicin
SR – Scavenger receptors
T reg – regulatory T cell
TB – Tuberculosis
TCR – T cell receptor
TGF – Transforming growth factor
TLR – Toll-like receptors
TNF – Tumor necrosis factor
Trp – Tryptophan
UPLC – Ultra-performance liquid chromatography
WHO – World Health Organization

CHAPTER 1

Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) is the causative agent of Tuberculosis (TB) in man. TB is one of the leading causes of death due to infectious disease in the world. In 2013, 9 million people fell ill with TB and 1.5 million people died of TB (1).

There are multiple existing diagnostic methods in use for TB. To date, however, there is no validated biomarker(s) that can differentiate patients with active TB disease from latent Tuberculosis infection (LTBI). Lack of a TB biomarker makes diagnosis and treatment very clumsy especially in TB endemic areas where most individuals are latently infected.

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of normal physiological or pathological process or pharmacological response to a therapeutic intervention (2). Biomarker(s) can be host or pathogen-specific and may provide insights about the pathogenic process in the host, including the current health status and future disease risk of the patient (3). In TB, there is an urgent need for a specific biomarker to classify patients at a single time point as having active TB disease, LTBI or no infection. Additionally, a reliable TB biomarker would help in identifying correlates of protection for TB vaccine development.

In the last two decades, a large number of candidate biomarkers for TB have been proposed and investigated. However, none have been validated for application in TB diagnosis or prognosis or monitoring anti-TB treatment. A highly plausible biomarker with prospects of better indicating or predicting TB is the activity of the enzyme indoleamine 2, 3 dioxygenase (IDO).

IDO was originally described for its antimicrobial role, depleting Tryptophan (Trp) essential for the growth of some microbes (4), but recent studies have found it to be a potent immunoregulatory enzyme. IDO is an intracellular enzyme that catalyses the breakdown of Trp to its immune-toxic metabolites collectively known as kynurenines (Kyn) along the kynurenine pathway (5). Through this pathway, IDO activity regulates certain physiological functions such as pregnancy and modulates the pathogenesis of diverse pathological conditions including some cancers and infectious diseases.

Recently, IDO activity has been proposed as a prognostic biomarker for TB (6-8). A study by Almeida et al. reported IDO mRNA expression was upregulated in sputum of TB patients and declined after anti-TB treatment (6). Serum IDO activity was also found to be significantly higher in TB patients compared to control subjects of the same age group by Suzuki et al. (7, 9). Additionally, Li et al. described upregulated IDO activity at the site of infection in TB pleurisy patients and inhibition of IDO activity restored local immune status through T cell cytokine secretion (10). Collectively, these data may imply that elevated IDO activity impairs cell mediated immunity (CMI), resulting in active TB disease.

To date, however, there is no longitudinal data to assess the clinical significance of elevated IDO activity in TB disease. This study investigates the clinical usefulness of IDO activity from a longitudinal study to ascertain whether it can act as a biomarker for diagnosing or predicting active TB disease.

1.1 Study hypothesis

This study hypothesizes that up-regulated levels of IDO activity may be a useful biomarker for active TB disease.

1.2 Aims and Objectives

1.2.1 Aims

- To determine plasma IDO activity in HIV infected patients who developed TB and compare with IDO activity in the months or years prior to and following the diagnosis of TB.

- To compare IDO activity in HIV infected patients with TB to an HIV infected control group who did not develop TB.

1.2.2 Specific Objectives

- To simultaneously measure plasma Kynurenine and Tryptophan by ultra-performance liquid chromatography- tandem mass spectrometry (UPLC-MS/MS) in stored plasma samples of HIV infected Tuberculosis patients and an HIV infected control group without Tuberculosis.

- To calculate IDO activity by using Kynurenine-to-Tryptophan ratio.

- To assess variability of serum IDO activity in HIV infected patients over time and assess correlations with other clinical factors including CD4 cell count, HIV viral load count, and C-reactive protein (CRP).

- To calculate sensitivity, specificity, positive and negative predictive values of IDO activity at various cut off values to diagnose TB in HIV infected individuals.

CHAPTER 2

History and epidemiology of Tuberculosis

2.1 History of Tuberculosis

TB has been traced back as far as the Egyptians in 2400 BC (11). For hundreds of years, this contagious disease killed people worldwide without any insight into its causative agent and pathogenesis. In 1720 Benjamin Marten first proposed that TB could be caused by "wonderfully minute living creatures", a truly revolutionary thought at the time. Then in 1882 Dr Robert Koch described the bacteria and successfully developed a staining protocol, allowing him to visualize *M. tuberculosis* for the first time (12). In 1921 *Mycobacterium bovis* Calmette-Guerin (*M. bovis* BCG) was administered as a vaccine for the first time. By early 1940s chemotherapeutic agents were being developed for use against *M. tuberculosis*, ushering in a new era in the fight against TB (13). Until the late 1980s when HIV/AIDS emergence brought a dramatic resurgence in TB cases particularly in the developing world, TB was drastically controlled. By early 1990s, because of HIV, TB won the fight against control measures (14). In 1993, the Centre for Disease Control and prevention (CDC) classified TB as an AIDS defining illness in HIV-infected patients and since then HIV has been the fuel for TB (15).

2.2 Epidemiology of Tuberculosis

Despite being an ancient disease, TB is still a worldwide problem. According to World Health Organization (WHO), one third of the world's population harbours *M. tuberculosis* infection. In 2013, 9 million people were infected with TB worldwide. Over 5 million were newly diagnosed cases of active disease and 1.5 million people died from TB (16).

As shown in Figure 2.1, most of the global burden of TB is borne by the developing world with 86% of cases occurring in South-East Asia, Africa and the Western Pacific (17). Even though TB affects all ages, it is more common in adult males than females. About 6% of the world's

TB burden is born by children. Over the past 15 years, South Africa is estimated as the country with the third highest TB burden (18).

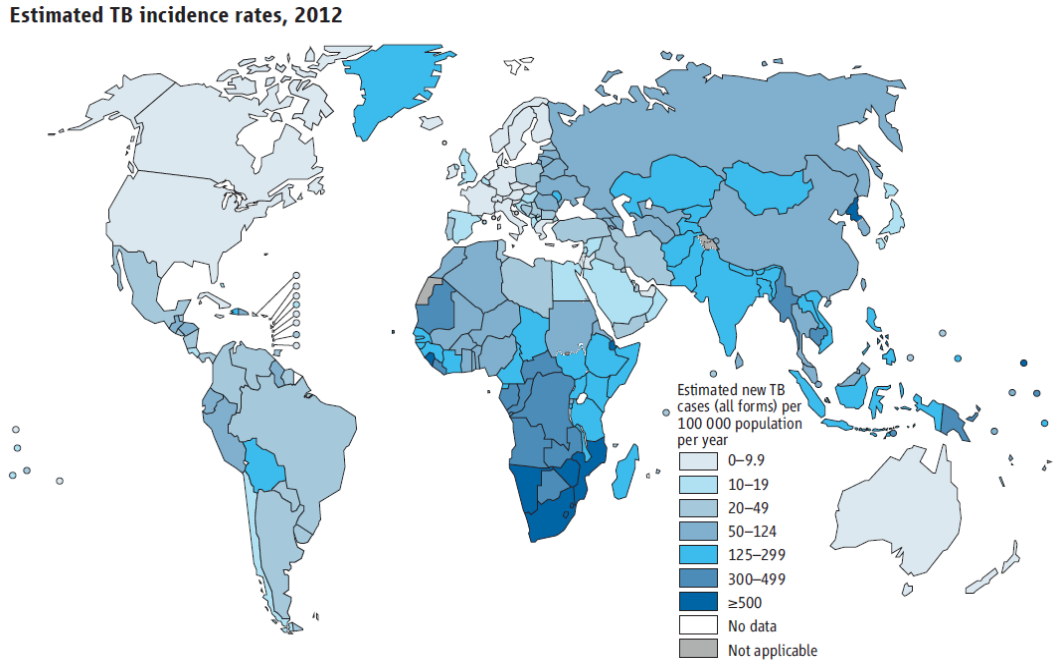


Figure 2.1: 2012 Worldwide distribution of Tuberculosis cases.
The map shows the regions with the highest prevalence of Tuberculosis cases to be within sub-Saharan Africa and Asia, the deep-blue shaded areas (16).

2.2.1 Epidemiology of Tuberculosis HIV co-infection

HIV infection is the fuel for the TB epidemic. Beginning in the 1980s, the HIV epidemic led to a dramatic upsurge in TB cases and mortality in Africa and most countries in the developing world. In 1993, centre for disease control and prevention (CDC) classified TB as an AIDS defining illness (14).

Over two decades on, 13% of TB cases worldwide are co-infected with HIV (19). The African region bears the highest TB-HIV co-infection burden. Overall, 39% of TB cases are estimated to be co-infected with HIV in Africa (20). In parts of Southern Africa, more than 50% of TB cases are co-infected with HIV. Malnutrition and poor living standards have been linked to developing TB during an HIV infection (14).

2.2.2 Epidemiology of Tuberculosis-HIV co-infection in South Africa

South Africa, like most sub-Saharan African countries, experienced the brunt of TB cases from the HIV epidemic in the 1990s. Two decades on, South Africa still has the largest absolute number of HIV infected individuals in a single country. In 2012, national household surveillance reported HIV prevalence of 30% among the 15-49 year age group. Among pregnant women attending antenatal clinics and women in the 30-35 year age group, HIV prevalence was found to be 43% (21). As a result of high HIV prevalence, South Africa, has the third highest annual TB incidence globally (993/100 000 population). About 1% of the South African population of about 50 million people develops TB every year. It is estimated that 67% of TB patients in South Africa are co-infected with HIV resulting in the highest absolute numbers of TB-HIV co-infected cases globally (20).

2.3 Tuberculosis-HIV mortality

Tuberculosis is the leading killer of HIV infected patients. Even though TB mortality has decreased significantly in 2013, deaths due to TB in patients co-infected with HIV are still high (20). TB infection among HIV infected patients presents an increased mortality rate with or without treatment. In a study by Elliott et al. at least 34% of HIV infected patients with smear positive pulmonary TB died from TB in the first 6 months of starting anti-TB treatment (22). In comparison with a study of the natural history of the disease among sputum smear-positive but HIV-negative patients, around 70% died within 10 years (23).

2.3.1 Tuberculosis-HIV mortality in South Africa

Highly Active Anti-retroviral treatment (HAART) for HIV prevents new cases of TB among HIV-positive patients (24). TB however, remains the leading opportunistic infection and the ultimate killer of HIV/AIDS patients receiving HAART (25). In South Africa, TB is the top-

ranked cause of death among adults and the fourth cause of death among children 0-14 years despite both TB and HIV being under reported on notification-of-death certificates (25, 26).

Conclusion

Improving control of TB in HIV infected patients is a priority health goal of CDC and WHO. WHO policies such as active TB case finding among HIV patients, prophylactic treatment with Isoniazid and antiretroviral therapy are measures to stop TB or reduce morbidity and mortality due to TB in HIV infected patients. Furthermore, the search for a Tuberculosis biomarker that can diagnose/predict TB before symptoms appear is a priority of the Tuberculosis Research Roadmap (107).

CHAPTER 3

Pathophysiology of Tuberculosis

3.1 Aetiological agent of Tuberculosis

TB results from infection by a pathogen belonging to the *M. tuberculosis complex*, primarily *M. tuberculosis* (Koch's bacillus). *M. tuberculosis* is a highly robust and sophisticated mycobacterial pathogen which resists and even subverts the host's protective immunity. *M. tuberculosis* is an obligate aerobe, intra-macrophage pathogen, which grows successfully in tissues with high oxygen content such as alveolar macrophages of the lungs (27).

3.1.1 *M. tuberculosis* bacilli

M. tuberculosis is a rod-shaped bacillus measuring about 2-5µm by 3 µm. It is a slow-growing, non-sporing, non-motile pathogen with high lipid content in its cell wall. Even though *M. tuberculosis* is a Gram-positive bacterium, it is not easily stained by the Gram-stain method. It is classified as an "acid fast bacillus" (AFB) due to its resistance to diluted acid decolourization during staining. The cell wall contains a high amount of mycolic acid and fatty acid covalently attached to the underlying peptidoglycan-bound polysaccharide arabinogalactan. *M. tuberculosis* is impervious to alcohol, diluted acids and alkali, drying and various basic dyes unless the dye is combined with phenol. The cell wall also confers antibiotic resistance and subversion of host's immune mechanisms. Another important component of the *M. tuberculosis* cell wall is lipoarabinomannan (LAM). LAM is a carbohydrate structural antigen on the outside of the organism. LAM facilitates the survival of the bacteria within macrophages and is immunogenic (28, 29). The cell wall of *M. tuberculosis* is depicted in figure 3.1 below.

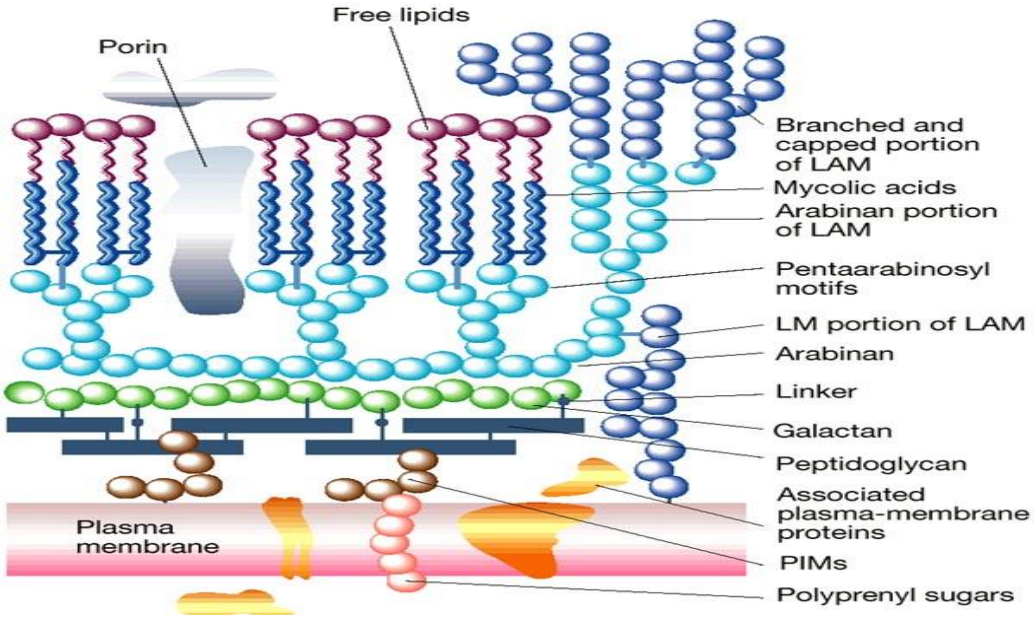


Figure 3.1: Structure of *M. tuberculosis* cell wall
Shows schematic arrangement of structural carbohydrates, LAM, mycolic acid and peptidoglycan (30).

3.2 Transmission of *M. tuberculosis*

M. tuberculosis is almost exclusively transmitted via the respiratory route, through inhalation of infectious droplet nuclei when a patient with active TB coughs, sneezes or otherwise transmits respiratory fluid. In the majority of people, the lungs are the primary site of initial infection. TB infection begins when *M. tuberculosis* reaches the alveoli of the lungs of a previously unexposed individual.

3.2.1 Primary Tuberculosis Infection

Primary TB infection results the first time a previously unexposed individual comes into contact with *M. tuberculosis*. Two-thirds of persons with primary TB remain asymptomatic (31). When *M. tuberculosis* is deposited into the lungs, it encounters endothelial cells, alveolar macrophages and dendritic cells (DCs). The alveolar macrophages and DCs are the first lines of defence within the lungs. Alveolar macrophages engulf the bacillus into a phagosome and attempt to destroy it via an array of antimicrobial pathways. The local inflammatory response

initiated by macrophages and DCs in an attempt to kill the bacilli induces the release of pro-inflammatory cytokines. These cytokines result in recruitment of more phagocytic cells such as monocytes, neutrophils and lymphocytes to the site. This primary lesion formed at the site of infection in the lung is called the Ghon focus. The infection may spread from the Ghon focus to involve draining lymph nodes in which case it is referred to as Ghon's Complex. There are four potential fates of the primary infection (32).

3.2.2 Healed Tuberculosis

In some fortunate individuals, the fate of primary TB may lead to remission or healed TB, in which case the Ghon's complex undergoes fibrosis and calcification. This is referred to as the Ranke complex (33).

3.2.3 Latent/Dormant Tuberculosis infection

In the majority of people, despite the sophiscated immune responses mounted by the individual's immune system, he/she often fails to completely eliminate the bacilli from the individual's system. *M. tuberculosis* may exist in a quiescent state for several years without causing disease. Such a state is called Latent Tuberculosis infection (LTBI). LTBI is characterised by the arrest of *M. tuberculosis* into a granuloma to wall-off the pathogen and truncate pathology (34). A granuloma consists of *M. tuberculosis* infected macrophages, usually at the centre, being walled off by giant cells (modified macrophages), neutrophils, B cells, T cells and fibroblasts. In the granuloma, cells stay in close and dynamic contact to physically restrict *M. tuberculosis* from dissemination. In the majority of people, *M. tuberculosis* may be in the granuloma life-long without causing disease until there is simultaneous infection or conditions (for example, HIV infection or diabetes) that cause waning CMI (35).

3.2.4 Primary progressive Tuberculosis

In persons with ineffective CMI the fate of primary TB infection may progress to serious active disease. Foci in infected lungs continue to enlarge and recruit more macrophages and other phagocytic cells. The centre of the primary focus may become necrotic with a cheesy-like appearance called caseous necrosis. The expanding lesions erode the bronchial architecture at the site of the foci and leave empty cavities which are referred to as cavitary TB lesions. Erosion and rupture of pulmonary vasculature can lead to haemorrhages within the respiratory parenchyma that is manifested by haemoptysis or collection of fluid in the pleural cavity. Also, in severely immunocompromised patients such as HIV/AIDS patients, lympho-hematogenous dissemination of *M. tuberculosis* can seed the organism into any tissue in the body resulting in extra-pulmonary Tuberculosis (EPTB).

3.2.5 Secondary Tuberculosis

Unlike primary TB, secondary TB develops in a previously exposed or sensitised individual. Secondary TB develops from either reactivation of primary TB or re-infection with *M. tuberculosis* (36). Reactivation of primary TB is more common in patients with waning immunity for example simultaneous infection by HIV. Reactivation of primary TB is more common in developed countries where TB is less prevalent. Secondary TB most commonly infects the apex of the lungs where there is high oxygen tension.

Re-infection TB may also occur after re-exposure to *M. tuberculosis* later. Re-infection TB is common in areas with high TB prevalence. In a study by Verver et al in Cape Town, South Africa, the risk of reinfection after successful treatment was about 2% per annum. This suggests that individuals who are successfully treated are at increased risk of developing TB again, rather than being protected (37).

3.3 Clinical presentation of Tuberculosis

The clinical presentation of TB differs according the strength of one's immune system. In immunocompetent individuals, the bacilli are driven into a state of latency. Individuals latently infected have no symptoms of the infection and the individual is not infectious because the bacilli are contained in the granuloma. The classical picture of TB is seen mainly in non-severely immunocompromised patients. Active TB disease may manifest in the lung in which case it is called pulmonary TB (PTB) or any other organ apart from the lungs which is referred to as extra-pulmonary TB (EPTB).

3.3.1 Pulmonary Tuberculosis

PTB accounts for over 80% of all TB cases in HIV negative patients. Patients with PTB usually have a cough, night sweats, weight loss and abnormal chest radiograph and may be infectious. Although the majority of TB cases are PTB, TB can occur in almost any anatomical site or as disseminated disease.

3.3.2 Extra-pulmonary Tuberculosis (EPTB)

EPTB occurs in places other than the lungs, including the pleura, the brain, the spinal cord, the kidneys, the bones and any other organ. In HIV-infected patients, EPTB disease may occur together with PTB. Patients with EPTB are usually not infectious unless they have PTB in addition to EPTB (38).

3.2.3 Miliary Tuberculosis (disseminated Tuberculosis)

Miliary TB results from a massive lympho-haematogenous dissemination of *M. tuberculosis* bacilli. Both primary and secondary progressive TB can give rise to miliary TB. Miliary TB is

rare but fatal if not diagnosed early and treated. Classically, the name "Miliary" refers to the appearance of millet-sized seed (1–2 mm) calcifications of TB bacilli in the lungs, as seen on the chest radiograph. Miliary TB is most common in severely immunocompromised persons. Among immunocompromised individuals, miliary TB accounts for about 20% of all EPTB cases (39).

3.4 Risk of Developing Tuberculosis over a lifetime

Without treatment, approximately 5% of persons who have been infected with *M. tuberculosis* will develop disease in the first two years after infection, and another 5% will develop disease sometime later in life. Thus, without treatment, approximately 10% of persons with normal immune systems who are infected with *M. tuberculosis* will develop TB disease at some point in their lives (31, 40). The risk of active disease is much higher in HIV infected individuals. Without ART, the risk of developing active TB disease in HIV infected individuals is estimated at 10% per year (39).

CHAPTER 4

Immunology of Tuberculosis

Immune responses mounted to *M tuberculosis* infection are generally successful in driving the bacteria into latency, but fail to eliminate the bacilli. As discussed previously, primary infection with *M. Tuberculosis* results in a small percentage of symptomatic disease. In most cases, latency extends for the lifetime of the individual. Reactivation of the latent infection, however, can occur in response to perturbations of the host's immune response (for example with simultaneous HIV infection) and active Tuberculosis ensues.

4.1 Innate immune responses to Tuberculosis

4.1.1 Recognition of *M. tuberculosis* by host innate receptors

Host cells utilize a multitude of receptors including pattern recognition receptors (PRRs), C-type lectins and integrins to interact with extracellular molecules. Host's PRRs recognise pathogen associated molecular patterns (PAMPs) of invading pathogens. *M. tuberculosis* is recognised by alveolar macrophages and DCs through multiple PRRs including mannose receptors (MRs), complement receptors (CRs), scavenger receptors (SR) and Toll-like receptors (TLRs) (TLR-2, TLR-4 and TLR-9) (41). Other intracellular PRR such as the nucleotide-binding oligomerization domain (NOD-) like receptors (NLRs), surfactant protein A receptors (Sp-A) and cholesterol receptors are involved in recognition of *M. tuberculosis* secreted antigens such as early secretory antigen 6 (ESAT 6) following endocytosis (42).

4.1.2 Interaction of macrophages, dendritic cells and other innate cells with *M. tuberculosis*

Macrophages

The interaction between mycobacterium surface protein LAM and the host's complement receptors (CR1, CR2, CR3 and CR4), MR, SR and CD14 results in endocytosis of the bacilli into macrophages. Within the macrophages the bacteria inhabit the phagosome, which traffics through the macrophage en route to fusing with the lysosome. The presence of the bacilli in the phagosome partially activate the macrophages to secrete interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12) and other Th1 cytokines to kill the pathogen. Activated macrophages increase Th1 cytokine secretion and decrease their surface receptor expression resulting in diminished ability of mycobacteria to adhere to macrophages (36).

Dendritic cells

The mycobacterium also interacts with DCs within the lungs through MRs, CRs, TLR-2, TLR-4 and C-type lectins. Through these cell surface receptors the bacteria is endocytosed. The bacterium is processed and presented via major histocompatibility complex (MHC) class II of DCs and trafficked to the lymph nodes for presentation to naïve T-cells. DCs can also present mycobacterial antigens via MHC class I and CD1 receptors to CD8+ T-cells, though this response is secondary to MHC class II mediated CD4+ T-cell activation (43).

Neutrophils

Neutrophils are among the first cells to respond to inflammatory stimuli by migrating to the site of infection. During the initial infection of *M. tuberculosis*, there is massive influx of neutrophils to the infection site (44). Even though there is conflicting evidence on the role of neutrophils in *M. tuberculosis* infection, it has been shown that neutrophils kill using their

antimicrobial peptides and molecules contained in their granules, including defensins, lactoferrin, cathelicidin, and lysozyme. Additionally, neutrophils exert efficient killing through the assembly of the NADPH oxidase in the phagosomal membrane, generation of superoxide and other reactive oxygen species (ROS) in the phagosome that lead to killing of the bacilli (45).

Natural killer cells

NK cells are granular lymphocytes of the innate immune system that have cytotoxic functions exerted through perforin and granzyme or granulysin (44). NK cells kill infected cells through direct lysis and also provide IFN-γ required for the partial activation of infected macrophages. NK cells are activated through complex interactions between a range of activating and inhibitory receptors and in the presence of IL-12, IL-18 and IFN-α. During early stages *M. tuberculosis* of infection, NK cell activity reduces the bacilli burden and also upregulates the antimicrobial capacity of macrophages (44, 46).

4.1.3 Fusion of the lysosome and phagosome

The lysosome of the cell contains acidic hydrolases that can kill bacteria and other pathogens that enter the cell. Endocytosed microorganisms are subject to degradation by intra-lysosomal acidic hydrolases upon fusion of the phagosome with the lysosome. This highly regulated event constitutes a significant antimicrobial mechanism of phagocytes. In *M. tuberculosis* infection, sometimes this fusion does not occur, and the pathogen resides within the macrophages. This will be discussed later.

4.1.4 How macrophages kill the bacteria

Macrophages employ two main effector functions in phagocytosis. They generate reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs). Both mechanisms are mediated by cytokines.

Reactive nitrogen intermediates (RNIs): Several reports have demonstrated the significance of nitric oxides in host defence against *M. tuberculosis* infection. Macrophages, upon activation by IFN-γ and TNF-α, generate nitric oxide (NO) and its related RNIs via inducible nitric oxide synthase (iNOS-2) using L-arginine as the substrate. In humans, the NOS-2 enzyme has been detected in the alveoli macrophages from Tuberculosis patients and contributes to killing of the bacilli (47).

Reactive oxygen intermediates (ROIs): Hydrogen peroxide (H_2O_2) is one of the ROI generated by macrophages through the oxidative burst pathway. This was the first identified effector molecule that mediated mycobactericidal effects of macrophages. While the ability of ROI to kill *M. tuberculosis* has been demonstrated in mice, its importance remains to be confirmed in humans.

Other types of cell death

Apart from the two effector functions described above, macrophages employ other effector mechanisms to kill pathogens. One of such mechanism is autophagy. Macrophages can kill infected pathogens through macro-autophagy. Autophagy is beneficial for the host as it reduces the number of viable mycobacteria through self-death of infected macrophages (48, 49).

4.1.5 Cellular chemotaxis

Early in *M. tuberculosis* infection, there is non-specific sequestration of phagocytic cells to the site. This result in the accumulation of neutrophils, monocytes, natural killer (NK) cells, dendritic cells, Gamma-Delta (γδ)-T cells and macrophages. These cells aggressively initiate phagocytosis and secretion of IL-12 and IFN-γ which activate macrophages and dendritic cells. Neutrophils and γδ-T cells contribute to killing by providing antimcrobial agents such as defensins, cathlicidin and lactoferrins. NK cells also secrete IFN-γ which activates the macrophages. Other components of innate immunity, such as natural resistance associated macrophage protein (Nramp), play a vital role in the first line containment of *M. tuberculosis*.

4.2 Adaptive immune response to *M. tuberculosis*

During the first few days of *M. tuberculosis* infection, very little is done to restrict bacterial growth although this is a highly dynamic stage of the infection. The expanding bacterial population spreads from cell to cell and increases the range of cell subsets that it infects. Alveolar macrophages and DCs are two predominant immune cell lines that are infected. Both cell types are found dispersed throughout the lungs sampling their local environment for invading pathogens. Infected DCs cells initiate adaptive immune responses to the pathogen because they shuttle between the infected site and the draining lymph nodes where they activate naïve T cells (36, 50). During early stages of *M. tuberculosis* infection, despite IFN-γ release from NK cells, alveolar macrophages do not reach full functionality until IFN-γ secreting helper T cells ($CD4^+$ T-cells) enter the lungs after about two weeks. It may be speculated that is one of the reasons that macrophages are not always fully capable of eliminating the intracellular bacilli.

4.2.1 *M. tuberculosis* antigen presentation by macrophages and dendritic cells

Antigen presentation plays an important role in activating the adaptive immune response against *M. tuberculosis*. Presentation of antigens from extracellular pathogens is usually achieved through uptake by an antigen-presenting cell (APC) such as a macrophage, DC or B cell. After bacilli are internalized, the bacteria are degraded within the phago-lysosomal compartment and mycobacterial peptides are loaded onto Major Histocompatibility Complex (MHC) class II. DCs with loaded MHCs are trafficked to draining lymph nodes where they present *M. tuberculosis* peptides to naïve T cells (36, 40).

Antigen presentation to the T cell receptor (TCR) by MHC class II in conjunction with costimulatory signals between B7 (CD80 or CD86) and CD28 and between CD40 and CD40L serves to activate $CD4^+$ T-cells within the lymph nodes. Chemokines produced by macrophages and DCs in response to infection within the lungs also aid T cell migration to the lungs (41). In *M. tuberculosis* infection, $CD4^+$ T cells entering the lungs are the primary responder cells to DCs and macrophages (36, 40). $CD4^+$ T cells are the secondary source of IFN-γ and TNF-α that fully activate macrophages, increasing their antimicrobial capacity in order to destroy the bacilli. Also, DCs can present mycobacterial peptides via MHC class I to $CD8^+$ T-cells. M. tuberculosis lipid antigens are presented via CD1 to NKT cells (51).

4.2.2 Adaptive immune cells in Tuberculosis

Immune responses to *M. tuberculosis* infection are T cell dependent. They comprise not only the conventional $CD4^+$ or $CD8^+$ T cells but a host of other phenotypes such as γδ-T cells and CD1 restricted T cells. Although different T cell populations are required for optimum cell-mediated immunity to the pathogen, T cells provide two main effector functions; a) macrophage activation via cytokine secretion and b) direct cytolytic activity by $CD8^+$ T cells.

Cytokine secretion is primarily undertaken by helper T cells and γδ-T cells whilst direct cytolysis is the function of $CD8^+$ cells (cytotoxic T cells).

4.2.3 Helper T cells

Helper T cells play a critical role in the adaptive immune response to *M. tuberculosis*, particularly via secretion of cytokines to activate infected macrophages, $CD8^+$ cells and B cells (Figure 4.1). Some helper T cell subsets, namely regulatory T cells (T regs), also help shut-down or suppress immune responses to prevent exacerbation or auto-reactivity after the immune response. Based on the pattern of cytokines secreted by activated macrophages or DCs upon antigen stimulation, naive helper T cells may proceed to differentiate into several $CD4^+$ T cell phenotypes (figure 4.1); Th1, Th2, Th17 or induced T regs.

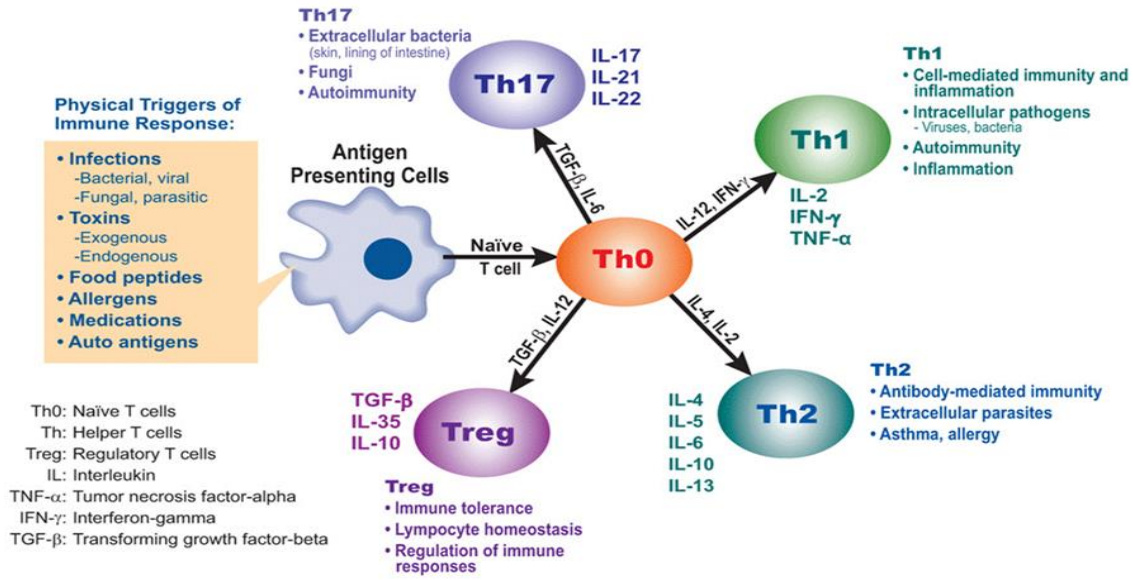


Figure 4.1: Helper T cell subsets
Image retrieved from Peterson (52)

Depending on the cytokine environment, $CD4^+$ T cells can mount a Th1 or a Th2 response against the invading pathogen. A Th1 response leads to the release of pro-inflammatory cytokines (IFN-γ, IL-12 and IL-18) which enhances killing of intra-macrophage mycobacteria

through NO and ROS production (53). The Th1 response also helps cytotoxic T cells ($CD8^+$) to kill the infected cell. A Th2 response on the other hand, leads to release of IL-4, IL-5, IL-10 and IL-13, promoting B lymphocyte activation leading to an antibody response, and promoting an anti-inflammatory macrophage response. Th17 cells secrete IL-17 which is thought to be pro-inflammatory (54).

4.2.4 Regulatory T cells (T reg)

T reg cells are a subset of $CD4^+$ helper T cells with immune modulatory functions. T regs shut down or put the brakes on the immune system after an immune response. They are characterized by expression of intracellular transcription factor forkhead box p3 (Foxp3) and some surface markers such as GITR and CD25. During infections, T reg cells appear to play a dichotomous role: on one hand, they benefit the host by curbing excessive inflammation that could be deleterious to host tissues and on the other hand, limit potentially protective immune responses which facilitates pathogen replication and persistence as observed in TB infection (55, 56). T reg cells are induced by the presence of anti-inflammatory cytokines such as IL-10 and TGF-β. T reg cells exert their effector functions by cell-to-cell contact. In *M. tuberculosis* infection, T reg activity has been shown to be elevated early and declined thereafter by the third week, perhaps to supress over-reactive immune response by the host to the bacilli (55, 56).

4.2.5 Cytotoxic T cells (CTLs) ($CD8^+$ T cells)

M. tuberculosis is an intra-macrophage pathogen. The mechanism by which *M. tuberculosis* proteins gain access to the cytosol and subsequently get presented by MHC I is poorly elucidated. According to Teitelbaum et al., *M. tuberculosis* induced pores or breaks in the endosomal membrane harbouring the bacilli that allow antigens to escape into the cytosol of the

infected cell, allowing these antigens to eventually get presented on MHC I (57). *M. tuberculosis* peptides presented by infected macrophages and DCs via MHC I activate cytotoxic T cells with specific TCRs to kill the infected cell via the FAS/FAS-Ligand pathway (58). CTLs can also kill intracellular bacterial through perforin and granulysin/granzyme pathways after being activated by IL-2 through attachment.

4.2.6 Non-classically restricted $CD8^+$ T cells and γδ-T cells in *M. tuberculosis* infection

CD1 molecules are non-polymorphic antigen presenting molecules usually found on DCs. CD1 molecules present lipids or glycolipids antigens to NKT cells. *M. tuberculosis* bacilli have a high lipid content, components of which are presented via CD1 receptor to NKT cells (59).

γδ-T cells are non-MHC restricted antigen presenting cells and function largely as cytokine secreting cells. γδ-T cells secrete cytokines that are involved in granuloma formation and maintenance in latent Tuberculosis (60).

4.2.7 B cells and antibodies responses

The role of B cells and a humoral response in protection against *M. tuberculosis* infection is poorly explained. Reports have long dismissed their importance because of the intra-macrophage localization of *M. tuberculosis*. However, evidence from experimentally infected animals suggests that an antibody response can have beneficial roles to the host through the classical functions of antibody including; neutralization, opsonisation, complement fixation and promoting antibody-dependent cellular cytotoxicity (ADCC) during the extracellular life stage of *M. tuberculosis* (61).

4.3 Cytokine and chemokines in immune response to *M. tuberculosis*

Cytokines and chemokines are small protein molecules that regulate immunological responses at the cellular level. They stimulate and recruit a wide range of cells involved in immunity and inflammation. The actions of cytokines can be pleiotropic, where one cytokine has the ability to act on different cell types, or redundant where multiple cytokines have the same functional effect. Other cytokines have a cascade effect in which one cytokine manipulates the manufacture and actions of other cytokines. They may also have antagonistic action where the effect of one cytokine opposes the action of others, or synergistic effects where two different cytokines work together. Cytokines can be autocrine, in which case the cytokine acts on the cell that secreted it. Cytokines can also be paracrine, where they act on neighbouring cells. On very few occasions, a cytokine acts at endocrine level where it acts on a target cell far away from the cell that produced it.

The actions of chemokines can be homeostatic where they guide cells during immune surveillance for pathogens by interacting with antigen presenting cells residing in these tissues. Some chemokines are inflammatory and they function mainly as chemo-attractants for leukocytes from the blood to sites of infection or tissue damage.

The protective response to *M. tuberculosis* is complex and multifaceted involving many components of the immune system, mainly the result of productive cooperation between macrophages and T-cell populations. Several studies from both humans and animals have firmly established that cytokines and chemokines have a major role in determining the outcome of infection with *M. tuberculosis* (62, 63).

In both mice and human studies, IFN-γ, TNF-α and IL- 12 are the key cytokines involved in the control of *M. tuberculosis* infections. Anti-inflammatory cytokines such as IL-4, IL-10, IL-13, and TGF-β are known not to be protective in *M. tuberculosis* infection (62-64).

M. tuberculosis induces elevated levels of a variety of chemokines, including IL-8 (CXCL-8), monocyte chemoattractant protein 1 (MCP-1) (CCL-2), MCP-3 (CCL-7), MCP-5 (CCL-12), regulated on activation normal T cell expressed and secreted (RANTES/ CCL-5), MIP1-α (CCL-3), MIP1-β (CCL-4), MIP-2 (CXCL-2) and IFN γ-inducible protein-10 (IP-10/CXCL-10) and their receptors such as CCR5 and CXCR4 (65). The role of such chemokines in Tuberculosis pathology is unclear.

4.4 Macrophage activation in *M. tuberculosis* infection

Macrophages are myeloid cells that play an essential role in inflammation and host defense. Macrophages regulate immune responses and maintain tissue homeostasis. In *M. tuberculosis* infection, macrophages dominate in the immune response from recognition of the bacilli to granuloma formation. Alveolar macrophages are the first line of defense against invading pathogens. Depending on the microenvironment, macrophages can polarize to two distinct phenotypes; M1 phenotype (classical activation pathway) and M2 phenotype (alternative activation pathway) (66). Macrophages are activated via the M1 pathway in the presence of pro-inflammatory stimuli and via M2 pathway in the presence of anti-inflammatory stimuli. Bacterial cell wall components, lipopolysaccharides (LPS) and pro-inflammatory cytokines such as IFN-γ promote the differentiation of the M1 phenotype. M1 activated macrophages produce high levels of oxidative metabolites (eg. nitric oxide) and other pro-inflammatory cytokines like IL-2 (67) that are essential for host defense against *M. tuberculosis*.

4.4.1 Alternatively activated macrophages in *M. tuberculosis* infection

Conversely, activating macrophages in the presence of anti-inflammatory cytokines such as IL-4, IL-10, IL-13 or TGF-β promotes an M2 differentiated macrophages (36, 67, 68). Other enzymes such as arginase and indoleamine 2, 3 dioxygenase activity also switch on M2 polarized macrophages (69, 70). The M2 polarized macrophages attenuate antimycobacterial destructive immunity and promote matrix remodelling and angiogenesis (71).

4.5 The granuloma and latency in Tuberculosis

Upon infection by mycobacteria, a granuloma forms through successive recruitment of both innate and adaptive immune cells by means of complex cytokine and chemokine signals. The granuloma represents the intersection of innate and adaptive immunity. In humans, the granuloma is the hallmark of chronic inflammation. The primary goal of granuloma formation is thought to be to contain the bacilli, truncate pathology and prevent bacilli dissemination (35).

However, granuloma formation need not be thought of as a mere host strategy for entrapping the bacteria. In a successful granuloma, there is a cycle of immune activation and suppression to constantly contain the bacilli and prevent immunopathology (44). The granuloma is also a prerequisite for the necrosis, tissue damage and spread of the mycobacteria observed when containment fails due to immune suppression for example, simultaneous infection by HIV.

For a long time, the granuloma was viewed as beneficial to the host since it coincided with the onset of adaptive immunity and reduction of bacterial growth in the lung. Recent knowledge in animal model have indicated that mycobacteria also benefit from the granuloma (44). The granuloma centre becomes caseous in active disease, containing necrotic macrophages which in

advanced TB form cavities in the lung. Infectious bacilli spill into the airways, especially in cavitary TB, when the structure ruptures. Bacilli are then expelled in respiratory fluid (72).

The granuloma is thought to play a major role in maintaining LTBI and avoiding reactivation of infection through complex immune interactions. For instance, the hypoxic core of the granuloma is thought to induce a dormant bacillary state where there is little or no replication of the bacilli (73). In the granuloma there is a dynamic interplay between dormant mycobacteria and host immune cells. The continuous recruitment of host cells into the granuloma is required to maintain its integrity to prevent reactivation of the bacilli and development of active TB (74).

The granuloma is a characteristic of LTBI infection. During this stage, *M. tuberculosis* remains within the host not inducing disease but also not being eliminated by the host immune response. Two hypotheses of how latency is maintained exist; the classical or "static" hypothesis and the "dynamic" hypothesis (75). The proponents of the classical model of latent infection maintain that the bacilli have the ability to enter a dormant phase, where they are capable of remaining in a state of slow replication and low metabolic activity (76). On the other hand, the dynamic hypothesis suggests that naturally slow growing *M. tuberculosis* disseminate from the granuloma, possibly in foamy cells or epithelioid cells at a very low level. These bacilli reach the alveolar space where they begin to multiply but are killed by the primed immune system (75).

Contrary to the classical model of complete bacillary dormancy in the granuloma, it is becoming clear that LTBI is not a static state with a homogenous population of non-replicating bacilli. One piece of evidence for this is the fact that treatment with Isoniazid, which is only

active against replicating bacilli, for a prolonged period, can have a sterilizing effect (77). This is in line with the constant activation of the bacilli and supports the theory that reactivation negatively correlates with the period of time after infection (75). Even though the exact response of the bacilli during the latent stage has not been fully characterized within humans, the immunological responses have been studied within the many animal models available (78).

4.5.1 Maintaining the granuloma

During the latent stage, expression of TNF-α is needed for maintenance of the granuloma and the continued sequestration of the bacilli. The anti-inflammatory cytokines interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) have been shown to affect the ability of the host to fully eliminate the bacilli. TGF-β primarily functions to maintain T-cell tolerance to self and abundant antigens, while IL-10 performs more of a negative feedback mechanism in response to inflammatory activity. TGF-β expression within the granuloma may serve to inhibit ROI and RNI production within macrophages by reducing the effectiveness of IFN-γ and by reducing T-cell proliferation (78).

4.5.2 Evasion of immune response by *M. tuberculosis*

The *M. tuberculosis* bacillus is a minute infectious agent, but its ability to subvert the host immune response and persist is very complex. The mycobacterium has evolved multiple immune evasion strategies to manipulate its cellular niche to its advantage. *M. tuberculosis* employs many mechanisms to evade both innate and adaptive immune responses to survive and perpetuate its pathology. These immune evasion strategies range from subversion of recognition by the initial innate response to modulation of cell mediated immunity and regulation of its own gene expression. As discussed previously, *M. tuberculosis* has the ability to inhibit some host innate immune functions such as prevention of fusion of the phagosome

and lysosome, and alteration in nitric oxide synthase expression of the host. Mycobacteria also use enzymes such as superoxide dismutase and catalase to binds to and neutralize the effector functions of RNIs and ROIs. Mycobacterial components such as sulphatides, LAM and phenolic- glycolipid I (PGL-I) are potent oxygen radical scavengers (79).

4.6 Dysfunctions in adaptive immune response during *M. tuberculosis* Infection

While no single reason has surfaced for the incomplete elimination of the invading *M. tuberculosis* bacilli, a host of immunological dysfunctions have been proposed as contributing to the ability of *M. tuberculosis* to persist within the host. One major candidate for the inability of the host to fully combat the bacteria is a dysregulation of the normal functionality of DCs within the lungs. Since DCs play a central role in priming the adaptive immune system, subverting the normal functionality of the DC provide the bacterium an avenue to modulate the adaptive immune system to suit its survival needs. The first dysfunction within the host response that indirectly affects the DC occurs immediately after DCs ingest live *M. tuberculosis* bacilli. Once the bacteria are phagocytosed, *M. tuberculosis* has the ability to inhibit phago-lysosomal fusion (72). By blocking this pathway the bacteria reduces the amount of antigen available to the DC to present on MHC I or II, thus reducing DC activation and priming of T-cells. This strategy of immune evasion allows the bacteria to avoid recognition by DCs and limits their ability to activate $CD4^+$ T-cells, which are very important steps in controlling the infection (72). Also, matured DCs and macrophages appear to be diminished in their ability to present antigens to $CD4^+$T cells, which leads to these important cells being persistently infected.

4.6.1 Anti-inflammatory cytokines dampen adaptive immunity to *M. tuberculosis*

Another immune subversion mechanism by *M. tuberculosis* is the production of cytokines that counter-balance the pro-inflammatory responses mediated by $CD4^+$ T-cells, IFN-γ, TNF-α and

the classically activated macrophage (M1) phenotype. During *M. tuberculosis* infection, the secretion of anti-inflammatory cytokines such IL-10 and TGF-β have immunomodulatory effects that dampen the pro-inflammatory milieu. These cytokines function in many ways, both constructively regulating the immune response to the host's advantage and also over-dampening the immune response to the pathogen's advantage and promoting remodelling of the infection site (71)

A major anti-inflammatory player in regulation of inflammation in Tuberculosis is IL-10. In mouse studies, over-expression of IL-10 within resistant mouse strains leads to a decrease in $CD4^+$ T-cell populations within the lungs and reactivation of disease (80). IL-10 may lead to a constructive dampening of inflammation by counteracting the Th1 response from becoming over active and destroying the host through immunopathology. IL-10 may also however, even further down-regulate the Th1 mediated inflammatory responses necessary to control the growth of the bacteria, leading to reactivation of the disease. While it is clear that completely eliminating IL-10 is detrimental to the host, it also appears the bacteria is able to harness this anti-inflammatory cytokine to allow its persistence within the host. In humans, monocytes have been shown to produce IL-10 in response to *M. tuberculosis* infection (80).

4.7 Tuberculosis - HIV immunopathology

Both HIV and *M. tuberculosis* preferentially infect and replicate in macrophages and DCs. HIV infects cells that expresses the CD4 surface marker while *M. tuberculosis* infect host cells through a multitude of cell surface receptors including; MR, CR, SR and TLRs. Macrophages and DCs share these surface markers to permit infection by both pathogens. HIV is an intracellular pathogen whiles *M. tuberculosis* is an intra-macrophage pathogen residing in a phagosome. Infection by both pathogens has a profound influence on the progress of each

disease. As a result, Tuberculosis thrives on the heels of HIV induced immunosuppression while HIV progression to AIDS is accelerated by Tuberculosis (14).

HIV, even though not a typical cytolytic virus, kills infected $CD4^+$ T cells (36, 81) within the granuloma and compromises the granuloma in co-infected patients. HIV infection brings about reduction in the quantity and functions of both $CD4^+$ T cells and $CD8^+$ T cells which play a vital role in immunity to Tuberculosis (81). Recent data shows macaques infected with simian immunodeficiency virus (SIV) had fewer $CD4^+$ T cells in granuloma tissues than those with active Tuberculosis alone (82). Also, HIV-positive patients with lower $CD4^+$ T cells count are more susceptible to Tuberculosis than those with relatively higher $CD4^+$ T cells count (81). In HIV infection, infected macrophages and DCs are killed by CTLs through the FAS/FAS Ligand pathway or the perforin/granzyme/granulolysin pathway. The increased replication of HIV at the site of *M. tuberculosis* or the granuloma disrupts the immunological synapse and induces anergic T cells that impair infected macrophages and DCs from becoming activated to kill and clear the pathogens (81).

Conversely, some cytokines secreted in *M. tuberculosis* infection also activate HIV viral replication. For instance, TNF-α, which is critically secreted by activated macrophages within a Tuberculosis granuloma, acts as a potent activator of HIV replication. *M. tuberculosis* infected macrophages also upregulate CXCR4 (a surface receptor) which facilitates HIV infection (83).

Thus there is a mutual interaction between both pathogens in their pathophysiology. Clinically, HIV modifies the presentation of Tuberculosis and its management because the clinical features of HIV infected patients with Tuberculosis are often non-specific and diagnosis of Tuberculosis can be difficult.

4.8 Effect of HIV on the Tuberculosis granuloma

As previously discussed, the granuloma serves to contain the bacilli and acts as an immune microenvironment for cellular interactions that limit *M. tuberculosis* replication. However, simple formation of a granuloma is not sufficient to control infection. Instead, the granuloma must have optimal immunologic function to physically barricade the bacilli. In latent infection, the host and bacillus coexist with the granuloma serving as the site of bacterial persistence and host resistance. Disruption of the structure or function of the granuloma is what leads to reactivation of LTBI, dissemination of the bacilli and active disease (84). It has been proposed that the increased Tuberculosis pathology associated with HIV infection is caused by functional disruption of the local immune response within the granuloma (81, 84). The disruptions presumably decrease the ability of the granuloma to contain *M. tuberculosis*, leading to increased bacterial growth with more mycobacterial dissemination and severe pathology. The causes of disruption in the granuloma during HIV infection or immunosuppression can be categorized as; (i) a decrease in absolute number and function of $CD4^+$ T cells (ii) an increase in HIV viral load and viral replication at the granuloma microenvironment in the infected person (iii) a disruption of macrophage function and (iv) a perturbation of *M. tuberculosis*-specific T cell function that lead to functional and detrimental changes within granulomas (81, 82, 84). In the granuloma, $CD4^+$ T cells play a critical role by their presence and cytokine functions. Decrease in $CD4^+$ T cells leads to a compromised integrity and the ultimate dissolution of the granuloma.

Conclusion

Immune response to *M. tuberculosis* is complex, comprising different cells and molecules of both innate and adaptive immunity. Although different cells and molecules are required for

protection against Tuberculosis, there is a lack of comprehensive understanding of what exactly constitutes protective immunity.

CHAPTER 5

Diagnosis and Treatment of Tuberculosis

TB is typically a pulmonary infection but it can affect other parts of the body. PTB is diagnosed by demonstrating the presence of the bacilli through microbiological methods and EPTB is diagnosed by finding the bacilli in cytological/histopathological preparation of appropriate clinical samples. Additionally, other methods such as immunological methods can be employed in TB diagnosis. Such methods detect the presence of immune responses to the bacilli. Radiological imagining techniques are used as surrogate diagnostic modalities. This dissertation will not discuss cytological or histological methods for TB diagnosis nor radiological imaging techniques.

5.1 Microbiological diagnosis of *M. tuberculosis* infection

Diagnosis of PTB is usually made by microscopy, bacteriological culture or molecular analysis of patient sputum. Microbiological diagnostic tools are the only direct identification of mycobacteria in clinical specimens. Sputum is the preferred clinical sample for direct *M. tuberculosis* diagnosis in PTB.

5.1.1 Sputum Smear Microscopy

The most widely used method for direct identification of mycobacteria in clinical specimens in developing countries is sputum-smear microscopy. The purulent part of sputum is smeared on a microscopic slide and stained with Ziehl-Neelsen's staining technique to detect Acid-fast bacilli (AFB). The technique is rapid, inexpensive and simple to perform, but it has poor sensitivity. Accurate results also strongly depend on the skill of the microscopist. It is used mainly for suspected PTB cases and is an insensitive technique that performs poorly in young children and individuals who are severely immunocompromised (85).

The need for sputum as a diagnostic sample is a great limiting factor for diagnostics due to the challenges of collecting it from patients and also its complex composition/matrix. The viscosity of the material restricts test sensitivity, increases sample-to-sample heterogeneity and increases costs and labor associated with testing (86). Moreover, sputum is not easy to collect from paediatric patients and severely immunocompromised (HIV/AIDS) patients. These patients often have paucibacillary sputum. Also, up to 30% of suspected TB patients are unable to produce sputum, especially common in children (87). Also sputum become unavailable for follow-up investigation once the patient improves. Therefore, while sputum smear microscopy remains a valuable rapid diagnostic tool in resource-limited settings, it should not be used to exclude TB disease.

A further concern with sputum smear microscopy is the inability to differentiate between non-tuberculous mycobacteria (NTM) and *M. tuberculosis*. NTM are ubiquitous in the environment, diagnosis requires that the bacilli are isolated and identified. For instance, among a group of sputum ZN-positive patients from Cape Town, South Africa, 10% had NTM cultured from their sputum (88).

5.1.2 Bacteriological culture of *M. tuberculosis*

Culturing *M. tuberculosis* from a clinical specimen is regarded as the gold standard for confirming a case of Tuberculosis. Culture for *M. tuberculosis* remains the most definitive means for diagnosis of active Tuberculosis. Culture confirms the presence of live bacilli in the patient. It is able to detect 10 to 100 bacilli per millilitre of specimen making it superior to sputum smear microscopy (89). Culture is more sensitive (70-80%) than smear microscopy and culture detects a higher proportion of cases among patients with symptoms. Bacteriological culture has the advantage of being used for speciation and drug sensitivity testing. However,

mycobacterial culture is an expensive and relatively slow diagnostic technique as it can take about 4 weeks to get results on traditional media (33).

Traditional culture uses a solid medium such as coagulated egg (e.g. Löwenstein-Jensen media) or agar (e.g. Middlebrook 7H10) as a base. Solid media are simple and cost effective to use. However, disadvantages include slow bacterial growth (3-4 weeks) and errors due to manual reading of results. Recent development of more sensitive liquid medium culture techniques that are automated, has allowed for the more rapid detection of TB bacilli, within 7 to 14 days. The mycobacterial growth indicator tube (MGIT) is currently the preferred culture system in high-throughput settings as it is automatable. A major disadvantage of liquid culture is that it is more prone to contamination than solid culture.

Culture of clinical specimens also allows for drug-susceptibility testing (DST), mycobacterial speciation and epidemiological studies through strain typing. Culture-based DST is considered the most significant determinant of drug susceptibility as it can define resistance irrespective of the molecular mechanism responsible for resistance. Bacterial culture requires expensive reagents, extensive biosafety facilities and well-trained workforce to maintain it. This makes it very difficult to establish in resource-poor settings where Tuberculosis is endemic.

5.2 Molecular diagnosis of *M. tuberculosis*

About two decades ago, there were only two tests available for diagnosing Tuberculosis in clinical samples; Sputum smear microscopy test to detect AFBs and the microbiological culture of *M. tuberculosis* on solid media. Due to poor sensitivity and specificity of these conventional methods, a search for a more reliable diagnostic tool for TB led to the introduction of the molecular diagnostic techniques. Molecular diagnosis (genotype-based diagnosis) has a number

of potential advantages over phenotypic-based (bacteriological-based) methods. Bacterial genotype, like in all other living organisms leads to its phenotype, and if the genotype of a particular phenotype is well-described, the genotype may be used to infer the phenotype without the need to culture the bacteria. Molecular methods are more specific and can be performed more rapidly than the conventional bacteriological methods. Molecular applications also allow other sample matrices to be tested. Molecular methods are applied often in drug susceptibility testing and speciation for epidemiological purposes.

5.2.1 Nucleic acid amplification test (NAAT)

NAATs are more specific and more sensitive that the conventional methods. Results are available within a few hours. Beginning from 2009, CDC recommended that NAAT be performed on at least one respiratory specimen for each patient with signs and symptoms of pulmonary TB. NAATs can be performed on direct clinical specimens; sputum, CSF, lymph node aspirates, pleural effusion etc. in AFB smear negative patients. NAATs have the ability to confirm rapidly the presence of *M. tuberculosis* in about 50-80% of AFB smear negative but culture positive specimens. In cases where both AFB smear and NAAT are negative, the likelihood of TB becomes very low. For AFB smear positive patients in a setting with high rates of NTM, a negative NAAT result in many instances can obviate the need for contact investigation.

There are two commercially available NAATs; Xpert MTB/RIF (often called GeneXpert) and Amplified mycobacterium direct test (MTD, Gene-Probe)

5.2.2 Xpert MTB/RIF (GeneXpert)

The development and introduction of the GeneXpert polymerase chain reaction (PCR) technology in TB diagnosis has been a game changer. The technique can detect *M. tuberculosis* in clinical samples and results are rapidly available in less than 2 hours, allowing treatment

initiation on the same day. GeneXpert also has the added value of detecting drug-resistance strains in *M. tuberculosis* diagnosis before initiating treatment. GeneXpert MTB/RIF assay is a real-time PCR technology developed by Cepheid Inc., Sunnyvale, CA, USA (90). In 2010, GeneXpert MTB/RIF was endorsed for used in the investigation of patients suspected of Tuberculosis in regions where MDR Tuberculosis and HIV infection are common (91).

A large-scale multicenter study by Boehme et al. in 2010 showed that the GeneXpert MTB/RIF assay is able to detect *M. tuberculosis* complex with 98.2% sensitivity in smear-positive clinical samples and 72.5% in smear-negative cases. This reduced sensitivity in smear-negative cases was improved to 90% when the assay was repeated three times. The method also showed 99.1% sensitivity and 100% specificity as compared with culture for detecting the most common mutations occurring in the *rpoB* gene conferring rifampicin resistance (92). This makes it an ideal method for rapidly identifying multi-drug resistance (MDR)-TB patients.

In a study by Scott et al., 2011, authors reported Xpert MTB/RIF may provide a more accurate rapid diagnosis of TB in HIV co-infected patients than smear microscopy and other currently available NAATs in TB endemic populations. Xpert MTB/RIF has a sensitivity of 85% and a specificity of 97% for TB diagnosis in sputum samples (93). In a study by Tortoli et al. in Europe, Xpert MTB/RIF showed a diagnostic sensitivity and specificity of 81% and 99% in patients suspected of EPTB (94).

5.2.3 Gene-probe *M. tuberculosis* direct test (MTD)

Gene-Probe MTD test is a transcription-mediated amplification test that targets the ribosomal RNA (rRNA) of *M. tuberculosis*. Gene-Probe was approved in 1995 for use in AFB smear positive specimens. Subsequently enhanced-MTD (E-MTD) test was also approved for used on

AFB smear negative specimens. In smear negative specimens, Gene-probe MTD has a sensitivity of about 70% and specificity of about 98% (95). Gene-Probe can be performed on direct clinical samples.

It should be noted however that, all NAATs fails to discriminate dead bacilli from live bacilli and hence results cannot be used to follow up cases. Also, the presence of inhibitors in the clinical sample matrix can give rise to a false negative result. Other limitations of NAATs are high financial cost, requirement for highly skilled technical staff to operate and interpret data and the potential cross contaminations in open-tube based assays.

5.3 Immunological methods

Immunological diagnosis of TB is based on the host's immune response against *M. tuberculosis*. When individuals are exposed to *M. tuberculosis*, they mount a robust immune response to either kill and clear the pathogen or establish latency. Individuals with LTBI have an immunological memory of the infection through sensitised/primed T cells whose response can be detected. Even though, these sensitised T cells or other inflammatory mediators of *M. tuberculosis* infection can be detected, immunological tests largely cannot discriminate latency from active disease or indicate the site of the infection (96). Immunodiagnostic tools employed in TB diagnosis measure nonspecific mediators of inflammation secreted by both innate and adaptive immune cells, some aspects of the T cell-mediated immune response to *M. tuberculosis* antigens or the detection of specific antibodies against some *M. tuberculosis* antigens by serological tests. Because immunological diagnosis of TB is solely based on immune responses of the host, conditions and chronic illnesses that affect the immune system may hamper the quality of results from these methods.

Immunological diagnosis can detect both active and LTBI, PTB and EPTB but cannot distinguish or classify them (96). Also, immunological diagnosis also fails to differentiate infection by *M. tuberculosis* from NTM.

5.3.1 Tuberculin skin test (also known Mantoux test)

The TB skin test (TST) is an *in-vivo* immunological test used to determine if someone has developed an immune response to *M. tuberculosis*. Immune response can occur if someone currently has active disease or LTBI or had been exposed to *M. tuberculosis* in the past (like those cured of TB disease). A milder response can be seen simply if the patient received the BCG vaccine against TB.

Although Koch in 1890 administered it as a substance that should cure TB, the purified protein derivative (PPD), a refined variant of Koch's 'old tuberculin', has been used in the diagnosis of LTBI in the TST for more than 100 years (97). The TST over the years had been used as a diagnostic aid for LTBI and mainly employed as an epidemiological tool in many settings. The test plays a mostly circumstantial role in diagnosing active disease, often in children (96).

The principle of the TST is based on the fact that infection with mycobacteria produces a delayed type hypersensitivity reaction to certain components of the mycobacterium. The components of the organism are contained in extracts of culture filtrates and are the core elements of the classic tuberculin PPD. Reaction in the skin to tuberculin PPD begins when T cells, which have been sensitized by prior infection, are recruited by the immune system to the skin site where they release cytokines. The result is a hard, raised area with clearly defined margins at and around the injection site referred to as induration.

The TST has several shortfalls including poor sensitivity and specificity, poor reader's objectivity and test results interpretation based on many permutations such as presence or

absence of HIV and or prior BCG vaccination. However, TST still remains the most widely used method to identify TB infection without active disease.

5.3.2 Interferon-gamma release assays (IGRAs)

The discovery of the diagnostic potential of the *M. tuberculosis*-specific antigens, early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) led to the development of the IFN-γ-release assays (IGRAs) (98). The IGRA methods are employed as surrogate markers of *M. tuberculosis* infection and primarily indicate a cellular immune response to *M. tuberculosis*. IGRAs cannot distinguish between LTBI and active TB disease, and should not be used as a sole method for diagnosis of active Tuberculosis (99). A positive IGRA result may not necessarily indicate active TB; however, a negative IGRA result rules out the possibility of both active and latent tuberculosis. The detection of both latent and active disease has been markedly improved by employing IGRAs.

IFN-γ is a key cytokine that plays a critical role in resistance to *M. tuberculosis* infection. *M. tuberculosis* infected individuals respond to *M. tuberculosis* antigen stimulation by releasing copious amount of this cytokine from effector memory T cells. The IGRA assays measures IFN-γ produced after re-stimulation of T cells (96). There are two IGRA methods commercially available; Enzyme-linked immunospot assay (ELISpot) (T-SPOT.TB, developed by Oxford Immunotec, Oxford, UK) and the Enzyme-linked immunosorbent assay (ELISA) (QuantiFERON-TB Gold In-Tube (QFT-GIT), developed by Cellestis, Carnegie, Australia). Both IGRAs have high sensitivity and specificity for detecting exposure to Tuberculosis

The improved sensitivity and specificity of IGRAs over TST are due to the use of antigens encoded by regions of difference 1 (RD1) in the *M. tuberculosis* genome which are absent in

the BCG vaccine or the NTM (96). Since IGRAs are not affected by Bacille Calmette-Guérin (BCG) vaccination status, they are useful in evaluation of LTBI in BCG-vaccinated individuals, particularly in settings where BCG vaccination is administered after infancy or multiple (booster) BCG vaccinations are given.

The merits of IGRAs include i) the test requires a single patient visit to draw a blood sample, ii) results can be available within 24 hours, iii) IGRAs do not boost responses measured by subsequent tests as seen with TST, iv) they are not subjected to reader bias as occurs in TST, and v) they are not affected by prior BCG vaccination (100).

IGRAs also have some limitations which include i) the blood samples must be processed within few hours after collection to ensure while white blood cells are still viable, ii) errors in collecting or transporting blood specimens or in running and interpreting the assay can decrease the accuracy, iii) false positive results can occur with infection other than MTB since, ESAT-6 and CFP-10 antigens are shared with NTM (100), iv) results for certain IGRAs depend on absolute relative lymphocyte number per millilitre of whole blood (96, 100).

5.4 Treatment of Tuberculosis

Treatment of TB is achieved with a multi-drug chemotherapeutic treatment regimen to eliminate the bacteria. Isoniazid (INH) and rifampicin (RMP) are the two most common first line anti-TB drugs, commonly used with pyrazinamide (PZA) and ethambutol (EMB). Standard treatment for presumably drug-susceptible Tuberculosis consists of a two month high intensity multi-drug chemotherapy regimen followed by four months of lower intensity multi-drug chemotherapy. The standard treatment of both PTB and EPTB uses the same multi-drug chemotherapy but different lengths of time. Each of these drugs varies in their capacity to kill *M. tuberculosis* and prevent the emergence of drug resistance. INH and RMP are the most potent bactericidal drugs, killing greater than 90% of bacilli within 7 days of starting treatment

(14). They kill all metabolically active bacilli. PZA, although bactericidal, is used mainly for its sterilizing effect. It is effective for killing bacilli sequestrated by macrophages in an acid environment. EMB is less potent and is bactericidal at high concentrations (14).

Since 1995, combination chemotherapy is been at the heart of the WHO "directly observed treatment, short course" (DOTS) strategy has been the corner stone in the treatment of TB. The goal of DOTS at the individual level was to ensure drug regimen compliance and adherence. DOTS at the national level ensured a political commitment to treatment from each country through efforts to increase case detection, maintain a constant drug supply for those in need and to improve international monitoring and epidemiology. Since 1995 DOTS has been adapted by most endemic countries and is making great strides in detection and treatment (20).

Unfortunately, even though DOTS has been successful and praised by many as a single intervention increasing detection and treatment of Tuberculosis, some regions have seen an increase in multi-drug resistant (MDR) and extensively drug resistant (XDR) and recently total-drug resistance (TDR) strains. MDR Tuberculosis is defined as Tuberculosis that is resistant to INH and RMP, while XDR Tuberculosis is additionally resistant to one or more fluoroquinolones and one or more injectable drugs and TDR Tuberculosis is total complete resistance to all anti-tuberculous drugs available (20).

The fluoroquinolones; ciprofloxacin, levofloxacin, moxifloxacin and ofloxacin, and the injectables; capreomycin, kanamycin and amikacin, are known as second line drugs. These second line drugs are reserved for use when primary treatments fail since they are more toxic or less efficacious. Due to natural mutations such as single nucleotide polymorphisms within the mycobacteria, poor drug adherence practices and irrational use of antibiotics, these resistant strains have been able to spread when they should be kept in check (101). The XDR epidemic

in KwaZulu-Natal, South Africa is a reminder of how dangerous and how rapidly this disease can strike if not properly monitored and treated (102).

CHAPTER 6

Biomarkers in Tuberculosis

Prompt and accurate diagnosis of TB and its effective treatment are fundamental to curtailing the spread of the infection. Furthermore, tackling the large reservoir of latent infection is the cornerstone to TB control since about 10% (higher in HIV-infected individuals) of people with LTBI develop active TB in their lifetime (103). However, the existing approach of diagnosis, treatment and prevention of TB remains inadequate. Biomarkers have the potential to contribute significantly in the fight against TB given that latently infected individuals remain healthy until control mechanisms fail. This suggests that there are protective responses in LTBI individuals that control the pathogen and when this protection fails the individual progress to disease (104). Even though the transition from LTBI to active disease is poorly explained, it remains critical to find a marker that can predict at one point the progress of LTBI to active disease. Biomarkers or biological markers play an important role in this situation and could improve clinical practice by helping decision-making both in diagnosis and treatment.

6.1 What is a biomarker/biological marker?

A biomarker is a characteristic that can be objectively measured and assessed as an indicator of a normal biological process, pathogenic process, or pharmacological response to a therapeutic intervention (2). Biomarkers are molecular substances that can be used to indicate health or disease. Biomarkers can provide information about disease status, risk of progression, likelihood of response to treatment or of drug toxicity and protective immunity after vaccination (3). Biomarkers span from simple characteristics, such as high blood pressure as an indicator of risk of stroke, to more complex characteristics such as gene mutations as an indicator of risk of particular cancers.

Biomarkers can be either host or pathogen-specific and may advance knowledge by providing information about the pathogenic process, including the current health status and future disease risk of the patient. In clinical care, biomarkers offer the possibility of a surrogate endpoint that substitutes for a clinical endpoint. The most valuable biomarker measures an event that is directly involved in pathogenesis or protection or which changes early during therapy and is related to the pharmacologic or pharmacokinetics of the therapy.

In TB, there is an urgent need for a specific biomarker to classify patients at a single time point as having active disease, LTBI or no disease. Other potential applications for biomarker(s) in TB include predicting risk of reactivation and monitoring treatment response. Biomarkers may also provide pragmatic endpoints for clinical trials by serving as surrogate markers of cure following Tuberculosis treatment. Another application could be indicating protective efficacy following TB vaccination.

6.2 Clinical application of Tuberculosis biomarker(s)

A potential application for biomarker(s) in TB is the ability to classify patients at diagnosis or early during treatment according to their risk of relapse. This might then allow resources to be focused on patients with higher likelihood of poor outcome and also promote policies to shorten treatment duration of good responders. The decreased or shortened treatment regimen will help to promote drug compliance in TB treatment. Similarly, biomarkers that accurately indicate the risk of reactivation of LTBI in specific individuals might facilitate the targeted application of Isoniazid prophylactic treatment in endemic areas (105).

The diagnosis and monitoring of PTB in children has historically posed unique challenges due to children's inability to produce sputum. This problem has been amplified by the HIV/AIDS epidemic, which has increased the number of children with TB and made its clinical and

laboratory diagnosis more difficult (106). Also it is difficult to get sputum from adults when they improve after treatment initiation. IGRAs are not useful for monitoring of treatment. A biomarker with the prospects of diagnosing TB and monitoring treatment would be of enormous value in diagnosing TB in children and monitoring treatment response (104).

In research, the lack of a simple biomarker to indicate or predict the different clinical outcomes of *M. tuberculosis* has been given as an important reason for the failure of developing new drugs and vaccines superior to the BCG against TB (107). A TB biomarker would serve as surrogate endpoint and also identify correlates of protection to accelerate TB vaccine development (104).

6.3 Candidate biomarkers in Tuberculosis

A large number of candidate biomarkers for TB have been proposed and investigated. Biomarker studies have utilized blood, urine, saliva, exhaled breath and sputum samples to identify molecules for indicating or predicting the different clinical outcomes of *M. tuberculosis* infection (108). Both host specific and pathogen specific markers have been investigated in these clinical specimens. However, among these clinical sample matrices, peripheral blood remains an attractive sample type due to the ease with which this sample can be obtained in all suspected patients. In blood samples; genes, proteins, lipids and metabolites can be measured for biomarker analysis (3).

6.3.1 Blood-based biomarkers for Tuberculosis

In blood, markers such as non-specific pro-inflammatory cytokines, TNF-α, *M. tuberculosis* specific $CD4^+$ T cells, *M. tuberculosis* specific antibodies and non-specific immune activation markers have been investigated as potential biomarkers for diagnosing or predicting Tuberculosis.

Non-specific immune activation markers

Several studies have examined non-specific markers of immune activation as predictors of TB disease outcome. Serum neopterin is a soluble marker of macrophage activation that is a recognised prognostic marker in HIV/AIDS (3). Levels of serum neopterin are increased at the time of diagnosis in TB and decline during and after treatment. In HIV negative patients with TB, serum neopterin was raised after completion of anti-TB treatment and was associated with relapse (41).

Another activation marker, intracellular adhesion molecules-1 (ICAM-1), has also been shown to rise at baseline and fall with treatment. ICAM-1 is a ligand for leucocyte integrins that are mainly expressed by endothelial cells. A soluble form (sICAM) is released into the blood stream. sICAM levels are raised in TB patients at diagnosis in proportion to disease extent, and decrease in response to anti-TB treatment (3).

Serum urokinase plasminigen activator receptor (suPAR)

This is a cell surface receptor involved in cell adhesion and motility that is mainly expressed by macrophages and monocytes. The soluble form of this receptor is raised in active TB patients and relates directly with number of bacilli in sputum. The levels fall with treatment (109).

Specific pro-inflammatory cytokine levels

Serum cytokines like IL-2, IL-9, IL-13, IL-17, and TNF-α have been reported as adjunctive biomarkers for active TB from LTBI and NTM disease (110). Whole blood cells stimulated by *M. tuberculosis* antigen in vitro have higher TNF-α and IL-12 production in TB patients

compared with LTBI controls (111). Again, these cytokine markers warrant further validation before clinical application.

Serum C - reactive protein (CRP)

CRP is an acute phase protein produced by the liver that promotes phagocytosis. Serum CRP levels are increased in TB, particularly in patients with advanced disease and rescind with treatment (3, 111). Serum CRP level is elevated in multiple conditions with acute or persisting inflammation other than TB.

Erythrocyte sedimentation rate (ESR)

ESR is a non-specific indicator of chronic inflammation. ESR is increased in TB and rescinds after treatment. Although an elevated ESR may be expected in both adults and children with TB, several reports have found one-third of children with TB have normal ESR at the time of diagnosis, and consequently there seems to be little value in using ESR as a diagnostic test for childhood TB (111).

Adenosine deaminase (ADA)

ADA is an enzyme of purine metabolism which catalyses adenosine into inosine. It is found in most tissues particularly in lymphoid tissues. ADA estimation in pleural fluid has particularly been found useful in the diagnosis of Tuberculosis pleurisy, and high levels in CSF are often to be associated with Tuberculosis meningitis (112). High ADA levels have also been reported in effusions due to rheumatoid arthritis, lymphoma, chronic lymphocytic leukaemia, empyema and other host of chronic diseases other than Tuberculosis pleurisy (113).

6.3.2 TNF-α *M. tuberculosis* specific $CD4^+$ T cells

Previous investigation of T-cell responses in *M. tuberculosis* infection showed that the presence of single-positive TNF-α *M. tuberculosis*-specific $CD4^+$ T cells is a strong predictor of diagnosis of active disease versus latent infection (114). A validation study shows that the sensitivity and specificity of the flow cytometry-based assay are 67% and 92%, respectively, with positive and negative predictive values of 80% and 92%, respectively. As such, the proportion of single-positive TNF-α *M. tuberculosis*-specific $CD4^+$ T cells may be a new reference for the rapid diagnosis of active Tuberculosis. Other studies also have also shown similar findings and suggest combined measurement of T-cell phenotype and function as a highly discriminatory biomarker of TB disease activity. This approach requires validation in large-scale prospective studies (114, 115).

6.3.3 *M. tuberculosis* Specific antibodies as biomarker

Some mycobacterial antibody levels are raised at diagnosis and may be modulated during treatment. Azzurri et al., 2006, examined antibody levels to 10 mycobacterial antigens in 168 patients before, during and at completion of treatment. Household contacts were used as controls. 10 patients failed therapy. Antibodies to ESAT-6 and Rv2626c were higher in patients than controls and decreased with treatment (116). The limitation was that antibodies failed to distinguish treatment failures those cured. Nonetheless, a number of antibody-based TB diagnostic tests have been developed and are commercially available, although clinical validation is usually absent and current test performance is inconsistent.

6.4 Sputum-based biomarkers for Tuberculosis

Unlike blood-based markers, which are easily influenced by systemic inflammation or infection, materials in the sputum are more directly from the disease site in PTB. In sputum,

both whole mycobacteria and its antigens or proteins have been investigated as biomarkers to diagnose PTB.

6.4.1 Two (2) months sputum culture

Sputum culture after 2 months of anti-TB treatment had been widely investigated as a predictor of cure of Tuberculosis patients (3). This marker has been examined at different levels: the individual level, across trials and within trials. At the individual level, 2 month culture positivity was an independent predictor of relapse. However, the marker was relatively insensitive as it identified only half of all relapse patients and lacked adequate positive predictive value for use as a guide to treatment of the individual (3, 41).

6.4.2 Levels of *M. tuberculosis* Antigen85 in sputum

M. tuberculosis specific markers, such as Ag85 in sputum, have also been investigated as biomarkers for TB. Ag85 complex is a 30-32kDa family of three proteins (Ag85A, Ag85B and Ag85C). By virtue of their strong potential to induce a Th1 immune response, members of the Ag85 family are ranked among promising biomarkers of TB infection. Several studies have evaluated its application as a biomarker in TB treatment response. The magnitude and duration of increases in Ag85 during the first week of therapy could predict relapse in treatment (3, 41).

In a study by Willis et al., 2009, MTBAg85 was found to be induced by the administration of Isoniazid. Concomitant administration of benzoxinorifampicin prevented the induction of MTBAg85. Also, induction of Ag85 was impaired in isoniazid-resistant strains, potentially limiting its application as a marker for clinical purposes (3).

6.5 Urine-based biomarkers

Urine represents a clinical sample that is easy to collect from both adults and children. Urine specimens have been used extensively to evaluate several mycobacterial antigens and DNA products for diagnosis of *M. tuberculosis* infection (117). Mycobacterial antigens including LAM are known to be shed into urine of TB patients (3, 118). Some studies have indicated a correlation of urinary LAM antigen with sputum bacillary burden at the time of diagnosis; this may indicate a potential role as a biomarker. Currently, there are commercially available assays that can detect LAM in the urine of active TB patients. Although the sensitivity of this test is disappointing in HIV negative patients, moderate sensitivity and high specificity are observed in HIV positive patients with advanced immunodeficiency (3, 118).

6.5.1 *M. tuberculosis* IS6110 DNA (trDNA)

M. tuberculosis IS6110 DNA is a small fragment of MTB DNA that is shed in urine. These DNA fragments, termed transrenal DNA (trDNA), are thought to arise due to apoptosis of host cells. Due to the ease and safety with which urine samples can be obtained, there is a growing interest in detection of pathogen markers that can be measured and quantified in urine. Some studies have reported the presence of MTB IS6110 DNA in urine of patients with pulmonary TB but not in healthy controls (118). Monitoring of MTB trDNA may be particularly useful in situations where sputum cannot be readily obtained, such as in children.

6.6 Volatile organic compounds produced by *M. tuberculosis*

Some volatile organic compounds produced by MTB have been detected in exhaled breath of patients with active TB (3). The potential of these compounds in diagnosis and monitoring of treatment are being vigorously pursued.

6.7 Challenges of Tuberculosis biomarker search

Identifying a simple, reliable and sensitive biomarker for TB is challenging due to lack of a gold standard for infection or defined protective immunity. Currently, our understanding of what strictly constitutes protective immunity to TB is incomplete. Most of the current understanding of *M. tuberculosis* immunopathology is generated from animal models. Although the animal models have yielded considerable information on the mechanisms of pathogenesis, innate and adaptive immunity to the pathogen, a clear understanding of human pathogenesis of *M. tuberculosis* is not clearly defined. It is clear that the animal models mimic some immunopathology but not all. Studies of the human immune responses to *M. tuberculosis* most often compare responses in healthy latently infected individuals with those with active disease, but these studies have not yet identified a clear mechanisms or correlates of effective immunity to human *M. tuberculosis* infection (111).

Also immune response to *M. tuberculosis* is very complex, comprising different cells and molecules of both innate and adaptive immunity. The lack of comprehensive understanding of what exactly constitutes protective immunity hampers the definition of what a good biomarker should be, and whether it should be host specific or pathogen specific. The understanding that a pro-inflammatory cytokine milieu is protective against *M. tuberculosis* was criticized when copious amounts of INF-γ did not confer protection against *M. tuberculosis* infection after MVA85 vaccine challenge (119). Other factors such as host genetic diversity, presence of environmental microbes like helminths, presence or absence of infections by NTM and other metabolic/inflammatory conditions eg. Diabetes, weight and others (105) play a key role in determining the clinical outcome of *M. tuberculosis* infection in an individual.

The search for a TB biomarker has been challenging because validation of a candidate biomarker requires validation against clinical endpoints. This is difficult because TB endpoints are poorly defined.

6.8 Tuberculosis biomarker and other diseases

An ideal TB biomarker would predict the disease risk from latent stage, allow monitoring of anti-TB therapy and indicate vaccine efficacy and protection. Currently, most candidate biomarkers for TB are general indicators of intracellular infection and subsequent immune activation rather than a specific marker for TB. This is anticipated since other pathogens activate pathways associated with immune activation and inflammation. At the cellular level, infection with any pathogen activates inflammation in similar ways irrespective of the nature of pathogen. It is therefore not surprising a large number of biomarkers relying on similar host responses would be shared between similar disease processes.

6.9 A plausible biomarker for Tuberculosis

Even though most of these biomarkers have been intensely investigated, none have been validated for clinical application in TB disease. The lack of a validated, simple, reliable and sensitive biomarker for TB currently hampers the progress in the global fight to eliminate TB. A highly plausible biological biomarker with prospects of better diagnosing, prognosing and monitoring TB treatment response is the activity of the enzyme, indoleamine 2, 3 dioxygenase (IDO). The concept that cells expressing IDO can supress T cell responses and promote tolerance is relatively new perspective in immunology. However, there is considerable evidence that IDO activity impairs T cell functions and contributes to TB pathogenesis.

Conclusion

In conclusion, a biomarker for TB is urgently needed to step-up the fight against TB. A simple, reliable and sensitive biomarker would improve clinical decision making, diagnosis and monitoring anti-TB, and eventually accelerate development of an improved vaccine other than the BCG vaccine.

CHAPTER 7

Indoleamine 2, 3 dioxygenase

Indoleamine 2, 3-dioxygenase (IDO) is a cytosolic enzyme that catalyses the initial rate-limiting step of Tryptophan (Trp) degradation along the Kynurenine pathway (120). Trp is essential amino acids in mammals (ie. it cannot be synthesized *de novo*). At the cellular level, Trp is required for the synthesis of essential cellular factors such as nicotinamide adenine dinucleotide (NAD+) and serotonin involved in important metabolic functions. Trp depletion by IDO consumption through the Kynurenine pathway inhibits T cell maturation, proliferation and activation. Furthermore, products of Trp catabolism collectively known as kynurenines potently inhibit CMI to induce immune tolerance (121).

In the last few years, IDO has been extensively investigated for its immunoregulatory roles in certain physiological and pathological conditions. Induction of IDO has been demonstrated to play a crucial in immune tolerance during pregnancy, oncogenesis, transplantation, and infectious diseases (122, 123).

In humans, IDO activity was originally described to contribute to maternal-foetal tolerance, an example of foreign antigen which the immune system must tolerate. IDO activity was first found constitutively expressed by human extra-villous trophoblast at the maternal-foetal interface (123, 124). In infectious diseases, IDO was initially considered to play an antimicrobial role by acting through local depletion of Trp, which is essential for the growth of some microbial pathogens. Recently, upregulated IDO activity has been demonstrated to significantly contribute to the pathogenesis and severity of some infectious diseases or cancers through induction of peripheral tolerance to pathogens and tumor cells (125).

7.1 IDO and immune suppression

Historically, IDO has been considered to be part of innate host defense against invaders during inflammation. It is now clear that not all the roles of IDO in immune response are beneficial to the host. As part of its functions, IDO works to control the primary immune responses from exacerbation through acting to inhibit T cell activation. By this, IDO functions as negative feedback control mechanism to counteract the primary immune activation which is beneficial to the host. However, overexpression of IDO may result in immunosuppression and tolerance to the invading pathogen or tumor cell (123). Chronic inflammation that presents with persistent immune activation releases high level of pro-inflammatory cytokines including INF-γ. INF-γ is by far the most potent inducer of IDO expression (126). Thus chronic immune activation may have resulted in chronic suppression (127) incapable of abrogating the inflammatory responses, in which case IDO's immunomodulatory role is not beneficial to the host anymore but the invading pathogen or tumor cell.

Several mechanistic studies from the mouse model have demonstrated that IDO activity suppresses immune responses especially cell-mediated immunity. In a mouse model of pregnancy, administration of 1-Methyl-tryptophan (1-MT), a synthetic inhibitor of IDO, led to rejection of the pregnancy due to inhibited IDO activity in vivo (124, 126).

In humans, overexpression of IDO activity has been associated with escape and poor prognosis of some malignancies including colorectal cancer, ovarian carcinoma and endometrial carcinoma (121, 128). In particular to colorectal cancer, tumor cells express IDO through induction of IFN-γ in the microenvironment. By this, tumor cells supresses infiltrating tumor specific T cells through IDO activity to create a tolerogenic microenvironment to evade host's immunity (129).

Also, IDO activity plays a critical role in the escape of some infectious pathogens from immune destruction. For instance in infection caused by intra-macrophage pathogens including *M. tuberculosis* and *Chlamydia pneumoniae* where pathogens are rarely cleared by the primary immune responses, suppression of T cell immunity provides the milieu for pathogen escape from quiescence to cause severe pathology (130).

7.2 IDO suppression of adaptive immune responses

IDO activity supresses the adaptive immune responses through attenuation of T cell functions and through IDO's effect on APCs. IDO activity supresses T cells responses in two ways; First through Tryptophan depletion and secondary through generation of Kynurenine metabolites such as athranilinic acid, quinolinic acid and oxygen free radicals which regulate T cell proliferation and survival (120, 121).

The first effect of IDO activity on T cells is the rapid consumption of Trp from the local microenvironment. Trp depletion acts as a potent regulatory signal through the molecular stress-response pathways such as general control nonderessible (GCN) 2 kinase and the mammalian target of rapamycin (mTOR) that respond to amino-acid. It is evident that local Trp depletion as well as increased Kyn act by up-regulating the GCN2 kinase which promotes cell cycle arrest and impairs T cell maturation, proliferation and functions leading to generation of anergic T cells. Excess addition of Trp reverses and restores T cell functions (126, 131).

Secondly, Trp catabolism via the IDO catalysed pathway generates Kynurenines (Kyn) (figure 7.1). Accumulation of Kyn potently inhibits T cell functions and promotes differentiation of naïve T cells into T regs and T cell apoptosis (123).

IDO activity has an indirect effect on APCs especially macrophages and DCs through Trp depletion and downstream Kyn production. Even though antigen presenting capacity is not attenuated by IDO, the effect of responding T cell cells is diminished. This affects the full activation of cells such macrophages (120, 132).

7.3 "IDO-competent" cells and induction in tissues

Although various cells can express IDO, the expression of IDO is often restricted to certain specific cell subsets of APCs that appear specialised for rapid, high level upregulation of the IDO gene in response to inflammatory stimuli. Macrophages and DCs in this regard are the only "IDO-competent" cells with the capacity to up or downregulate IDO expression in response to external stimuli depending on the microenvironment (121). DCs are by far the most potent cells in terms of IDO expression. Other cells types such as fibroblasts, endothelial cells, lung epithelial cells and some tumor-cells line can also express IDO usually after exposure to IFN-γ (122). In humans, IDO is highly expressed in organs such as lung, epididymis, small intestine and placenta. In most other organs IDO expression is low and is usually in response to IFN-γ (126).

Pro-inflammatory signals such as IFN-γ and TNF-α as well as signals from T cells and bacterial components like LPS are known to induce IDO expression in APCs. Also, ligation of CD40 on APCs up-regulates anti-inflammatory pathways including IDO expression. Recently, constitutive expression of IDO by human myeloid cells has been reported (133).

7.4 IDO and human pathologies

Even though the contribution of IDO-expressing cells to specific disease states have not yet been mechanistically defined, elevated IDO activity has been linked to the escape of the invading agent. Based on studies from both humans and the mouse models, IDO might have a

pathogenic role in certain disorders in which the immune system paradoxically fails to respond in a protective way by killing and clearing the invader from primary inflammation or immune activation. IDO-induced tolerance is implicated in the continual sustenance of the invader and the resulting chronic disease. Recent data indicates IDO participates in a broad spectrum of immune responses during chronic infections, immune-escape of cancer cells, tissue inflammation, transplant rejection and autoimmunity (122). This thesis will discuss only IDO in infectious diseases and IDO in cancer immunopathology.

7.4.1 IDO activity in infectious disease

In infectious diseases, IDO activity can have opposing roles in host defense against pathogens. First, IDO activity can play a dominant role in directly suppressing pathogen replication through Trp depletion as is observed during Toxoplasmosis or Chlamydial infections and some viral infections. Secondly, IDO can also dampen host protective immunity, thus indirectly leading to increased pathogen burdens and escape as occurs during leishmaniasis and TB (10, 134).

The inhibitory roles of IDO activity on bacteria, virus, fungi and protozoa have been described since the early 1980s. Early reports on IDO activity demonstrated its role as an antimicrobial agent towards intra-macrophage pathogens such as *Toxoplasmas gondii* and *Chlamydia species*. Pathogens such as *Chlamydia species* are unable to synthesize Trp on their own from biosynthetic precursors. The pathogen depends entirely on the host for Trp to replicate. IDO depletion of Trp in the microenvironment acts as an anti-microbial mechanism that kills the pathogen. This represents an innate mechanism of pathogen elimination (135, 136).

On the other hand, pathogens such as *M. tuberculosis* seem to leave nothing up to chance. *M. tuberculosis* contains the entire biosynthetic toolset for all 20 amino acids (137). *M.*

tuberculosis is not affected by the change in Trp concentration in the microenvironment. *M. tuberculosis* takes advantage of the depleted Trp-induced immunosuppression to reactivate and cause active disease.

7.4.2 IDO in Cancer immunopathology

IDO is expressed in many cancers, and high IDO expression is associated with poor prognosis in a variety of cancer types. During tumor development, cancer cells acquire certain cell-intrinsic characteristics such as immortalization, growth signal self-sufficiency, resistance to apoptosis mechanisms as well as properties that are defined through the interaction with host environment (cell extrinsic). Among these properties, the capacity of cancer cells to interact with the immune system represents a crucial step in the process of malignant transformation. Indeed, the "immune system-tumor cell" interaction plays a vital and dual role in tumor development both by eliminating tumor cells and by facilitating tumor escape from immune control.

It is increasingly clear that tumor cells induce tolerance to their own antigens (138). Tumor cells can also evade local destruction despite the presence of tumor-reactive T cells. IDO participates in evasion by two ways; a) by directly supressing immune effector T cell functions in the tumor microenvironment (123) and b) as a tolerogenic mechanism expressed by host APCs that cross present tumor antigens. Also inhibition of clonal expansion and survival of antigen-specific T cells in tumor draining lymph nodes in myeloma has been shown to be due a population of IDO expressing plasmacytoid DCs (123).

In humans, IDO expression has been correlated with poor prognosis in patients with prostate cancer, endometrial carcinoma, and colorectal cancer. For example, in colorectal cancer, IDO-expressing-tumor cells function to reduce the effector functions of tumor-reactive T cells (129).

7.5 The Kynurenine pathway of Tryptophan metabolism

Trp cannot be synthesised *de novo* and thus has to be obtained through external sources mainly diet. Once absorbed from diet, Trp travels round peripheral circulation either bound to albumin or in free form. In humans, Trp is predominantly metabolised by the brain tissue. Trp is metabolised via the Kynurenine pathway and is essential in several fundamental physiological processes including neuronal excitability, cell maturation and proliferation in various cell types particularly lymphocytes.

As shown in Figure 7.1, Trp is catabolised into Kyn by two haem-containing enzymes namely IDO and Tryptophan 2, 3 dioxygenase (TDO). IDO is responsible for the oxidative metabolism of Trp to serotonin or Kynurenines (139). Interestingly, enhanced Trp metabolism via the Kynurenine pathway is known to starve serotonin production which is responsible for the induced depression and mood changes associated with chronic infections such as Tuberculosis and HIV-infection (140).

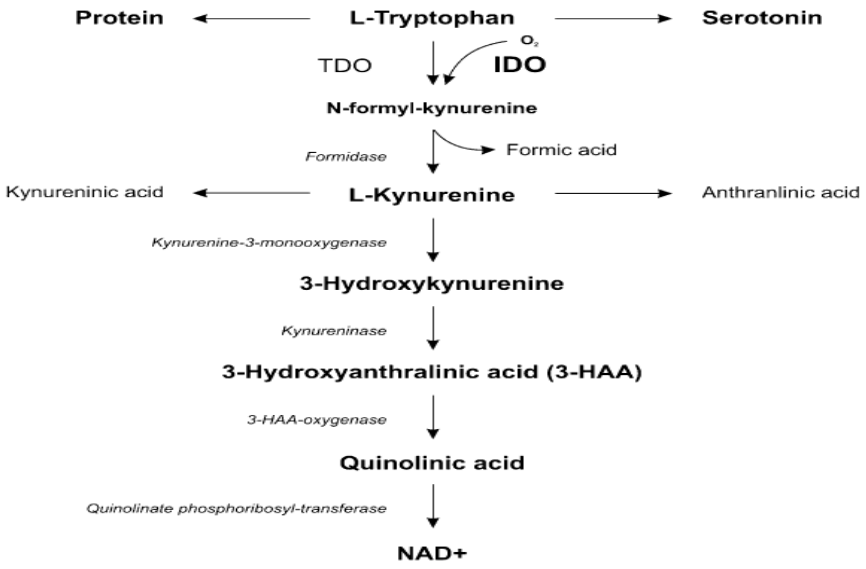


Figure 7.1: A simplified diagram of the kynurenine pathway
Diagram indicates the principle enzymes, indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO), and the subsequent formation of kynurenine and its metabolites from tryptophan (140).

7.6 IDO biochemistry and gene regulation

There are two genes that encode unrelated heme-containing enzymes that catalyse oxidative degradation of tryptophan: IDO and TDO. Each enzyme catalyses the same reaction, oxidative cleavage of the 2,3 double bond in the indole ring, which is the first and rate limiting step in Trp degradation. TDO expression and activity is mainly confined to hepatocytes. TDO expression is not induced or regulated by signals from the immune system. On the other hand, as previously discussed, IDO-expressing cells are found in many tissues, and expression is highly induced in APCs. IDO is also subjected to a complex array of immunological signal regulations.

At the mRNA level, IDO transcription is promoted by factors such as Foxp3 and interferon regulatory factor (IRF)-8. Signal transducer and activator of transcription 1 (STAT 1) and IFN-regulatory factor 1 (IRF1) are also known to function cooperatively to mediate the induction of IDO expression by IFN-γ. Also, the presence of Interferon Stimulated Response Element (ISRE) and IFN-γ Activated Sequence (GAS) elements in the proximal gene promoters of

mammalian IDO genes are associated with IDO expression and induction. Other molecular signals from cytokines, such as transforming growth factor beta (TGFβ), IL10, TLRs, B7, CD200, glucocorticoid-induced TNFR family related gene (GITR), 0X40, programmed cell death(PD)-l, and Aryl Hydrocarbon Receptor (AhR) ligands, may also promote IDO expression (141). At post-translational level, the regulatory factor, suppressor of cytokine signal 3 (SOSC3), binds to IDO and targets the IDO protein for ubiquitination and rapid degradation.

7.7 Regulation of functional IDO activity in tissue

IDO is a cytosolic enzyme and is not secreted. The metabolic effect of IDO begins as inherently local signals. However, the immunological effects of IDO are not confined only to the cells expressing IDO. Neighbouring cells may sense and respond to secreted Kyn and also to the reduction in access to Trp in the microenvironment. By these mechanisms professional APCs expressing IDO can affect both the APC itself and also neighbouring T cells that interact with the APC.

IDO activity is tightly controlled *in vivo* even though the mechanisms of post-translation regulations are not well explained. Regulation of protein stability and inhibitors of the enzyme active site may be involved. Nitric oxide is also believed to be directly involved in inactivating and promoting proteasome-mediated degradation of the IDO protein.

Conclusion

In conclusion, IDO has diverse physiological roles and not all the roles may be beneficial to the host. IDO participates in both innate and adaptive immune responses. When expressed by APCs, IDO is centrally positioned to link the two arms of the immune system to create peripheral tolerance to avoid immune overreaction. Alternatively, overexpression of IDO is

deleterious to the host since it provides the enabling milieu for pathogen and tumor cells to escape. In chronic infections where the pathogen evades being killed and cleared such as *M. tuberculosis,* the pathogen may take advantage of elevated IDO activity and induced immune suppression to escape protective immunity to cause severe pathology.

CHAPTER 8

Role of IDO in Tuberculosis and HIV infections

Long before HIV/AIDS was first reported, it was known that measles virus could render individuals immunosuppressed and that TB commonly occurred following an outbreak of measles. In this era, HIV infection is leading cause of immunosuppression in individuals and the driver of TB. Co-infections with both *M. tuberculosis* and HIV are addictive on the immune system. Both infections induce and sustain chronic inflammation.

Recent studies have found elevated IDO-mediated Trp breakdown and its immunoregulatory effect in both infections (7, 9, 142, 143). By reducing the local Trp concentration and producing immuno-modulatory metabolites, IDO potently inhibits T cell functions, generates T regs and anergic T cells, leading to immune suppression and escape of the pathogen.

Infections with both HIV and *M. tuberculosis* appear to have great impact on each other, accelerating deterioration of immune functions. HIV infection causes severe impairment of T cell responses by depletion of functionally competent $CD4^+$ T helper cells and CD8+ T cells. Even though the exact mechanisms of impairment are not fully understood, some inhibitory molecules on T cells are known to be activated (144). Also, elevated levels of IFN-γ and Kynurenines in HIV/TB patients point toward a participation of IDO in suppression of T cell functions. Furthermore, increased IDO mRNA levels measured in peripheral blood mononuclear cells (PBMCs) of HIV/TB co-infected patients also supports the participation of IDO activity in the induced immunosuppression and immunopathology of both infection. Bosso et al. reported that both $CD4^+$ and $CD8^+$ T cells are suppressed by IDO activity TB-HIV infection. Inhibition of IDO activity by 1-methyl tryptophan (1-MT) reversed and restored both $CD4^+$ and $CD8^+$ T-cell functionality (6, 10, 142).

8.1 Diagnostic significance of IDO activity in Tuberculosis

Recent studies have shown IDO activity to be a novel prognostic factor not only in cancer patients but also in TB. In 2012, Suzuki et al. reported PTB patients had significantly higher IDO activity at the time of diagnosis than controls and high IDO activity correlated with time to death. The same group also reported in 2013 that pleural fluid from TB pleurisy patients showed significantly higher IDO activity compared with pleural effusion from non-TB pleurisy (7, 9).

Almeida and colleagues reported upregulated IDO mRNA expression and activity in sputum of PTB patients among a host of biomarkers invested for TB. Out of the numerous biomarkers investigated, including cytokines and other immunological genes, IDO was the best maker of TB infection and response to therapy. IDO mRNA expression decreased over 500 fold from baseline within two (2) weeks of starting anti-TB treatment, and IDO activity normalized to that of the control group by the end of the first month into TB treatment (6).

Several other studies have linked increased IDO activity to immunosuppression and subsequent progress to active TB disease. Most of these studies have focused on the association of increased IDO activity and pathogenesis. Despite their notable findings, the above studies did not comment on the diagnostic usefulness of elevated IDO activity in their discussion. To date however, the clinical significance and the potential of elevated IDO activity in Tuberculosis has not received much attention, even though it is linked to pathogenesis, severity and progress to death.

In conclusion, this study investigates the clinical usefulness of IDO activity in a longitudinal study to determine if IDO activity could be used as a diagnostic/prognostic biomarker for active TB disease.

CHAPTER 9

Analytical method selection

9.0 Introduction

There are multiple available methodologies and specific types for IDO determination. IDO gene expression, protein and activity have been determined in tissue biopsies, blood (serum/plasma), sputum, pleural fluid and other appropriate clinical specimens. IDO mRNA expression and protein have been studied using reverse-transcriptase PCR (rtPCR), western blot and radio-immunoassay or immunofluorescence methods (6, 145, 146). Recently, flow cytometric methods have also been described (146). The enzymatic activity of IDO can be determined through Kynurenine-to-Tryptophan ratio in blood using liquid chromatographic-mass spectrometry (LC-MS) methods or ELISA (7, 9, 128, 145, 147).

IDO mRNA or protein expression does not always agree with IDO activity in vivo. This is because IDO activation in tissue is post-translationally regulated. However, since IDO activity leads to the breakdown of local Trp, IDO activity influences the concentration of Trp metabolites (Kynurenines) in blood. Therefore, for clinical/diagnostic purposes determination of Kyn-to-Trp ratio is currently used as a reliable marker to monitor IDO activity. In this study, we determined IDO activity in plasma by simultaneously measuring plasma Kyn and Trp concentrations using ultra-performance liquid chromatography mass spectrometry (UPLC-MS/MS). The objective of this section was to optimize and validate a simple, rapid, selective (specific) and sensitive UPLC-MS/MS method for determination and quantitation of IDO activity in plasma.

9.1 Method and instrument selection

Determination of IDO activity by LC-MS represents a preferable alternative to conventional methods such as the enzyme immunoassay methods (eg. ELISA). LC-MS methods have high

analytical specificity and analytical sensitivity and allow simultaneous measurement of both Kyn and Trp in blood (plasma/serum). LC-MS methods also have an added advantage of being able to determine the main metabolite (Kynurenine) and its isoforms due to the high analytical sensitivity of mass spectrometry (148). The conventional immunoassay method suffers relatively low analytical specificity and sensitivity due to non-specific binding of antibodies and also its relative high cost of assay reagents compared to LC-MS.

The recent advances in mass-spectrometry have enabled most mass spectrometer devices to be linked to high pressure separation instrument such as UPLC or HPLC systems. Hyphenated systems (LC-MS) are a combination of two selective techniques that allow analytes of interest in highly complex matrices to be separated by chromatography and quantified by mass-spectrometry. Liquid chromatography differentiates compounds by their physico-chemical properties whiles mass-spectrometers differentiate compounds by their masses (ie. mass-to-charge ratio). It is this dual selectivity that makes hyphenated applications such a powerful analytical tool. LC-MS also allows a low detection limit with minimal sample volume and generates more information on the analyte such as isoform quantification.

9.2 Mass spectrometry

Mass spectrometry is an analytical tool in which analytes of interest are converted to charged ions and subsequently separated according to their mass-to-charge ratio. The resulting mass spectrum is a plot of the relative abundance of generated ions and their mass-to-charge (149). Mass spectrometry is one of the most sensitive analytical techniques currently available. It can detect very low concentration of analytes in matrices. The most critical components of mass spectrometry are the ionization techniques and the mass analyser.

There are many types of mass spectrometers available for interfacing with LC systems. One of the more common systems used for LC–MS is the single quadrupole mass spectrometer; this system provides a mass spectrum for each chromatographic peak that elutes from the LC column and is analyzed by the MS system. The triple quadrupole MS/MS system is most commonly used for the detection of small molecules and metabolites (148). Other common mass spectrometry analysers include time-of-flight (TOF) systems, which provide a higher mass resolution spectrum from each component that is assayed enabling the detection of intact proteins. A Triple-Quadrupole tandem mass spectrometer (MS/MS) was used in this study. The operational principles will be discussed later under instrumentation.

9.3 Chromatography

Chromatography is a physical separation technique in which components to be separated are selectively distributed between two phases; a mobile phase flowing through a stationary phase bed. Chromatography may be a preparative step for MS analysis or analytical in itself. Chromatographic techniques are named after the mobile phase used for the separation. For example, liquid chromatography means the mobile phase used is liquid and gas chromatography means the mobile phase used is gas. During the chromatographic process, the components of mixtures/matrices are separated according to their physico-chemical properties. Separation occurs as a result of repeated absorption/desorption steps during movement of analytes along the face of the stationary phase. Improvements in chromatographic resolution and techniques brought about the advances in liquid chromatography and the introduction of more powerful separation systems such as the UPLC systems is now common place in clinical laboratories.

9.3.1 Ultra-performance liquid chromatography (UPLC)

The UPLC is a new development from the high-performance liquid chromatography (HPLC) system. Typically, the UPLC system is an HPLC with smaller (sub-2µm) particle size as the stationary phase packing and it operates at elevated pressures to achieve higher separation speed, resolution and sensitivity than the conventional HPLC systems (151, 152). The UPLC system has also overcomes previous limitations of HPLC such as high sample injection volume, longer assay run time, analyte carry-over and inefficient temperature control (153). Hence UPLC coupled to MS/MS was chosen to provide for required fast, high-resolution separation.

9.4 Instrumentation

UPLC-MS/MS (shown in Figure 9.1 below) is a hyphenated technique, combining high separation power of UPLC with the detection power of triple-quadrupole tandem mass spectrometry. Even though the mass spectrometer has high analytical sensitivity and selectivity, UPLC is still useful in removing interfering substances that would impact the ionization of analytes. The goal is to separate the analyte of interest from sample matrix using LC. Co-elution of unwanted substances may cause ion suppression/enhancement that can compromise the quality of assay results (154).



Figure 9.1: UPLC-MS/MS system
Acquity UPLC system (right) and Micromass Quattro Mass spectrometer (left) Supplied by Waters, Milford, MA, USA.

9.4.1 UPLC column

In UPLC, a smaller column packing particles are used. A 1.7µm or 1.8µm particle packed column provides significant improvement in resolution because efficiency is better. Several columns are commercially available for used on the UPLC system. A 1.8µm phenyl Fortis column was used for UPLC separations in this study. This column has the advantage of providing the widest pH range for separations.

9.4.2 Tandem mass spectrometry

The technique of tandem mass spectrometry (MS/MS), as the name implies, involves two stages of mass spectrometry. In the first stage of MS/MS, ions of a desired *m/z* are isolated

from the rest of the ions emanating from the ion source. These isolated ions are termed parent ions or precursor ions. These parent ions are induced to undergo a chemical reaction that changes either their mass (*m*) or charge (*z*). The resulting ions are termed product ions and these are analysed in the second stage of MS/MS. MS/MS is particularly useful when analysing complex matrices such as plasma/serum (155).

9.5 Operational principle of the triple quadrupole mass spectrometry (QqQ MS)

QqQ MS (As shown in Figure 9.2) is a mass analyser consisting of 3 quadrupoles arranged in series. Each quadrupole (Q) is composed of 4 circular rods placed in parallel to which an oscillating electric field is applied. Q1 and Q3 are responsible for filtering sample ions according to their mass to charge (m/z) ratio. In between there is Q2 which serves as a non-linear collision cell. The ions are selected or scanned in Q1 and Q3 based on the stability of their paths in the electric field. Once they reach Q2, they are accelerated by the electric field and are collided with a neutral gas (e.g. N2, Ar) to produce small fragments (156). Employing the QqQ provides enhanced selectivity, better accuracy, and greater reproducibility; all of which are limited in single quadrupole mass analyzers (150, 156, 157).

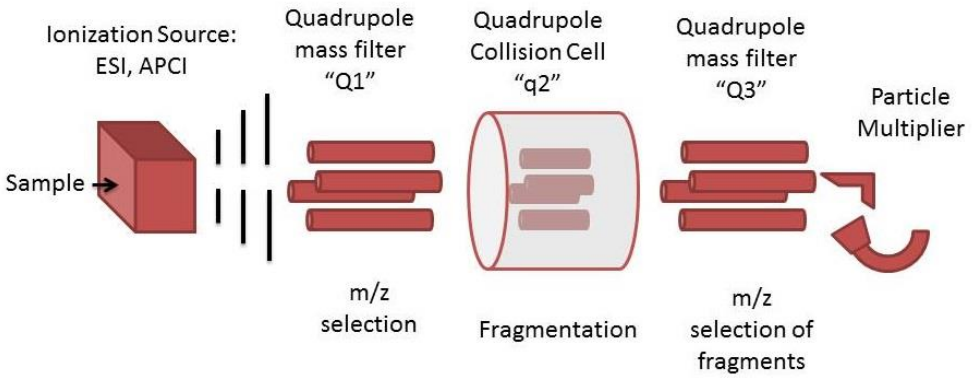


Figure 9.2: Arrangement of the triple quadrupoles rods in the Triple-quadrupole mass spectrometer
Picture was adapted from an article linked to Johnson et al. (156)

It should be noted that to interface UPLC with MS, there are some restrictions on the flow rate and mobile phases that can be used. Hyphenated systems use some combination of water and either methanol or acetonitrile as the mobile phase. Furthermore, there are limitations on mobile phase modifiers; for example, mobile phase modifiers should be volatile. Mobile phase modifiers are chemicals added to the mobile phase that are used primarily to improve the chromatography. Typical mobile phase modifiers include; ammonium acetate, acetic acid and formic acid (154, 158).

9.6 Chemicals and reagents

Tryptophan and Kynurenine reference compounds were purchased from Sigma-Aldrich, South Africa. Tryptophan with molecular mass of 204.22g/mol is an essential amino acid found in diet and blood circulation. Kynurenine with molecular mass of 208.22g/mol is a metabolite of Tryptophan breakdown found in blood circulation.

Tryptophan-d5 (Trp-d5) and Kynurenine-d4 (Kyn-d4) were used as internal standards and were obtained from Separation Scientific, South Africa. Trp-d5 and Kyn-d4 are deuterated form of

L-Tryptophan and L-Kynurenine with molecular mass of 209.25g/mol and 212.23g/mol respectively. We chose Trp-d5 and Kyn-d4 as internal standards because they have similar physical and chemical properties as Tryptophan and Kynurenine during mass analysis. Other reagents used were Acetonitrile, methanol and ortho-Phosphoric acid, all HPLC/analytical graded.

9.6.1 Standard solutions

Kynurenine and Tryptophan standard stock solutions were prepared at 10.8mg/20ml and 8.5mg/20ml in 50% Acetonitrile and stored at $4^{o}C$ in brown bottles. The concentrations of Kynurenine and Tryptophan pure standard compounds were informed by previous exploratory work in our laboratory (personal communication, Tracy Snyman). Stock solutions of internal Standards (IS) of Trp-d-5 and Kyn-d-4 were also prepared at 1mg/ml in 50% Acetonitrile. All stock solutions were stored at $4^{o}C$ and daily working solutions were prepared in HPLC/analytical grade distilled water.

9.6.2 Mobile phase and mobile phase modifier

Analytical-grade Acetonitrile was used as mobile phase with Ammonium acetate, formic acid in water (5:95V/V) mobile phase A and Acetonitrile 0.1% formic acid for mobile phase B. See appendix D for reagent preparation.

9.6.3 Precipitation agents

Ortho-phosphoric acid and absolute methanol was used as protein precipitants at difference stages of the method development and optimization.

9.7 Analytical method optimization

Analytical method optimization is a routine task in bioanalytical laboratories. Both sample preparation and analytical techniques in the LC-MS/MS platform are common targets for method optimization. The main goal is to be able to obtain optimum sensitivity, selectivity and test method robustness for the proposed analytical methods. Widner and colleagues in 1997 described an HPLC method to determine Kynurenine and Tryptophan in serum simultaneously with use of an external albumin-based calibrator and an internal calibrator (159). We modified their method and optimised it for this study. For instance, Trichloroacetic (TCA) acid, used as protein precipitant, was replaced with ortho-phosphoric acid and later changed to absolute methanol at different stages of method optimization.

9.7.1 Blood samples for method optimization

Both serum and plasma samples were used in the method optimization process. In all, nine (4 HIV-negative and 5 HIV-positive) samples were used for optimization and method validation. Blood samples were obtained from 4 HIV-negative healthy individuals into plain vacutainer blood collection tubes for serum and EDTA anticoagulated vacutainer blood collection tubes for plasma. HIV-infected samples were selected from the main study group (see chapter 11).

9.7.2 Sample preparation

Frozen serum/plasma specimens were thawed at room temperature. 300µL of sample was spiked with 100µL internal standard and vortexed for 30 seconds. Protein was precipitated with 1200µL of 0.5 Normal (0.5N) ortho-phosphoric acid. The capped tubes with the precipitant were immediately vortexed and centrifuged for 10 min at 5752 relative centrifugal force (rcf). 200µL of the supernatants were transferred into new tubes and dried down at 60^{o}C. The dried

residue was reconstituted with 100µL acetonitrile and transferred to the micro-vials for analysis on LC-MS/MS system.

Even though this extraction procedure produced reliable and reproducible results, it required high sample volume of 300µL (serum/plasma) which was a disadvantage. Also, the ortho-phosphoric acid upon dry-down turned into black precipitates. This produced intolerable blackening on the UPLC system. Again, the drying-down step increased sample processing time since it took approximately 16 hours (overnight) to dry-down before analysis.

Therefore, the method was revised and extraction agent was changed. The ortho-phosphoric acid was replaced with absolute methanol (100% methanol) as a precipitating agent. Methanol has an advantage of quick drying after precipitation and does not cause a black residue. This also allowed the comparison of both freshly precipitated samples and dried-down samples. The sample volume was also reduced to 100µL. As part of the revised method the drying-down step was omitted from the sample preparation procedure to improve the sample processing time.

In the revised extraction procedure, 100µL of sample (plasma/serum) was spiked with 100µL internal standard and vortexed for 30 seconds. Proteins were precipitated with 1200µL of absolute methanol. The capped tubes with the precipitant were immediately vortexed for 10 min at 3156 RCF. 200µL of the supernatants were transferred to the micro-vials and placed into the auto-sampling platform on the LC-MS system for analysis.

9.8 Optimization of chromatography

The Acquity UPLC system (Waters, Milford, MA) consisting of an autosampler, sample manager, binary gradient pump, a degasser and a column oven (60 °C) were used for separation. All compounds were separated in one single gradient run. A 50 × 2.1 mm i.d. Fortis phenyl column with 1.8 µm particles (Phenomenex) was used for separation of the compounds.

This system has a pre-filter installed before the column. Mobile phase eluents were analytical-grade water/Acetonitrile at 5:95V/V as mobile phase A and absolute Acetonitrile(0.1% formic acid) as mobile phase B, both containing 2mM Ammonium Acetate and 2mM formic Acid as mobile phase modifiers. The flow rate was 0.5ml/min and the run time for each was 2.01minutes. The column temperature was 40^{o}C and sample temperature of 4^{o}C were used for all analytes.

9.9 Optimization of mass spectrometry

Analytes were detected by Multiple Reaction Mode (MRM) using electrospray ionization mass spectrometry (ESI MS) on the micromass Quattro Micro MS. During the run, the system was in positive ESI mode. The precursor ion/product ion (m/z) transitions was set at 204.9>187.7 for Trp, 208.7>191.1 for Kyn, 209.1>191.5 for Trp-d5 and 213.0>196.0 for Kyn-d4. The cone voltage and collision energy were 35V and 20eV, for both Trp and Trp-d5 respectively, and 35V and 10eV for Kyn and Kyn-d4. The source temperature was 450 °C for all analytes. Nitrogen was used both as nebulizing and drying gas. The capillary voltage was 2500 V. For each compound 2 MRM-transitions were monitored. The retention time was 2 minutes.

CHAPTER 10

Results of analytical method validation

As defined by the US Food and Drugs Authority (FDA), analytical method validation is establishing documented proof that a specific analytical method and the instruments used in the method, will consistently yield results that accurately reflect the quality characteristics of the analyte tested (160). Analytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given sample matrix, such as blood (plasma or serum) is reliable and reproducible for the intended use.

Currently, there is no consensus guideline for assay validation of endogenous substances such as Kynurenine or other IDO metabolites. Method validation therefore was performed using guidelines adopted by the Department of Chemical Pathology, Charlotte Maxeke Johannesburg Academic Hospital for bioanalytical methods. This guideline in itself was adopted from the international conference on harmonization (IHC) guidelines for validation of bioanalytical methods (161). The method was validated in terms of assay linearity, specificity, sensitivity, intra-day and inter-day precision, accuracy, limit of detection (LOD) and limit of quantitation (LLOQ), extraction efficiency and recovery.

Each analytical run included a double blank sample (human plasma without internal standard), a blank sample (human plasma with internal standard), 10 standard concentrations of Trp or Kyn for calibration curve, and replicate sets of plasma samples from healthy individuals and or HIV-positive plasma spiked at low, medium and high concentrations to assess accuracy at different concentrations. Six validation runs were used to demonstrate method accuracy and precision using three (3) sets of control samples (low, medium and high) in plasma from HIV-

positive and HIV-negative individuals. A one day assay of six replicates was used for assessing intra-day precision. Inter-day precision was also assessed using six replicates assayed each day for 6 days.

10.1 Preparing calibration curves for Tryptophan and Kynurenine

Stock concentrations of Kyn and Trp standard were prepared each day for the calibration curve. An eight (8) point calibration curve was prepared by serially diluting the stock concentration with distilled water and spiking into plasma or serum.

10.2 Extraction efficiency or recovery

Extraction efficiency is the proportion of analyte that is extracted from a sample matrix during the sample extraction process. Extraction efficiency of Kyn and Trp from plasma samples were determined and evaluated using surrogate analyte compounds (Trp-d5 and Kyn-d4) from the supplier. Kyn-d4 and Trp-d5 were chosen because they have the same physicochemical properties as the analyte in plasma and should have a very similar extraction recovery and mass analysis on the system if thoroughly mixed with the sample before extraction. Known concentrations of these surrogate compounds were prepared at high and low concentrations and spiked into plasma. Samples were extracted using the procedure described earlier for one set of samples and another set without the precipitation step (non-extracted). The extraction efficiencies were determined using peak areas of Trp, Kyn, Trp-d5 and Kyn-d4. The total volume for both extracted and non-extracted during injection was the same, the extraction efficiency (%) was calculated as;

$$Extraction\ efficiency\ (\%) = \frac{Peak\ areas\ of\ extracted\ standard}{Peak\ area\ of\ nonextracted\ standard} * 100$$

Table 10.1: Extraction efficiency of Trp-d5 and Kyn-d4 in extracted plasma and non-extracted plasma

Analyte	Spiking Conc. (μM/L)	Peak area in extracted plasma (n = 3)	Peak area in non-extracted plasma (n = 3)	Relative peak area difference (%)
Trp-d5	105	116249	82276	20
	50	61554.6	38345	16
Trp	105	108426	4526	24
	50	58809	7124	8
Kyn-d4	125	37445	9124	4
	50	21012	2456	9
Kyn	125	35432.1	6125	6
	50	12377	3021	4

The mean extraction efficiency for Trp-d5 and Kyn-d4 in extracted plasma has high recovery than non-extracted plasma. This indicates that the extraction procedure yielded more analyte for ionization and detection than non-extracted. Similar results were found using charcoal striped plasma (treated plasma) and non-treated plasma. The extraction efficiency was greater 90%.

10.3 Matrix effect

Sample matrix refers to any component of a sample other than the analyte of interest. The sample matrix can have a considerable effect on the quality of the results obtained either by causing ion suppression or enhancement during ionization and mass analysis, and such effects are called matrix effects. Matrix effect was assessed by spiking internal standard (i.e Kyn-d4 or Trp-d5) in plasma from healthy individuals after extraction and in distilled water. Ionization suppression or enhancement was assessed by comparing the peak area of Trp-d5 and Kyn-d4 in plasma to peak area in the absence of plasma matrix (i.e. distilled water). From the table

below, there was no significant ionization suppression or enhancement in Trp-d5 or Kyn-d4 in human plasma as compared to pure water. The relative peak area difference is close to 1.

Table 10.2: Matrix ionization or suppression of Trp-d5 and Kyn-d4 in plasma and distilled water

Analyte	Spiking Conc. (µM/L)	Peak area in Distilled water (n = 3)	Peak area in plasma (n = 3)	Relative peak area difference
Trp-d5	105	452354	467702	1.03
	50	117321	118256	1.00
Kyn-d4	125	161824	159203	0.98
	50	65131	60123	0.92

10.4 Analytical specificity or selectivity

Analytical specificity is the complete discrimination of the analyte of interest by the method in the presence other matrix components. The analyte must be detectable in the presence of components that may be expected to be present in the plasma matrix. There should be no cross-interference between Kyn and Trp and internal standards using the proposed extraction procedure and UPLC-MS/MS conditions.

The specificity/selectivity of the assay method was investigated by using extracted internal standard of Trp-d5 or Kyn-d4 to demonstrate the absence of interference with the elution of the analyte. Assay specificity was assessed by confirming the detected Trp or Kyn in plasma were similar to Trp-d5 or Kyn-d4 using two different transitions in multiple reaction mode (MRM). For example, the transition of Trp in plasma was 205.1>187.72 whilst that of Trp-d5 was set at 209.01>191.59. As shown in figure 10.1 below, the peak obtained for Trp in the plasma was same as that for the surrogate analytes Trp-d5.

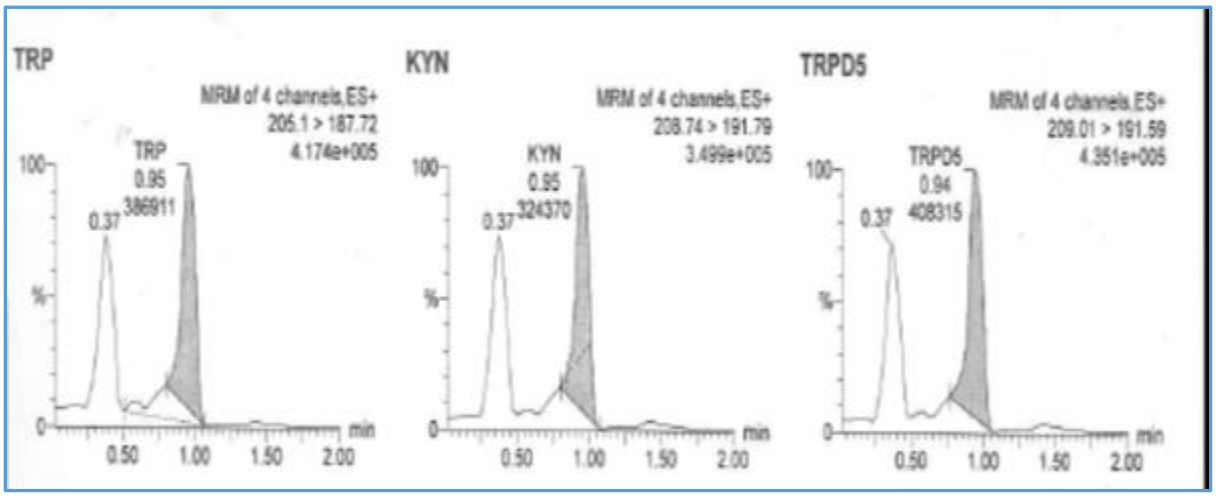


Figure 10.1: UPLC-MS/MS chromatogram for analysis of Tryptophan and Kynurenine and Trp-d5 in plasma

10.5 Linearity and analytical sensitivity

Linearity of an analytical method is the ability of the test method to produce analytical responses (peak area or peak height or optical absorbance) that is directly proportional to the concentration of the analyte in sample within a particular range (161). Analytical sensitivity is also the ability of the method to produce a change in signal for a defined change in analyte concentration.

Assay linearity was assessed by using standard solutions prepared from pure compounds. Standard solutions were prepared at ten (10) different target concentrations. Three individually prepared replicates at each concentration were analysed. A calibration curve was constructed by plotting the observed analytical response (y-axis) against the expected concentration (x-axis). The calibration curve was fitted using a least-square linear regression model provided on the UPLC-MS/MS system with the MassLynx 4.1 software and also where relevant, Excel data analysis was used.

10.6 Linearity of Tryptophan

Tryptophan concentrations showed good linear relationship with observed analytical response as shown in figure 10.2 below. The assay was linear from a concentration of 21µmol/L to 115.5µmol/L. The regression co-efficient was more than 0.99 with the equation of the line being Y = 6121.8x + 69970. Even though the linear range of Tryptophan could be extended from the highest concentration to about 140µmol/L, previous exploratory assays in our laboratory had demonstrated that the current ranges are sufficient to cover expected tryptophan concentrations in the study population.

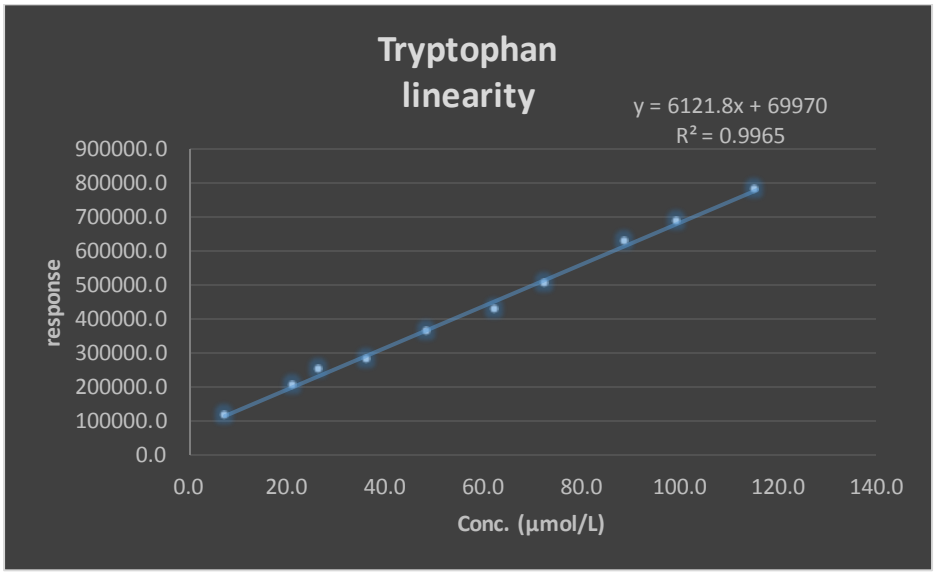


Figure 10.2: Linearity of Tryptophan
Linearity of Tryptophan response with co-efficient of determination (R^2) of > 0.99

10.7 Linearity of Kynurenine

Kynurenine concentrations showed good linear relationship with observed analytical responses as shown in figure 10.3 below. The correlation co-efficient was greater than 0.99 with the equation of the line being Y = 769,68x + 562, 64. The assay was linear from 2.5µM/L to 120µmol/L. Even though the linear range of Kynurenine could be extended from the highest concentration to about 140µmol/L, previous exploratory assays in our lab has demonstrated the current ranges are sufficient to cover expected kynurenine concentrations in the study samples.

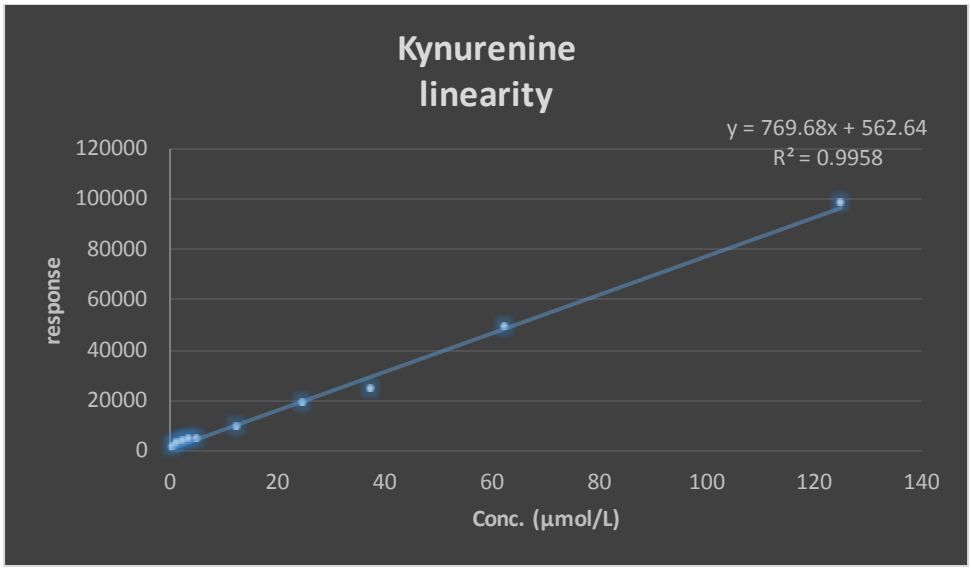


Figure 10.3: Linearity of Kynurenine
Linearity of Kynurenine response with co-efficient of determination (R^2) of > 0.99.

10.8 Lower Limit of Detection and Lowest limit of Quantitation (LOD & LOQ)

The LOD is the lowest concentration of an analyte in a sample which can be detected but not necessarily quantitated as an exact value. The LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy in a sample matrix. In chromatographic methods, LOQ is the concentration of the analyte that gives a signal-to-noise (SNR) of 10:1 (a peak with height at least 10x as high as the baseline noise level). According to ICH guidelines, LOD and LOQ can be determined by visual evaluation of the chromatogram, signal-to-noise ratio and determining the LOD & LOQ from the standard deviation of response and the slope of the linear curve.

Mathematically, LOD and LOQ may be expressed as:

- LOD = 3.3 x σ/S
- LOQ = 10 x σ/S
- where σ = the standard deviation of the response
- S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The value of σ may be taken from the standard deviation of analytical background responses of an appropriate number of blank samples. Alternatively, the standard deviation of a regression line or standard deviation of the analytical response can be used as σ if the regression line was obtained from samples containing an analyte in the range of LOD and LOQ.

The LOD and LOD were determined by visually inspecting the signal-to-noise ratio on the chromatogram and the calibration curve.

Table 10.2: Lower Limit of Detection and Lowest limit of Quantitation (LOD & LOQ)

	LOD (µM/L)	**LOQ (µM/L)**
Tryptophan	**4.00**	**14.00**
Kynurenine	**3.00**	**10.00**

The LOD for Tryptophan and Kynurenine were estimated at approximately 4.00µmol/L and 3.00µmol/L respectively based on signal-to-noise ratio ≥ 3 and the standard deviation of the response and the slope. The LOQ is also 14µmol/L and 10µmol/L for Tryptophan and Kynurenine respective based on the above criteria.

10.9 Assay accuracy

The intra and inter day accuracy was expressed as the percentage difference between the measured concentration and the expected concentration. The % Accuracy was calculated by the using the formula;

$$\%\ Accuracy = \frac{Measured\ concentration}{Expected\ concentration} \times 100$$

10.10 Precision

Precision of an analytical method expresses the degree of scatter between a series of replicate measurements of the sample under the same conditions. Intra-day assay precision was evaluated using plasma samples from healthy individuals as well as pure standard solutions prepared at six different concentrations. Inter-day assay precision was evaluated using plasma samples from healthy individuals, HIV-positive patients and pure standard solutions, at six

different concentrations for six days. Both intra- and inter-day precision was determined as percentage of co-efficient of variation (%CV) and percentage difference (%Diff).

$$\%\ CV = \frac{Standard\ Deviation\ (SD)}{Mean} \times 100$$

$$\%\ Diff = \frac{Mean\ of\ measured\ Conc. - Expected\ conc.}{Expected\ conc.} \times 100$$

10.11 Tryptophan intra-day precision

Intra-day precision of Tryptophan was evaluated by using three replicates of six standard solutions (105µmol/L, 100µmol/L, 75µmol/L, 50µmol/L, 37.5µmol/L and 25µmol/L) and healthy plasma samples spiked with low and high (62.5µM/L and 105µM/L) concentrations of pure Tryptophan. The mean %CV of Tryptophan standard at different concentrations was less than 5% and in healthy plasma was about 10%.

Table 10. 3: Intra-day precision of Tryptophan in spiked plasma and pure standard solutions

	Std conc. (µM/L)	Run 1 (µM/L)	Run 2 (µM/L)	Run 3 (µM/L)	Mean (µM/L)	SD	% Diff	% CV	%Accuracy
Pure standard solution									
standard 1	105	105	115	110	**110,0**	**5,0**	**4,7**	**4,5**	**104,7**
standard 2	100	98,5	100	105	**101,1**	**3,4**	**1,1**	**3,3**	**101,1**
standard 3	75	77,5	75	75	**75,8**	**1,4**	**1,1**	**1,9**	**101,1**
standard 4	50	50	50	51	**50,3**	**0,5**	**0.6**	**1,1**	**100,6**
Standard 5	37,5	45,5	45	44	**44,8**	**0,7**	**19.5**	**1,7**	**119,5**
Standard 6	25	23,5	25	25	**24,5**	**0,8**	**-2.6**	**3,5**	**98,0**
Spiked healthy plasma									
Low	62,5	91	100,5	100,5	**97,3**	**5,48**	**55,7**	**5,6**	**155,7**
High	105	134,5	143,5	144,5	**140,8**	**5,51**	**34,1**	**3,9**	**134,7**

**Std = Standard, Conc. = µM/L*

.

10.12 Inter-day precision of Tryptophan

Inter-day assay precision was evaluated by using six standard solutions and three plasma (3 healthy and 3 HIV-positive) samples spiked with low, medium and high (25µM/L, 62.5µM/L and 105µM/L respectively) concentrations of pure Tryptophan. As shown in the Table 10.3 below, the mean %CV of replicates for six days was between 3% to 12% in standard solutions and plasma samples (healthy and HIV-positive). This results indicates that the method has good precision for measuring Tryptophan in plasma.

Table 10. 4: Tryptophan inter day precision

	Std conc (µM/L)	Day 1 (µM/L)	Day2 (µM/L)	Day 3 (µM/L)	Day 4 (µM/L)	Day 5 (µM/L)	Day 6 (µM/L)	**Mean (µM/L)**	**SD**	**%Dff**	**%CV**	**%Accuracy**
Pure standard solution												
std 1	105	105	115	115	110,5	107,5	107,5	**110,08**	**4,19**	**4,84**	**3,80**	**104,84**
std 2	100	95	90	105	102,5	104,5	104,5	**100,25**	**6,27**	**0,25**	**6,25**	**100,25**
std 3	87,5	82,5	95	90	90	84,5	84,5	**87,75**	**4,72**	**0,29**	**5,38**	**100,29**
std 4	75	77,5	75	75	85	60	81	**75,58**	**8,55**	**0,78**	**11,31**	**100,78**
std 5	50	50	50	50	50	63	48,5	**51,92**	**5,46**	**3,83**	**10,52**	**103,83**
std 6	25	23,5	25	25	29	21,5	29	**25,50**	**3,00**	**2,00**	**11,76**	**102,00**
**Std = Standard*												
Spiked healthy plasma												
Low	25	28,5	30,5	29,5	30	31,5	25,5	**29,25**	**2,09**	**17,00**	**7,15**	**117,00**
Med	62,5	68	80	80	70,5	65,5	66	**71,67**	**6,69**	**14,67**	**9,34**	**114,67**
High	105	171,5	156	160,5	160	170,5	170	**164,75**	**6,68**	**56,90**	**4,06**	**156,90**
Spiked HIV-positive plasma												
Low	25	29,3	31	28,5	30	30,5	29,5	**29,80**	**0,89**	**19,20**	**3,00**	**119,20**
Med	62,5	65,5	63	65	63,5	64,5	64,5	**64,33**	**0,93**	**2,93**	**1,45**	**102,93**
High	105	115,5	116	111	116,5	100	116	**112,50**	**6,45**	**7,14**	**5,73**	**107,14**

Accuracy of Tryptophan

As shown in Table 10.5, the intra-day accuracy of Tryptophan was between 98-119% in pure standard solutions and between 134-196% in spiked healthy plasma. This was because the concentration of Tryptophan in plasma was not known before assay, so the measured concentration is a reflection of both endogenous Tryptophan and spiked concentration. This result indicates the method has good precision and accuracy for determining Tryptophan in plasma.

10.13 Intra-day Precision of Kynurenine

Intra-day assay precision of kynurenine was evaluated by using three replicates of a one day assay of six standard solutions (125µM/L, 112.5µM/L, 100µM/L, 75µM/L, 62.5µM/L and 37.5µM/L) and healthy plasma samples spiked with low, medium and high (12.5µM/L, 75 µM/L and 125µmol/L respectively) concentrations of pure Kynurenine. As shown in Table 10.6, the mean %CV was less than 10% in both plasma and standard compound solutions. This results indicates the method has good precision for measuring Kynurenine in plasma.

Table 10. 5: Kynurenine intra-day precision assay

	Std Conc.	Rep 1 (µM/L)	Rep 2 (µM/L)	Rep 3 (µM/L)	**Mean** (µM/L)	**SD**	**% Diff**	**% CV**	**%Accuracy**
Pure standard solution									
standard 1	125	130	120	131,5	**127,1**	**6,2**	**1.7**	**4,9**	**101,7**
standard 2	112,5	109,5	109,5	115	**111,3**	**3,1**	**-1.4**	**2,8**	**98,9**
standard 4	75	75	75	70	**73,3**	**2,8**	**-2,2**	**3,9**	**97,7**
standard 5	62,5	60	60	62	**62,0**	**1,1**	**-0,8**	**1,8**	**99,2**
standard 6	37,5	39,5	39,5	35	**38,0**	**2,6**	**1,3**	**6,8**	**101,3**
Spiked healthy plasma									
Low	12,5	12,5	11,5	11	**11,6**	**0,7**	**-6,6**	**6,5**	**93,3**
Med	75	70	80	70	**73,3**	**5,7**	**-2,2**	**7,8**	**97,7**
High	125	124	119,5	112	**118,5**	**6,0**	**-5,2**	**5,1**	**94,8**

** Rep = Replicate*

10.14 Inter-day assay precision of kynurenine

Similarly, inter-day assay precision of kynurenine was assessed by evaluating six standard solutions of pure kynurenine, healthy plasma and HIV-positive plasma. The %CV of kynurenine was less 12% in all samples (Table 10.7).

Table 10. 6: Kynurenine inter-day assay precision

	Std conc (µM/L)	Day 1 (µM/L)	Day2 (µM/L)	Day 3 (µM/L)	Day 4 (µM/L)	Day 5 (µM/L)	Day 6 (µM/L)	Mean (µM/L)	SD	%Dff	%CV	%Acc.
Pure standard solutions												
std 1	125,0	126	127	131,5	120	125	119,5	**124,8**	**4,5**	**-0,1**	**3,6**	**99,8**
std 2	112,5	108,5	109,5	113	105	110,5	115,5	**110,3**	**3,6**	**-1,9**	**3,3**	**98,0**
std 3	87,5	84,5	84	90	85	85	83	**85,3**	**2,4**	**-2,5**	**2,8**	**97,4**
std 4	62,5	60.0	60	60	75	60	66,5	**63,6**	**6,1**	**1,7**	**9,7**	**101,7**
std 5	50,0	53,5	55	45	55	55	54	**52,9**	**3,9**	**5,8**	**7,4**	**105,8**
std 6	37,50	39,5	39,5	35	39,5	35	39	**37,9**	**2,2**	**1,1**	**5,9**	**101,1**
Spiked healthy plasma												
Low	12,5	12	13,5	13,5	13	12	12	**12,7**	**0,7**	**1,3**	**5,9**	**101,3**
Med	75	60,5	70,5	81	71	68,5	59	**68,4**	**8,0**	**-8,7**	**11,7**	**91,2**
High	125	169	130	155	156	155	135	**150,1**	**14,7**	**20,0**	**9,8**	**120,0**
Spiked HIV-positive plasma												
Low	12,5	17	17	14,75	15	16	16	**15,9**	**0,9**	**27,6**	**5,9**	**127,6**
Med	75	96	89,8	90,5	91	90,5	75	**88,8**	**7,1**	**18,4**	**8,0**	**118,4**
High	125	155	166,5	145,7	155	160,5	155,5	**156,4**	**6,8**	**25,1**	**4,4**	**125,1**

**std = standard*

Accuracy of Kynurenine

Similarly, intra-day accuracy of kynurenine was also 97-102% in pure standard compounds and 93-95% in spiked healthy plasma from Table 10.6. This is because, normal Kynurenine concentration in individuals without disease is very low. However, the inter-day assay accuracy in spiked HIV-positive plasma was between 91-128% (Table 10.7). This is similar to other findings with high plasma Kynurenine in HIV infection (162).

Conclusion

From the validation results above a simple, specific and reproducible UPLC-MS/MS method has been optimised and validated for determination of Tryptophan and Kynurenine in plasma. It covers the concentration range of interest to this study and has a satisfactory accuracy, precision and limit of quantification.

CHAPTER 11

Study design

11.0 Study design

This was a retrospective analysis of a longitudinal cohort study conducted on HIV-infected patients who developed Tuberculosis in Soweto, Johannesburg, South Africa.

11.1 Description of sample cohort

The study subjects were drawn from the Lung Cohort study which existed from 2008 to 2012. The cohort recruited HIV infected patients who were naïve to anti-retroviral drugs at the Perinatal Health Research Unit at Chris Hani Baragwaneth Hospital in Soweto, South Africa. About 400 HIV-infected adults who lived within the study area (Soweto) were recruited and followed-up semi-annually for a period of about 48 months to detect interval events. Thirty-five (35) patients developed active Tuberculosis disease during the follow up period. These subjects who developed TB had no history and clinical signs compatible with active TB disease at the time of recruitment. Diagnosis of TB was based on the highest attainable standards for routine clinical practice in South Africa. TB was confirmed in these subjects by the use of standard Tuberculosis diagnostic algorithm in South Africa at the time of the study which included the use of microbiological techniques as well as clinical signs and chest X-ray.

11.2 Ethical approval

Ethical approval for use of the stored samples for the purposes of this Masters of Science (MSc) project was reviewed by Human Research ethics committee of Wits Medical School, Johannesburg. (Clearance certificate M140488, see appendix A)

Ethical clearance for the main study was obtained from the Committee for Research on Human Subjects at the University of the Witwatersrand (Clearance certificate M03-05-68).

11.3 Inclusion criteria

From the Lung Cohort study database, patients (ie. those who developed TB) and controls were selected by matching age, gender and CD4 test results. We selected only patients who made at least three (3) or more visits to the study with clinical data and blood samples available for analysis. Enrolment criteria into the main study were; a positive HIV diagnosis, age 18 years or older, residence within one hour journey of the study centre. All participants provided consent to the main study before enrolment.

11.4 Exclusion criteria

From the Lung Cohort study database, all patients who had history of TB or signs compatible with TB at enrolment into the study, or with less than three (3) visits, were not selected for this study.

11.5 Study subjects

Thirty-five (35) patients developed active TB disease during the follow up period. We evaluated blood samples from 32 out of 35 patients who developed TB during follow-up. This was because 2 patients were diagnosed with non-tuberculous mycobacteria (NTM) and the other patient's sample could not be readily retrieved from the sample bank.

Tuberculosis diagnosis in these patients was either bacteriologically confirmed Tuberculosis or clinical Tuberculosis according to the standard Tuberculosis diagnostic algorithm in South Africa. Bacteriologically confirmed TB was defined as a patient with signs and symptoms compatible with TB and a positive *M. tuberculosis* culture or AFB. Clinical TB had compatible clinical and chest X-ray parameters but lacked a positive bacteriological test for *M.*

tuberculosis. At the time of the main study (ie. LCS), GeneXpert was not in routine use and samples were not available for molecular testing.

All TB patients received standard TB treatment for six (6) months according to the South African Tuberculosis treatment guidelines. There was no data readily available on TB treatment or sputum follow-up results since all TB cases were treated at sites other than the study site.

This study included blood samples collected at the various time-points during patients' evaluation in the main study. We included samples from enrolment which served as baseline as well as from 6 months after enrolment, the time-point at which TB was diagnosed and all available time-points after Tuberculosis diagnosis. About 40% of the TB patients were available for follow-up in the main study at 24 months after enrolment, some patients were followed until 36 months.

11.6 Selection of control subjects

We selected seventy (70) control subjects with age, gender and CD4 test results matched to those of the Tuberculosis patients. We included samples from enrolment, 6, 12, 18 and 24 month visits.

11.7 Statistical analysis

Discrete variables were expressed as counts (percentage). Normally distributed (parametric) data were shown as mean ± standard deviation whereas, non-normally distributed (non-parametric) data were expressed as median with the interquartile range [IQR]. The student's t test was performed to assess difference between two parametric groups. The Mann-Whitney test was used to compare differences between non-parametric unpaired groups whereas the

Wilcoxon test was used to compare paired groups for statistical differences. The Kruskal-Wallis or Dunn's posttest (non-parametric ANOVA) was used to compare multiple non-parametric groups at specific time points. IDO activity fold change was plotted to assess within-person variation over five time points. Sensitivity, specificity, positive and negative predictive values were calculated using samples from all time-points per patient. A receiver operating characteristic (ROC) curve was performed to evaluate the most suitable IDO activity cut-off value that discriminated between those with TB and those without TB. The optimal cut-off value was selected as that which gave the best combination of sensitivity and specificity. For correlation calculations, Spearman's correlation coefficient was used. Analyses were two-sided and the confidence intervals (CI) were set to 95%. Results were considered to be significant when the p-value was less than 0.05. All statistical analyses were done using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA).

CHAPTER 12

Study results

12.1 Demographic characteristics of Tuberculosis cases and controls

This study evaluated thirty-two (32) HIV-infected individuals who developed active TB during follow-up of a longitudinal cohort and compared them with seventy (70) control subjects, age and CD4 cell count matched, in the same HIV infected cohort who did not develop TB. The demographic and clinical characteristics of one-hundred and two (102) patients are summarized in Table 12.6.

Table 12.7: Clinical and demographic characteristics of TB cases and control

Characteristic	*Value*		
Demographic data [a]	**TB cases (n = 32)**	**Control (n = 70)**	***P value***
Sex (Male/Female) (n)	10M, 22F	18M, 52F	
Sex (%)	31%M : 69%F	26%M : 74%F	
Age (years)	38 ± 8	36 ± 6	0.2178
TB diagnosis			
Acid Fast Bacilli (AFB) smear positive only	13 (41%)	NA	
M. tuberculosis culture	4 (13%)	NA	
AFB smear & Culture positive	1 (3%)	NA	
Clinical symptoms & chest radiograph only	14 (44%)	NA	
Extra-pulmonary TB	1	NA	
MDR-TB*	2	NA	
Clinical data			
Baseline [b]			
Body mass index (BMI) (kg/m^2)	23 [20-27]	26 [23-30]	**0.0280**
CD4 cell count (cells/ml)	329 [240-403]	372 [273-511]	0.0569
C-reactive protein (mg/L)	6 [1-15]	3 [1-11]	0.3287
HIV viral load count (copies/ml)	21289 [49-41870]	1581 [49-8338]	**0.0140**
Time of TB diagnosis [b]	**TB patients**	**Controls (V3)**	
Body mass index (BMI) (kg/m^2)	22 [16-27]	25 [16-31]	**0.0297**
CD4 cell count (cells/ml)	249 [211-401]	417 [300-593]	**0.0119**
C-reactive protein (mg/L)	9 [2-14]	5 [1-10]	**0.0293**
HIV viral load count (copies/ml)	5368 [101-49022]	525 [49-6813]	**0.0148**

[a] Mean ± SD *Multi-drug resistant TB [b] Median [IQR] [b] P value determined by Mann-Whitney test [b] Visit 3 (V3)

Among HIV infected patients who developed active TB disease (TB cases), 10 (31%) were males and 22 (69%) were females with mean age of 38 ± 8 years with age range of 27-56 years. There was no statistically significant difference between the mean ages of males and females who developed TB (mean ages; 40 ± 7 versus 38 ± 8, P value = 0.3923). The control subjects consisted of 18 (26%) males and 52 (74%) females with mean age of 36 ± 6 years with age range of 22-51 years. It should be noted that age was a parametric variable hence, we reported the mean and standard deviation. Other variables were non-parametric, hence we reported the median and interquartile ranges.

Among those who developed TB (TB cases), active TB disease was either laboratory confirmed (bacteriologically proven) TB or clinical TB. Laboratory confirmed TB was defined as clinical and chest x-ray findings compatible with active TB and a positive *M. tuberculosis* culture or acid-fast bacilli (AFB) smear. On the other hand, clinical TB was defined as a patient with compatible clinical and radiograph signs suggestive of TB but lacking laboratory confirmation of a positive results.

Out of the 32 TB cases, 18 were laboratory confirmed TB whilst 14 were clinically diagnosed TB (clinical TB). Only one person was diagnosed with extra-pulmonary TB (skin TB). Two patients were diagnosed with multi-drug resistant TB (MDR-TB). All TB patients received standard anti-TB treatment for appropriate duration according to the South African TB treatment guidelines. Both TB cases and controls were naïve to ART drugs at the time of enrolment into the main study and none of them died during the observational period. Data for length of time on ART was not available to us.

12.2 Clinical parameters in TB cases and controls

To monitor interval changes that may be associated with HIV/AIDS progression, all study participants were clinically evaluated at the time of enrolment into study and semi-annually during the entire study period. Clinical evaluation included assessment of patient's CD4 cell count, HIV viral load, CRP and other relevant laboratory tests. These tests were performed at NHLS diagnostic facilities in and around the main study centre.

Comparing clinical parameters between TB cases and controls at the time of enrolment, there was no statistically significant differences in CD4 cell count. The median baseline CD4 cell count for TB cases and controls were 329 [IQR 240-403] cells/ml and 372 [IQR 273-511] cells/ml respectively (P value = 0.0569). Also, there was no significant difference in CRP results between TB cases and controls (median, 6 [IQR 1-15] mg/L versus 3 [IQR 1-11] mg/L, P = 0.3287]) at baseline. However, there was a statistically significant difference in HIV viral load count between TB cases and control at baseline (median, 21289 [IQR 49-41870] copies/ml versus 1581 [IQR 49-8338] copies/ml, P = 0.0149). TB cases also had smaller body mass than controls (median, 23kg/m^2 versus 26kg/m^2, P = 0.0280).

At the time of TB diagnosis, TB cases had significantly lower CD4 cell count than controls at the third visit (V3) (median, 249 [IQR 211-401] cells/ml versus 417 [IQR 300-593] cells/ml, P = 0.0119) and significantly higher HIV viral load count compared to controls (median, 5368 [IQR 101-49022] copies/ml versus 525 [49-6813] copies/ml, P = 0.0148). In addition, TB cases had significantly higher CRP levels than controls at time of TB diagnosis (median, 9 [IQR 2-14] mg/L and 5 [IQR 1-10] mg/L respectively with a P = 0.0293). Furthermore, TB cases had

further decreased in body mass than controls (median, 22 [16-27] kg/m^2 versus 25 [16-31] kg/m^2, P = 0.1235).

12.3 Plasma IDO activity in HIV infected TB cases and controls

Plasma IDO activity was evaluated from 12 months before (-12m) TB diagnosis (TBdx) to 12 months after (+12m) TB diagnosis. Plasma IDO activity was determined as the ratio of plasma Kynurenine concentration to plasma Tryptophan concentration (Kyn/Trp). Figure 12.1 (A & B) shows plasma IDO activity variability in HIV infected patients who developed TB and controls. For the controls, the median IDO activity over 5 time points (2 and half years) was 0.16 [IQR 0.11-0.24]. The reproducibility of IDO activity measurement was determined to be 0.10 with a %CV of 55%. The median IDO activity for TB cases over the same period of two and half years was 0.30 [IQR 0.15-0.82]. Figure 12.1C shows the comparison of plasma IDO activity in HIV infected TB cases and controls at each time point.

Plasma IDO activity was significantly higher in TB cases than controls at the time of TB diagnosis (median, 1.35 [IQR 1.15-1.60] versus 0.21 [IQR 0.14-0.30], P < 0.0001). At 6 months prior to TB diagnosis, plasma IDO activity was significantly elevated in those who progressed to TB compared with controls at the second visit (V2) (median, 0.35 [IQR 0.29-0.60] versus 0.17 [IQR 0.12-0.23], P < 0.0001). However, IDO activity declined in all TB patients after anti-TB treatment to levels comparable to that of the controls at fourth visit (V4) (median, 0.20 [IQR 0.16-0.28] versus 0.17 [IQR 0.13-0.23], P > 0.05).

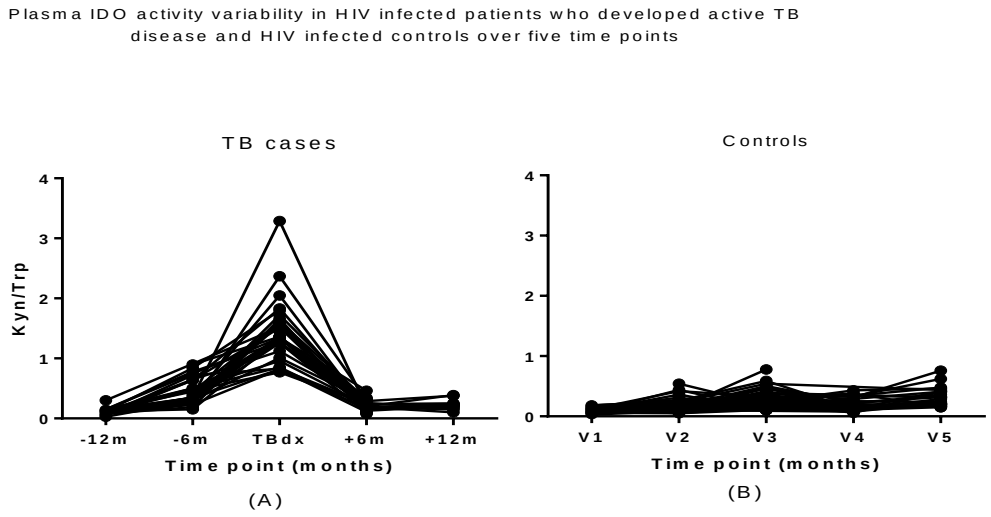


Figure 12.1: Plasma IDO variability in HIV infected patients who developed TB (A) and controls (B) over 5 time points

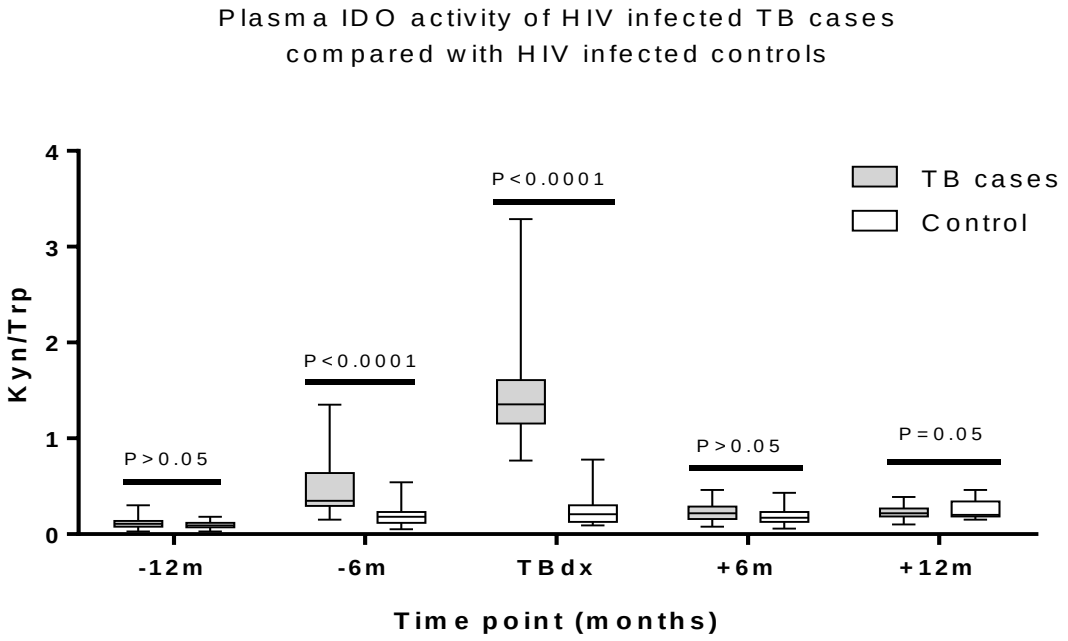


Figure 12.1:Plasma IDO activity in HIV infected TB cases compared to HIV infected controls (3C)

*P value was determined by Dunn's multiple comparison test. The lower and upper portion of the boxes indicates 25th and 75th percentiles respectively whilst the horizontal lines within the box plots indicates median.

12.4 Plasma IDO activity in laboratory confirmed TB and clinical TB

Plasma IDO activity in laboratory confirmed TB cases were compared to patients with clinical TB (Figure 12.2). This was to evaluate whether IDO activity in patients with laboratory confirmed TB significantly varied from patients with clinical TB. The results indicated that there was no statistically significant difference in plasma IDO activity between lab confirmed TB and clinical TB cases (P = 0.7289, determined by Mann-Whitney test). The median IDO activity in laboratory confirmed TB was 1.35 [IQR 0.99-1.67] while clinical TB was 1.34 [IQR 1.22-1.58].

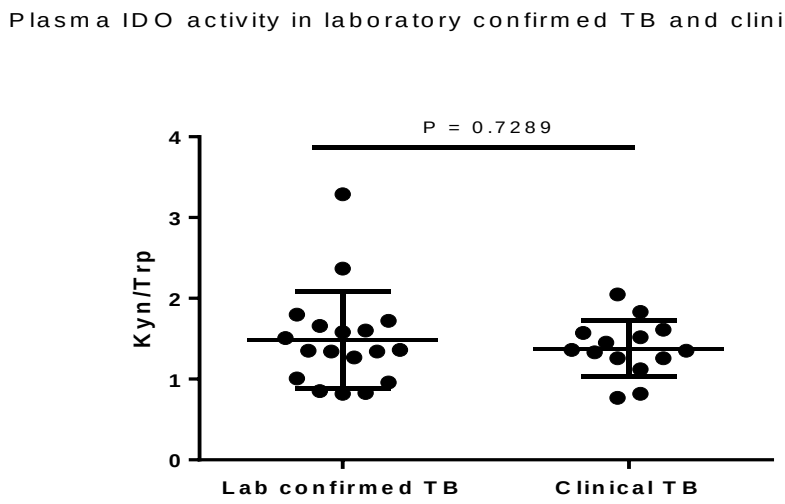


Figure 12.2: Plasma IDO activity in Laboratory confirmed TB and clinical TB

* P value was determined by Mann-Whitney test. The lower and upper portion of the scatter plots indicates 25th and 75th percentiles respectively whilst the horizontal lines in the middle indicate the median.

12.5 Plasma concentration of Kynurenine in HIV infected TB cases and controls

To evaluate whether IDO activity elevations in TB patients were due mainly to Kyn, Trp or both analytes, we evaluated Kyn and Trp individually. Plasma concentrations of Kyn in TB cases and controls are presented in Figure 12.3. At baseline (-12 months before TB diagnosis in those who developed TB and first visit (V1) of controls), there was no statistically significant (P > 0.05) difference between TB cases and controls. However, at the time of TB diagnosis,

there was a statistically significant difference between those who developed TB and the controls ($P < 0.0001$). The median Kyn concentrations of those who developed TB at time of TB diagnosis and the controls were 20.50 [IQR 17.22-24.72] µM/L and 6.54 [IQR 5.08-8.63] µM/L respectively. In addition, plasma Kyn concentrations were statistically significantly higher in TB cases than controls at the second visit (V2) 6 months prior to TB diagnosis ($P < 0.0001$). The median Kyn levels of TB cases were 8.68 [IQR 6.71-10.39 µM/L] whilst that of the controls was 5.98 [IQR 4.69-6.60 µM/L] at the second visit. However, Kyn concentrations declined in all TB patients after anti-TB treatment to levels comparable to that of the controls. The median Kyn concentration of TB cases after TB treatment (+6m) and controls were 9.19 [IQR 6.90-11.53 µM/L] versus 6.54 [IQR 5.08-8.63 µM/L] respectively after TB treatment (+6m). No statistically significant difference was found between TB cases and control up to 12 months after anti-TB treatment.

Comparing the baseline (-12m) Kyn concentration in patients who developed TB to kynurenine concentration after TB treatment, Kyn concentrations remained significantly higher after anti-TB treatment compared to baseline Kyn level in TB patients ($P < 0.0001$, using Wilcoxon matched-paired signed rank test). The median Kyn concentration at 6 months after (+6m) TB treatment was 9.17 [IQR 6.90-11.53 µM/L] compared to 3.97 [IQR 2.86-4.85 µM/L] at baseline. Kynurenine concentration remained high even after treatment.

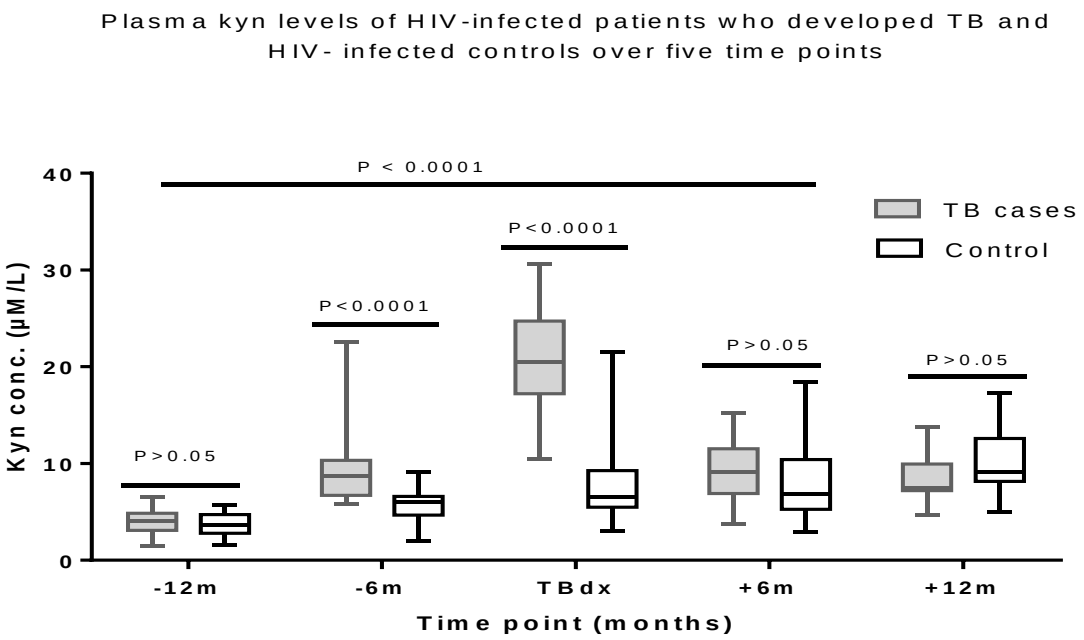


Figure 12.3: Plasma Kynurenine levels of HIV infected patients who developed TB and HIV infected controls over 5 time points

*P value was determined by Dunn's multiple comparison test. The lower and upper portion of the boxes indicates 25th and 75th percentiles respectively whilst the horizontal lines within the box plots indicates median.

12.6 Plasma Tryptophan concentration in HIV infected TB cases and controls

Again like Kyn, plasma Trp levels were evaluated from 12 months before (-12m) TB diagnosis to 12 months after TB diagnosis in HIV infected TB cases and compared with controls as presented Figure 12.4. Plasma Trp concentrations at baseline (-12m) did not vary significantly in TB cases and controls (median, 38.33 [IQR 28.76-46.42 µM/L] versus 41.14 [IQR 35.20-46.57 µM/L], P = 0.1119). However, plasma Trp concentrations decreased significantly at the time of TB diagnosis in TB cases compared to controls at the third visit (V3) (median, 15.43 [IQR 12.46-18.74 µM/L] versus 39.36 [IQR 31.03-53.48 µM/L], P < 0.0001). At 6 months prior to TB diagnosis, Trp levels were significantly decreased in those who developed TB compared with controls at the same time point (median, 23.13 [IQR 18.39-28.24 µM/L] versus 32.74 [IQR 26.58-40.45 µM/L], P < 0.0001).

After anti-TB treatment, plasma Trp levels were significantly higher in TB cases than controls. The median Trp level of TB cases was 47.24 [IQR 43.75-53.59 µM/L] which was relatively higher than the median Trp levels in the control (median, 36.68 [IQR 31.60-41.54 µM/L], P < 0.0001). Plasma Trp levels remained fairly higher in TB cases after anti-TB treatment even up to 12 month (+12m) after TB diagnosis and treatment.

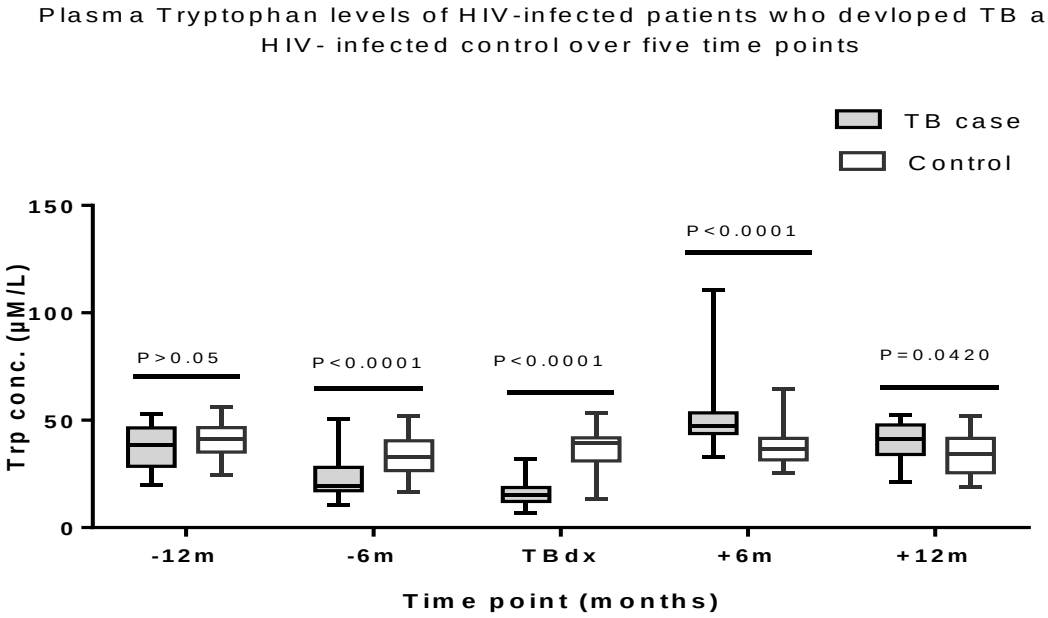


Figure 12.4: Plasma Tryptophan level in HIV infected who developed TB and HIV infected control over 5 time points
*P value was determined by Dunn's multiple comparison test. The lower and upper portions of the boxes indicate 25th and 75th percentiles respectively whilst the horizontal lines within the box plots indicates median.

12.7 Plasma Kynurenine or Tryptophan concentrations in laboratory confirmed TB and clinical TB

To determine if Kyn or Trp levels in laboratory confirmed TB cases significantly varied from clinical TB, we performed a Mann-Whitney test. Figure 12.5 (A & B) shows the comparison of Kyn or Trp in patients with laboratory confirmed TB and clinical TB.

The results indicated that neither Kyn nor Trp showed a significant statistical difference between confirmed TB and clinical TB cases. The median Kyn level in patients with laboratory confirmed TB and clinical TB were 20.50 [IQR 17.13-24.12 µM/L] versus 20.47 [IQR 16.27-

25.52 µM/L], P = 0.6733). Whilst the median Trp levels in laboratory TB and clinical TB patients were 14.74 [IQR 12.23-18.51 µM/L] versus 15.73 [IQR 12.43-18.82 µM/L] respectively with a P value of 0.4816.

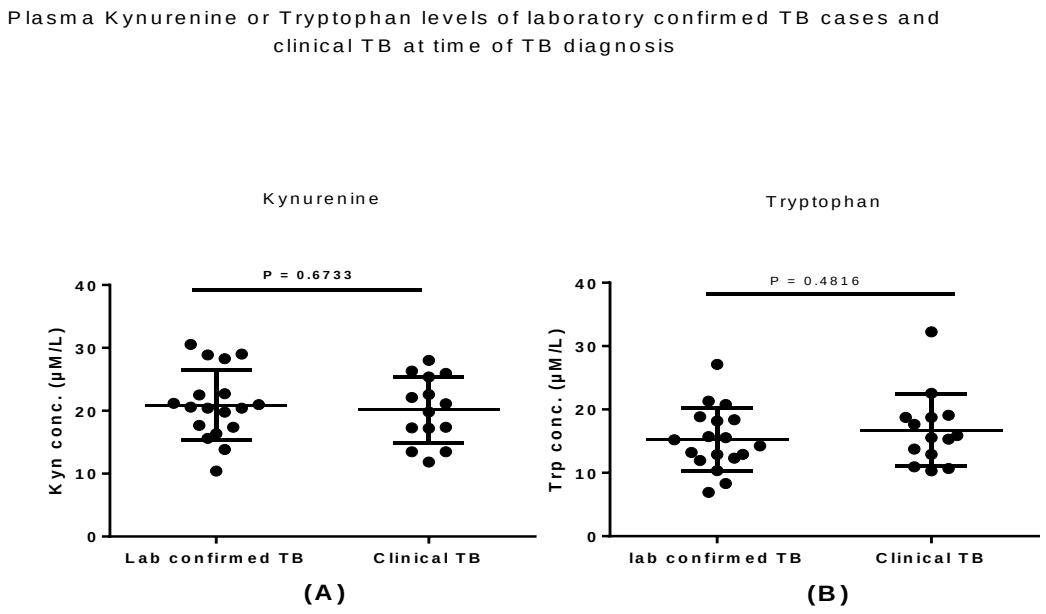


Figure 12.5 1: Plasma Kynurenine or Tryptophan in confirmed TB cases and clinical TB

* P value was determined by Mann-Whitney test. The lower and upper portions of the scatter plots indicates 25th and 75th percentiles respectively whilst the horizontal lines in the middle indicates the median.

12.8 Plasma IDO activity fold change in HIV infected TB cases and controls

To evaluate IDO activity change in months prior to TB diagnosis and its prognostic significance, the IDO activity fold change was determined by using the 12 months before TB diagnosis IDO activity level (-12m) as the reference/baseline time point. IDO activity fold change was defined as the ratio of IDO activity level at a particular time point to the activity level at baseline. For controls, baseline was defined as the first visit.

$$IDO\ activity\ fold\ change = \frac{(IDO\ activity\ at\ any\ time\ point)}{(IDO\ activity\ at\ baseline\ (-12m))}$$

For controls, average IDO activity fold change over 5 time points (2 and half years) was 4 with the range of 0-13 from baseline. Among the TB cases, the average IDO activity fold change over 5 time points (2 and half years) was 16 with the range of 5-58 from baseline.

At the time of TB diagnosis, plasma IDO activity had increased by about 12 fold from baseline in TB patients (median 12 [IQR 9-20]) compared to about 2 fold increase in controls at the third visit (V3) with IQR [1-4]. Even at 6 months before TB diagnosis, the median IDO activity fold change in TB patients was 4 [IQR 2.49-4.62] compared to 2 [IQR 1.40-2.58] in controls at second visit (V2). The results indicate that plasma IDO activity significantly increased in TB cases compared to controls in months prior to TB diagnosis. Plasma IDO activity increased to a peak of about 12 fold at TB diagnosis and declined significantly after treatment to two times baseline.

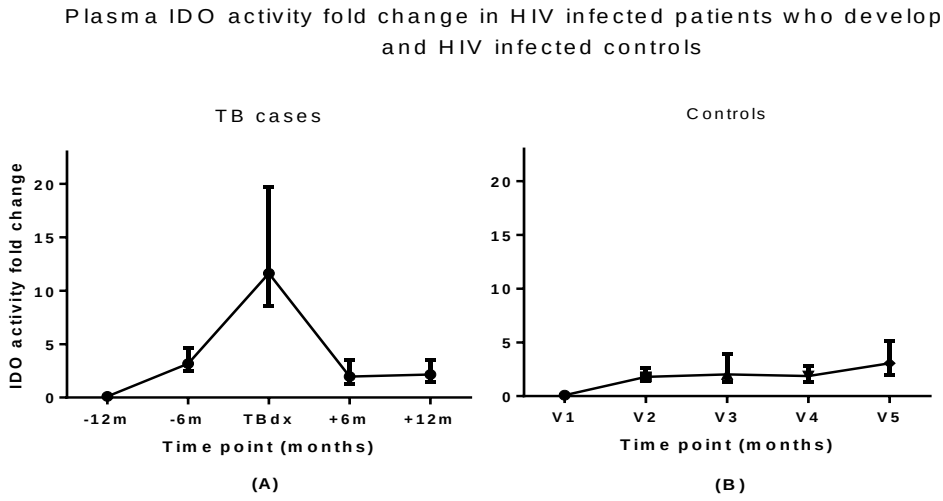


Figure 12.6 2: Plasma IDO activity fold change in controls and TB cases
*This is a plot of median IDO activity fold change with the upper and lower bars indicating interquartile ranges

12.9 Diagnostic significance of plasma IDO activity

To evaluate the diagnostic significance of elevated plasma IDO activity, we selected different cut-off to determine diagnostic sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) at various time points (Table 12.2). Using both laboratory

confirmed and clinical TB at the time of TB diagnosis as gold standard positives (n = 32) and true negatives as all IDO activity results at all control time points, TB cases at baseline, -6m, +6m and +12m (controls n = 335). Plasma IDO activity at a cut-off of 0.70 gave a diagnostic sensitivity of 100% and a specificity of 98.57% with Positive and Negative predictive values of 96.97% and 100% for detecting active TB cases. A receiver operative characteristics (ROC) curve was performed to determine the optimum threshold that gives the best sensitivity and specificity to indicate TB. At an optimum cut-off of 0.70, at the time of TB diagnosis, IDO activity showed the highest area under the curve (AUC) of 0.99 (Figure 12.7).

Table 12.2: Diagnostic significance of IDO activity at TB diagnosis time point

Using confirmed & clinical TB as gold standard positive (n = 32) and true negatives included controls at all time points and TB patients at time points other than TB diagnosis (TBdx) (n = 335)

	At time of TB diagnosis			
Cut off value	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
0.50	100	96.7	74.4	100
0.70	100	98.5	86.4	100
0.80	96.8	99.4	93.9	99.7
0.85	84.3	99.7	96.4	98.5

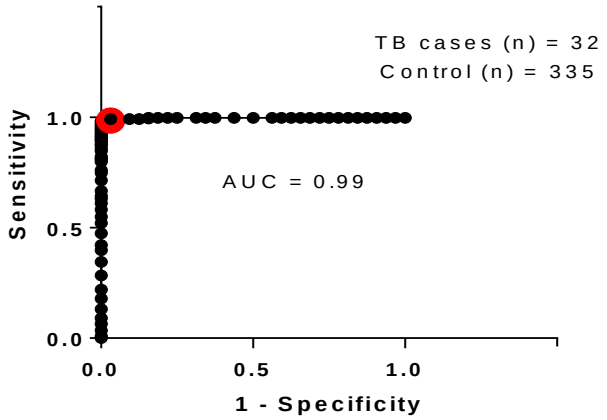


Figure 12.7: ROC curve: using plasma IDO activity results at TB diagnosis time point

*At a cut-off value of 0.70, plasma IDO activity showed a sensitivity of 100% and specificity of 98.5%

We repeated the analysis using laboratory confirmed and clinical TB at time of TB diagnosis as gold standard positives (n = 32) and all control time points together this time with TB cases at baseline, +6m and +12m as controls but excluding IDO activity results for TB cases at 6 months before TB diagnosis (-6m) from the analysis (controls, n = 308). Plasma IDO activity showed variable sensitivities and specificities at various thresholds (Table 12.3). Plasma IDO activity gave a diagnostic sensitivity of 100% and specificity of 99.6% with PPV and NPV of 96.9% and 100% respectively at a cut-off of 0.70 for detecting TB. A ROC curve analysis to determine the optimum cut-off gave the highest AUC of 0.99 at this time point (Figure 12.8).

Table 12.3: Diagnostic significance of IDO activity at TB diagnosis time point

Using confirmed & clinical TB as gold standard positives (n = 32) and true negatives included all controls at all time points and TB patients at time points other than TBdx and -6m (n = 308)

At time of TB diagnosis				
Cut off value	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
0.50	100	98.7	88.8	100
0.70	**100**	**99.6**	**96.9**	**100**
0.80	96.8	100	100	99.6
0.85	84.3	100	100	98.4

ROC curve: Using plasma IDO activity results at TB diagnosis time and excluding 6 months prior to TB diagnosis results

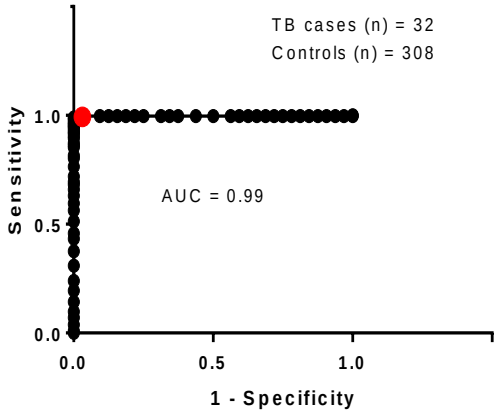


Figure 12.8: ROC curve: Using plasma IDO activity results at TB diagnosis time point excluding -6m results
*cut-off of 0.70 (red dot) plasma IDO activity showed a sensitivity of 100% and specificity of 99.6%

Furthermore, we repeated the analysis this time considering IDO activity results at TB diagnosis and 6 months prior to TB diagnosis (-6m) as gold standard positives (n = 59), and using controls at all-time points as well as TB patients baseline, 6 months (+6m) and 12 month after treatment (+12m) as true negatives (controls, n = 308). IDO activity gave a diagnostic sensitivity of 60% and a specificity of 99.0% with Positive and Negative predictive values of 94.0% and 92.4% for detecting active TB cases at a cut-off of 0.70 (Table 12.4).However, at a cut-off of 0.28, IDO activity gave a higher sensitivity and specificity of 93% and 91%

respectively. A ROC curve analysis of results at this time point to determine the optimum threshold gave the highest AUC of 0.9567 (see Figure 12.9) with a $P < 0.0001$.

Table 12.4: Diagnostic significance of IDO activity using -6m and TB diagnosis time point as gold standard positives

Using confirmed & clinical TB as Gold standard (n = 59) and true negatives includes all control time points together with TB patients at -12m, +6m and +12m (n = 308)

	At time of TB diagnosis			
Cut off value	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
0.25	95.0	87.0	59.0	99.0
0.28	**93.0**	**91.0**	**67.0**	**98.0**
0.50	78.0	98.0	88.0	95.0
0.70	60.0	99.0	94.1	92.4

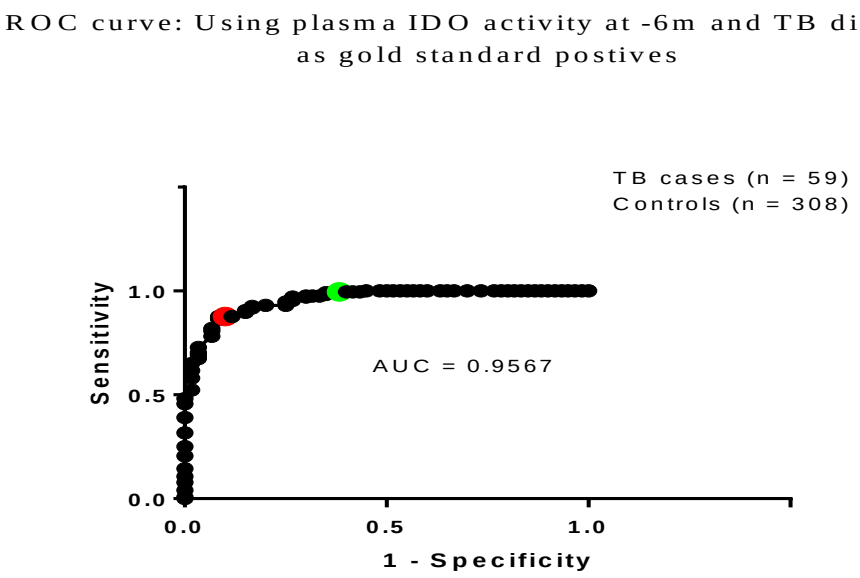


Figure 12.9: ROC curve: Using plasma IDO activity at -6m and TB diagnosis as gold standard positives
At a cut-off of 0.20 (red dot), plasma IDO activity showed a sensitivity of 93% and specificity of 91%
At a cut-off of 0.70 (green dot), plasma IDO activity showed a sensitivity of 60% and specificity of 99%

12.10 Prognostic significance of plasma IDO activity in HIV infected TB cases and HIV infected controls

To determine if plasma IDO activity at 6 months prior to TB diagnosis could be used to diagnose TB disease 6 months ahead of clinical symptoms (Table 12.5), we determined the

diagnostic value of plasma IDO activity at 6 months prior to TB. We used laboratory confirmed TB and clinical TB at 6 months prior to TB diagnosis as gold standard positive for this time point (n = 27), and all control time points and TB cases at baseline, +6m and +12m as controls (n = 181). Excluding TB cases at time of TB diagnosis, at a cut-off of 0.28, plasma IDO activity gave a diagnostic sensitivity, specificity, PPV and NPV of 93.8%, 81.4%, 66.6% and 97.1% respectively (Table 10.10). At a cut-off of 0.70, IDO activity gave a sensitivity less than 50% but a perfectly identified all controls as true negatives. A ROC curve analysis at 6 months prior to TB diagnosis (Figure 12.10), showed the greatest AUC of 0.93 at a cut off threshold of 0.28 with a p value < 0.0001.

Table 12.5: Diagnosing TB using IDO activity at 6 months prior TB diagnosis time point

Using confirmed & clinical TB as Gold standard (n = 27) and Control (n = 180)

	At 6 months before TB diagnosis			
Cut off value	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
0.25	85.1	91.6	60.5	97.6
0.28	81.4	93.8	66.6	97.1
0.30	74.0	93.8	64.5	96.0
0.40	62.9	97.7	89.9	94.6
0.70	26.0	100	100	90.0

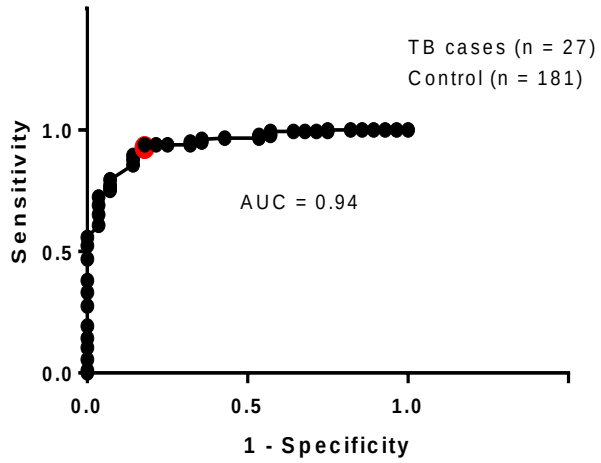


Figure 12.10: ROC curve: Using plasma IDO activity at 6 months prior to TB diagnosis
*At a cut off of 0.28 (red dot), plasma IDO activity showed a sensitivity of 81% and specificity of 93%

12.11 Plasma IDO activity and other clinical parameters

To evaluate whether plasma IDO activity results correlated with other clinical evaluation parameters, we determined if IDO activity at baseline or at time of TB diagnosis correlated with CD4 cell count, HIV viral load count or CRP.

In a spearman correlational analysis, IDO activity showed no significant correlation with CD4 cell count, HIV viral load count or CRP levels at baseline or time of TB diagnosis (Table 12.6).

Table 12.6: Correlation of plasma IDO activity with CD4 cell count, HIV viral load & CRP

Clinical parameter	Correlation co-efficiency (r)	P value
	Baseline	
CD4 cell count	0.0527	0.7939
HIV viral load count	-0.3822	0.0590
CRP	-0.0796	0.6990
	Time of TB diagnosis	

CD4 cell count	0.2762	0.1329
HIV viral load count	-0.1303	0.4697
CRP	-0.3404	0.0708

*Spearman correlation

Figure 12.11 (A - C) show scatter plots of the correlation between plasma IDO activity and CD4, HIV viral load and CRP at baseline (-12m) and time of TB diagnosis.

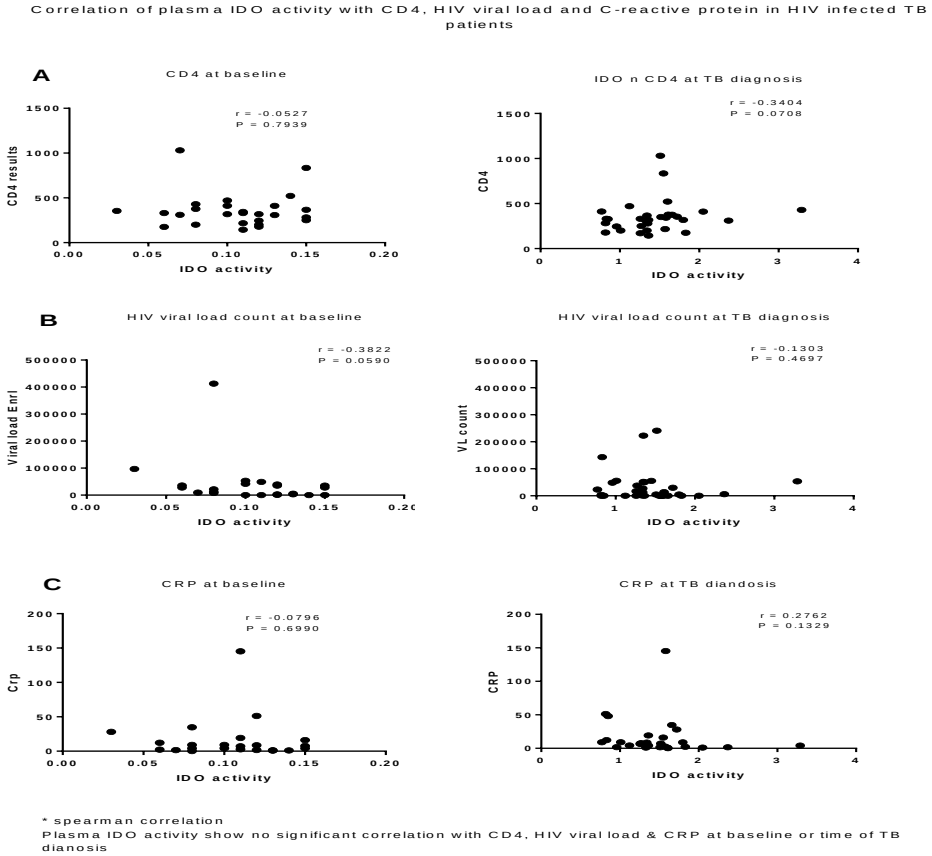


Figure 12.11: Plasma IDO correlation with CD4, HIV viral load & CRP

12.12 Plasma IDO activity in patients with pulmonary TB and extra-pulmonary TB

To evaluate whether plasma IDO activity varied between PTB and EPTB patients, we compared plasma IDO activity in patients with PTB to EPTB. Even though our cohort had 2 EPTB cases, we could only gain access to blood samples of only 1. There was no noticeable difference in plasma IDO activity between PTB patients and the EPTB patient. The average

IDO activity in PTB was 1.43 (0.50) with a median of 1.40 [IQR 1.2-1.6], which spanned the result of 1.6 in the EPTB patient. As there was only one EPTB patient we could not compare results for statistical difference.

CHAPTER 13

Discussion and conclusion

We evaluated IDO activity in plasma as a potential TB biomarker by measuring plasma Kyn and Trp concentrations in HIV infected patients who developed active Tuberculosis in a longitudinal cohort.

At baseline, there was no significant difference in CD4 cell count between controls and those who progressed to develop TB. The median CD4 cell count in controls and those progressed to TB was 372 cells/ml [IQR240-403] versus 329 cells/ml [IQR 240-403] respectively. This observation was because we selected our controls by matching CD4 cell count, age and sex to the TB cases at baseline. However, those who progressed to develop TB had significantly lower BMI and higher HIV viral load count compared to controls at baseline. Lower BMI and high HIV load in HIV infection are significant predictive risk to early progression to AIDS (163).

At the time of TB diagnosis, TB cases had significantly lower CD4 cell count (median, < 250 cells/ml) with higher HIV viral load (median > 5000 copies/ml) and increased CRP levels compared to controls. CRP is a marker of acute inflammation which in HIV infection is linked to sustained immune activation. Low CD4 cell count with high HIV viral load and lower BMI are associated with a weak immune system and progression to AIDS in HIV infection (163, 164). TB is usually the first opportunistic infection to develop in AIDS infection.

In controls, IDO activity showed good reproducibility over 5 time points (two and half years), with standard deviation (SD) of 0.10 and %CV of 55%. The median IDO activity in controls was 0.16 ranging from 0.04 to 0.76.

The normal biological variation in IDO activity over time in controls was about two fold (range, 1-4) change from the baseline. This gives an indication of intra-individual variability. In TB cases, the median IDO activity fold change was 12 fold (range, 9-20) from baseline in months preceding TB diagnosis. After anti-TB treatment, IDO activity in TB patients declined to almost same levels as that of the controls. Additionally, even 6 months prior to diagnosis, those who developed TB had significantly higher IDO activity compared to controls.

For controls, average IDO activity fold increase was 4 with the range of 0-13 from baseline over 5 time points. However, among the TB patients, the average IDO activity fold change over the same period increased to 16 with the range of 5-58 from baseline. The finding of significantly elevated IDO activity in TB patients at the time of TB diagnosis is consistent with recent literature (6, 7, 9, 165). Although elevated IDO activity was due to increased Kyn concentration and decreased Trp concentrations, neither Kyn alone or Trp alone showed strong sensitivity and specificity on its own for TB diagnosis.

IDO is a potent immune regulatory enzyme involved in modulating immune cell activation and phenotype. IDO is chiefly induced by the pro-inflammatory cytokine IFN-γ at the site of infection. The downstream metabolites of IDO along the Kyn pathway have been shown to potently impair CMI. The variability of IDO activity in HIV infected patients who develop TB disease in months or years after HIV diagnosis has not been previously assessed. In this regard, we evaluated IDO activity in a longitudinal cohort from 12 months prior to TB diagnosis to 12 months after TB diagnosis in HIV infected patients. IDO activity increased in the months prior to TB diagnosis and declined during anti-TB treatment.

To evaluate the diagnostic significance of elevated IDO activity, we calculated the diagnostic sensitivity, specificity, positive and negative predictive values of plasma IDO activity at various cut-off thresholds. Using laboratory confirmed TB and clinical TB at time of TB diagnosis as gold-standard positives (n=32) and true negatives as all control visits at all-time points and TB cases at all-time points other than TB time points but excluding 6 months before TB results (-12m, +6m and +12m) (controls n = 335), the diagnostic sensitivity, specificity, PPV and NPV was 100%, 99.6%, 96.9% and 100% respectively at a cut-off of 0.70. In a receiver operating characteristic (ROC) curve, at 0.70 as the optimal cut-off, IDO activity gave the highest area under the curve (AUC) of 0.99 with a P value < 0.0001.

This results means that if we use IDO activity at the time of TB diagnosis in HIV infected patients as a test for the presence of TB, IDO activity correctly classified all TB patients (sensitivity of 100%) as true positives with a PPV of about 97%. A negative result for IDO activity at a cut-off 0.70 means that the patient does not have TB (NPV of 100%). Also, IDO activity has a high specificity for diagnosing TB. Among the controls (true negatives), IDO activity has a diagnostic specificity of 99.9%. This means that IDO activity at a cut-off of 0.70 correctly classified 307 out of 308 control subjects as not having TB (true negatives) with NPV of 100%. The 100% NPV means that those with a negative IDO activity test at a cut-off of 0.70 have a probability of 1 being truly negative of TB. This result implies that IDO activity is an excellent diagnostic test for ruling out TB in suspected patients at a cut-off of 0.70. IDO activity is an excellent diagnostic test for indicating the presence of TB in HIV infected patients. So both a rule-in and rule-out test for TB, IDO activity shows stronger diagnostic potentials than the conventional sputum smear microscopy and sputum culture test for TB in HIV infected individuals.

Both sputum smear microscopy and microbiological culture have poor diagnostic sensitivity and specificity and suffer significant limitations due to their due over-reliance on sputum samples as the preferred sample for analysis. In advanced HIV disease, where patients are unable to produce quality sputum for analysis these methods have shown tremendous variability in sensitivity and specificity (164, 166, 167). The problem is compounded by the increased prevalence of EPTB in advanced HIV infected patients (14, 41). An advantage of IDO activity in plasma is that it is a blood based test and blood is more readily available than sputum. Also, it is easier to get blood in these patients than to get sputum.

To further evaluate the potential of IDO activity as a biomarker for predicting TB disease ahead of appearance of symptoms, we evaluated the diagnostic significance of elevated IDO activity at 6 months prior to TB diagnosis. Using only IDO activity results at 6 months prior to TB diagnosis as true positive for diagnosing TB (n=25), and using all controls at all-time points and TB cases at -12m as controls (controls n = 180), at a cut off of 0.28, IDO activity has a sensitivity and specificity of 81% and 94% respectively with PPV and NPV of 67% and 97% for predicting TB disease in HIV infected patients. A ROC curve gave the highest AUC of 0.94. However, at the diagnostic cut-off of 0.70, IDO activity performed poorly with a sensitivity of <50% but specificity of 100%. This results indicates that IDO activity may be a good diagnostic/predictive test for ruling-in HIV patients who will progress to develop TB. IDO activity failed to indicate the presence of TB in about 20% of patient when the test was applied to them 6 months ahead of being diagnosed as having TB by the gold standard test. However, IDO activity correctly classified about 80% of HIV infected patients who went on to develop TB within 6 months. IDO activity may thus be a useful biomarker for predicting progress to active TB disease 6 months before the appearance of active disease symptoms.

IDO activity may be applied using two cut-off values. A cut-off of 0.70 is optimal for diagnosing TB and 0.28 for predicting progress to TB in months ahead. In between these cut-off values, IDO activity showed variable diagnostic sensitivity and specificity as well as PPV and NPV. However, HIV infected patients with IDO activity above 0.28 could be closely monitored for progress to TB and patients with IDO activity above 0.70 with clinical signs could be said to have active TB. We chose 0.70 as a diagnostic cut-off because of high sensitivity and high positive predictive value.

In addition, we investigated whether IDO activity could differentiate laboratory proven TB and clinical TB and classify PTB and EPTB at the time of TB diagnosis. IDO activity showed no difference between laboratory confirmed TB and clinical TB nor between PTB and EPTB, although our sample size numbers were small. This suggests IDO activity may be useful in indicating the presence or absence of TB disease in either site, and like the IGRAs fail to classify the site of infection. Unlike IGRAs, IDO activity discriminates between active TB disease and latent or cured infection.

IDO activity declined to levels comparable to that of controls in response to anti-TB treatment. Plasma IDO activity declined drastically (about 15 fold change from baseline) in TB patients after treatment to levels comparable to that of the controls. This finding is also consistent with the findings of Almeida et al. who reported IDO activity deceased in TB patients after anti-tuberculous therapy (6). This implies that plasma IDO activity may perhaps be used to monitor anti-tuberculous treatment in TB-HIV infected patients. We had no post treatment relapses with which to assess whether IDO activity could be used for monitoring successful versus unsuccessful treatment. Currently, TB treatment response is monitored by 2 months sputum conversion. Usually it becomes difficult for TB patients to produce sputum for follow-up

analysis when they improve upon starting anti-TB treatment. The IGRAs also fail to discriminate active disease from previous exposure and cannot be used to monitor treatment because of such limitations (168). In this regard, IDO activity offers a great potential to monitor anti-TB treatment response since it is a blood based rather than sputum based test. This aspect requires follow-up studies of larger cohorts, including patients with treatment failures.

IDO activity showed no significant correlation with CD4 cell count or HIV viral load count. This implies that even though HIV patients with low CD4 and high viral load counts are likely to get TB disease, the low CD4 or high viral load is unlikely to be the cause of increased plasma IDO activity. This finding is in agreement with the study by Cheng et al., 2014 which found elevated IDO activity does not correlate with CD4 cell count in HIV infected patients as the disease progressed.

An improved TB biomarker would be of great impact in the fight against TB especially in predicting TB disease early in infected persons, indicating TB at the time of diagnosis and potentially monitoring anti-TB therapy. Most biomarker studies on early prediction of TB disease are focused on using microbiological diagnosis, chest radiograph or clinical manifestations (105). In this regard, the advantage of plasma IDO activity to all other proposed diagnostic biomarkers is it is elevated months before development of clinical signs in HIV infected patients. Early identification or prediction of TB in HIV infected patients would be critical in helping to reduce the occurrence of contagious pulmonary TB in HIV infected patients and limit disease transmission. Future studies may investigate IDO as a surrogate endpoint for development of a better TB vaccine other than the current BCG.

Most potential TB biomarkers identified so far are indicators of general intracellular infection and subsequent immune activation rather than highly specific for TB disease. It should be noted however that there is a large overlap with biomarkers reported in other inflammatory diseases including sarcoidosis and melioidosis (105, 169). Even though Sarcoidosis and melioidosis cannot be easily discriminated from TB based on clinical manifestations, additional existing laboratory diagnostic tests are used to rule-out these diseases. Furthermore, it is not surprising that most proposed TB biomarkers overlap with other chronic inflammatory conditions or infections. This is because most immune activation pathways are represented by common genes at the cellular level. In this regard, it is to be anticipated that a large number of candidate biomarkers would be shared between disease pathology that rely on similar host responses. A limitation of our study to date is that we have not yet analysed samples from patients with lung infections other than TB.

In infectious diseases, very little is known about the precise role of IDO activity. Initially, IDO was considered for its antimicrobial roles in infectious diseases especially in diseases caused by tryptophan-requiring intracellular pathogens, such as some *Chlamydia* species, *Leishmania donovani* and *Toxoplasma gondii* (135, 170, 171). IDO activity also exerts potent immunosuppressive functions to curb exacerbation of immune response. It is not clear whether complete inhibition of IDO expression in HIV infected patients would be beneficial or harmful to the host immune defences. There are various studies that has explored inhibition of IDO expression in animal models. For instance, in a study by Jung et al., blocking IDO expression was beneficial to mice since it protected them against LPS-induced endotoxin shock (172). However, blocking IDO expression in sheep or cattle did not enhance proliferation or restoration of immune cells. These discrepancies in animal models might be due the differences in infectious pathogesn and the severity of infections (136, 172, 173). With regards to TB in

humans, there is an emerging body of evidence that imatinib (a tyrosine kinase inhibitor used in the treatment of multiple cancers, most notably chronic myelogenous leukemia (CML)) inhibits IDO *in vivo*. Future studies would be needed to ascertain the prospects of IDO inhibition as potential/novel therapy in HIV infected patients.

There are several strengths of this study. First, this retrospective cohort was from a longitudinal study and patients had been evaluated from about 12 months prior to TB diagnosis to 12 months after TB diagnosis (two and half years). Secondly, both study subjects and controls were all HIV infected patients who were naïve to therapy at the time of recruitment into the main study.

Limitations to this study were that the study was conducted prior to the nationwide implementation of molecular diagnostic methods such as GeneXpert in routine TB diagnostic services. As a result, there was no molecular confirmation of TB cases. Also, there was no data on TB case treatment outcome (e.g. sputum conversion). The TB patients were treated in clinics outside the main study centre. Furthermore, because our cohort was adult HIV infected patients, it is not clear whether our results also apply to HIV uninfected or paediatric populations. We have not yet analysed results of plasma IDO activity in patients with other lung infection other than TB. These samples are available and due to be analysed in the near future.

Conclusion

In conclusion, we report that IDO activity, as measured by Kyn-to-Trp ratio using LC-MS/MS at a recommended cut-off of 0.70 could be used as a biomarker with a diagnostic sensitivity of 100% and a specificity of 98.5% with positive and negative predictive values of 96.9% and

100% for detecting active TB cases in HIV infected individuals. Furthermore, IDO activity may be a useful biomarker for predicting progress to active TB disease.

Future studies are required to assess the behaviour of IDO activity prospectively, including in patients with other lung diseases, and to assess utility of IDO activity in monitoring response to TB treatment.

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Appendix A

Ethics clearance certificate

R14/49 Mr Clement Adu-Gyamfi

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140488

NAME: (Principal Investigator)	Mr Clement Adu-Gyamfi
DEPARTMENT:	Molecular Medicine and Haematology Centre for Vaccines and Immunology National Communicable Diseases, Johannesburg
PROJECT TITLE:	Serum Indoleamine 2,3-Dioxyginase Activity, A Potential Biomarker for Active Tuberculosis Infection
DATE CONSIDERED:	25/04/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Melinda Suchard
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	23/05/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B

Study cohort

TB cases

Patient ID	Sex	Age	Date of enrolment	Visit number	Date of diagnosis	Mode of Diagnosis
170012	1	38	3-Dec-08	12	18-Mar-2010	SSM (++)
170100	2	28	16-Feb-09	12	25-Jan-2010	SSM (+)
170374	2	31	14-Oct-09	12	18-Oct-2010	SSM (+)
170467	2	31	8-Mar-10	12	24-Mar-2011	SSM (+++)
170541	2	27	6-May-10	6	2-Nov-2010	SSM (+)
170553	2	32	18-May-10	18	16-Nov-2011	SSM (+)
170557	2	29	19-May-10	12	5-Aug-2011	SSM (+)
170648	1	46	4-Aug-10	12	11-Aug-2011	SSM (+)
170704	1	46	17-Sep-10	6	15-Mar-2011	SSM (++)
170705	2	36	20-Sep-10	12	20-Sep-2011	SSM (+)
170707	1	40	20-Sep-10	12	23-Aug-2011	SSM (+)
170727	2	42	30-Sep-10	18	21-Jul-2010	SSM (+)
170750	2	45	26-Oct-10	6	13-Aug-2009	SSM (+)
170123	1	56	25-Feb-09	12	25-Feb-2010	Culture
170559	2	28	20-May-10	12	30-May-2011	Culture
170583	2	50	7-Jun-10	24	6-Jun-2012	Culture
170177	1	32	7-May-09	12	7-May-2010	SSM & Culture
170053	2	46	20-Jan-09	18	21-Jul-2010	Suggestive
170082	2	29	4-Feb-09	12	4-Feb-2010	Suggestive
170121	1	41	25-Feb-09	12	25-Feb-2010	Suggestive
170146	2	43	16-Apr-09	18	15-Apr-2010	Suggestive
170212	2	38	26-May-09	12	26-May-2010	Suggestive
170251	2	53	24-Jun-09	12	9-Nov-2010	Suggestive
170265	2	51	30-Jun-09	12	2-Jul-2010	Suggestive
170324	2	31	26-Aug-09	6	14-Jun-2010	Suggestive
170340	2	46	7-Sep-09	6	21-Jun-2010	Suggestive
170515	1	33	20-Apr-10	18	19-Oct-2011	Suggestive
170584	2	39	8-Jun-10	12	2-Jul-2011	Suggestive

170588	1	41	10-Jun-10	6	6-Apr-2011	Suggestive
170715	2	43	27-Sep-10	12	18-Mar-2010	Suggestive
170067	1	32	29-Jan-09	6	13 Aug-2009	*LDH 556
170127	2	40	6-Apr-09	12	6-Oct-2010	Skin TB

**SSM = Sputum smear microscopy Culture = M. tuberculosis culture positive LDH = Lactate dehydrogenase*

Controls

Patient ID	Sex	Age	Date of enrolment
170005	1	36	27-Nov-08
170010	2	37	02-Dec-08
170013	2	50	03-Dec-08
170024	2	48	11-Dec-08
170025	2	41	11-Jun-09
170027	2	42	18-Dec-08
170046	2	29	18-Jun-09
170069	2	33	18-Dec-09
170070	2	32	29-Jan-09
170084	2	36	30-Jul-09
170089	2	37	29-Jan-10
170090	1	43	30-Jul-10
170092	2	45	29-Jan-11
170101	2	44	31-Jul-11
170104	2	33	17-Feb-09
170119	1	42	18-Aug-09
170122	2	31	17-Feb-10
170125	2	30	18-Aug-10
170131	1	40	17-Feb-11
170142	2	32	19-Aug-11
170150	2	33	17-Feb-12
170159	2	40	17-Feb-09
170160	2	51	18-Aug-09
170170	2	28	17-Feb-10

170176	2	41	07-May-09
170192	2	36	05-Nov-09
170194	1	48	07-May-10
170195	2	41	05-Nov-10
170196	2	36	07-May-11
170205	2	43	06-Nov-11
170207	2	35	06-May-12
170214	2	35	07-May-09
170220	1	39	05-Nov-09
170247	2	27	07-May-10
170253	2	37	05-Nov-10
170258	2	32	07-May-11
170260	2	32	08-May-09
170266	2	32	06-Nov-09
170274	2	36	08-May-10
170284	2	38	06-Nov-10
170301	2	39	05-Aug-09
170306	2	44	03-Feb-10
170315	1	26	05-Aug-10
170324	2	31	03-Feb-11
170349	2	26	05-Aug-11
170376	1	48	06-Aug-09
170392	2	30	04-Feb-10
170403	1	45	06-Aug-10
170408	2	34	06-Aug-09
170456	1	22	04-Feb-10
170457	2	38	02-Mar-10
170494	2	27	31-Aug-10
170505	2	23	02-Mar-11
170507	1	47	31-Aug-11
170510	2	28	01-Mar-12
170531	1	38	02-Mar-10
170563	2	41	31-Aug-10
170577	1	37	03-Mar-10
170579	2	44	01-Sep-10

170593	2	33	03-Mar-11
170603	1	36	01-Sep-11
170605	2	27	03-Mar-10
170636	2	39	04-Mar-10
170640	2	45	02-Sep-10
170643	1	37	04-Mar-11
170655	1	34	02-Sep-11
170669	1	39	04-Mar-10
170726	1	49	02-Sep-10
170749	2	38	04-Mar-11
170752	2	26	02-Sep-11

**n = 70 Sex 1 = Male, 2 = Female*

Appendix C

Study results

TB cases

	Enrolment (-12m)			6 months before TB (-6m)			Time of TB diagnosis (TBdx)			Post treatment (+6m)			Post treatment (+12m)		
PT ID	**Kyn**	**Trp**	**Kyn/Trp**	**Kyn**	**Trp**	**Kyn/Trp**	**Kyn**	**Trp**	**Kyn/Trp**	**Kyn**	**Trp**	**Kyn/Trp**	**Kyn**	**Trp**	**Kyn/Trp**
170012	5,14	45,03	0,11	10,28	21,18	0,48	29,00	21,33	1,36	11,18	47,24	0,24	11,56	52,28	0,25
170053	3,51	29,36	0,12	8,68	18,39	0,50	17,68	18,39	0,96	7,68	49,17	0,22			
170067				10,5	26,92	0,39	17,39	12,92	1,35	12,34	47,57	0,26	7,53	43,86	0,17
170082	5.00	36,22	0,14	8,44	18,85	0,45	21,19	13,22	1,60	10,26	54,22	0,19			
170100	3,29	42,28	0,08	6,71	19,30	0,35	22,73	6,90	3,29	9,08	51,02	0,18	10,07	41,29	0,24
170121	2,08	38,11	0,05	9,32	17,40	0,64	28.00	15,30	1,83	5,32	53,76	0,19	7,20	37,4	0,21
170123	4,27	28,90	0,15	8,92	11,41	0,78	19,78	15,55	1,27	5,68	43,75	0,24	7,20	32,17	0,22
170127	2,26	27,68	0,08	7,86	24,73	0,32	22,11	13,76	1,61	9,17	38,29	0,31			
170146	4,02	38,54	0,10	6,77	10,40	0,65	19,81	17,66	1,12	13,8	47,24	0,29	5,82	36,05	0,38
170177	2,61	35,49	0,07	6,45	28,35	0,23	30,56	12,88	2,37	9,67	45,99	0,32			
170212	4,56	43,42	0,11	6,62	32,36	0,26	17,20	10,96	1,57	6,43	41,53	0,18	7,36	48,49	0,15
170251	4,01	35,10	0,11	6,51	21,48	0,30	13,49	10,67	1,26	10,28	110,53	0,09			
170265	3,94	26,73	0,15	5,8	17,15	0,34	26,33	32,24	0,82	6,76	63,85	0,11			
170324				6,51	21,48	0,30	11,86	15,55	1,52	8,5	67,35	0,13	7,36	37,30	0,20
170340				13,16	27,68	0,48	13,49	18,74	1,26	9,01	47,24	0,19	4,70	47,24	0,10
170374	3,08	26,92	0,11	5,80	17,15	0,34	16,36	10,34	1,58	9,17	45,99	0,20	8,29	21,06	0,39
170467	5,87	49,9	0,15				10,43	12,32	0,85	6,17	41,29	0,15			
170515	3,79	52,7	0,11	9,85	28,35	0,35	21,12	10,32	2,05		53,07	0,14	7,43	32,17	0,23
170541				21,14	23,57	0,90	25,38	18,74	1,36				9,81	49,48	
170553	4,85	41,81	0,12	7,67	50,79	0,15	20,43	15,23	1,34	14,67	43,76	0,34			
170557	4,85	41,81	0,12	6,47	27,92	0,23	15,62	18,19	0,82	7,33	64,17	0,16			
170559	1,44	48,57	0,03	8,44	38,85	0,29	20,57	11,95	1,72	13,8	49,48	0,28			
170583	5,14	50,28	0,15				20,43	14,24	1,34	15,19	32,93	0,46			
170584		19,73		8,68	18,39	0,47	17,29	22,56	0,77	9,87	41,29	0,24			
170588							22,57	15,90	1,45						
170648	4,01	48,57	0,08	9.00	29,57	0,3	20,98	20,80	1,01	7,33	34,17	0,30			
170704				17,39	22,68	0,45	17,39	12,92	1,35	5,28					
170705	3,85	45,7	0,08	9,67	29,79	0,19	13,85	8,33	1,66	3,78	46,36	0,08			
170707	4,85		0,12	22,57	25,90	0,84	28,28	15,72	1,80						

170715	3,55	28,33	0,13	6,71	19,30	0,35	25,94	19,07	1,33	10,13	64,17	0,16
170727	2,51	34,69	0,07	9,32	15,30	0,64	28,88	18,86	1,51	12,66	43,76	0,29
170750				19,97	27,90	0,72	22,5	27,11	0,83	11,64	49,48	0,24

**Kyn = Kynurenine Trp = Tryptophan Kyn/Trp = IDO activity Conc. µM/L*

Controls

	Visit 1 (V1)			Visit 2 (V2)			Visit 3 (V3)			Visit 4 (V4)			Visit 5 (V5)		
Pt ID	**Kyn**	**Trp**	**Kyn/Trp**	**Kyn**	**Trp**	**Kyn/Trp**	**Kyn**	**Trp**	**Kyn/Trp**	**Kyn**	**Trp**	**Kyn/Trp**	**Kyn**	**Trp**	**Kyn/Trp**
170005	4,78	41,47	0,12	6,06	31,37	0,22	6,96	29,47	0,24	6,96	34,73	0,43	9,35	37,3	0,46
170010	3,22	53,48	0,06	5,84	42,36	0,12	5,08	32,36	0,16	5,08	49,51	0,14	6,8	39,28	0,17
170013	3,62	27,17	0,13	2,52	28,69	0,09	4,02	31,03	0,13	4,02	29,11	0,21	8,29	23,3	0,36
170024	3,39	49,04	0,07	5,91	37,84	0,16	7,47	33,75	0,22	7,47	53,32	0,15			
170025	3,98	25,09	0,16	4,62	17,01	0,27	8,63	29,23	0,3	8,63	41,29	0,38	5,64	38,5	0,15
170027	3,13	40,93	0,08	5,87	34,03	0,17	10,24	32,36	0,32	10,24	40,11	0,11	6,59	43,26	0,15
170046	3,2	39,36	0,08	6,54	28,12	0,23	4,67	41,79	0,11	4,67	44,17	0,16	8,15	41,53	0,20
170069	4,12	35,2	0,12	9,11	21,56	0,42	4,67	50,79	0,09	4,67	43,76	0,24			
170070	2,05	37,48	0,05	6,63	25,25	0,26	19,53	33,38	0,59	19,53	33,93	0,12	8,71	29,9	0,29
170084	3,7	41,14	0,09	7,85	21,79	0,36	3,03	29,38	0,1	3,03	29,01	0,17	8,15	41,53	0,2
170089	2,51	34,69	0,07	8,69	19,55	0,44	5,08	39,14	0,13	5,08	41,53	0,08	9,08	41,46	0,22
170090	2,64	42,96	0,06				6,15	29,36	0,21						
170092	2,79	49,9	0,06				6,54	25,09	0,26	6,54	27,11	0,19			
170101	2,54	48,04	0,05	7,56	29,49	0,26	7,52	41,47	0,23	7,52	26,25	0,06	10,68	25,84	0,41
170104	1,9	35,41	0,05	7,47	33,75	0,22	15,91	53,48	0,27	15,91	37,94	0,23	8,29	25,53	0,32
170119				8,92	16,41	0,54	4,62	38,84	0,19				8,15	41,53	0,2
170122	3,37	44,55	0,08	6,91	29,45	0,23	5,87	29,36	0,54				8,29	34,41	0,44
170125	3,33	38,76	0,09	7,47	33,25	0,22	6,54	27,17	0,17	6,54	32,56	0,15			
170131	3,87	38,67	0,1	5,19	34,22	0,15	4,62	49,04	0,12	4,62	40,46	0,13	5,01	25,11	0,20
170142	2,19	31,41	0,07	3,63	29,23	0,12	6,54	13,5	0,48	6,54	26,18	0,25	8,15	52,22	0,24
170150	3,52	35,47	0,1	5,19	32,23	0,17	9,27	41,14	0,11	9,27	43,76	0,18	5,54	41,53	0,2
170159	5,31	48,35	0,11	6,62	35,4	0,19	6,54	40,93	0,16	6,54	40,46	0,13			
170160	2,59	46,39	0,06	5,51	38,43	0,14	9,27	51,97	0,18	9,27	33	0,15	7,53	33,86	0,17
170170	2,77	54,66	0,05	2,04	26,41	0,08	7,52	41,87	0,3	7,52	26,18	0,23			
170176	1,55	44,31	0,04	5,24	42,36	0,12	6,54	45,70	0,46	6,54	43,76	0,16			
170192	3,6	40,61	0,09	5,51	36,23	0,16	8,63	25,09	0,26						
170194	4,52	40,97	0,11	5,51	39,43	0,14	20,47	31,75	0,78						
170195	4,3	39,85	0,11	3,09	26,15	0,12	8,63	29,23	0,3	8,63	33,12	0,19			
170196	3,67	37	0,1	5,24	40,79	0,13	10,24	32,36	0,32	10,24	25,35	0,12			

170205	1,93	39,17	0,05							5,70	31,69	0,18			
170207	3,85	31,76	0,12							5,14	64,38	0,08			
170214	5,25	46,57	0,11	7,47	33,25	0,22	6,54	40,91	0,16	6,54	36,43	0,15			
170220	3,22	46,1	0,07	5,8	36,23	0,16	4,62	29,36	0,39				5,31	17	0,32
170247	5,14	50,28	0,1	3,03	29,38	0,1	5,87	27,17	0,17				17,33	42,26	0,41
170253	4,87	47,38	0,1	6,17	34,05	0,18	5,87	47,52	0,12	5,87	26,97	0,25			
170258				4,67	50,79	0,09	4,62	43,5	0,5	4,62	41,29	0,12	9,08		0,42
170260	4,06	44,75	0,09	6,47	47,92	0,14	6,54	41,14	0,11	6,54	32,17	0,2	7,53		0,17
170266	2,38	47,32	0,05	6,62	35,4	0,26	9,27	40,93	0,16	9,27	37,26	0,15			
170274							8,25	52,12	0,37	8,25			8,29		0,32
170284	4,64	47,29	0,1	6,67	50,79	0,19	10,02	41,87	0,2	10,02					
170301	5,03	32,3	0,16	3,03	25,49	0,12	6,54	47,22	0,42	6,54	49,22	0,15			
170306	2,72	33,13	0,03	4,67	50,79	0,09	6,54	25,09	0,26	6,54					
170315	4,71	36,57	0,13	6,17	27,08	0,23	8,63	39,75	0,37	8,63	25,35	0,34	11,19		0,48
170324	4,12	28,08	0,15	6,47	31,29	0,21	10,24	29,23	0,3	10,24	31,6	0,33			
170349	4,2	46,29	0,09	5,8	21,87	0,27	5,08	32,36	0,32	5,08	31,69	0,33	14,26		0,62
170376	2,65	31,7	0,08	4,67	50,79	0,09	9,73	39,14	0,13	9,73	34,24	0,31	14,26		0,76
170392	5,54	31,4	0,18	6,17	24,05	0,26	9,73	41,57	0,23	9,73	36,43	0,24			
170403	4,67	50,79	0,09	4,67	50,79	0,09									
170408	3,23	56,21	0,06	6,47	27,92	0,23	5,01	38,31	0,37	5,01	39,54	0,13			
170456	4,68	37,88	0,12	6,17	34,05	0,18	5,02	33,24	0,15	5,02	26,97	0,17			
170467	4,03	32,28	0,12	4,67	50,79	0,09	5,87	46,01	0,13	5,87	41,29	0,12			
170494	5,73	37,61	0,15	6,47	27,92	0,23	8,25	41,87	0,13	8,25	29,54	0,16			
170505	5,54	31,4	0,18	6,62	25,4	0,26	4,62	41,29	0,2	4,62					
170507	3,22	24,39	0,13	6,17	26,08	0,31	6,54	39,41	0,11	6,54	32,17	0,20			
170510	5,52	48,67	0,11	2,04	41,41	0,05	6	40,17	0,15	6,00	49,48	0,17			
170531	4,85	41,81	0,12	6,62	25,4	0,26	10,87	45,86	0,24	10,87					
170563	3,22	46,1	0,07				6,54	25,09	0,24	6,54	43,76	0,11			
170577	3,55	28,33	0,13	6,47	27,92	0,23	5,08	32,36	0,26	5,08	31,6	0,17			
170593	5,25	46,57	0,11	4,67	51,79	0,09	4,02	31,03	0,16	4,02	39,22	0,13			
170593	4,96	51,23	0,1	4,67	50,79	0,09	7,47	33,75	0,13	7,47					
170603	3,55	28,33	0,13	3	39,38	0,08	8,63	29,23	0,22	8,63					
170605	2,51	34,69	0,07	3,03	29,38	0,1	5,24	39,36	0,30	5,24					
170636	2,64	42,96	0,06	3,09	26,15	0,12	4,67	41,79	0,26	4,67	55,35	0,10			
170640	2,79	49,9	0,06	2,04	41,41	0,05	6,54	43,5	0,11	6,54					
170643	3,29	42,28	0,08	6,47	37,92	0,17	4,62	42,28	0,50	4,62	36,92	0,23			
170655	5,25	46,57	0,11	6,47	27,92	0,23	6,54	41,14	0,11	6,54					
170669	3,22	46,1	0,07	6,47	27,92	0,23	4,62	40,93	0,11	4,62					
170726	4,82	35,39	0,14	6,62	26,4	0,25	6,00	53,48	0,16	6,00					
170749	2,64	42,96	0,06	4,67	50,79	0,09	6,54	36,38	0,18	6,54	41,57	0,24			
170752	4,82	44,62	0,14	6,17	41,41	0,19	4,62	41,14	0,18	4,62	37,45	0,24			

**Kyn = Kynurenine Trp = Tryptophan Kyn/Trp = IDO activity Conc.= µM/L*

TB cases

			Enrolment/baseline				Time of TB diagnosis			
Pt ID	Sex	Age	BMI	CD4 cell count	HIV viral load count	CRP	BMI	CD4 cell count	HIV viral load count	CRP
170012	1	38	19.81	275	49023	0.9	17.47	144	169	19,2
170053	2	46	24.44	378	35022	38	24.44	245	48587	1,8
170067	1	32	21.44	262	54523	1	21.48		51545	16,2
170082	2	29	25.37	668	49	0.9	26.38	522	49	1
170100	2	28	26.57	250	21289	1	22.48	429	53392	14
170121	1	41	21.33	136	35750	0.9	18.40	175	115	2,2
170123	1	56	20.27	354	35028	15	22.23	250	37535	7,6
170127	2	40	23.88	625	8325	2	40.56	377	13399	0,3
170146	2	43	40.56	428	49	6	17.90	470	49	4,2
170177	1	32	22.72	360	9364	2	21.84	311	5892	1,5
170212	2	38	21.84	157	49	1	18.35	217	49	2,8
170251	2	53	20.03	289	49	14	32.46	330	49	7,1
170265	2	51	33.35	1298	49	3	30.75	747	780	
170324	2	31	37.18	282	49	4	31.90	282	205	
170340	2	46	25.31	673		17.6	26.54	834	49	16,1
170374	2	31	20.42	350		7.1	18.93	350	240893	7,1
170467	2	31	28.83	253	21295	7	26.98	171	16704	6,6
170515	1	33	16.64	322	49	1	16.14	343	49	145,3
170541	2	27	25.39	250	49	9.2	21.64	328	49	48
170553	2	32	18.68	515	5129	0.9	18.47	410	123	11
170557	2	29	20.76	176	53257	30.2	23.63	318	50326	4
170559	2	28	38.54	207	1231	12.3	36.06	200	13244	8,6
170583	2	50	23.61	153	39473	0.6	26.31	178	3015	51,3
170584	2	39	18.17	263	96832	6.6	16.23	354	29921	28
170588	1	41	17.94	375	27942	3.6		367	27089	
170648	1	46	23.73	455	41870	4.8	20.04	412	23201	19
170704	1	46	20.38	50	342627	15.6	23.05		55270	17,5
170705	2	36	21.56	258	12418	169.9	19.18	201	55825	9
170707	1	40	20.09	259	245866	117	20.58	279	222895	4,3

170715	2	43	38.46	231	412812	11.1	33.96	376	61	34,7
170727	2	42	18.03	212	2723	18	18.72	318	4834	8,9
170750	2	45	29.28	373	2949	0.9	30.13	308	1250	1,1

**CRP = C – reactive protein CD4 = cells/ml HIV viral load = copies/ml*

Controls

			Visit 1				Visit 3			
Pt ID	**Sex**	**Age**	**BMI**	**CD4 cell count**	**HIV viral load count**	**CRP**	**BMI**	**CD4 cell count**	**HIV viral load count**	**CRP**
170005	1	36	21.78	208	14652	27	20.41	122	459	9
170010	2	37	24.08	514	88367	2	25.14	358	30880	6
170013	2	50	20.22	439	49	2	19.14	413	49	16
170024	2	48	24.44	595	2609	2	24.88	304	5924	6
170025	2	41	30.73	213	8172	0.9	29.86	345	49	3
170027	2	42	35.83	386	49	2	35.37	619	95	3
170046	2	29	36.80	875	926	19	38.77	522	591	16.1
170069	2	33	32.31	357	5662	1		896		
170070	2	32	32.31	1095	2653	13	45.16	1491	1443	39
170084	2	36	21.60	132	49	12	22.37	147	49	14.5
170089	2	37	24.11	171	49	41	21.50	219	49	23.4
170090	1	43		397	49	26		542		
170092	2	45	38.95	239	2575	70	37.22	227	981	8.3
170101	2	44	33.52	504	49	8	32.04	507	49	7.1
170104	2	33	28.73	398	49	1	27.28	438	49	28.3
170119	1	42	23.68	176	115707	0.9	21.49	177	198796	0.9
170122	2	31	24.31	463	49	4	22.03	440	49	2.2
170125	2	30	24.20	559	241	6		477	197	20
170131	1	40	23.38	301	342245	0.9	23.17	190	29577	11.2
170142	2	32	22.91	348	10145	0	22.22	407	49	0.9
170150	2	33	22.88	287	4433	1	24.65	254	2913	1.1
170159	2	40	23.00	203	383	4	23.12	247	2158	1.9
170160	2	51	25.56	404	49	5	25.05	460	49	2
170170	2	28	28.35	661	49	5	24.65	380	49	12.4
170176	2	41	42.84	623	49	12	42.65	598	49	9.7
170192	2	36	29.39	257	188727	1	28.62	430	49	0.5
170194	1	48	20.34	565	1978	2	20.06	394	871	4.6
170195	2	41	29.33	412	7935	14	28.39	394	7109	2.9

170196	2	36	38.37	750	823	8	37.46	1184	233	6.4
170205	2	43	26.08	338	49	2	29.73	303	728	0.9
170207	2	35	35.40	265	49	3	34.23	175	226	6.2
170214	2	35	35.35	264		2	36.01	208	3192	2.7
170220	1	39	11.83	447	2476	0	23.73	445	611	0.9
170247	2	27	24.44	641	49	0.9	23.62	448	800	1
170253	2	37	28.16	462	49	2	25.84	354	49	77.6
170258	2	32	24.46	271	7220	0.9	22.20	255	13546	0.2
170260	2	32	42.16		118	16	38.01	1094	660	5.8
170266	2	32	20.53	370	67	1	20.09	470	86	0.9
170274	2	36	20.46	226	6963	19	20.57	449	49	2
170284	2	38	22.58	154	29023	2	20.93	495	49	28.5
170301	2	39	32.55	371		30	32.62	878	49	15.3
170306	2	44	30.11				27.21	714	49	2
170315	1	26	21.94				20.00	420	322	28.1
170324	2	31	30.67				31.72	401	49	7
170349	2	26	28.16	485	49	7	24.51	539	49	10
170376	1	48	25.02	310	13570	2	24.87	223	46493	1.6
170392	2	30	25.39	873	269	4	27.03	813	259	2.8
170403	1	45	23.25	280	4178	2	19.66	385	8331	6.3
170408	2	34	29.56	312	1064	1	31.33	378	1288	0.9
170456	1	22	21.55	194	49	11.2	21.74	428	49	8.7
170457	2	38	31.97	313	4831	2.3	34.05	427	53576	2.1
170494	2	27	27.688	334	8393	4.1	28.12	309	11977	3.4
170505	2	23	24.179	621	1183	2.1	23.97	571	990	0.9
170507	1	47	19.267	310	4557	0.9	17.20	260	14059	0.9
170510	2	28	31.245	222	152728	0.6	32.95	164	75117	13.8
170531	1	38	21.096	289	12660	2.7	21.50	403	28449	1.3
170563	2	41	33.721	723	2301	1.9	32.23	598	3846	2.1
170577	1	37	20.281	366	77	13.5	21.12	285	8494	5.2
170579	2	44	24.835	485	187	4.1	22.47	596	144	2
170593	2	33	30.12	374	49	17.7	30.59	731	49	26.8
170603	1	36	26.19	497	11262	3.1	24.15	546	49	3
170605	2	27	19.72	743	43550	0.6	21.53	648	9266	3.9
170636	2	39	18.91	340	49	14.1	19.45	380	49	5.1
170640	2	45	44.53	238	62472	3.2	49.29	305	49	5.4
170643	1	37	28.80	513	317	1.4	27.55	439	1485	0.9
170655	1	34	15.78	456	34859	4	16.69	748	24936	0.1

170669	1	39	28.39	446	2347	1.5	27.07	340	49	4.5
170726	1	49	26.86				25.15	291	61155	0.9
170749	2	38	29.75	379	1101	3.7	31.29	424	850	9
170752	2	26	22.38	333	101368	13	21.01	252	500000	18.8

Appendix D

Reagents preparation

Preparation of standards and internal standards

Kynurenine and Tryptophan standard stock solutions were prepared at 10.8mg/20ml and 8.5mg/20ml in 50% Acetonitrile and stored at $4^{o}C$ in brown bottles. The concentrations of kyn and Trp pure standard compounds were informed from our previous laboratory exploration study in 2013 and personal communication with Tracy Snyama (Head, R&D lab, Chemical Pathology).

Internal Standards (IS) stock solutions of Trp-d5 and Kyn-d4 were also prepared at 1mg/ml in 50% Acetonitrile. All stock solution were stored at $4^{o}C$ and daily working solution were prepared in HPLC-analytical graded distil.

Preparation of mobile phase

Pure analytical grade Acetonitrile undiluted

Preparation of 1M ammonium acetate solution

Weigh 38.5g crystalline ammonium acetate into a beaker and dissolve it with 500ml of HPLC-graded Distilled water.

Preparation of 0.5N Ortho-Phosphoric acid (H_3PO_4)

To make a 0.5N (normal) solution of Ortho-Phosphoric acid, slowly add 570µL of stock solution of Ortho-Phosphoric (Conc. 85% w/w, Formula Weight of 98g/mol, and density of 1.685g/mL) to 12.5mL of analytical graded distilled water and adjust the volume to 50mL with same analytical graded distilled water.