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MEASUREMENT OF INDOLEAMINE 2, 3-DIOXYGENASE ACTIVITY, A POTENTIAL BIOMARKER FOR ACTIVE TUBERCULOSIS DISEASE IN SOUTH AFRICA

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Statement of original authorship

This concomitant thesis submitted for the degree of Doctor of Philosophy (PhD) has not been submitted to any university for any degree. This work constitutes research conducted by me under the supervision of my supervisors at the Department of Chemical Pathology at the University of the Witwatersrand and the National Institute for Communicable Diseases from January 2017 to November 2019. Every datum presented here is original or otherwise referenced in the text and bibliography.

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Dedication

This thesis is dedicated to my family

– *Kwasi, Ajah, Kwadwo, Jane, Abena, Mariam, Serwaa and Adusa.*

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2. **Adu-Gyamfi CG,** Snyman T, Makhathini L, Otwombe K, Darboe F, Penn-Nicholson A, Fisher M, Savulescu D, Hoffmann C, Chaisson R. 2020. Diagnostic accuracy of plasma kynurenine/tryptophan ratio, measured by enzyme-linked immunosorbent assay, for pulmonary tuberculosis. International Journal of Infectious Diseases 99:441 - 448. DOI: https://doi.org/10.1016/j.ijid.2020.08.028

3. **Adu-Gyamfi** Clement, Savulescu Dana, Mikhathani Lillian, Otwombe Kennedy, Nicole Salazar-Austin, Martinson Neil, George Jaya & Suchard Melinda. 2020. Plasma indoleamine 2, 3-dioxygenase, a highly sensitive blood-based diagnostic tool for Tuberculosis in HIV-infected pregnant women. Manuscript accepted for publication in *Clinical Infectious Disease.*

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1. **Adu-Gyamfi CG,** Snyman T, Makhatani L, Savulescu D, Hoffmann C, Chaisson R, Martinson N, George J, Suchard M. 2019. Plasma indoleamine 2,3-dioxygenase activity, a highly sensitive blood-based diagnostic tool for Tuberculosis in HIV-infected pregnant women. Conference presentation (Oral), National Institute for Communicable Diseases (NICD) Research Forum 2019, Johannesburg.

2. **Adu-Gyamfi CG,** Snyman T, Makhatani L, Savulescu D, Martinson N, George J, Suchard M. Plasma kynurenine/tryptophan ratio, a highly sensitive blood-based diagnostic tool for Tuberculosis in HIV-infected pregnant women. Conference presentation (Oral), South Africa Immunology Society conference (SAIS) 2019, Durban, South Africa

3. **Adu-Gyamfi CG,** Snyman T, Makhathini L, Savulescu D, Hoffmann C, Chaisson R. Martinson N, George J, Suchard M. Plasma indoleamine 2,3-dioxygenase activity, a potential biomarker for active Tuberculosis disease. Conference presentation (Oral), South Africa Matlosana Collaborative Centre for HIV/AIDS & TB (SoMCHAT) Emerging Researchers Conference 2017. Soweto, South Africa.

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5. **Adu-Gyamfi CG,** Snyman T, Makhathini L, Darboe F, Penn-Nicholson A, Fisher M, Savulescu D, Hoffmann C, Chaisson R, Martinson N, George J, Suchard M. Diagnostic accuracy of plasma kynurenine/tryptophan ratio, measured by enzyme-linked immunosorbent assay, for pulmonary tuberculosis. Conference presentation (Oral), Conference on Retroviruses and Opportunistic Infections (CROI), 2018 (Boston, USA).

6. **Adu-Gyamfi CG,** Snyman T, Makhathini L, Savulescu D, Hoffmann C, Chaisson R. Martinson N, George J, Suchard M. Plasma indoleamine 2,3-dioxygenase activity, a potential biomarker for active Tuberculosis disease. Conference presentation (virtual poster), International Congress of Immunology (ICI) 2017, Melbourne, Australia.

Abstract

The Global Action Plan to End Tuberculosis (TB) has set out a 90-90-90 strategy, aiming to reach at least 90% of people with TB, place at least 90% of them on appropriate treatment and ensure that 90% of those on treatment complete their treatment course. A key intervention to achieve this goal is the validation of a non-sputum-based diagnostic TB biomarker that can detect all forms of active TB disease.

TB biomarker studies have been ongoing for decades. HIV is a major risk factor for TB, but previous TB biomarkers have not performed well in HIV-infected patients. Recently, the activity of indoleamine 2,3-dioxygenase 1 (IDO), has attracted attention as a TB biomarker. IDO converts tryptophan (T), an essential amino acid, to kynurenine (K). In TB, IDO directs the host immune response to *Mycobacterium Tuberculosis* (Mtb) towards an anti-inflammatory profile.

In the first section of this work, we reviewed the literature regarding IDO in TB disease as well as in HIV infection. Broad evidence available indicates that IDO-mediated tryptophan catabolism via the highly regulated kynurenine-nicotinamide pathway represents a common axis upregulated in TB and HIV-infection. Evidence spanning over three decades suggests that elevated IDO activity (high K/T ratio) is a key factor marking progression from latent TB infection to active disease, particularly in HIV-infected individuals.

The gold standard method for detecting IDO activity is the measurement of the product (kynurenine)-to-substrate (tryptophan) (K/T) ratio using mass spectrometry, a time-consuming and costly procedure. In the second part of this study, we evaluated an enzyme-linked immunosorbent assay (ELISA) as an alternate method for plasma K/T ratio quantification. ELISA is a low-cost, high-throughput method, with the potential for automation, making it easy to use in peripheral laboratories. The assay requires small sample volumes and can be completed within a relatively short time. We found strong reproducibility and good agreement between plasma K/T ratio measured by ELISA and mass spectrometry. The mean bias between ELISA and mass spectrometry was 0.01 with 95% limits of agreement from -0.06 to 0.10.

We then used the ELISA method to determine plasma K/T ratio in two nested case-control studies, aiming to test whether plasma IDO activity measured by K/T ratio meets the proposed target product profile of a non-sputum-based TB biomarker.

In the first study, we used residual plasma samples from HIV-infected and uninfected individuals with and without active TB from Cape Town. We found that HIV-infected patients with active TB disease had higher plasma K/T ratio than those without TB (median 0.101 [interquartile ranges (IQR) 0.091-0.140] versus 0.061 [IQR 0.034-0.077], $p < 0.0001$). At a cut-off value of 0.080, plasma K/T ratio gave a sensitivity of 90%, specificity of 80%, a positive predictive value (PPV) of 82% and a negative predictive value (NPV) of 90%. In a receiver operating characteristic (ROC) analysis, K/T ratio had an area under the curve (AUC) of 0.93.

In HIV-uninfected patients, we also observed a higher plasma K/T ratio in those with active TB disease compared to those with latent TB infection (median 0.064 [IQR 0.040-0.088] versus 0.022 [IQR 0.016-0.027], $p < 0.0001$). A cut-off of 0.040 gave a sensitivity of 85%, a specificity of 92%, a PPV of 91% and an NPV of 84% with an AUC of 0.93.

In the third section of this work, we validated our findings using the Tshepiso HIV-infected pregnant women cohort and explored whether pregnancy is a confounder to the use of plasma K/T ratio as a diagnostic test for active TB disease. We found that plasma K/T ratio was significantly elevated during pregnancy compared with non-pregnant periods ($p < 0.0001$). At the time of TB diagnosis, plasma K/T ratio was markedly elevated in pregnant patients with active TB disease compared to individuals without TB ($p < 0{\cdot}0001$). Applying a previously optimised cut-off value of 0.080, plasma K/T ratio gave a diagnostic sensitivity of 96%, specificity of 86%, a PPV of 77% and an NPV of 99%. A ROC gave an area under the curve of 0.95. The optimal cut-off in HIV-infected pregnant women was however, calculated as 0.100 with a sensitivity of 94%, specificity of 90%, a PPV of 85% and an NPV of 98%. Thus, we assert that the higher cut-off is more appropriate in pregnancy. A novel finding from this work was that in our control group, pregnant women who received isoniazid preventative treatment (IPT) had lower plasma K/T ratio than those who did not receive IPT, suggesting that isoniazid alone decreases IDO activity.

Additionally, in both studies, we noted that plasma K/T ratio inversely correlated with body mass index (BMI) in TB patients, but not in controls. Relative loss in weight is a cardinal symptom of TB; thus, our findings suggest that elevated K/T ratio accompanies lower BMI as a factor associated with active TB.

Conclusion

In conclusion, plasma K/T ratio is a suitable blood-based biomarker of active TB disease in both HIV-infected and uninfected individuals. Plasma K/T ratio can be used as an initial blood-based test for active TB disease. Pregnancy was not a confounder to the use of plasma K/T ratio to diagnose active TB disease in HIV infected individuals, although a higher cut-off value of 0.100 rather than 0.080 was preferable. Plasma K/T ratio can be measured using a high-throughput, low-cost, widely available ELISA. Plasma K/T ratio should be evaluated prospectively in further studies to determine its impact on patient care.

Chapter 1

Background

In 2017, our group reported that plasma kynurenine/tryptophan ratio, largely mediated by indoleamine 2,3-dioxygenase (IDO) activity, is a potential biomarker of active Tuberculosis (TB) in human immunodeficiency virus (HIV)-infected patients in South African (1). In that study, we optimized and validated an ultra-high-performance liquid chromatography-mass spectrometry (UPLC-MS) for measuring plasma kynurenine and tryptophan concentration. We determined IDO activity by calculating kynurenine-to-tryptophan (K/T) ratio.

In this work, we have reviewed available literature regarding IDO activity (K/T ratio) in TB and HIV-infection. We then explored enzyme-linked immunosorbent assay (ELISA) in comparison with our previously published mass spectrometry results, as an alternative laboratory method for measuring plasma kynurenine and tryptophan concentration. Furthermore, we confirmed the clinical utility of K/T ratio measured by ELISA as a diagnostic TB biomarker in two patient groups including HIV-infected individuals and HIV-infected pregnant women.

1.1 Overview of TB pathology

After centuries of human co-existence with *Mycobacterium tuberculosis* (Mtb), TB pathogenesis remains incompletely understood. Active TB disease results from deleterious host-microbe interactions. After infection with Mtb, the individual may be asymptomatic, where the organism may be quiescent or there may be sub-clinical Mtb replication. In the presence of waning immunity, the individual may progress to active TB disease, which may lead to death if not treated (2, 3). Symptomatic or active TB disease may progress from primary TB infection or reactivation of latent TB infection (LTBI). Progression is usually within a couple of years but can occur even up to decades after primary infection. In individuals who were once cured of active TB disease, TB disease may develop again as a result of re-infection.

Infection with human immunodeficiency virus (HIV) is a significant risk factor for developing active TB disease, either from primary Mtb infection or from reactivation. For the past three decades, the HIV epidemic has greatly aggravated the TB burden (4). Immune suppression from ineffective T cell function has been proposed as a key factor leading to reactivation TB. Activity of a host enzyme involved in immune-modulation, indoleamine 2,3-dioxygenase-1 (IDO), has been associated with a poor immune response to Mtb (5-9).

1.2 Global TB burden

Historically, TB may be as old as man. TB was reported among the Egyptian mummies about seven thousand years ago (10). In the 1800's in Britain, TB was called consumption disease, the white death or great white plague (10). In the early 19th century, names that evoke despair or horror such as "the graveyard cough" and the "captain of all these men of death" were used to describe TB (10, 11). Throughout the history of mankind, until today, TB has killed more people than any other infectious pathogen (12, 13). In the early 21st century, TB was declared

a public health emergency in regions with low standards of living and poverty such as Africa (14). From the World Health Organization (WHO) estimation, about a third of the world's population is infected with Mtb and TB kills about 2 million people globally every year (15, 16). The majority of the global TB burden lies within the 15-50-year age group. As a result, TB is the disease with the most impact on social welfare and the poverty cycle (16). WHO data show that women and children are disproportionately affected by TB (17). Globally, TB kills more women than pregnancy-related deaths every year. Another group that is disproportionately affected is immune-compromised individuals, such as people living with HIV (16).

1.3 Current TB diagnostic toolkit

A case of TB is ascertained either by the presence of Mtb in a clinically appropriate sample or by the presence of immune response to Mtb or another surrogate test associated with the presence of classical TB symptoms. The current TB diagnostics have numerous limitations.

1.4.1 Sputum-based TB diagnostic tests

The sputum smear microscopy test, which allows visualization of acid-fast bacilli (AFB) from sputum is widely used both to diagnose and follow-up anti-TB therapy in resource-limited settings. Even though inexpensive and easy to perform, sputum smear microscopy has the lowest diagnostic sensitivity in high-risk groups (18). The overall sensitivity of smear microscopy is unacceptably low (<40%) among HIV infected patients (19-21). HIV-infected individuals and children are unable to adequately produce sputum and often have paucibacillary pulmonary or extra-pulmonary TB (22). As a result, sputum-based diagnostics

remain problematic (20, 23). Additionally, sputum smear microscopy is not able to differentiate between Mtb and nontuberculous mycobacteria (NTM) disease (24, 25).

More sensitive and specific sputum-based bacteriological tests, such as Mtb culture, are available as TB diagnostics. Mtb culture from sputum is regarded as the gold standard test for proving a case of PTB. However, sputum culture requires capital intensive infrastructure, experienced personnel, and most importantly delayed turn-around times for patient care. Presently, the Mtb culture test remains the only available tool to monitor anti-TB therapy, even though it is relatively insensitive, and lacks adequate positive predictive value to guide TB treatment (26, 27).

Recent developments in molecular TB diagnostics, including the Xpert MTB/RIF assay, have been a game-changer in TB diagnostics. Xpert MTB/RIF raised the hope for faster, sensitive and more accurate identification of active TB disease with even one visit to the clinic. Xpert MTB/RIF was designed to identify Mtb genes from sputum specimens. Xpert MTB/RIF can detect Mtb resistance genes to primary anti-TB drugs, which helps clinicians choose an appropriate TB treatment regimen from the start of therapy. Additionally, Xpert MTB/RIF is an automated real-time polymerase chain reaction test, meaning it poses less risk to laboratory personnel during specimen testing than traditional methods. The new Xpert MTB/RIF Ultra has advantages to TB detection such as improved sensitivity (28, 29). However, as a point-of-care test, there are still many issues that need to be addressed. Xpert MTB/RIF, just like smear microscopy, has limited applicability in following-up anti-TB treatment, as it cannot distinguish dead bacilli from live ones. Even though the WHO rolled-out Xpert MTB/RIF more than a decade ago, access to Xpert MTB/RIF testing devices remain restricted in countries

other than South Africa. The long-term sustainability of Xpert MTB/RIF in frontline TB diagnostics in developing countries hinges on donor support.

1.4.2 Non-sputum-based TB tests

By definition, sputum-based diagnostics are not useful in extra-pulmonary disease. The diagnosis of extra-pulmonary TB relies on samples collected mostly by invasive procedures. Non-sputum-based diagnostics help to rapidly rule-out the presence of active TB disease. Regarding blood-based Mtb tests, most have been unreliable and inaccurate. Several blood-based TB diagnostics proposed for clinical use have been rejected by the WHO (30).

The availability of tests to measure host factors released by immune cells during interaction with Mtb makes immune-based diagnostics a very promising option. TB immunodiagnostics measures non-specific mediators of inflammation secreted by both innate and adaptive immune cells. Immunodiagnostic assays such as interferon-gamma release assays (IGRAs) have performed well to diagnose prior exposure to Mtb. The IGRA is not a measure of mycobacterial activity nor its continuous presence but a response by the host cells. In principle, the IGRAs detect a memory immune response to Mtb antigens (31). In areas where TB is endemic, the IGRAs are not helpful due to the high prevalence of LTBI, and distinguishing LTBI from active TB disease thus remains a challenge.

Antibody tests for TB are not useful in diagnosis due to variabilities in test accuracy. In 2011, the WHO Expert Group issued guidelines for Mtb antibody testing (32, 33). WHO warned that most commercial Mtb antibody tests provided inconsistent and imprecise estimates of sensitivity and specificity. Broger and colleagues reported immunoglobulin G (IgG) antibody response in adults with pulmonary TB had a poor sensitivity of 35% with a 90% specificity

(34). Recently, studies combined 7 commercial Mtb antibody kits which detect either IgG or IgM to evaluate their combined performance (35). Even though the results provided a better clinical auxiliary diagnostic value, it has utility challenges. In patients with smear-negative pulmonary TB and extra-pulmonary TB, there was not much improvement over existing diagnostic methods.

The tuberculin skin test (TST), which detects a cellular immune response to Mtb, has been useful in diagnosing TB in infants in endemic countries and in adults where TB is not endemic. Although inexpensive, the test has severe limitations. TST has questionable sensitivity and specificity in detecting latent TB, particularly in populations in which the test is applied. The test result is affected by either HIV-infection or Bacillus Calmette-Guerin (BCG) vaccination status (36). Using TST in TB diagnosis presents a formidable challenge particularly in adults in endemic areas.

1.4 Other surrogate tools for TB diagnosis

The reliability of classic TB symptoms as diagnostic prompts plays a key role in case finding. Clinical signs and symptoms can indicate the likelihood of active TB, even in smear-negative individuals. Unfortunately, almost half of all PTB cases are smear-negative and they account for 15% of TB transmission (37). Prompt diagnosis of paucibacillary TB remains an urgent intervention to truncate transmission. Even though surrogate diagnostic tools perform variably in different risk populations they help in patient screening (38).

Chest X-ray, computed tomography (CT) and PET scanning are non-microbiological supportive diagnostic aids used in finding a case of TB (39). Chest X-ray findings are used in

TB screening programs despite their poor diagnostic specificity, sensitivity and reproducibility. Misdiagnosis using chest X-ray is common even when specialist radiologist services are available (40). Cavitation, patchy infiltrates, consolidation and pleural effusion are seen on the radiograph in variable proportions in smear-positive cases (41). Over time, new guidelines on reporting have proved helpful in specific population groups during the screening of patients (40), In many resource-limited setting where there are X-ray facilities, the chest radiograph remains a supportive diagnostic tool, and confirmation by other Mtb tests is required. The presence of severe HIV disease complicates the use of the chest x-ray in TB diagnosis.

The introduction of CT scanning for active TB diagnosis has improved TB diagnosis. CT scan has improved sensitivity compared with chest X-ray as surrogate TB diagnostic device (42). CT scans commonly show areas in the lung where the immune cells have walled-off the bacilli (42). Even though the CT scan does not show the microbe, CT scans are useful to show the degree of bronchogenic dissemination of Mtb. CT scan is helpful in the early identification of miliary TB (43). Improved imaging technology, such as F-fluoro-2-deoxy-glucose-positron emission tomography/computed tomography (FDG-PET/CT) is a research tool. PET/CT identifies areas of active inflammation by mapping where cells with high metabolic activity take up the radioactively labelled glucose analogue (FDG). PET/CT helps differentiate Mtb lesions from malignancy (44). These methods aid diagnosis of either pulmonary or extra-pulmonary TB, however; they are only found in tertiary hospitals because the technology is expensive and requires specialist services to interpret results.

TB diagnosis in the clinical setting remains challenging. Delays in TB diagnosis result in an increased risk of mortality and continuous TB transmission. Currently, by available TB

diagnostics, about 40% of people who have active TB disease are missed by health systems and continue to fuel the transmission cycle (45). There exists no TB test which is universally applicable to all patients, adult or pediatric, with pulmonary and extra-pulmonary TB. Host-derived blood-based markers associated with active TB thus hold promise, especially in situations where sputum diagnostics are problematic, including in children and HIV-infected individuals.

1.5 Overview of TB biomarkers

A biomarker is a measurable characteristic that can indicate health or disease or even a response to a therapeutic agent (2). Biomarkers give information about the presence or risk of disease or protection (3). For example, a simple measurement of arterial blood pressure can indicate a risk of cardiovascular disease in adults. A biomarker can be a host or pathogen-derived molecules, genes or enzymes. In TB, a desirable biomarker would be a characteristic that is directly involved in TB pathogenesis or one which changes early during anti-TB treatment. A TB biomarker is urgently needed at the point of diagnosis to classify patients as having active disease, LTBI or no disease. Also, a TB biomarker should be able to indicate the risk of progression to active disease or reactivation of quiescent infection. Another useful application would be to indicate the risk of relapse or the effectiveness of anti-TB therapy. A host-derived marker that satisfies these criteria would be a huge gain in TB diagnosis and would aid TB vaccine development.

1.6.1 Biomarkers for diagnosing active TB

The biomarker community have vigorously explored both host and Mtb molecules from the blood, urine, saliva, exhaled breath and sputum samples to identify either single or combined molecules that can be validated as biomarkers for TB (46). So far, the most attractive sample,

due to the ease with which it can be obtained from all suspected patients, is blood. In blood; genes, proteins, lipids and metabolites have been explored (3).

From the Mtb perspective, Mtb genes and cell wall components have been detected directly from sputum, urine and blood. In patients with PTB, sputum samples are obtained directly from the site of infection. Mtb or its antigens have been vigorously evaluated from sputum as TB diagnostic biomarkers. Mtb genes have been detected in blood and urine of PTB patients with improved sensitivity compared to Mtb culture from the same sample (47, 48). Some studies have also examined Mtb antigen85 (MtbAg85) in sputum as a diagnostic TB biomarker (26, 49). MtbAg85 test in blood and urine have shown highly variable performance in different studies. (50).

Lipoarabinomannan (LAM), a cell wall product of Mtb, has been proposed as a diagnostic biomarker particularly in HIV-infected patients. LAM is released from metabolically active or degenerating mycobacterial cells. In a study by Lawn *et al*, LAM was found to positively correlate with the bacillary burden in HIV-infected patients (51). Currently, there are commercially available assays that detect LAM in urine from active TB patients (52). In HIV-uninfected patients, the performance of the LAM test is poor compared to HIV-infected patients (51, 53). Although urine LAM test is unsatisfactory in HIV-uninfected individuals, it holds promise as a diagnostic biomarker in HIV-infected patients (52-54).

Among host-derived molecules as TB biomarkers, there are various proposed non-sputum-based assays for TB diagnosis. Most of these diagnostic TB biomarkers rely on stimulated blood, serum/plasma or urine as sample of choice. Specific and non-specific immune activation markers have been evaluated; for example, serum neopterin, intracellular adhesion molecule -

1 (ICAM-1) and complement fragment C3b rise at the time of TB diagnosis and drop after treatment. In HIV-uninfected patients, however, some of these markers did not decline after treatment, which raises questions as to their appropriateness (26, 55). Serum C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and adenosine deaminase also failed to demonstrate specificity as TB biomarkers (26, 27, 56). Although CRP does not meet the criteria as a diagnostic test for TB, it can be used as a triage test (rule-out) in individuals with HIV-infection (57, 58). Regarding antibodies to Mtb, due to heterogeneity of responses, it has not been promising as a diagnostic biomarker. WHO has strongly advised against using an antibody-based test for purposes of TB diagnosis (59). The evaluation of serum micro-RNA (mRNAs) has also shown different levels of accuracy for diagnosing active TB in both drug-sensitive and resistant TB (60-62). Even though the technology to detect mRNAs has been praised for its speed, accuracy and precision, a major concern of its application as TB diagnostics is still unclear as Mtb nucleic acids still persist even after the smear and culture becomes negative (63, 64).

Over the last 2 decades, TB biomarker studies have defined revealing biosignatures associated with active TB disease (65). Host-related biosignatures hold promise for prognosis, monitoring treatment responses and development of new vaccines. Biosignature studies have shifted from evaluating single markers to multiple integrated markers to improve the sensitivity and specificity (65, 66). Even though the biosignatures look promising in the research setting, implementation in clinical settings remains a challenge. A major drawback of biosignature implementation is complex result readout and interpretation.

Volatile organic compounds (VOCs) including metabolites of Mtb or product of oxidative stress from the host exhaled breath of patients with active TB have also been proposed as

diagnostic biomarkers. In symptomatic patients, a breath test was found to be 85% accurate in detecting active disease (67, 68). A particular concern with VOCs are that they are products of pathogen-host metabolism. Different stages of TB disease are characterized by dynamic metabolic changes within pathogen and host and their interactions, thus different stages of TB disease may generate different breath test results. Additionally, due to the high burden of LTBI, the use of VOCs as TB diagnostic have limited clinical applicability (25, 69).

Recently, a novel T cell activation marker-TB (TAM-TB) has been described for the diagnosis of active TB in children. TAM-TB assay is based on the relative intensity of fluorescence between $CD4^+CD27^+$ T cells and Mtb-specific $CD4^+CD27^+$ T cells. TAM-TB has been validated in small cohort and the technology cannot be applied in primary health care delivery.

1.6.2 Biomarkers to monitor TB therapy efficacy

Sputum culture conversion after 2 months of anti-TB treatment has been used as a predictive biomarker for response to therapy in patients with PTB (70). Mtb culture requires massive infrastructure which is often lacking in primary healthcare centres. In addition, sputum culture has no utility in patients with extrapulmonary TB. To accommodate these shortcomings, efforts are being made to identify alternative blood-based markers to monitor efficacy of TB therapy.

Several non-specific markers of inflammation have been shown to correlate with the extent of disease and/or treatment response. Serum markers including Neopterin, CRP, white blood cell (WBC) count and ESR have shown to decline after TB therapy (71-73). Also, a pro-inflammatory cytokine, IFN-ɣ-inducible protein (IP-10), secreted by antigen-presenting cells (APCs), has been evaluated from serum, plasma and urine as a marker of an effective response

to TB therapy. IP-10 levels were found to be higher in patients with active TB than controls, and decrease significantly after the completion of anti-TB treatment (74-76).

1.6.3 Biomarkers for latent TB identification

Regarding biomarkers for differentiating LTBI from active disease, several studies have evaluated plasma concentrations of epidermal growth factors, fractalkine, IFN-ɣ, interleukin 4 (IL4), monocyte chemoattractant protein (MCP) and IP 10 (74, 77, 78). IP10 has been suggested as an alternative marker to IFN-ɣ with high accuracy in HIV-infected patients (78, 79). A major concern of these blood-based biomarkers are that none have been confirmed in large independent studies.

1.6.4 Transcriptomic signatures for predicting and diagnosing active TB

Huge progress has been made in the pursuit of new and improved predictive markers of active TB using transcriptomic signatures. Transcriptional signatures of TB have enhanced our understanding of immunity to TB particularly in LTBI versus active TB (80, 81). Several studies of host blood transcriptomic signatures have shown that individuals who are uninfected with TB can be discriminated from those with LTBI and active disease (82). Additionally, transcriptomic signatures have been shown to discriminate TB from other chronic lung diseases (82, 83). Suliman *et al* reported the risk of progression to active TB disease among household contacts of individuals with active TB disease in one year before TB diagnosis (84). Despite this encouraging progress, the performance of these transcriptomic signatures for TB diagnosis and predicting active disease has not been synthesised and examined systematically in large cohorts.

Despite intensified efforts towards development and implementation of new TB diagnostics or biomarkers, there has been very little attention paid to the host's immune-modulatory enzymes and metabolites that can act as markers of active TB disease. One proposed enzyme is IDO. IDO is attractive as a TB biomarker as it is detectable in host-derived fluids including blood, sputum, pleural fluid, cerebrospinal fluid or urine. Previous studies have shown that IDO activity or mRNA expression is elevated at the time of TB diagnosis, and declines with treatment (6, 9, 49, 50).

1.6 Study aim

In this thesis, we aimed to define whether plasma IDO activity measured by product-to-substrate (kynurenine/tryptophan) ratio is a robust candidate blood-based biomarker for active TB disease in a TB/HIV endemic setting.

1.7 Study objectives

Our first objective was to review and converge available literature on IDO activity or K/T ratio in TB and HIV pathogenesis and diagnosis.

Secondly, we aimed to evaluate ELISA, a widely available, easier and relatively cheaper alternative laboratory method that can accurately measure plasma kynurenine and tryptophan concentration rather than use mass spectrometry for IDO activity quantitation.

Our third objective was to evaluate the diagnostic utility of plasma K/T ratio using two well-characterised, clinically relevant TB/HIV cohorts established in South Africa, the MRC-SHIP and Tshepiso pregnant women cohorts.

a) We used stored residual plasma samples from the MRC-SHIP study

- To measure plasma kynurenine and tryptophan concentrations using the ELISA method.
- To compare the ELISA method to the mass spectrometry method
- To determine the clinical significance of plasma K/T ratio in HIV-infected and uninfected patients with TB and LTBI by calculating diagnostic sensitivity, specificity, positive and negative predictive values for active TB diagnosis.
- To determine the optimum cut-off value for TB diagnosis using area under the receiver operating characteristic (ROC) curve.

b) We used stored plasma samples from the Tshepiso pregnant women cohort

- To measure plasma kynurenine and tryptophan using the ELISA method.
- To determine the diagnostic significance of elevated K/T ratio in HIV-infected pregnant patients with and without active TB by calculating diagnostic sensitivity, specificity, positive and negative predictive values for active TB diagnosis.
- To optimize a new cut-off value for IDO activity to diagnose active TB in pregnancy using a ROC curve.

1.8 Ethics

The Human Research Ethics Committee of the University of the Witwatersrand approved this nested study using residual samples (clearance number: M170476). The MRC-SHIP study and the Tshepiso study were approved by their respective institution's ethics committees.

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Chapter 2

Overview of IDO in TB and HIV

In this chapter, we have reviewed the literature regarding indoleamine 2,3-dioxygenase-1 (IDO) activity in Tuberculosis (TB) and Human immunodeficiency virus (HIV) pathogenesis. We converged a large body of evidence on IDO's immune-regulatory role in TB and HIV infection to facilitate understanding its role in the progression of latent TB infection (LTBI) to active TB disease. We have highlighted how the kynurenine pathway, which is largely mediated by IDO activity, is a common axis upregulated in TB/HIV pathology. We summarized the evidence for IDO activity (higher K/T ratio) as a potential diagnostic and prognostic biomarker for active TB disease, particularly in HIV-infection. We have published this work; our manuscript follows at the end of chapter 2.

2.1 The indoleamine 2,3-dioxygenase-1 (IDO) catalysed pathway

Tryptophan is an essential amino acid in mammals; humans cannot synthesize it *de novo*. As a result, tryptophan is obtained from the diet. In mammals, tryptophan is used in the biosynthesis of serotonin and melatonin. Alternatively, tryptophan can also be catabolized into kynurenine and other secondary metabolites (1-3), figure 1. Indoleamine 2,3-dioxygenase-1 (IDO) is one of the first enzymes in the kynurenine pathway (4). Two other enzymes with similar functions are tryptophan 2,3-dioxygenase (TDO) and IDO-2 (5-7). Tryptophan, the substrate for these enzymes, has an indole ring. The enzyme (IDO, TDO or IDO-2) first cleaves the carbon-carbon double bond across position 2 and 3 of the indole ring (8). After a series of conversions, nicotinamide adenine dinucleotide (NAD+) is formed as one of the end products of tryptophan break down (1). In human beings, the tryptophan-kynurenine-NAD+ pathway is responsible for about 90% of tryptophan use whilst the serotonin pathway accounts for about 1%. The rest goes into other protein syntheses. In mammals, elevated kynurenine metabolites have been reported in neurobehavioral conditions including mood swings, pain perception and the circadian cycle (sleep-wake-cycle). Also, tryptophan is required by T cells for maturation and effector functions (9).

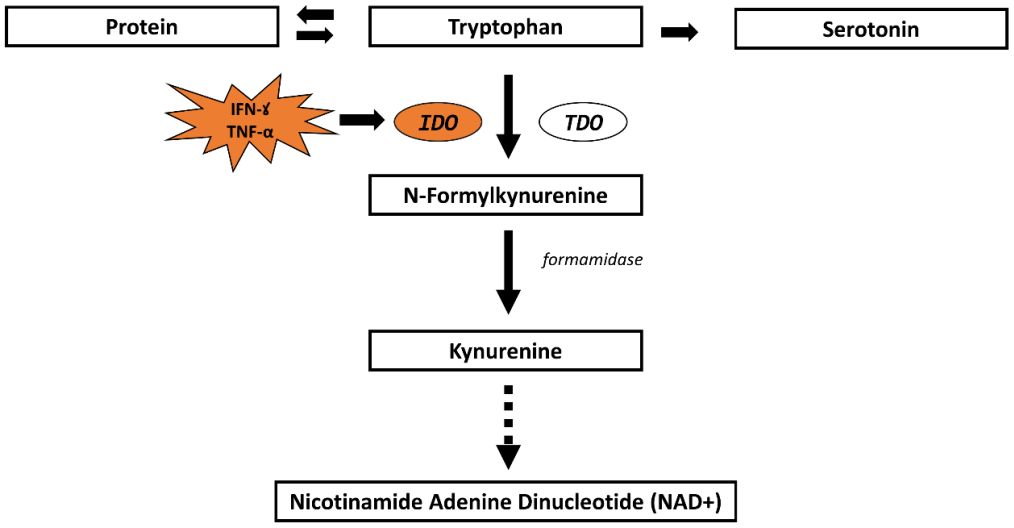

Figure 1.0: Pathway of Indoleamine 2,3-dioxygenase (IDO) activity.

Tryptophan may be converted to serotonin or kynurenine, depending on IDO activity. IDO is induced by the pro-inflammatory cytokines interferon-ɣ and tumour necrosis factor-α. IDO is expressed in antigen-presenting cells, including macrophages and dendritic cells. Tryptophan dioxygenase (TDO) is an IDO analogue expressed only in the liver. The dotted line represents intermediate steps that are not shown. Diagram adapted and modify from Dantzer 2008 (10).

2.2 IDO activation downregulates immune responses

IDO is expressed by antigen-presenting cells (APCs) including macrophages and dendritic cells during alternative activation (10). Other cells such as mesenchymal cells also express IDO activity (11). By far, interferon-ɣ and tumour necrosis factor-α are the most potent IDO inducers (2). IDO activation is a key feature of alternatively activated macrophages, while IDO activation is not seen in classically activated macrophages (1). Alternatively activated macrophages or dendritic cells have been suggested to down-modulate immune responses and may induce regulatory T cells with suppressive functions (12, 13). Tryptophan depletion

starves surrounding T cells of key nutrients, resulting in their inability to proliferate and respond effectively to infection (14, 15). IDO may also induce regulatory T cells, which down-modulate immune responses of other immune cell populations (12).

In a normal physiological state, IDO activation may function as a feedback mechanism to limit tissue damage from the overactive immune system (16, 17). However, chronic stimulation and overexpression lead to immune suppression (1). Available evidence shows that overexpression of IDO activity promotes the generation of ineffective T cell responses due to lack of tryptophan leading to peripheral immune tolerance (18). Secondary, by-products of IDO catabolism accumulated in the microenvironment of IDO activity are toxic to T cells. By-products including kynurenines may promote the generation of regulatory T cells (T regs) or induce T cell apoptosis, which down-regulate immunity (12). Macrophage expression of IDO leads to suppression of T cells through cell cycle arrest (19, 20). IDO induction switches macrophage phenotype from a pro-inflammatory to anti-inflammatory phenotype, which promotes immune tolerance (21-23). Alternatively, activated macrophages are typically associated with tissue repair, fibrosis and healing (20, 22). In dendritic cells, IDO expression promotes the generation of a regulatory dendritic cell phenotype (12, 13, 24). Several immune tissues including the lymph nodes, spleen and the tonsils with increased IDO activity function as immune suppressive sites (25).

2.3 IDO, immunity and TB

Mycobacterium tuberculosis (Mtb) has been shown to induce IDO activity *in vitro* and *in vivo* (26-28). Mtb can synthesize tryptophan (27, 29). Mtb can also survive in a tryptophan-deprived environment (30, 31). In the animal model, elevated IDO activity has been associated with a

poor immune response to Mtb (32). In HIV infected patients, chronic immune activation is sustained by high levels of pro-inflammatory cytokines such as interferon-ɣ. High levels of interferon-ɣ heighten IDO activity. Available evidence shows that IDO activity is implicated in the pathogenesis and early progression to severe HIV disease, of which TB is the first opportunistic infection (28, 33). Despite its high biological plausibility as a key checkpoint of immune responses in TB and HIV infection, very few studies have explored the clinical significance of elevated IDO activity as a TB biomarker.

For Mtb infection, the infected macrophage plays a critical role in containing the bacilli. Ineffective containment of the bacilli by the host macrophage results in severe pathology (34, 35). Therefore, immune or metabolic pathways associated with alternative macrophage activation may hold clues to understanding the switch from LTBI (contained TB) to active disease. The immune-suppressive functions of IDO have been intensively studied in cancer biology, where high IDO activity seems to correlate with a dampened immunity to cancer cells (1).

2.4 IDO activity as a potential diagnostic tool for active TB

In recent years, investigation of IDO activity as a key modulator of immune responses in TB has gained momentum. Almeida *et al* reported that among 20 immunological mediators examined in induced sputa, IDO mRNA was the marker most elevated in active TB patients compared to patients with other lung diseases and controls (36). Interestingly, within two weeks of beginning TB treatment, IDO mRNA levels decreased drastically more than 500-fold, suggesting that IDO may play a key role in TB pathogenesis as well as being a useful tool to monitor TB treatment efficacy.

Suzuki *et al* studied IDO activity in Japanese patients with active TB compared with controls. IDO was significantly elevated in TB patients, and IDO activity was an independent prognostic factor for death (37). Their cohort did not include patients with other lung diseases. Also, study limitations included a relatively old age of TB cases, and there were no HIV-infected patients included. In a subsequent study, Suzuki *et al* went on to show that IDO activity was elevated in both pleural fluid and serum of TB patients compared to patients with other lung diseases (38). Epithelioid granulomata in pleural tissue showed high IDO expression by immunohistochemistry, suggesting the involvement of IDO in the pathogenesis of TB disease.

Some metabolomics studies have also revealed variance in kynurenine, tryptophan or other pathway metabolites between TB patients and healthy controls (39). In patients with active TB, metabolites from amino acid and fatty acid catabolism pathways have been reported to be high (40-42). Feng *et al* reported that kynurenine, the first product of IDO activity, and quinolinic acid were among the most altered metabolites significantly upregulated in TB patients compared with those with other respiratory diseases. They reported ROC curves for kynurenine and quinolinic acid yielding areas under the curve (AUC) of 0.82 and 0.85 respectively for diagnosis of TB (40).

Weiner and colleagues explored more than 400 small molecules from sera of active TB patients, LTBI and healthy controls. They found that kynurenine was significantly elevated in patients with active TB disease compared with LTBI and controls. They confirmed their findings by showing elevation of IDO protein expression histochemically in pulmonary lesions of mice with TB and showing that cultured human monocyte-derived dendritic cells and

macrophages upregulated IDO gene expression following experimental infection with TB (41). Van Laarhoven *et al* found altered tryptophan metabolism in cerebrospinal fluid of patients with TB meningitis and an association between low tryptophan levels and survival (43).

Recently, Jeffrey *et al*, using high-resolution metabolomics reported that tryptophan metabolism along the kynurenine pathway is among the most regulated host metabolic response to TB disease and treatment. IDO transcript and kynurenine/tryptophan ratio was significantly elevated in both LTBI and active disease (44). Taken together, these data imply that IDO activity and K/T ratio is elevated in the course of TB pathogenesis and declines after therapy, suggesting K/T ratio may serve as a biomarker for active TB disease.

2. 5 IDO in TB and HIV co-infection

Co-infection with HIV and TB is responsible for about 40% of all death due to acquired immunodeficiency syndrome (45). Active TB disease in HIV-infection is thought to result from the imbalance between protective immunity and counter-protective immunity to Mtb.

To review the intersection of IDO activity with TB and HIV disease, we summarized evidence of altered IDO activity in both TB and HIV in a narrative mini review, attached at the end of this chapter. We highlight that IDO activity is moderately increased in HIV infection but markedly elevated in active TB disease. We have listed papers related to HIV in table 1.0 and those related to TB in table 2.0.

2.6 IDO activity in other microbial infections

Early studies considered IDO's activity as a component of the host's innate immunity to pathogens (46). This was because since tryptophan is a critical nutrient for microbial survival, tryptophan degradation by IDO-expressing cells represented an unfavourable environment which led to microbial death (47). Recent knowledge points to increasing evidence that IDO activity may exert pleiotropic effects, even with opposing outcomes (48). On one hand IDO activity directly suppresses the pathogen replication or spread, and on the other hand, it also acts to suppress host immune reactions (48, 49). Broad evidence clearly shows that elevated IDO activity is a hallmark of major human viral, bacterial, fungal and parasitic infections including Hepatitis B virus (HBV), Hepatitis C virus (HCV) influenza, Community Acquired Pneumonia (CAP) and *Listeria monocytogenes* (50-52). Suzuki *et al* reported increased kynurenine-to-tryptophan ratios as a prognostic marker for severity and morbidity of CAP caused by multiple pathogens (52). In addition, the patients showed reduced T cell ($CD4^+$ and $CD8^+$ T-cell) counts similarly pointing to an overall impairment of immune functions (52). Despite this, IDO activity could still diagnose active TB, and discriminate active TB from other undercurrent infections including pneumonia (53, 54). In other infections, IDO's effect is directly opposite. In fungal infection, Njau *et al* recently demonstrated that *C. pneumoniae* infection of human moDC-induced IDO expression in a TNFα-dependent manner and IDO was sufficient to restrain bacterial growth (55).

Table 1.0: Studies reporting elevated IDO activity, increased kynurenine or decreased tryptophan in human HIV-infection

Reference	Participant demographics	ART therapy	Sample type	Method of IDO quantification	Major conclusion (s)
Werner *et al*., 1988 (46).	HIV+ patients (n = 11) HIV- patients (n = 11)	Not specified	Serum	HPLC - MS	Elevated kynurenine/tryptophan ratio suggested increased tryptophan catabolism in HIV-infected patients. Increase tryptophan metabolism may have contributed to neurologic symptoms found among HIV-infected patients.
Larsson *et al*., 1989 (47).	HIV+ patients (n = 24) HIV- patients (n = 14)	No	Blood and CSF	HPLC - MS	HIV patients had significantly decreased tryptophan in blood and CSF compared to controls. HIV patients with severe AIDS-defining symptoms had significantly lower tryptophan concentrations.
Fuchs *et al*., 1990 (48).	HIV+ patients (n = 22) HIV- patients (n = 14)	Not specified	Serum and CSF	HPLC - MS	Decreased tryptophan levels were associated with chronic immune activation in HIV positive patients. IDO activity induced by interferon-ɣ may be the cause of reduced tryptophan concentration in HIV infected patients.
Wiegand *et al*., 1991 (49).	HIV+ patients (n = 14) HIV- patients (n = 14)	95% on Zidovudine monotherapy	Serum and CSF	HPLC - MS	Lower tryptophan concentration in HIV-infected patients was associated with sleep disturbances.

Fuchs *et al*., 1991 (50).	HIV+ patients (n = 42) HIV-Controls were from 3 different cohorts	38% on only Zidovudine	serum	HPLC - MS	Decreased tryptophan concentration in HIV-infected patients may be due to chronic immune activation which results in increased IDO activation.
Heyes *et al*., 1991 (51) and 1992 (52).	HIV+ patients (n = 126) Controls HIV- (n = 28)	No	Serum and CSF	HPLC - MS	Tryptophan concentration was decreased with increased kynurenines in serum and cerebrospinal fluid.
Hortin *et al*., 1994 (53).	HIV+ patients (n = 20) HIV- patients (n = 20)	Majority on ARV	Plasma	Flow cytometry and ELISA	Plasma tryptophan concentration decreased in response to HIV infection.
Gisslen *et al*., 1994 (54).	HIV+ patients (n = 14)	Yes	Blood and CSF	HPLC–MS	Initiation of antiretroviral therapy was associated with decreased immune activation and decreased tryptophan breakdown in HIV-infected patients.
Huegsberg *et al*., 1998 (55).	HIV+ patients (n = 206) HIV- patients (n = 72)	Not specified	Serum	HPLC–MS	Severe HIV disease correlated with increased tryptophan degradation and increased IDO activity.
Look *et al*., 2000 (56).	HIV+ patients (n = 17) HIV- patients (n = 55)	Yes	Serum	GC – MS	HIV-infected patients had increased kynurenine/tryptophan ratio compared to controls. ART treatment mitigated immune activation in HIV disease and led to a decrease in IDO activation.

Murray *et al*., 2001 (57).	HIV+ patients (n = 4)	Yes	Plasma	HPLC – MS	Plasma tryptophan increased 40% in HIV-infected patients following treatment with antiretroviral medications, however, other amino acids concentrations remained fairly unchanged in plasma.
Zangerle *et al*., 2002 (58).	HIV+ patients (n = 45) HIV- patients (n = 40)	Yes	Plasma	HPLC – MS	Tryptophan degradation was increased in HIV-infection, and ART partially reversed it.
Atlas *et al*., 2007 (59).	HIV+ patients (n = 22) HIV- patients (n = 22)	Yes	CSF	HPLC – MS	HIV-infected patients had elevated kynurenine concentration compared to controls; increased kynurenine levels were associated with the altered mental state of HIV patients.
Favre *et al.*, 2010 (60).	HIV+ patients (n = 85)	Yes	Plasma, CCS, Tissue, PBMCs	LC - MS/MS	IDO mRNA was upregulated in myeloid dendritic cells from HIV+ patients IDO activity was increased in progressive HIV infection
Byakwaga *et al*., 2014 (61).	HIV+ patients (n = 435)	Yes	Plasma	LC - MS/MS	IDO activity independently predicted poor CD4+ T cell recovery Elevated IDO activity was associated with increased mortality among HIV-infected patients.
Chen *et al*., 2014 (62).	HIV+ patients (n = 76) HIV- patients (n = 16)	Yes	Plasma	HPLC - MS	IDO activity significantly declined after treatment; however, it did not reach normal activity.

Page *et al.*, 2014 (63).	HIV+ patients (n = 36) Controls (n = 16)	40% on ART	Plasma	HPLC - MS	IDO activity was significantly elevated in HIV-infected patients not receiving antiretroviral therapy compared to patients on treatment.
Jenabian *et al.*, 2015 (64).	HIV+ patients (n = 116) Controls (n = 12)	Yes	Plasma	HPLC - MS/MS	Early phase of HIV-infection, and/ or patients not receiving ART has high IDO activity. IDO activity declined after initiating treatment. IDO activity was positively associated with CD8+ T cell activation
Bipath *et al*., 2015 (65).	HIV+ patients (n = 105) HIV- patients (n = 60)	78% on ART	Plasma	GC - MS	Tryptophan depletion directly correlated with the severity of immune deficiency.
Gaadbo et al., 2015 (66).	HIV+ patients (n = 41)	Yes	Plasma	LC - MS/MS	IDO activity decreased after antiretroviral therapy initiation. High IDO activity was associated with increased of T regs, low naïve T cells, low CD4/CD8 ratio.
Gelpi *et al*., 2017 (67).	HIV+ patients (n = 100) HIV- patients (n = 16)	Yes	Plasma	LC - MS/MS	Early initiation of antiretroviral therapy has a beneficial effect on kynurenine/tryptophan ratio set point, and this is associated with reduced immune stimulation.
Chen *et al.*, 2018 (68).	HIV+ patients (n = 127) HV- patients (n) = 25	Yes	Whole blood	UPLC - MS	HIV viral load positively correlated with IDO activity, immune activation and T cell exhaustion.

Human Immunodeficiency Virus (HIV), Indoleamine 2, 3-dioxygenase (IDO), Positive (+), Negative (-), High-performance liquid chromatography-mass spectrometry (HPLC-MS/MS), Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS), Gas chromatography-mass spectrometry, Real-time polymerase chain reaction (RT-PCR), Cell culture supernatant (CCS), Cerebrospinal fluid (CSF), Antiretroviral therapy (ART), regulatory T cells (Tregs).

Table 2.0: Studies reporting altered IDO activity, kynurenine or tryptophan in human active TB disease

Reference	Participant demographics	Sample type	Method of IDO Quantification	Major conclusion (s)
Almeida *et al*., 2009 (69).	TB cases (n = 30) HIV+, active TB disease (n =4) Other lungs diseases (No-TB, HIV- n = 11), Healthcare workers (n = 16)	Induced Sputum	RT-PCR	Active TB patients expressed significantly higher IDO mRNA compared with patients with other lung disease and health care workers. IDO expression declined over 500-fold change 15 days post-treatment with anti-TB medication.
Li *et al*., 2011 (70).	TB cases (n = 31) Controls (n = 35)	Pleural effusion	Cell culture	Inhibiting IDO reversed cytokine production and restored T cell functions *in vitro.*
Weiner *et al*., 2012 (71).	Active TB cases (n = 44) LTBI (TST+) (n = 46) No-TB (TST-) (n = 46)	Blood	UPLC/GC–MS/MS Cell culture	Metabolic profiles showed IDO activity was increased in TB

Suzuki *et al*., 2012 (72).	TB case (n) = 174) Controls (n = 85)	Serum	LC - MS/MS	Patients with pulmonary TB have elevated serum IDO activity compared to controls. Higher IDO activity independently predicted the death of TB patients.
Suzuki *et al*., 2013 (73).	TB cases (n = 92) TB pleurisy (n = 34), Malignant pleuritis (n = 36) Parapneumonic effusion (n = 15)	Pleural effusion	LC - MS/MS	IDO activity may be strongly involved in the pathogenesis of TB pleurisy. IDO activity holds diagnostic significance in TB pleurisy.
Feng *et al*., 2015 (74).	TB cases (n = 120) Non-TB (n = 146) Healthy controls (n = 105)	Blood	UPLC – MS	Kynurenine and quinolinic acid, which are products of IDO-mediated tryptophan metabolism were among 20 metabolic profiles that indicated active TB disease.
Adu-Gyamfi *et al*., 2017 (75).	TB-HIV cases (n = 32) Controls (HIV+, No-TB) (n = 70) Pneumonia (HIV positive) (n = 37)	Plasma	UPLC– MS/MS	IDO activity is a plausible diagnostic active TB in HIV infected patients. IDO activity predicted active TB disease months ahead of major TB symptoms, IDO activity declined in all patients after standard TB treatment.
Van Laarhoven *et al*., 2018 (76).	Discovery cohort: TBM, HIV- patients (n = 33) Controls (n = 22)	CSF and Serum	UPLC - MS/MS	Increased tryptophan metabolites were found among TB meningitis patients, and correlated strongly with mortality.

	Validation cohort: TBM patients (n = 101) Controls (HIV-, no TBM) (n = 17)			
Wen Shi *et al.*, 2019 (77).	Drug susceptible TB cases (n = 16) MDR-TB cases (n = 16), Controls (No-TB n = 11, Lung cancer n = 6) No patient or control had HIV infection.	Plasma	LC-MS/MS	Plasma IDO activity could distinguish multi-drug resistance – TB (MDR-TB) from drug-susceptible TB and lung cancer. Higher plasma IDO activity can indicate a higher risk of MDR-TB.
Adu-Gyamfi *et al*., 2020 (78).	HIV+ with TB (n=20) HIV+ with LTBI (n=25) HIV- with TB (n=20) HIV- with LTBI (n=25)	Plasma	ELISA	Plasma IDO activity measured as K/T ratio was elevated at the time of TB diagnosis in both HIV-infected and uninfected patients with active TB disease.
Collins et al., 2020 (44).	HIV- with TB (n=89) Control (No TB, HIV- n=57) HIV+ with TB (n=85) Control (No Tb, HIV- n=57)	Plasma	HRMS	Tryptophan metabolism to kynurenine is highly upregulated in active TB disease.

Tuberculosis (TB), Tuberculous meningitis (TBM), Human Immunodeficiency Virus (HIV), Indoleamine 2,3-dioxygenase (IDO), latent tuberculosis infection (LTBI), Positive (+), Negative (-), High-performance liquid chromatography-mass spectrometry (HPLC-MS/MS), Ultra-performance liquid chromatography-

mass spectrometry (UPLC-MS/MS), Gas chromatography-mass spectrometry, High-resolution mass spectrometry (HRM), Enzyme-linked immunosorbent assay (ELISA), Real-time polymerase chain reaction (RT-PCR).

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Indoleamine 2, 3-Dioxygenase-Mediated Tryptophan Catabolism: A Leading Star or Supporting Act in the Tuberculosis and HIV Pas-de-Deux?

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Progression from latency to active Tuberculosis (TB) disease is mediated by incompletely understood host immune factors. The definitive characteristic of progressive human immunodeficiency virus (HIV) disease is a severe loss in number and function of T lymphocytes. Among the many possible mediators of T lymphocyte loss and ineffective function is the activity of the immune-modulatory enzyme indoleamine 2,3-dioxygenase (IDO). IDO is the rate-limiting enzyme converting tryptophan to kynurenine. IDO activity was initially recognized to mediate tolerance at the foeto-maternal interface. Recently, IDO activity has also been noted to play a critical role in immune tolerance to pathogens. Studies of host immune and metabolic mediators have found IDO activity significantly elevated in HIV and TB disease. In this review, we explore the link between IDO-mediated tryptophan catabolism and the presence of active TB disease in HIV-infected patients. We draw attention to increased IDO activity as a key factor marking the progression from latent to active TB disease in HIV-infected patients.

Keywords: IDO, latent TB, active TB, kynurenine/tryptophan ratio, nicotinamide

INTRODUCTION

Recently, the effect of indoleamine 2,3-dioxygenase-1 (IDO) activity in various diseases, including tuberculosis (TB) and human immunodeficiency virus (HIV) disease has generated considerable interest. Tryptophan, an essential amino acid in the body,

can either be converted to serotonin or oxidized to kynurenines. IDO is the rate-limiting enzyme in the conversion of tryptophan to kynurenines (Higuchi and Hayaishi, 1967; Katz et al., 2008). IDO activation plays a crucial role in macrophage and dendritic cell modulation toward alternate activation and immune tolerance (Nagamatsu and Schust, 2010; Martinez and Gordon, 2014). Two theories have been postulated to explain the mechanism of tolerance induced by IDO. Firstly, depletion of tryptophan in the microenvironment starves T cells of a vital micronutrients, which results in the inability of T cells to proliferate and function effectively (Fallarino et al., 2006; Sharma et al., 2009; Yan et al., 2010).

Secondly, the accumulation of kynurenine and its downstream metabolites promotes T cell stress, anergy and apoptosis (Frumento et al., 2002; Fallarino et al., 2006; Mezrich et al., 2010; Platten et al., 2012). IDO activity plays a crucial role in T cell hypo-responsiveness during pregnancy and evasion of malignant cells from immune surveillance (Munn et al., 1998; Mellor and Munn, 1999, 2004; Nagamatsu and Schust, 2010; Platten et al., 2012). IDO is expressed in many malignancies and high IDO expression is associated with poor prognosis in a variety of cancer types (Brandacher et al., 2006; Ino et al., 2006; Creelan et al., 2013; Wei et al., 2018). Prior to 1998, IDO activity was considered to be anti-microbial, acting via depletion of available tryptophan, which is required for microbial growth (Däubener and Mackenzie, 1999). IDO activity, however, may have opposing roles, either by dominantly suppressing pathogen replication or providing a comfortable immunological niche for the pathogen to thrive in and to evade immune-mediated killing (Leonhardt et al., 2007; Almeida et al., 2009; Divanovic et al., 2011; Makala et al., 2011; Yeung et al., 2012). Both HIV-infection and TB disease are associated with upregulation of IDO activity (Divanovic et al., 2011; Blumenthal et al., 2012; Chen et al., 2014). In this review, we highlight evidence examining the role of elevated IDO activity in HIV infection and active TB disease.

TRYPTOPHAN AND ITS CELLULAR METABOLISM

Essential micronutrients are those which cannot be produced endogenously and must be acquired through dietary sources. Humans, like other mammals, cannot synthesize tryptophan, and therefore, must obtain it from their diet and internal protein turn-over (Brown, 1996). Foods such as banana, spinach, soyabeans, fish, egg, milk, and cheese are rich sources of tryptophan (Sainio et al., 1996; Friedman, 2018). Human beings require about 150–250 mg tryptophan from the diet, and this amount is exceeded in regular household meals (Peters, 1991). Dietary tryptophan is absorbed and delivered through the hepatic portal system, where it enters the bloodstream for protein synthesis and other cellular functions.

In certain cell types, tryptophan can be converted to serotonin or melatonin by tryptophan hydroxylase (Ruddick et al., 2006). Alternatively, tryptophan can be catabolised to kynurenines (Higuchi and Hayaishi, 1967; Takikawa, 2005; Katz et al., 2008). Two cytosolic enzymes, IDO and Tryptophan 2,3-dioxygenase (TDO), are responsible for tryptophan catabolism in specific cell types (Takikawa, 2005). IDO and TDO are intracellular, nonsecreted enzymes (Yamazaki et al., 1985). While TDO expression and activity is confined to hepatocytes, IDO is expressed by a wider variety of cells, but most potently by macrophages, dendritic and mesenchymal stem cells (Thomas et al., 2001; Dai and Zhu, 2010; Francois et al., 2010). IDO comprises two related, but distinct enzymes encoded by two different genes, namely *IDO1* and *IDO2* (Yamazaki et al., 1985; Takikawa, 2005; Bilir and Sarisozen, 2017). Both enzymes are expressed by epithelial and antigen-presenting cells (Munn et al., 1998; Nagamatsu and Schust, 2010; Zhu, 2010). In this review, IDO1 is referred to as IDO. IDO or TDO initiate catabolism of tryptophan by oxidizing the indole ring to produce N-formylkynurenine (Liu et al., 2016). N-formylkynurenine can be further converted to stable end-products including kynurenic acid, anthranilic acid and 3hydroxykynurenine. N-formylkynurenine can also be further oxidized to nicotinamide adenine dinucleotide (NAD+), a vital co-factor in energy production, DNA synthesis and cellular homeostasis (Bryleva and Brundin, 2017). The tryptophankynurenine-NAD+ pathway represents the *de novo* pathway for NAD+ synthesis when niacin intake is limited in the diet (Higuchi and Hayaishi, 1967; Takikawa, 2005).

IDO EXPRESSION AND FUNCTION

Munn and colleagues observed IDO expression and activity at the foeto-maternal interface in pregnant mice (Munn et al., 1998). Their findings suggested that IDO activity mediated immune tolerance to the fetus by the mother. Interestingly, blocking IDO activity in allogeneic pregnancy led to a break in the tolerogenic state that protected the fetus from the maternal immune system (Munn et al., 1998; Mellor and Munn, 1999). Since then, several studies have shown that IDO is expressed in various tissues including liver, lymph nodes, spleen and tonsils (Mellor and Munn, 2004; Bilir and Sarisozen, 2017). Antigen-presenting cells, particularly macrophages and dendritic cells, can express IDO in response to cytokines such as IFN-γ and TNF-α (Robinson et al., 2005; Nagamatsu and Schust, 2010). During infection or inflammation, IDO is stimulated in

polymorphonuclear cells, endothelial cells, epithelial cells, eosinophils and fibroblasts by pro-inflammatory cytokines (Hayashi et al., 2004; Robinson et al., 2005; Xu et al., 2008).

IDO-MEDIATED TRYPTOPHAN METABOLISM AND HOST IMMUNITY

Tryptophan depletion and subsequent generation of kynurenine metabolites have considerable effects on the host's immunity. The IDO pathway plays a significant role in T cell hypofunctionality, a critical concept in immune tolerance to pathogens (Mellor et al., 2017). In acute inflammation, induction of IDO may act as a regulatory feedback loop to prevent over-reaction of the immune response (Grohmann et al., 2003). In chronic inflammatory conditions, elevated IDO activity may dampen the host's protective immunity (Leonhardt et al., 2007; Divanovic et al., 2011).

Immune cells, in particular macrophages and dendritic cells, are sites of IDO production. IDO expression and activation creates a local immunosuppressive micro-environment, which allows pathogens to escape sterilizing immunity or containment (Nagamatsu and Schust, 2010). IDO induction is a feature of alternative macrophage activation, which favors a tolerogenic or anti-inflammatory state, rather than a pro-inflammatory phenotype. While a pro-inflammatory phenotype leads to killing and elimination of a pathogen, an alternatively activated phenotype leads to immune tolerance, induction of regulatory T cells, tissue repair and fibrosis (Nagamatsu and Schust, 2010; Mellor et al., 2017). Macrophage phenotypes have been termed M1 and M2, however, plasticity and intermediate types exist (Das et al., 2015). The alternative pathway of macrophage activation is shown in **Figure 1**.

Downstream metabolites of the IDO pathway such as quinolinic acid, are neurotoxic and have been implicated in HIV-associated dementia (Gostner et al., 2015). Increased tryptophan oxidation leads to a net increase in circulating niacin, which increases the basal metabolic rate (Murray, 2003a). Kynurenines also activate the aryl hydrocarbon receptor (AhR), a transcription factor, key in sensing external environmental triggers and modulating gene expression in response (Lewis et al., 2014; Schmidt and Schultze, 2014; Salazar et al., 2017). Lipopolysaccharides, viral proteins such as HIV-Tat, HIV-Nef, and HIV-p17, and pathogen-associated molecular patterns, stimulate IDO expression to influence survival and pathogenesis (Murray, 2003b, Schmidt and Schultze, 2014). In malignancies, where IDO activity has been extensively studied, IDO activity correlates with dampened immunity, and poor prognosis (Ino et al., 2006; Hornyák et al., 2018).

TRYPTOPHAN METABOLISM IN MICROBIAL INFECTIONS

Amino acids are critical micronutrients for all pathogens. Microbes either synthesize amino acids or depend on their hosts for amino acids for growing and multiplying. The ability of the host to deprive pathogens of essential micronutrients is a crucial feature of innate defense. Tryptophan, just like carbon, iron, and nitrogen are critical for microbial survival (Rohmer et al., 2011). Conventionally, tryptophan depletion has long been known to have anti-microbial actions for pathogens, which cannot synthesize it from smaller molecules (Russell, 2013; Zhang and Rubin, 2013; Zhang et al., 2013). Tryptophan deprivation has been shown to inhibit Chlamydia and Toxoplasmosis *in vitro* (Byrne et al., 1986; Murray et al., 1989). Several *in vitro* studies have reported that measles, influenza, cytomegalovirus, and herpes simplex viral infections are also susceptible to tryptophan depletion (Obojes et al., 2005; Zhang and Rubin, 2013; Schmidt and Schultze, 2014). On the other hand, mycobacteria possess all the complete set of tools to synthesize any essential amino acid, including tryptophan, required for their survival, a feature which gives them an evolutionary advantage (Zhang and Rubin, 2013; Zhang et al., 2013).

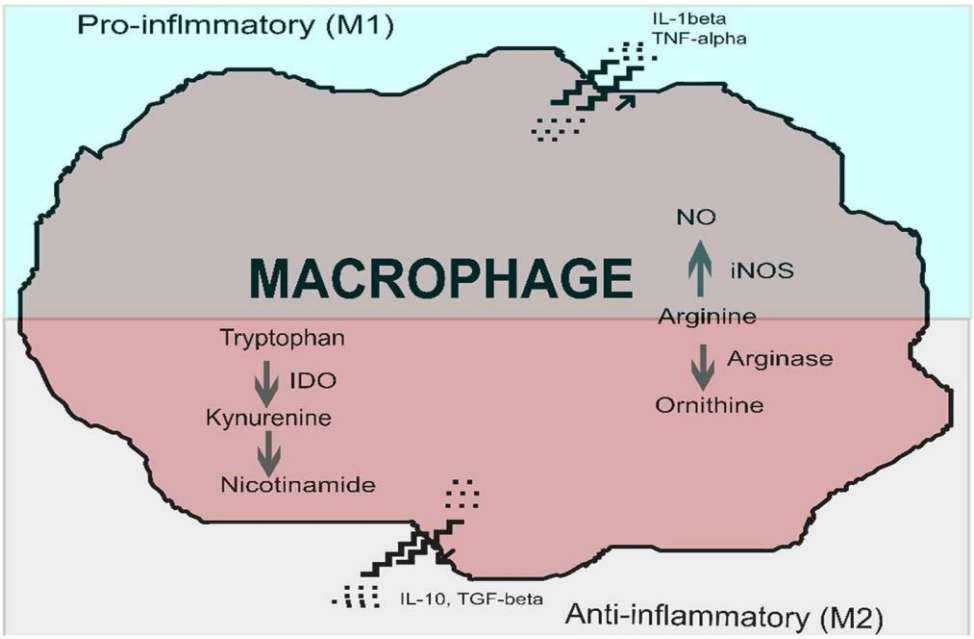

FIGURE 1 | Macrophage activation, in the presence of indoleamine 2,3-dioxygenase (IDO), favors polarization to an immunosuppressive phenotype (alternatively activated macrophage) over that of a pro-inflammatory macrophage (classically activated macrophage). Classically activated macrophages produce pro-inflammatory cytokines such as interleukin 1β (IL-1β) at the time of macrophage activation. Alternatively activated macrophages produce immune-suppressive cytokines such as interleukin 10 (IL-10) and transforming growth factor-beta (TGF-β). Key enzymes involved in classical activation include inducible nitric oxide synthase (iNOS), which converts arginine into nitric oxide. Two critical enzymes are involved in the alternative activation pathway: arginase converts arginine to ornithine, and indoleamine 2,3-dioxygenase converts tryptophan to kynurenines. Image adapted and modified from Nagamatsu and Schust (2010) with permission.

ANIMAL STUDIES OF IDO ACTIVITY IN TB PATHOGENESIS

In animal models, increased tryptophan metabolism via the IDO pathway has been associated with poor immune responses to M. TB (O'Connor et al., 2009; Mehra et al., 2012; Zhang and Rubin, 2013; Zhang et al., 2013). IDO activation has been reported in mycobacterial infection in both mice and non-humanprimates (Mehra et al., 2012; Foreman et al., 2016; Gautam et al., 2018). In mice, increased IDO activation has been associated with poor TB outcomes (Moreau et al., 2005; O'Connor et al., 2009). In macaques, Mehra and colleagues demonstrated that IDO induction in the periphery of the granuloma correlates with active TB disease (Mehra et al., 2012). IDO induction was significantly higher in mock-vaccinated compared with BCG-vaccinated animals, and was seen predominantly in nonlymphocytic cells within the ring structure of the granuloma, lining the area of central necrosis (Mehra et al., 2012). Gautam et al. (2018) also showed that M. TB induces IDO expression and activity in the lung macrophages of macaques with active TB disease (Mehra et al., 2012). Based on evidence from both human and animal studies, M. TB infection is associated with high expression of IDO in the granuloma, leading to compromised T cell function, which promotes mycobacterial survival and growth (Mehra et al., 2012; Seo et al., 2015; Gautam et al., 2018). Blocking IDO expression and activity led to reduced pathogen burden, minimal TB pathology and mproved survival (Gautam et al., 2018). Furthermore, IDO inhibition was accompanied by restoration of T cell quantity and functions (Gautam et al., 2018). Collectively, these findings indicate that IDO activation plays a crucial role in M. TB pathogenesis in animal models.

TRYPTOPHAN CATABOLISM IN HIV INFECTION

When co-infecting a host, HIV and M. TB influence each other, accelerating deterioration of immune function. HIV infection causes severe loss of T cells, both in number and function (Douek et al., 2001; Alimonti et al., 2003; Schindler et al., 2006). Specific mechanisms of immune impairment are beyond the scope of this review; however, it is noted that aspects of T cell exhaustion persist in the face of chronic immune activation (Brenchley et al., 2006; Doitsh et al., 2014; Doitsh and Greene, 2016; Sokoya et al., 2017). In the last three decades, a wealth of information has indicated that IDO activation and its associated metabolites play a role in human HIV infection and early progression to AIDS (Fuchs et al., 1990; Hunt et al., 2014; Jenabian et al., 2015; Mehraj and Routy, 2015; Gelpi et al., 2017). Tryptophan catabolism and its immune-regulatory effect are notable during HIV progression (Almeida et al., 2009; Mehraj and Routy, 2015; Gelpi et al., 2017; Gautam et al., 2018). There is a strong association of elevated IDO activity with AIDS-defining illnesses (Nagamatsu and Schust, 2010; Catalfamo et

al., 2012; Martinez and Gordon, 2014; Sokoya et al., 2017). Furthermore, gut microbial translocation sustains increased immune activation, and chronic immune activation results in a pro-inflammatory cytokine storm, which activates the IDO pathway (Vazquez-Castellanos et al., 2015; Chen et al., 2018; Lu et al., 2018).

Werner et al. (1988) reported that HIV-infected patients have high serum kynurenine-to-tryptophan ratio (IDO activity). The elevated kynurenine-tryptophan ratio in HIV-infected patients correlates with advanced HIV disease (Huengsberg et al., 1998). Huengsberg et al. (1998) observed a striking association between the kynurenine-tryptophan ratio, declining CD4 T cells and progression to AIDS. Antiretroviral therapy (ART) improved the kynurenine-tryptophan ratio and correlated with decreased viral load and improved CD4 cell count (Zangerle et al., 2002). Hunt et al. (2014) showed that the kynurenine-tryptophan ratio was an independent predictor of death. Multiple studies have reported a similar finding of increased IDO activity or kynurenine metabolites in HIV infection (**Table 1**). We speculate that increased IDO-mediated tryptophan catabolism in HIV infection may be more than merely an association, but play a causal role in susceptibility to disease, particularly active TB.

IDO ACTIVITY IN HUMAN TUBERCULOSIS DISEASE

Despite extensive work in murine and macaque models of TB, only a few studies have investigated IDO-mediated tryptophan catabolism and its metabolites in human TB (**Table 2**). M. TB has been shown to induce IDO activity *in vitro* (Blumenthal et al., 2012) and *in vivo* (Gautam et al., 2018). Almeida et al. (2009) studied 20 immunological mediators by reverse transcriptase-polymerase chain reaction (rt-PCR) in induced sputa of patients with active TB disease, patients with other lung diseases and controls. The authors found significantly elevated IDO expression in TB patients. The IDO levels decreased more than 500-fold within 2 weeks of beginning of TB treatment. Their work suggested that IDO might be a useful tool to diagnose active disease and monitor the efficacy of TB treatment; however, their studies did not include HIV-infected patients (Almeida et al., 2009). Weiner et al. explored more than 400 metabolites from the serum of latently infected and active TB patients and found that IDO activity was elevated in active TB patients (Weiner 3rd et al., 2012). Li et al. found increased IDO expression and activity in pleural fluid from TB patients (Li et al., 2011). We and others have shown that IDO activity is elevated at the time of TB diagnosis and declines after TB treatment (Almeida et al., 2009; Suzuki et al., 2012, 2013; AduGyamfi et al., 2017) (**Table 2**). Both HIV-infected and uninfected patients with active TB had elevated IDO activity compared with latent TB infection (Almeida et al., 2009; Suzuki et al., 2012; Adu-Gyamfi et al., 2017; Chen et al., 2018). Additionally, IDO activity showed potential to predict the onset of active disease ahead of major TB symptoms (Adu-Gyamfi et al., 2017) and could be useful in early diagnosis of multi-drug resistance TB (Shi et al., 2019).

LABORATORY MEASUREMENT OF IDO ACTIVITY

IDO can be studied quantitatively by measuring mRNA expression or protein levels or enzymatic activity (productto-substrate ratio; Li et al., 2011; Mehra et al., 2012; Suzuki et al., 2012, 2013; Huang et al., 2013; Bipath et al., 2015; Zhang et al., 2019). IDO activity can be detected in host fluids including blood, sputum, pleural fluid, cerebrospinal

TABLE 1 | Studies reporting elevated IDO activity, increased kynurenine or decreased tryptophan in human HIV infection.

References	Participant demographics	ART therapy	Sample type	Method of IDO quantification	Major conclusion (s)
Werner et al. (1988)	HIV+ patients (*n* = 11) HIV– patients (*n* = 11)	Not specified	Serum	HPLC-MS	Elevated kynurenine/tryptophan ratio suggested increased tryptophan catabolism in HIV-infected patients. Increase tryptophan metabolism may have contributed to neurologic symptoms found among HIV-infected patients.
Larsson et al. (1989)	HIV+ patients (*n* = 24) HIV– patients (*n* = 14)	No	Blood and CSF	HPLC-MS	HIV patients had significantly decreased tryptophan in blood and CSF compared to controls. HIV patients with severe AIDS-defining symptoms such as markedly lower CD4 count had significantly lower tryptophan concentrations.

Fuchs et al. (1990)	HIV+ patients (*n* = 22) HIV– patients (*n* = 14)	Not specified	Serum and CSF	HPLC-MS	Decreased tryptophan levels were associated with chronic immune activation in HIV positive patients. IDO activity induced by interferon-γ may be the cause of reduced tryptophan concentration in HIV infected patients.
Wiegand et al. (1991)	HIV+ patients (*n* = 14) HIV– patients (*n* = 14)	95% on Zidovudine monotherapy	Serum and CSF	HPLC-MS	Lower tryptophan concentration in HIV-infected patients was associated with sleep disturbances.
Fuchs et al. (1991)	HIV+ patients (*n* = 42) Controls were HIV– from three different cohorts	38% on only Zidovudine	serum	HPLC-MS	Decreased tryptophan concentration in HIV-infected patients may be due to chronic immune activation which result in increased IDO activation.
Heyes et al. (1991, 1992)	HIV+ patients (*n* = 126) Controls HIV– (*n* = 28)	No	Serum and CSF	HPLC-MS	Tryptophan concentration decreased with increased kynurenines in serum and cerebrospinal fluid.
Hortin et al. (1994)	HIV+ patients (*n* = 20) HIV– patients (*n* = 20)	Majority on ARV	Plasma	Flow cytometry and ELISA	Plasma tryptophan concentration decreases in response to HIV infection.
Gisslén et al. (1994)	HIV+ patients (n = 14)	Yes	Blood and CSF	HPLC-MS	Initiation of antiretroviral therapy was associated with decreased immune activation and decreased tryptophan breakdown in HIV-infected patients.
Huengsberg et al. (1998)	HIV+ patients (*n* = 206) HIV– patients (*n* = 72)	Not specified	Serum	HPLC-MS	Kynurenine/tryptophan ratio showed reciprocal relation to tryptophan. Severe HIV disease correlated with increased tryptophan degradation and increased IDO activity.
Look et al. (2000)	HIV+ patients (*n* = 17) HIV– patients (*n* = 55	Yes	Serum	GC-MS	HIV-infected patients had increased kynurenine/tryptophan ratio compared to controls. ART treatment mitigated immune activation in HIV disease, and led to a decrease in IDO activation.
Murray et al. (2001)	HIV+ patients (*n* = 4)	Yes	Plasma	HPLC-MS	Plasma tryptophan increased 40% in HIV-infected patients following treatment with antiretroviral medications, however, other amino acids concentrations remained fairly unchanged in plasma.
Zangerle et al. (2002)	HIV+ patients (*n* = 45) HIV– patients (*n* = 40)	Yes	Plasma	HPLC-MS	Tryptophan degradation was increased in HIV-infection, and ART partially reversed it.
Atlas et al. (2007)	HIV+ patients (*n* = 22) HIV– patients (*n* = 22)	Yes	CSF	HPLC-MS	HIV-infected patients had elevated kynurenine concentration compared to controls; increased kynurenine levels were associated with altered mental state of HIV patients.
Favre et al. (2010)	HIV+ patients (*n* = 85)	Yes	Plasma, CCS, Tissue, PBMCs	LC-MS/MS	IDO mRNA is upregulated in myeloid dendritic cells from HIV+ patients IDO activity was increased in progressive HIV infection
Byakwaga et al. (2014)	HIV+ patients (*n* = 435)	Yes	Plasma	LC-MS/MS	IDO activity independently predicted poor CD4+ T cell recovery Elevated IDO activity was associated with increased mortality among HIV-infected patients.
Chen et al. (2014)	HIV+ patients (*n* = 76) HIV– patients (*n* = 16)	Yes	Plasma	HPLC-MS	IDO activity significantly declined after treatment; however, it did not reach normal activity.

(Continued)

TABLE 1 | Continued

References	Participant demographics	ART therapy	Sample type	Method of IDO quantification	Major conclusion (s)
Page et al. (2014)	HIV+ patients (*n* = 36) Controls (n = 16)	40% on ART	Plasma	HPLC-MS	IDO activity was significantly elevated in HIV-infected patients not receiving antiretroviral therapy compared to patients on treatment.
Jenabian et al. (2015)	HIV+ patients (*n* = 116) Controls (*n* = 12)	Yes	Plasma	HPLC-MS/MS	Early phase of HIV-infection, and/ or patients not receiving ART has high IDO activity. IDO activity declined after initiating treatment. IDO activity was positively associated with CD8+ T cell activation
Bipath et al. (2015)	HIV+ patients (*n* = 105) HIV– patients (*n* = 60)	78% on ART	Plasma	GC-MS	Tryptophan depletion directly correlated with the severity of immune deficiency.
Gaardbo et al. (2015)	HIV+ patients (*n* = 41)	Yes	Plasma	LC-MS/MS	IDO activity decreased after antiretroviral therapy initiation. High IDO activity was associated with increased of Tregs, low naïve T cells, low CD4/CD8 ratio.
Gelpi et al. (2017)	HIV+ patients (*n* = 100) HIV– patients (*n* = 16)	Yes	Plasma	LC-MS/MS	Early initiation of antiretroviral therapy has a beneficial effect on kynurenine/tryptophan ratio set point, and this is associated with reduced immune stimulation.
Chen et al. (2018)	HIV+ patients (*n* = 127) HV– patients (*n* = 25)	Yes	Whole blood	UPLC-MS	HIV viral load positively correlated with IDO activity, immune activation and T cell exhaustion.

Human Immunodeficiency virus (HIV), Indoleamine 2, 3-dioxygenase (IDO), Positive (+), Negative (–), High-performance liquid chromatography-mass spectrometry (HPLC-MS/MS), Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS), Gas chromatography-mass spectrometry, Real-time polymerase chain reaction (RT-PCR), Cell culture supernatant (CCS), Cerebrospinal fluid (CSF), Antiretroviral therapy (ART), regulatory T cells (Tregs).

fluid and urine (Almeida et al., 2009; Li et al., 2011; Suzuki et al., 2012, 2013; Bipath et al., 2015; Adu-Gyamfi et al., 2017; Gautam et al., 2018; Yarbrough et al., 2018). IDO mRNA expression has been studied using rt-PCR, and IDO protein has been measured by western blot, radioimmunoassay and immunofluorescence methods (Almeida et al., 2009; Soliman et al., 2013; Zhang et al., 2019). Recently, intracellular staining methods have also been described (Sørensen et al., 2011). For purposes of clinical diagnosis, the current gold standard method for measuring IDO activity is using liquid chromatographymass spectrometry (Suzuki et al., 2012; Huang et al., 2013; AduGyamfi et al., 2017). Even though the chromatographic-mass spectrometry method has been reliable, it requires substantial laboratory infrastructure and high-grade expertise; hence the facility is not available in frontline diagnostics, especially in resource-constrained, high burden countries (Tiwari et al., 2007). Newer, higher throughput, lower-cost method–enzyme-linked immunosorbent assay (ELISA) are available (Ziklo et al., 2018). The quantitative ELISA method can be performed using cell culture supernatant, serum or plasma. We recently evaluated the ELISA method in comparison with the mass spectrometry method for measuring IDO activity. The ELISA showed good reproducibility and agreement, and could replace mass spectrometry as the analytical method for clinical use. ELISA has an added advantage of small sample volume and the potential of being fully automated compared with mass spectrometry. IDO detection in plasma has shown good discrimination between patients with active compared to latent TB, and may reflect a cumulative picture of intracellular IDO activity over time (Adu-Gyamfi et al., 2017).

IDO ACTIVITY AS A NOVEL ACTIVE TB BIOMARKER IN HIV-INFECTION

A biomarker is defined as any characteristic that can be objectively measured as an indicator of health or disease process or as a response to a therapeutic intervention (Group et al., 2001; Phillips et al., 2010; Mcnerney et al., 2012; Walzl et al., 2018). In an infectious disease, biomarkers can be host or pathogen-derived parameters (Phillips et al., 2010; Mcnerney et al., 2012; Wallis et al., 2013). In TB, the lack of a biomarker indicating risk of progression to active disease, particularly in individuals with compromised immunity, presents a massive gap in consolidating efforts to eliminate the disease. Similarly, the absence of a diagnostic TB biomarker or a marker to monitor response to anti-TB

therapy cripples the efficiency of global TB control strategies. Despite increased research aimed at finding a reliable, inexpensive and easily quantitated TB biomarker, our lack of full understanding of factors mediating the switch of latent TB to active TB disease is partly to be blamed for lack of an improved TB vaccine and new therapeutics.

Most TB studies have relied on animal models or *ex vivo* M. TB challenge in cells or tissue from active or latently infected patients to understand TB pathology. Some studies have examined metabolic profiles or genes from active TB patients to identify metabolites or genes that are switched on or off, which may be implicated in the transition from latency to active disease in both HIV-infected and uninfected individuals (Weiner 3rd et al., 2012; Scriba et al., 2017; Suliman et al., 2018). Despite dynamic efforts in the search for a sensitive, specific, and reliable TB biomarker, there has been insufficient attention paid to the host pathway of tryptophan metabolism, which is a central player in both HIV infection and TB disease. M. TB, an organism able to synthesize tryptophan, thrives during tryptophan depletion in its microenvironment, while proliferating T cells die *in vitro* (Zhang et al., 2013; Berney and Berney-Meyer, 2017; Gautam et al., 2018). The high level of interferon-γ during TB infection activates IDO-mediated tryptophan catabolism in macrophages (Giacomini et al., 2001; Khan et al., 2016). This phenomenon may result in M. TB's ability to hijack IDO's immunosuppressive actions on the host immune system to cause pathology.

It remains to be determined whether elevated IDO activity is causative of, or only associated with, progressive HIV and TB disease. Some researchers postulate that human co-existence with latent TB infection may be a human evolutionary adaptation to increase host tryptophan in times of low dietary tryptophan availability (Williams and Dunbar, 2013; Williams and Hill, 2017). Intriguingly, M. TB can produce nicotinamide from tryptophan (Fricker et al., 2018). An older laboratory test for distinguishing M. TB from non-tuberculous mycobacteria was known as the niacin test (Ogawa et al., 1968). Isoniazid (INH), one of the most effective anti-TB drugs was developed as a nicotinamide analog for the treatment of TB (Kushner et al., 1952; Murray, 2003a, Mckenzie et al., 1948). Serendipitously, nicotinamide was discovered to be anti-tuberculous even though the mechanism of action remained unknown (Mckenzie et al., 1948; Kushner et al., 1952; Murray, 2003a). Accordingly, these pieces of evidence support the fact that elevated flux through the tryptophan-nicotinamide pathway results in M. TB inhibition.

Currently, several clinical trials are exploring blocking IDO activity for therapeutic use. During infection with *Chlamydia trachomatis*, an intra-macrophage pathogen, which can synthesize tryptophan, blocking IDO activity with levo-1methyl-tryptophan led to improved susceptibility to doxycycline (Ibana et al., 2011). In TB, mechanistic studies have demonstrated that blocking IDO activity in Simian immunodeficiency virus (SIV) infection improved T cell number and functions (Boasso and Shearer, 2007; Boasso et al., 2009). Blocking IDO activity in combination with antiviral therapy significantly reduced the SIV viral load in plasma and lymph nodes of treated animals (Boasso et al., 2009). Recently, Gautam et al. (2018) demonstrated *in vivo* that M. TB induces IDO expression in the lungs of macaques and mice with active TB disease. In the macaque, inhibition of IDO activity led to a reduced M. TB burden, pathology and improved animal survival (Gautam et al., 2018). Together, these results suggest that there exists a potential for using IDO inhibitors as an effective and host-driven therapy in TB. IDO inhibition as a therapeutic strategy against various cancers has yielded encouraging results (O'brien et al., 2003; Muller et al., 2005; Hanafi et al., 2014; Spranger et al., 2014; Austin and Rendina, 2015; Prendergast et al., 2017, 2018). IDO inhibitors are thus readily available and include some agents already licensed by the US Food and Drug Administration (Jeong et al., 2013).

In summary, IDO-mediated tryptophan catabolism via the kynurenine-nicotinamide pathway represents a common axis upregulated in HIV infection and TB disease. Rather than being uniquely elevated in TB, cumulative elevation of IDO in HIV and TB may explain the extraordinarily high rate of progression to active TB in HIV-infected patients. It is unclear whether elevated IDO is causative of progression to active disease or a compensatory response to the microbe. There is much promise in using metabolites from the IDO-mediated tryptophan pathway to diagnose active TB and monitor antiTB treatment. IDO holds promise as a TB biomarker in both HIV-positive and negative patients, but further confirmatory studies are urgently needed. Regarding new TB drug targets, it remains speculative whether IDO inhibition would be beneficial. Further exploration of this pathway is necessary to better understand TB pathogenesis and discover novel host biomarkers and potential drug targets for either TB or HIV disease.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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TABLE 2 | Studies reporting IDO activity or either kynurenine or tryptophan in human active TB disease.

References	Participant demographics	Sample type	Method of IDO quantification	Major conclusion (s)
Almeida et al. (2009)	TB cases (*n* = 30) HIV+, active TB disease (*n* =4) Other lungs diseases (No-TB, HIV– *n* = 11), Healthcare workers (*n* = 16)	Induced Sputum	RT-PCR	Active TB patients expressed significantly higher IDO compared with patients with other lung disease and health care workers. IDO expression declined over 500-fold change 15 days post-treatment with anti-TB medication.
Li et al. (2011)	TB cases (*n* = 31) Controls (*n* = 35)	Pleural effusion	Cell culture	Inhibiting IDO reversed cytokine production and restored T cell functions *in vitro.*
Weiner 3rd et al. (2012)	Active TB cases (*n* = 44) LTBI (TST+) (*n* = 46) No-TB (TST-) (*n* = 46)	Blood	UPLC/GC–MS/MS Cell Culture	Metabolic profiles showed IDO activity increased in TB pathogenesis.
Suzuki et al. (2012)	TB case (n = 174) Controls (*n* = 85)	Serum	LC-MS/MS	Patients with pulmonary TB has elevated serum IDO activity compared to controls. Higher IDO activity independently predicted death of TB patients.
Suzuki et al. (2013)	TB cases (*n* = 92) TB pleurisy (*n* = 34), Malignant pleuritis (*n* = 36) Parapneumonic effusion (*n* = 15)	Pleural effusion	LC-MS/MS	IDO activity may be strongly involved in the pathogenesis of TB pleurisy. IDO activity holds diagnostic significance in TB pleurisy.
Feng et al. (2015)	TB cases (*n* = 120) Non-TB (*n* = 146) Healthy controls (*n* = 105)	Blood	UPLC-MS	Kynurenine and quinolinic acid, which are products of IDO-mediated tryptophan metabolism were among 20 metabolic profiles that indicated active TB disease.
Adu-Gyamfi et al. (2017)	TB-HIV cases (*n* = 32) Controls (HIV+, No-TB) (*n* = 70) Pneumonia (HIV positive) (*n* = 37)	Plasma	UPLC-MS/MS	IDO activity is a plausible diagnostic active TB in HIV infected patients. IDO activity predicted active TB disease months ahead of major TB symptoms, IDO activity declined in all patients after standard TB treatment.
Van Laarhoven et al. (2018)	Discovery cohort: TBM, HIV– patients (*n* = 33) Controls (*n* = 22) Validation cohort: TBM patients (*n* = 101) Controls (HIV-, no TBM) (*n* = 17)	CSF and Serum	UPLC-MS/MS	Increased tryptophan metabolites were found among TB meningitis patients, and it correlated strongly with mortality.
Shi et al. (2019)	Drug susceptible TB cases (*n* = 16) MDR-TB cases (*n* = 16), Controls (No-TB *n* = 11, Lung cancer *n* = 6) No patient or control had HIV infection.	Plasma	LC-MS/MS	Plasma IDO activity could distinguish multi-drug resistance—TB (MDR-TB) from drug-susceptible TB and lung cancer. Higher plasma IDO activity can indicate a higher risk of MDR-TB.

Tuberculosis (TB), Tuberculous meningitis (TBM), Human Immunodeficiency virus (HIV), Indoleamine 2,3-dioxygenase (IDO), latent Tuberculosis infection (LTBI), Positive (+), Negative (–), High-performance liquid chromatography-mass spectrometry (HPLC-MS/MS), Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS), Gas chromatography-mass spectrometry, Real-time polymerase chain reaction (RT-PCR).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 3

Methods

IDO has been studied quantitatively by measuring mRNA expression, protein levels or enzymatic activity (product-to-substrate ratio). Each approach has yielded encouraging results in studies of animal and human TB (1, 2). IDO mRNA expression has been analysed using reverse transcriptase polymerase chain reaction (rt-PCR). IDO protein levels have been quantitated with western blot, radioimmunoassay, immunohistochemistry or immunofluorescence methods (3-5). Recently, some researchers have reported intracellular staining of IDO by flow cytometry (3, 5, 6). IDO mRNA is post-translationally regulated, hence measuring IDO mRNA expression or protein levels may offer very little for purposes of clinical diagnosis.

For clinical diagnostic purposes, plasma kynurenine-to-tryptophan (K/T) ratio has been used to assess IDO activity (7, 8). Related enzymes including tryptophan 2,3-dioxygenase (TDO) and IDO-2 as well as enzymes encoded by gut-resident bacteria also convert tryptophan to kynurenine. Elevated K/T ratio, therefore, reflects not only IDO-1 activity but a composite picture of host and microbial tryptophan metabolism. Measurement of product-to-substrate ratio however remains the preferred clinical approach to quantitatively detecting shifts in IDO activity.

3.1 Laboratory method selection

The gold standard method for the quantitation of K/T ratio is high-performance liquid chromatography-mass spectrometry (HPLC-MS) (9, 10). The HPLC-MS method measures the concentration of kynurenine and tryptophan from clinical samples to calculate the product to

substrate ratio. In our previous work published in 2017, we optimized and validated an ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) method (8). Even though the UPLC-MS method yielded reliable and accurate results, it not a widely available frontline TB method, especially in resource-constrained, high TB burden countries. The cost and the required expertise also exclude it from being a primary care tool. We, therefore, sought to explore an alternative widely available method that could replace UPLC-MS for detection of kynurenine and tryptophan concentration from blood samples.

3.2 Determination of kynurenine and tryptophan concentration by using enzyme-linked immunosorbent assay (ELISA)

In this study, we explored a commercial enzyme-linked immunosorbent assay (ELISA) kit as an alternative method for measuring kynurenine and tryptophan concentration in plasma. ELISA has the advantage of being available in many facilities, as well as being less expensive and time-consuming than UPLC-MS. ELISA also has relatively few maintenance costs and can potentially be automated. ELISA can be performed using serum or plasma and requires small sample volume. The ELISA competitively determines kynurenine and tryptophan concentration in the sample. We performed an assay verification for the ELISA test by comparison with mass spectrometry.

3.2.1The ELISA method

We purchased a commercial kit from ImmuSmol, Inc. France. The ELISA package contains two test kits, one for kynurenine and one for tryptophan. It is designed for the quantitative determination of L-kynurenine and L-tryptophan in samples using a competitive ELISA procedure. The kit can measure kynurenine and tryptophan concentration from cell culture

supernatant, serum or plasma. In this study, we used EDTA-anticoagulated plasma to measure kynurenine and tryptophan concentration.

3.2.2 ELISA test principle

For kynurenine, as per package insert, plasma samples were first acylated with 2-(N-Morpholino) ethane sulfonic acid. Acylation allows the small molecule kynurenine to be recognized by the antibody to allow for quantitation. Acylated samples were then made to compete with the solid phase-bound kynurenine for a fixed number of antibody binding sites over a 15-hour (overnight) incubation period at 2 – 8 °C. Free antigen and free antigen-antibody complexes were removed by washing 4 times with the wash buffer (non-ionic detergent at physiological pH). The antibody bound to the solid phase was detected by an anti-rabbit IgG-peroxidase conjugate using 3,3',5,5'-tetramethylbenzidine as a substrate. The reaction was monitored at 450 nm using a microplate reader. Quantification of unknown samples was achieved by comparing their absorbance with a reference curve prepared with known standards provided with the kit.

Per manufacture instructions, plasma tryptophan was derivatized using dimethyl sulfoxide. Derivatization treatment was required to allow the tryptophan to be recognized by the commercial antibody. Derivatized plasma tryptophan competed with solid-phase bound antigen for a fixed number of antibodies over a 15-hour (overnight) incubation period at 2 – 8 °C. Free antigen and free antigen-antibody complexes were washed off using a non-ionized detergent at a physiological pH for 4 times as wash buffer. The antibody bound to the solid phase was then detected by an anti-rabbit IgG-peroxidase conjugate using 3,3',5,5'-tetramethylbenzidine as a substrate. The reaction was monitored at 450 nm using a microplate

reader. Kynurenine and tryptophan were quantified from samples by comparing their absorbance on an ELISA reader (Gen5, Biotek Inc, USA) with a standard curve generated from known standards supplied by the manufacturer.

3.2.3 Plasma IDO activity (kynurenine/tryptophan ratio)

We calculated IDO activity as the ratio of kynurenine (product) to tryptophan (substrate).

To facilitate calculation of the K/T ratio, we converted both kynurenine and tryptophan to the same units of measurement. Plasma K/T ratio was determined as kynurenine concentration (µmol/L) / tryptophan concentration (µmol/L). Therefore, plasma K/T ratio was written with no unit throughout.

3.3 ELISA test verification

We performed a verification of the ELISA test using plasma samples from healthy volunteers who were HIV-negative as well as samples from HIV-positive patients.

3.3.1 ELISA test reproducibility using HIV-uninfected samples

First, we verified the ELISA test kit using plasma samples from HIV negative healthy individuals. We determined intra- and inter-run reproducibility of the assay using 6 plasma samples. For intra-run reproducibility, each sample was tested in triplicate in the same run to determine the mean, standard deviation (SD) and percentage coefficient of variation (%CV). In HIV-negative healthy individuals, of the six samples, kynurenine gave a mean SD of 0.06 µmol/l with average %CV of 3.20%, table 3.0. Tryptophan showed a mean SD of 1.80 µmol/l with an average %CV of 2.52%, table 3.0. Plasma K/T ratio showed a mean SD of 0.001 with average intra-assay %CV of 3.77%, table 3.0.

Table 3.0: Intra-run reproducibility – Plasma samples from healthy individuals (HI)

Kynurenine						
Sample no.	**Result 1 umol/L**	**Result 2 umol/L**	**Result 3 umol/L**	**Mean umol/L**	**Std Dev**	**CV (%)**
HI 1	1,926	2,007	1,922	1,952	0,048	2,458
HI 2	2,039	2,118	2,049	2,069	0,043	2,079
HI 3	1,931	1,936	2,083	1,983	0,086	4,345
HI 4	2,154	2,172	1,970	2,099	0,112	5,327
HI 5	1,801	1,836	1,807	1,815	0,019	1,031
HI 6	1,963	2,097	1,956	2,005	0,079	3,963
Average					**0,065**	**3,202**
Tryptophan						
HI 1	74,518	72,399	75,832	74,250	1,732	2,333
HI 2	69,051	72,892	76,309	72,751	3,631	4,991
HI 3	59,369	58,553	56,924	58,282	1,245	2,136
HI 4	70,775	69,314	66,085	68,725	2,400	3,492
HI 5	75,108	76,740	76,010	75,953	0,818	1,076
HI 6	87,037	85,401	85,302	85,913	0,974	1,134
Average					**1,800**	**2,527**
Kynurenine/tryptophan ratio						
HI 1	0,030	0,030	0,029	0,030	0,001	1,946
HI 2	0,030	0,033	0,031	0,031	0,002	4,875
HI 3	0,036	0,037	0,039	0,037	0,002	4,092
HI 4	0,032	0,031	0,028	0,030	0,002	6,863
HI 5	0,024	0,024	0,024	0,024	0,000	0,000
HI 6	0,023	0,025	0,023	0,029	0,001	4,879
Average					**0,001**	**3,776**
Standard deviation (std), coefficient of variation (CV)						

For inter-run reproducibility in HIV-negative individuals, each sample was tested in duplicate on three different days. Duplicate results were averaged to give three readings per sample. For each sample, we calculated the SD and %CV of the 3 readings. Kynurenine gave a mean SD of 0.09 µmol/l with average %CV of 4.48%, whilst tryptophan gave a mean SD of 0.98 µmol/l with average %CV of 1.43%, table 4.0. K/T ratio gave a mean SD of 0.001 with an average CV of 4.47%, table 4.0.

Table 4.0: Inter-run reproducibility – Plasma samples from healthy individuals (HI)

Kynurenine						
Sample no.	**Day 1 umol/L**	**Day 2 umol/L**	**Day 3 umol/L**	**Mean umol/L**	**Std Dev**	**CV (%)**
HI 1	2,128	2,267	1,937	2,111	0,166	7,850
HI 2	2,057	2,160	2,079	2,099	0,054	2,585
HI 3	1,931	2,078	2,108	2,039	0,095	4,646
HI 4	1,927	1,891	2,031	1,950	0,073	3,729
HI 5	2,325	2,203	2,002	2,177	0,163	7,493
HI 6	2,569	2,600	2,579	2,583	0,016	0,613
Average					**0,094**	**4,486**
Tryptophan						
HI 1	67,604	66,883	68,976	67,821	1,063	1,568
HI 2	70,084	72,670	71,742	71,499	1,310	1,833
HI 3	54,282	54,754	56,360	55,132	1,089	1,976
HI 4	68,725	69,619	67,557	68,634	1,034	1,507
HI 5	75,108	76,010	76,740	75,953	0,818	1,076
HI 6	87,037	86,113	86,013	86,388	0,565	0,654
Average					**0,980**	**1,435**
Kynurenine/tryptophan						

HI 1	0,031	0,034	0,029	0,031	0,003	8,032
HI 2	0,029	0,030	0,029	0,029	0,001	1,968
HI 3	0,036	0,038	0,037	0,037	0,001	2.703
HI 4	0,028	0,027	0,030	0,028	0,002	5,391
HI 5	0,031	0,029	0,026	0,029	0,003	8,779
HI 6	0,030	0,030	0,030	0,030	0,000	0,000
Average					**0,001**	**4,479**
Standard deviation (std), coefficient of variation (CV)						

3.3.2 ELISA test reproducibility using HIV-infected samples

Using HIV-positive samples, we repeated the ELISA test verification. For intra-run reproducibility, each sample was assayed in triplicate in the same run. Again, we determined SD and %CV. Kynurenine gave a mean SD of 0.17 µmol/l with average %CV of 4.68%, table 5.0. Tryptophan showed a mean SD of 3.31 µmol/l with an average %CV of 7.97%. K/T ratio showed a mean SD of 0.005 with average intra-assay %CV of 7.792%, table 5.0.

Table 5.0: Intra-assay reproducibility – Plasma samples from HIV-infected patients

Kynurenine						
Sample no.	**Result 1 umol/L**	**Result 2 umol/L**	**Result 3 umol/L**	**Mean umol/L**	**Std Dev**	**CV (%)**
Sample 1	2,439	2,220	2,233	2,279	0,123	5,348
Sample 2	4,940	4,996	4,673	4,870	0,172	3,544
Sample 3	4,546	4,161	4,401	4,369	0,195	4,450
Sample 4	4,604	4,351	4,477	4,477	0,127	2,825
Sample 5	4,034	4,171	3,824	4,010	0,175	4,359
Sample 6	3,216	3,319	2,865	3,133	0,238	7,601
Average					**0,172**	**4,687**

Tryptophan						
Sample 1	31,027	30,437	30,758	30.074	0,295	0,961
Sample 2	41,333	35,990	33,547	36,957	3,982	10,775
Sample 3	51,629	43,046	42,830	45,835	5,019	10,950
Sample 4	57,514	60,446	66,590	61,517	4,632	7,530
Sample 5	58,282	51,619	54,298	54,733	3,352	6,125
Sample 6	43,642	39,067	39,221	40,643	2,598	6,392
Average					**3,313**	**7,976**
Kynurenine/tryptophan ratio						
Sample 1	0,079	0,072	0,073	0,074	0,004	5,070
Sample 2	0,120	0,139	0,121	0,127	0,011	8,442
Sample 3	0,088	0,097	0,103	0,096	0,008	7,864
Sample 4	0,080	0,072	0,067	0,073	0,007	8,983
Sample 5	0,069	0,081	0,070	0,073	0,007	9,080
Sample 6	0,074	0,081	0,073	0,076	0,004	5,735
Average					**0,005**	**7.792**
Standard deviation (std), coefficient of variation (CV)						

For inter-run reproducibility using HIV-positive samples, each sample was tested in duplicate on three different days. Duplicate results were averaged to give three readings per sample. For each sample, we determined the SD and %CV of the 3 readings. Kynurenine gave a mean SD of 0.21 µmol/l with average %CV of 5.91%, whilst tryptophan gave a mean SD of 1.69 µmol/l with average %CV of 3.847%, table 6.0. K/T ratio gave a mean SD of 0.005 with an average of %CV of 4.708%.

Table 6.0: Inter-day reproducibility –Plasma samples from HIV-infected samples

Kynurenine						
Sample no.	**Day 1 umol/L**	**Day 2 umol/L**	**Day 3 umol/L**	**Mean umol/L**	**Std Dev**	**CV (%)**
Sample 1	2,231	1,965	2,377	2,191	0,209	9,534
Sample 2	4,670	4,033	4,749	4,484	0,392	8,755
Sample 3	4,546	4,737	4,492	4,592	0,128	2,803
Sample 4	4,604	4,337	4,402	4,448	0,139	3,130
Sample 5	4,034	3,966	4,093	4,031	0,064	1,577
Sample 6	3,216	3,602	3,906	3,575	0,346	9,674
Average					**0,213**	**5,910**
Tryptophan						
Sample 1	32,741	32,512	31,978	32,410	0,392	1,208
Sample 2	36,957	33,203	31,394	33,851	2,838	8,383
Sample 3	45,835	49,192	49,602	48,210	2,066	4,286
Sample 4	61,517	63,485	66,771	63,924	2,655	4,153
Sample 5	54,733	55,752	56,195	55,560	0,750	1,350
Sample 6	40,643	39,627	37,771	39,347	1,456	3,701
Average					**1,693**	**3,847**
Kynurenine/tryptophan ratio						
Sample 1	0,068	0,060	0,074	0,068	0,007	10,431
Sample 2	0,126	0,121	0,130	0,126	0,005	5,588
Sample 3	0,099	0,096	0,091	0,095	0,004	4,239
Sample 4	0,075	0,068	0,066	0,070	0,005	6,783
Sample 5	0,074	0,071	0,073	0,073	0,002	2,102
Sample 6	0,089	0,091	0,093	0,091	0,002	2,197
Average					**0,005**	**4.708**
Standard deviation (std), coefficient of variation (CV)						

3.4 Comparison of ELISA to measure Kynurenine and tryptophan to mass spectrometry

For comparison of the ELISA to mass spectrometry, we used 70 plasma samples on whom we had previously published mass spectrometry results (11). The plasma samples were drawn from the Lung Cohort Study established by the Perinatal HIV Research Unit in Soweto, Johannesburg. The LCS enrolled HIV-infected adults from 2008 to 2012 (11).

The demographics of the samples used for the ELISA method comparison are shown in Table 7.0. All the samples included were from HIV-infected patients. Due to the availability of samples, we used samples from study enrolment time-point, at which time no participants had TB disease. Participants had not been tested for TB latency but had been recruited from an area of high TB endemicity.

Table 7.0: Demographic characteristics of lung cohort study subjects used for method comparison

Total number of subjects	n = 70
Women (%)	52 (74%)
Age (years)	36 ± 6
HIV status	All positive
TB disease status	All negative

3.4.1 Agreement of ELISA to mass spectrometry (MS)

To assess agreement between ELISA and MS (gold standard method), we performed a Bland-Altman's test and correlation plots for plasma K/T ratio to determine the limits of agreement using data from our previously published MS work (11). For kynurenine, the mean bias

between ELISA and MS was -0.01 µmol/l with standard deviation (SD) of 1.4 µmol/l and limit of agreement from -2.8 µmol/l to 2.8 µmol/l, figure 3A. For tryptophan, the mean bias between ELISA and MS was 7.0 µmol/l with SD of 14.5 µmol/l and limits of agreement from -21.4 µmol/l to 35.5 µmol/l figure 2.0. For plasma K/T ratio there was a mean bias of 0.01 with standard deviation (SD) of 0.04 and limits of agreement from -0.6 to 0.10 between the two methods figure 3.0.

We further explored the correlation of the difference between the two methods and the average values (figure 3C). For kynurenine, the difference between ELISA and mass spectrometry showed outliers at high kynurenine values, although there was no significant correlation overall between the difference of the methods and the average value (r=-0.0424, P=0.7083). Tryptophan showed a significant positive correlation between the difference between the methods and the average tryptophan value (r=0.3203, P=0.0038). For plasma K/T ratio, there was a weak positive correlation (r=0.2983, P=0.0093) between the difference of the methods and average K/T value, with outliers at high K/T value. Despite the low r value (r=0.29), the P value showed significant correlation. The low r value may be due the relatively small number of data outlier points. Additionally, a possible reason for the fair agreement of kynurenine assay between the ELISA and the mass spectrometry may be due to interference by kynurenine derivatives in the competitive ELISA method, which did not interfere with mass spectrometric measurement.

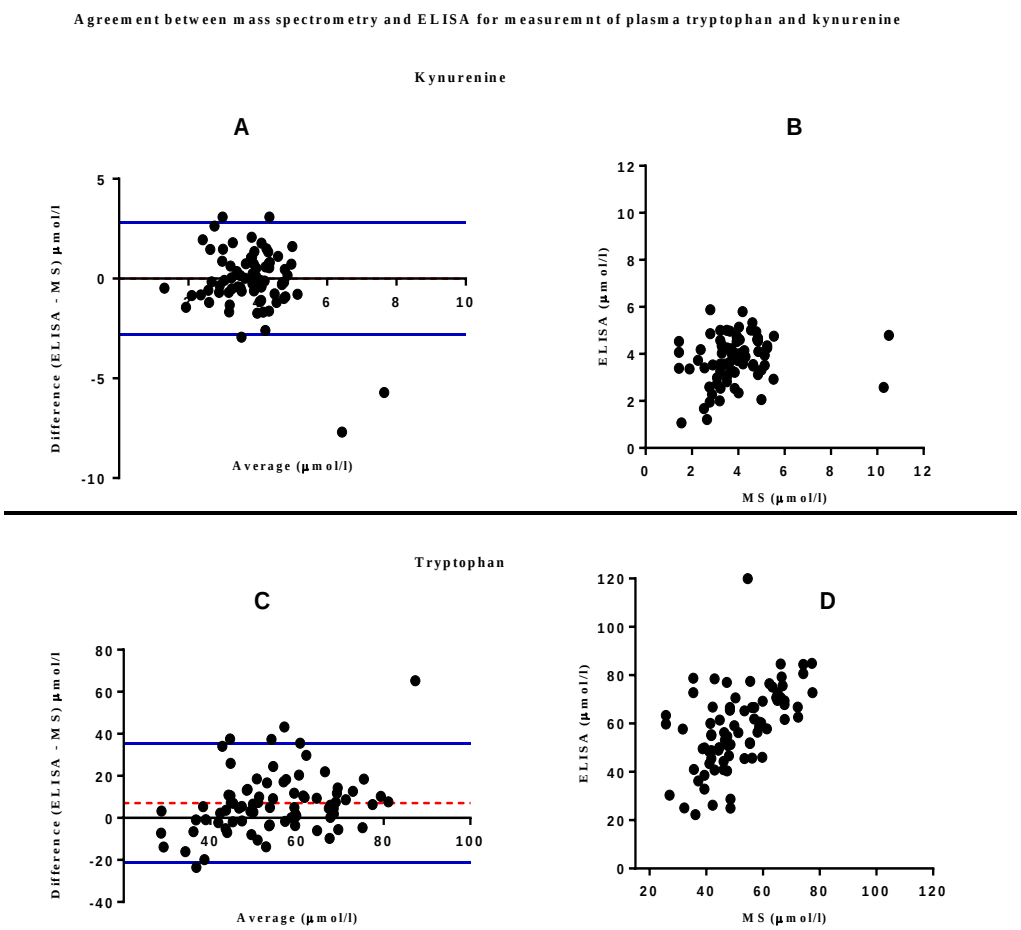

Figure 2.0: Comparison of mass spectrometry and ELISA measurement.

Agreement between mass spectrometry (MS) and ELISA for measurement of kynurenine (A, B) and tryptophan (C, D) using the lung cohort study.

For kynurenine, we plotted the difference between ELISA and MS versus the average between the two tests in a Bland-Altman plot (A). Plasma kynurenine measured by MS and ELISA showed fair agreement with a mean bias of -0.01µmol/l, standard deviation (SD) of 1.4µmol/l and limits of agreement from -2.8 to 2.8µmol/l. Spearman's correlation (B) showed a significant association between plasma kynurenine measured by ELISA and MS ($r = 0.25$, $P = 0.0226$). For kynurenine, there was only moderate concordance with some outliers.

For tryptophan, we plotted the difference between ELISA and MS versus the average between the two tests in a Bland-Altman plot (C). There was a good agreement between plasma tryptophan measured by ELISA and MS. The mean bias was 7.0µmol/l with SD of 14.5µmol/l, and limits of agreement from -21.4 to 35.5µmol/l. A Spearman's correlation showed a significant association between the two methods ($r = 0.57$, $P < 0.0001$).

The red dotted line represents the mean bias whilst the blue lines indicate the upper and lower limits of agreement.

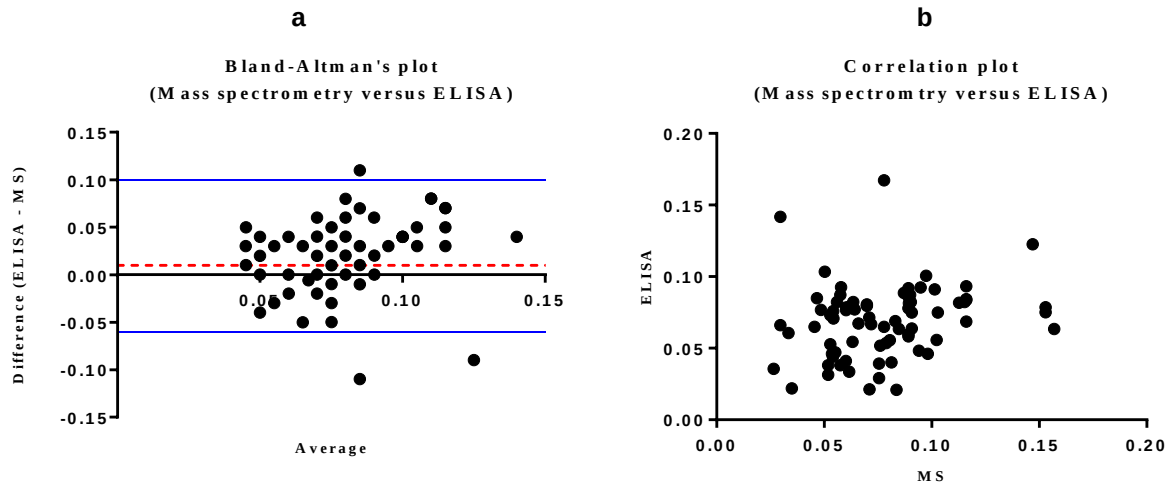

Figure 3.0. Comparison of plasma K/T ratio measured by mass spectrometry and ELISA

Comparison of plasma K/T ratio measured by mass spectrometry (MS) and ELISA using samples from the Lung cohort study. Bland-Altman's plot (a): we plotted the difference between ELISA and MS versus the average between the two tests, n = 70. There was a good agreement between the methods with a mean bias of 0.01 (SD ± 0.04), 95% limit of agreement from -0.06 to 0.10. The red dotted line represents the mean bias whilst the blue lines indicate the upper and lower limits of agreement. In a Spearman's correlation (b), there was a significant association between K/T ratio measured by ELISA and MS (r = 0.29, P = 0.0122, n = 70). All units are in (µmol/l)/(µmol/l)

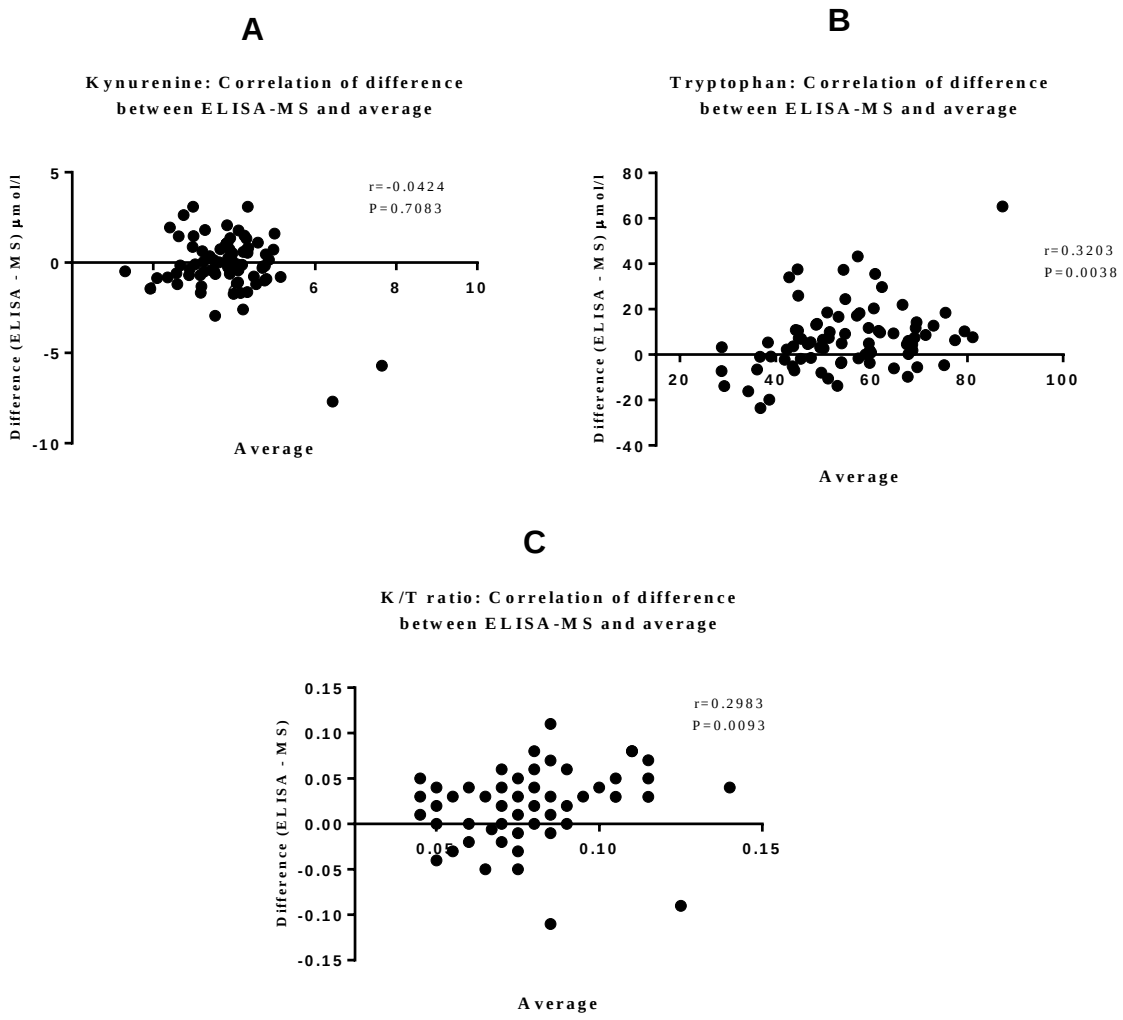

Figure 4.0: Correlation of difference between mass spectrometry and ELISA versus average.

Spearman's correlation of the difference between the two methods plotted against the average values for kynurenine (A), tryptophan (B) and K/T ratio (C). For kynurenine, the difference between ELISA and mass spectrometry showed outliers at high kynurenine values, although there was no significant correlation overall between the difference of the methods and the average value (r=-0.0424, P=0.7083). Tryptophan showed a significant positive correlation between the difference between the methods and the average tryptophan value. For plasma K/T ratio, there was a weak positive correlation (r=0.2983, P=0.0093) between the difference of the methods and average K/T value, with outliers at high K/T value.

3.5 Conclusion

From our verification data showed above, there was strong reproducibility and good agreement between tryptophan measured by the ELISA and mass spectrometry. For kynurenine, there was moderate concordance with some outliers. A possible reason may be due to interference by kynurenine derivatives in the ELISA method that was not present with the mass spectrometric method. ELISA is low-cost, relatively high-throughput methods with the potential of automation, which could be used in peripheral laboratories. ELISA also requires small samples volume and can be completed within a relatively short period. We therefore evaluated the ELISA assay further as a potential diagnostic test for TB using two clinical cohorts.

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Chapter 4

Evaluation of ELISA as a diagnostic test for TB in HIV-infected and uninfected patients

In this section, we used the ELISA method to determine the diagnostic significance of plasma kynurenine/tryptophan (K/T) ratio in patients with active TB disease compared with those with latent TB. We accessed residual plasma samples from South African Tuberculosis Vaccines Initiative (SATVI), University of Cape Town, South Africa. We included samples from HIV-uninfected and HIV-infected individuals. We sought to evaluate whether the same cut-off value could apply to HIV-uninfected and HIV-infected patients.

4.1 Study subjects and cohort description

We received residual plasma samples from the MRC-SHIP study established by SATVI. The MRC-SHIP study measured blood-based TB risk signatures in HIV-infected and uninfected adults (2). Two groups of participants were enrolled from the SATVI clinical field site in Worcester, Boland, Overberg, Western Cape, South Africa. The first group were HIV-positive participants with active TB or latent TB infection (LTBI). The second group were HIV-negative participants with active TB or LTBI.

TB cases had been identified by approaching individuals with respiratory symptoms who attended clinics in the Worcester area for assessment. Inclusion criteria for active TB included age ≥ 18 years with a positive Xpert MTB/RIF (Cepheid, USA) on sputum. All active TB cases had pulmonary TB. Participants with severe anaemia, low body mass index (<17) or chronic disease other than TB had been excluded. Controls were invited to participate through a visit to homes, community centres and word of mouth. For controls, to be eligible for inclusion,

subjects had to be 18 years or older with a positive QuantiFERON-TB Gold In-Tube (QFT) test (Qiagen, USA). All individuals with a negative QFT test (IFNγ <0.35 IU/ml) were excluded.

Due to sample availability, we took all samples collected from HIV-infected and uninfected patients with active TB and selected same number of controls chronologically. Ninety (90) samples were included in our nested study. Fifty (50) were HIV-negative; 25 with active TB disease and 25 with LTBI, whilst 40 were HIV-positive; 20 with active TB disease whilst the other 20 had LTBI. We compared patients with active TB disease to those with LTBI in both the HIV-negative and HIV- positive group.

Analysing HIV positive and negative patients together, K/T ratio was markedly elevated in patients with active TB disease compared to LTBI, figure 5.0.

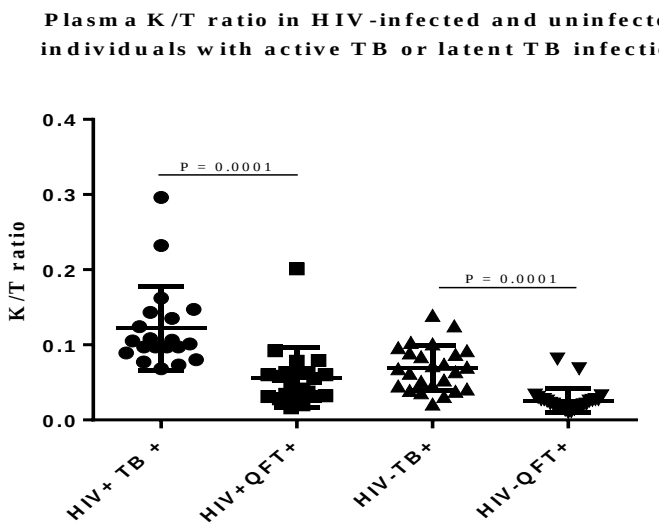

Figure 5.0: Plasma K/T ratio in HIV-infected and uninfected individuals with active TB and latent TB infection.

Plasma kynurenine/tryptophan (K/T) ratio measured using ELISA, in HIV-infected active TB patients (n=20), HIV-infected individuals with latent TB (n=20), HIV-uninfected active TB patients (n=25) and HIV-uninfected individuals with latent TB (n=25) from the MRC-SHIP cohort. Patients with active TB had significantly higher K/T ratio than individuals with latent TB (Mann Whitney test, P values shown, Kruskal-Wallis test overall P-value < 0.0001). The upper and lower horizontal lines indicate 25th and 75th percentiles, respectively, with the median, is indicated.

4.2 Plasma K/T ratio in all patients and controls

Analysing HIV-infected and uninfected groups together, patients with active TB disease had significantly higher plasma K/T ratio than patients with latent TB infection (median, 0.090 [IQR 0.063-0.105] versus 0.030 [IQR 0.020-0.056], P < 0.0001, figure 6.0. In a ROC curve analysis, plasma K/T ratio had AUC of 0.89 (P < 0.0001, figure 6B). Table 4B (*appendix 2*) shows the diagnostic significance of plasma K/T ratio in patients with active TB disease at

various cut-offs. At a cut-off 0.040, K/T ratio gave a diagnostic sensitivity 90% [CI 76-96%], specificity 70% [CI 53-81%], PPV 74% and NPV of 90%.

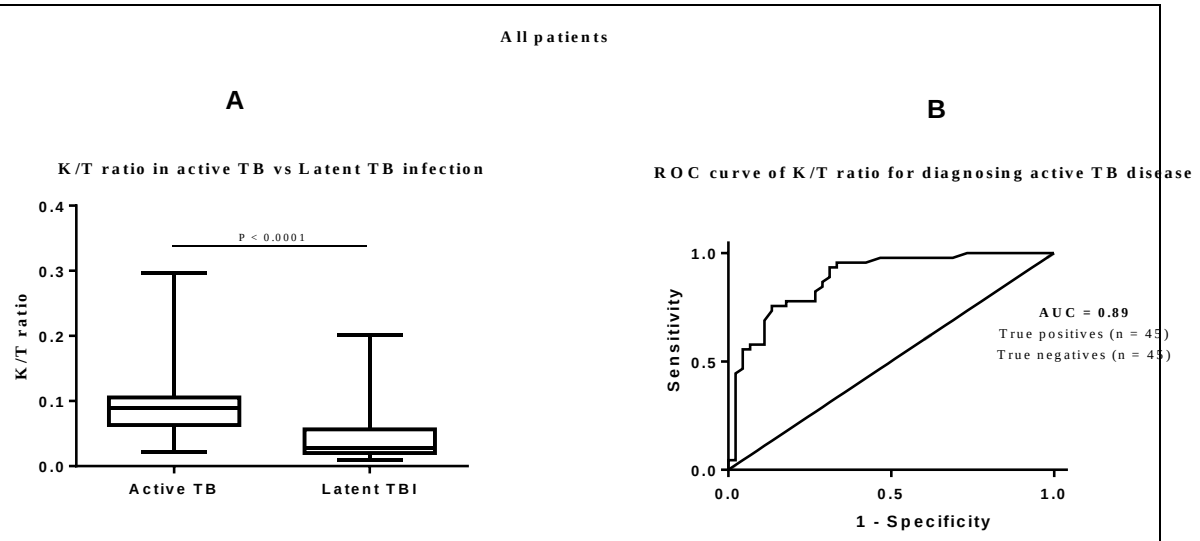

Figure 6.0: In the MRC-SHIP cohort

A; Plasma K/T ratio in active TB patients compared with patients with latent TB infection (controls). Both cases and control groups included patients with and without HIV-infection. Patients with active TB had significantly elevated K/T ratio compared with patients with latent TB (P < 0.0001). Using a cut-off of 0.040, plasma K/T ratio had a diagnostic sensitivity of 90% (CI 76-96%), specificity 70% (CI 53-81%), PPV 74% and NPV of 90%. B; A ROC had an area under the curve of 0.89 (CI 0.81-0.95).

4.3 Plasma K/T ratio in HIV negative patients with active TB

In HIV-uninfected patients alone, patients with active TB disease had significantly higher K/T ratio than those with latent TB infection (median, 0.064 [IQR 0.040-0.088] versus 0.022 [IQR 0.016-0.027], P < 0.0001, figure 7.0). A ROC analysis gave AUC of 0.93 (95% CI 85-100, P < 0.0001, figure 4C). At a cut-off of 0.040, plasma K/T ratio showed diagnostic sensitivity of 85% (95% CI 59-93%), specificity of 92% (95% CI 73-99%), PPV 91% and NPV 84% (table 4C, *appendix 2*).

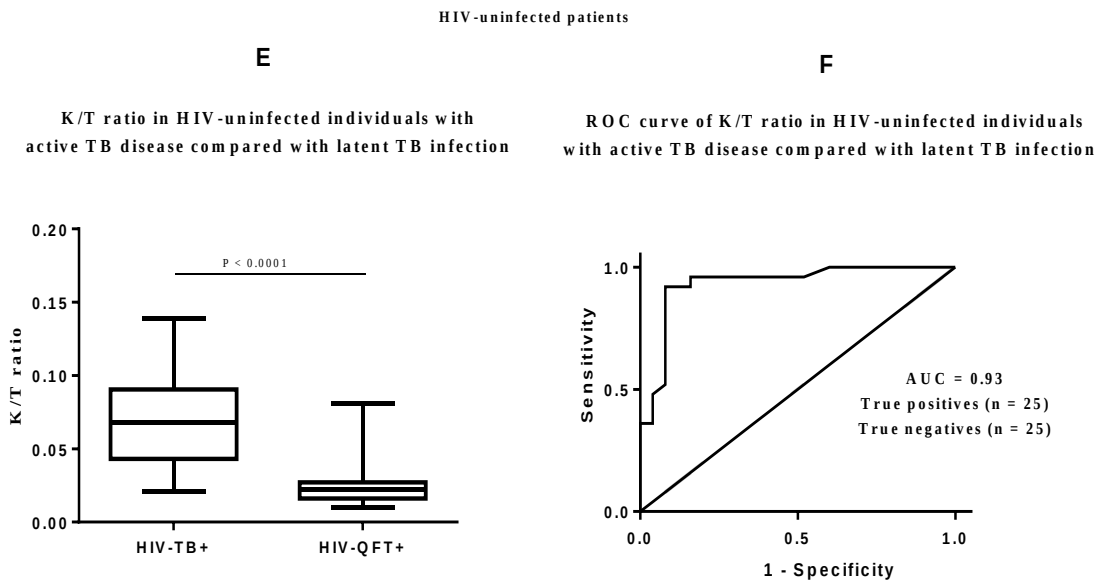

Figure 7.0: In the MRC-SHIP cohort – Plasma K/T ratio in HIV-uninfected patients alone

E; Plasma K/T ratio in HIV-uninfected individuals with active TB disease compared with HIV-uninfected individuals with latent TB infection (controls). HIV-uninfected patients with active TB disease had significantly higher K/T ratio compared with HIV-uninfected patients with latent TB infection ((P < 0.0001). At a cut-off of 0.040, plasma K/T ratio had a diagnostic sensitivity of 85% (CI 59-93%), specificity 92% (CI 73-99%), PPV 91% and 84%. F; Area under the ROC curve was 0.93 (CI 0.85-1.0).

4.4 Plasma K/T ratio in HIV-infected patients with active TB

To investigate whether the same cut-off would be appropriate for HIV-positive samples, we evaluated K/T ratio in HIV-infected group. HIV-infected patients with active TB disease had significantly higher K/T ratio than HIV-infected patients with latent TB (median, 0.101 [IQR 0.091-0.140] versus 0.061 [IQR 0.034-0.077], P < 0.0001, figure 8.0). A ROC curve showed AUC of 0.93, P < 0.0001 (figure 4D). At a cut-off of 0.080 K/T ratio gave a diagnostic sensitivity 90% [95%CI 62-96%], specificity 80% [95%CI 68-98%], PPV 82% and NPV 90% (table 4D, *appendix 2*).

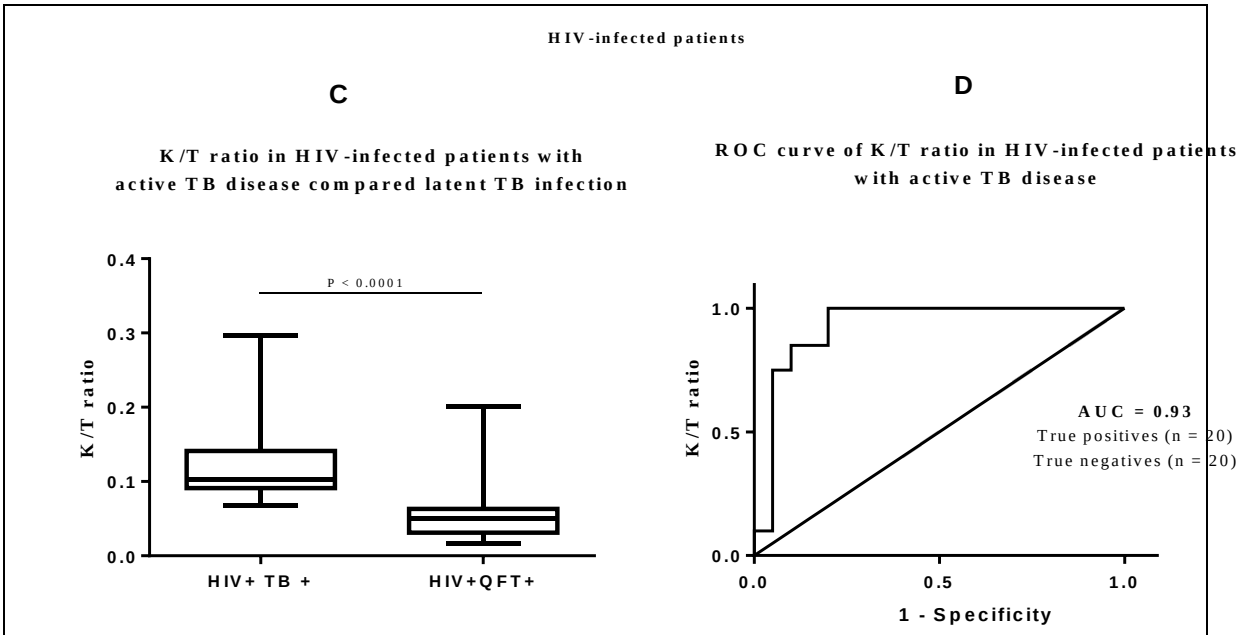

Figure 8.0: In the MRC-SHIP cohort - Plasma K/T ratio in HIV-infected patients alone

Plasma K/T ratio in HIV-infected patients with active TB disease compared with HIV-infected patients with latent TB patients (controls). HIV-infected patients with active TB disease had significantly higher K/T ratio (P < 0.0001) than HIV-infected patients with latent TB infection. At a cut-off of 0.080, plasma IDO activity had a diagnostic sensitivity of 90% (CI 62-96%), specificity 80% (CI 68-98%), PPV 82% and 90%. D; A ROC curve had an area under the curve of 0.93 (CI 0.83-1.0).

4.5 Association of plasma K/T ratio to gender and body mass index (BMI)

Analysing K/T ratio by gender in the MRC-SHIP samples, we found no significant difference between males and females. BMI negatively correlated with K/T ratio (rho = -0.249, P = 0.0180), however, in subgroups (HIV-infected with or without TB; HIV negative with or without TB), there were no significant correlations between BMI and K/T ratio, although a trend was seen in the patients with active TB (figure 9.0).

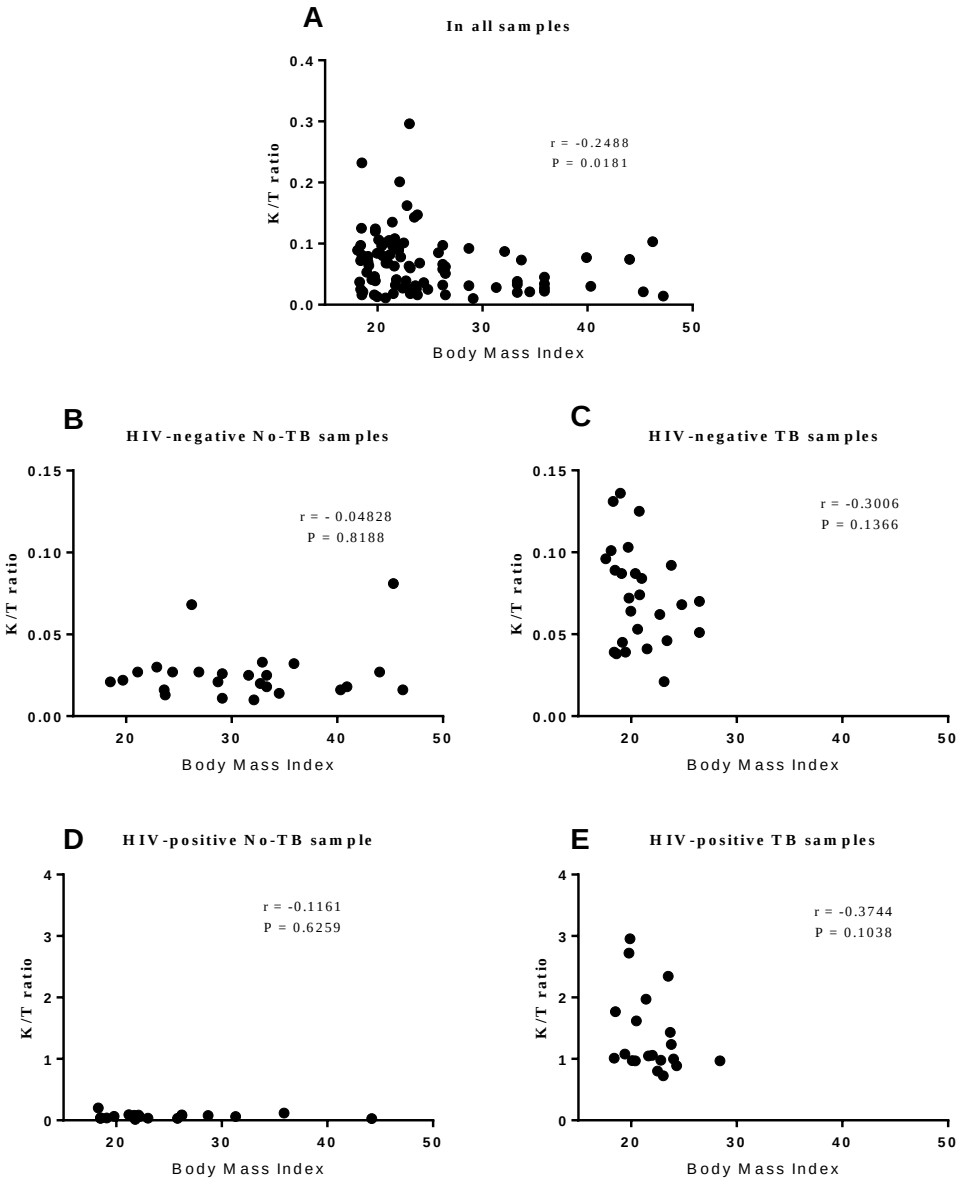

Figure 9.0: Spearman's correlation of body mass index (BMI) with plasma K/T ratio using samples from the MRC-SHIP cohort.

A: All samples combined, B: HIV-negative samples with no TB, C: HIV-negative samples with TB, D: HIV-positive samples with no TB and D: HIV-positive with TB. In all samples together, plasma K/T ratio was inversely correlated with body mass index (r = -0.2488, P = 0.0181). In sub-groups (B, C, D and E) plasma K/T ratio shows no significant correlation with BMI although a trend to the negative association was apparent in the TB samples.

4.6 Discussion and conclusion

We evaluated plasma kynurenine/tryptophan ratio as a blood-based diagnostic biomarker for active TB disease in HIV-infected and uninfected individuals.

Using the ELISA method, we analysed patients with active TB disease compared with individuals with latent TB infection, defined as QuantiFERON-positive individuals. In the HIV-uninfected group, patients with active TB disease had significantly elevated K/T ratio compared to individuals with latent TB infection. Additionally, HIV-infected patients with active TB disease also had significantly higher K/T ratio compared to HIV-infected individuals with latent TB, confirming previous findings (3). A cut-off ratio of 0.040 for HIV-uninfected patients gave a sensitivity of 85%, specificity 92%, PPV 91% and NPV 84% for diagnosing active TB disease.

For HIV-infected patients, raising the cut-off value to 0.080, plasma K/T ratio gave a diagnostic sensitivity of 90% and specificity of 80%, with high PPV of 82% and NPV of 90% in diagnosing active TB. If HIV status was unknown, a cut-off 0.040 would detect 90% of all active TB patients with a specificity of 70%, PPV of 74% and NPV of 90%. Thus, if used as an initial diagnostic test, at a cut-off of 0.040, K/T ratio would detect 100% of HIV-infected and 85% of HIV-uninfected individuals who could have appropriate confirmatory TB testing.

These findings agree with previous reports showing that plasma K/T ratio is elevated in active TB patients at the time of TB diagnosis (3-6). Furthermore, HIV-infected patients have higher

plasma K/T ratio than HIV-uninfected individuals. The size of the HIV viral reservoir impacts plasma K/T ratio (7, 8). Broad evidence indicates the K/T ratio is mildly increased in HIV-infection, and highly increased in active TB (8, 9). Separate cut-offs for HIV uninfected and infected individuals appear appropriate.

K/T ratio is thus a promising diagnostic TB biomarker according to the WHO TPP guidelines (10). K/T ratio meets the minimum criteria of the TPP for a non-sputum-based screening (triage) and diagnostic test for active pulmonary TB (10). The only disadvantage for the ELISA assay is the time to results as it requires overnight (16 hours) incubation period for sample processing.

For evaluation of the assay as a TB diagnostic, inclusion of participants with LTBI as our control group reflects the realities of using a blood-based *Mtb* assay in diagnosis in a TB endemic area and is a strength of our study.

A diagnostic test showing a sensitivity of 90% with NPV of 90% in HIV-infected patients, holds great potential to improve patient care. A blood-based tool has the potential to detect patients with difficulty producing sputum, potentially including children and extra-pulmonary TB cases, although this aspect requires further investigation. Additionally, a blood-based tool would improve infection control practices, alleviating the need for sputum collection. Other conditions, including pregnancy or malignancy, should be investigated as potential confounders of plasma K/T ratio, as they are conditions in which IDO activity is reportedly upregulated (20).

Interestingly, we noted an inverse correlation between plasma K/T ratio and BMI, particularly apparent in TB patients (supplementary figure), however; this correlation was not significant in the subgroup or multivariable regression analysis. Loss of weight is a cardinal TB symptom; thus, any TB biomarker might be expected to show an inverse correlation with BMI. Multiple regression analysis showed that older age and positive HIV status were associated with higher K/T ratio, but the strongest association was the presence of active TB.

A limitation of our study is that we did not include individuals with other lung diseases, although we have previously reported good discriminatory power of K/T ratio even when pneumonia patients were included (3). Similarly, other studies have shown kynurenine as one of the most increased metabolites during active TB when compared with other lung diseases (6, 21-23). Also, our reported positive predictive values may be overestimated due to the case-control study design in which the prevalence of the disease is approximately half. Furthermore, related enzymes, tryptophan 2,3-dioxygenase (TDO), IDO-2 and other enzymes encoded by gut-resident bacteria also convert tryptophan to kynurenine. Therefore, elevated K/T ratio reflects not only IDO-1 activity but a composite picture of host, microbial and mycobacterial tryptophan and kynurenine metabolism. Measurement of kynurenine to tryptophan ratio remains the preferred clinical approach to quantitatively detecting shifts in IDO activity. CD4 count and HIV viral load data were not available as they were not recorded in the MRC-SHIP study. Nevertheless, plasma K/T ratio shows a strong potential advantage over conventional diagnostic methods for high-burden TB settings. Furthermore, it is of relevance that plasma K/T ratio can be measured by high thorough-put, low-cost, ELISA methodology.

In summary, we showed that plasma K/T ratio is a promising plasma-based tool for identifying active TB disease in HIV-infected or uninfected adults. K/T ratio results above a cut-off of

0.040, when HIV status is unknown, could be used to triage patients requiring confirmatory TB testing. We propose that plasma IDO activity (K/T ratio) should be evaluated in large, well-designed prospective studies as a blood-based screening test for active TB disease. This work has been published and the manuscript is attached (hereafter).

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Diagnostic accuracy of plasma kynurenine/tryptophan ratio, measured by enzyme-linked immunosorbent assay, for pulmonary tuberculosis

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ABSTRACT

Introduction: The World Health Organization has identified the need for a non-sputum-based test capable of detecting active tuberculosis (TB) as a priority. The plasma kynurenine-to-tryptophan (K/T) ratio, largely mediated by activity of the enzyme indoleamine 2,3-dioxygenase, may have potential as a suitable biomarker for active TB.

Method: We evaluated a commercial enzyme-linked immunosorbent assay (ELISA) in comparison to mass spectrometry for measuring the K/T ratio. We also used ELISA to determine the K/T ratio in plasma from patients with active TB compared to latently infected controls, with and without HIV. Results: The two methods showed good agreement, with a mean bias of 0.01 (limit of agreement from 0.06 to 0.10). Using ELISA, it was found that HIV-infected patients with active TB disease had higher K/T ratios than those without TB (median, 0.101 [interquartile range (IQR), 0.091–0.140] versus 0.061 [IQR, 0.034–0.077], $P < 0.0001$). At a cutoff of 0.080, the K/T ratio produced a sensitivity of 90%, a specificity of 80%, a positive predictive value (PPV) of 82%, and a negative predictive value (NPV) of 90%. In a receiver operating characteristics analysis, the K/T ratio had an area under the curve of 0.93.

HIV-uninfected patients with active TB also had higher K/T ratios than those with latent TB infections (median, 0.064 [IQR, 0.040–0.088] versus 0.022 [IQR, 0.016–0.027], $P < 0.0001$). A cutoff of 0.040 gave a sensitivity of 85%, a specificity of 92%, a PPV of 91%, and an NPV of 84%.

Conclusion: The plasma K/T ratio is a sensitive biomarker for active TB. The K/T ratio can be measured from blood using ELISA. The K/T ratio should be evaluated as an initial test for TB.

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Introduction

Tuberculosis (TB) remains a leading infectious cause of death worldwide. The early diagnosis of TB is essential in guiding TB treatment, but 40% of new TB cases remain undiagnosed, which fuels transmission (World Health Organization, 2019). TB diagnosis is difficult in immunocompromised patients, children, and patients with extra-pulmonary TB, as most current diagnostic tools rely on sputum as the sample of choice. The World Health Organization (WHO) has identified as a critical priority the urgent need for a non-sputum-based TB test capable of detecting all forms of TB disease, both pulmonary and extra-pulmonary TB, as a tool to reach TB control goals (Stop, 2015). Novel blood-based diagnostic tools that differentiate active disease from latent TB infection (LTBI) predict the onset of active TB disease, or allow monitoring of TB therapy would clearly aid TB control efforts.

Current TB diagnostic methods have multiple limitations. Mycobacterial culture tests are capital-intensive and have prolonged turnaround times. Molecular tests such as Xpert MTB/RIF (Cepheid, USA) have been a game-changer due to their rapidity and detection of resistance to primary anti-TB drugs (World Health Organization, 2017);

however, they are sputum-based tests, require specialized equipment, and are unsuitable for monitoring treatment (Nicol, 2013). Blood-based Mycobacterium tuberculosis (Mtb) antibody tests are unreliable either as a screening or a diagnostic tool, and several have been rejected by WHO (World Health Organization, 2011). Interferon-gamma release assays fail to distinguish active disease from previous TB exposure or LTBI, and therefore they have limited utility in TB-endemic areas (World Health Organization, 2010). Several host biosignatures have been explored in TB diagnosis (Scriba et al., 2017; Suliman et al., 2018). Although some are promising, the lack of clear guidelines on their utility hinders their use in diagnosis. Despite the renewal of efforts toward the development of new TB diagnostics, relatively little attention has been paid to the activity of the host's immunemodulatory enzyme indoleamine 2,3-dioxygenase (IDO) as a marker of active disease.

IDO, the interferon-g inducible enzyme, breaks tryptophan down to kynurenine. IDO is expressed in antigen-presenting cells such as macrophages and dendritic cells (Dantzer et al., 2008). IDO-mediated tryptophan depletion and the accumulation of kynurenines lead to T cell anergy and apoptosis (Almeida et al., 2009; Murray, 2003a; Nagamatsu and Schust, 2010; Yeung et al., 2015). The immunoregulatory effect of IDO is not limited to T cells but extends to other immune cells, leading to dampened immune response (Mezrich et al., 2010a, b; Prendergast et al., 2014).

Mtb has been shown to induce IDO activity in macrophages (Blumenthal et al., 2012; Gautam et al., 2018). IDO activity is in turn associated with shifts in macrophage metabolism (Eleftheriadis, 2018; Suchard et al., 2020). Other cells in the lungs, such as the epithelia and endothelial cells, also express IDO in response to Mtb infection (Desvignes and Ernst, 2009). Increased IDO activation results in an elevated kynurenine to tryptophan (K/T) ratio, which has been postulated to play a key role in the immune suppression noted in HIV infection (Scheri et al., 2017; Boasso et al., 2008). Recently, we reported increased K/T ratios in HIVinfected adults with TB (Adu-Gyamfi et al., 2017). We found that the plasma K/T ratio was elevated months ahead of clinical TB symptoms and that it declined after treatment (Adu-Gyamfi et al., 2017). Increased K/T ratios and IDO activity expression in active TB have been reported by other groups using serum and sputum (Li et al., 2011; Suzuki et al., 2013; Suzuki et al., 2012; Collins et al., 2020). Collins et al. recently found that tryptophan catabolism along the kynurenine pathway was highly activated during TB, and it was normalized after effective TB treatment (Collins et al., 2020). Additionally, elevated kynurenine has been seen in the metabolic profiles of TB patients (Desvignes and Ernst, 2009; Feng et al., 2015; Weiner et al., 2012; Zhou et al., 2015). The K/T ratio thus has potential as a TB biomarker to predict, diagnose, and monitor antiTB therapy.

The aim of this retrospective, nested, case-control study was to evaluate enzyme-linked immunosorbent assay (ELISA), a widely available method, in comparison to mass spectrometry (MS), for the detection of the K/T ratio from peripheral blood. We further evaluated the diagnostic significance of an increased K/T ratio using ELISA in HIV-infected and uninfected patients with an active compared with LTBI. We have reported this study according to the Standards for Reporting Diagnostic Accuracy Studies (STARD) guidelines (Bossuyt et al., 2015). Methodology

Comparison of ELISA to MS

To compare ELISA to MS, we used 70 plasma samples, conducted by the Lung Cohort Study conducted by the Perinatal HIV Research Unit in Soweto, Johannesburg. The LCS enrolled HIVinfected adults from 2008 to 2012. Details of the study demographics are shown in Supplementary Table 1, which has been previously published (Adu-Gyamfi et al., 2017). Only samples at the time point of study enrolment were available for testing, at which time no participants had TB. We performed ELISA testing and compared its results with those of MS.

Diagnostic utility of the ELISA method

For the evaluation of the diagnostic utility of the K/T ratio, residual plasma samples were obtained from the MRC-SHIP study conducted by the South Africa Tuberculosis Vaccine Initiative (SATVI), University of Cape Town, South Africa. The MRC-SHIP study measured blood-based TB risk signatures in HIV-infected and uninfected adults (Darboe et al., 2018). Two groups of participants were enrolled from the SATVI clinical field site in Worcester, Boland, Overberg,

Western Cape, South Africa. The first group were HIV-positive participants with active TB or LTBI. The second group were HIV-negative participants with active TB or LTBI. TB cases were identified by approaching for assessment individuals who had respiratory symptoms who were attending clinics in the Worcester area. Inclusion criteria for active TB included age 18 years with a positive Xpert MTB/RIF (Cepheid, USA) on sputum. All active TB cases had pulmonary TB. Participants with severe anemia, low body mass index (<17), or chronic disease other than TB were excluded. Controls were contacted for participation through home visits, community center visits, and through word of mouth. To be eligible for inclusion, a control subject had to be 18 years old or older with a positive QuantiFERON-TB Gold in-Tube (QFT) test (Qiagen, USA). All individuals with a negative QFT test (IFNg < 0.35 IU/mL) were excluded.

Due to availability, only 90 samples were included in our nested study, of which 50 were HIV-negative, 25 with active TB disease and 25 with LTBI, and 40 were HIV-positive, 20 with active TB and 20 with LTBI. We compared patients with active TB to those with LTBI in both the HIV-negative and HIV-positive groups.

Determination of K/T ratio with enzyme-linked immunosorbent assay (ELISA)

EDTA-anticoagulated plasma (20 mL) that had been stored at 70 C was used to measure kynurenine and tryptophan concentrations with competitive ELISA using commercial kits (ImmuSmol, Inc., France) at the National Institute for Communicable Diseases, Johannesburg, in 2018 to 2019. The kits contained kynurenine or tryptophan antigen bound to the solid phase of a microplate. The plasma samples were first precipitated in an extraction process. Kynurenine was acylated, and tryptophan was derivatized. Kynurenine and tryptophan analytes from the sample then competed with solid-phase-bound antigens at a fixed number of antibody binding sites for a 16-h incubation period. At equilibrium, unbound antigen and antibody complexes were removed by washing. Antibody bound to the solid phase was detected by anti-rabbit IgG-peroxidase conjugate using 3,30,5,50tetramethylbenzidine as a substrate, and the reaction was read at 450 nm. Kynurenine and tryptophan were quantified from samples by comparing their absorbance with a curve generated from standards supplied by the manufacturer. The measurement range for kynurenine was from 0.5 mmol/L to 50 mmol/L. The measurement range for tryptophan was from 15 mmol/L to 150 mmol/L. All samples yielded measurable results and are included in the data presented. The K/T ratio was calculated as an indication of IDO activity, as kynurenine (mmol/L)/tryptophan (mmol/L). The units are not mentioned again in the manuscript.

Determination of K/T ratio by MS

Plasma kynurenine and tryptophan were measured with an Acquity TQ-S UPLC–tandem mass spectrometer (Waters, USA), using reference compounds (L-kynurenine and L-tryptophan) and deuterated tryptophan-d5 and kynurenine-d4 as internal standards (Sigma-Aldrich, South Africa). The plasma samples stored at 70 C were thawed at room temperature, followed by spiking with internal standards, deproteination with absolute methanol, and vortexing for 10 min. The samples were then centrifuged at 3156 rcf (relative centrifugal force) for 10 min, and 10 mL supernatant was injected in the UPLC-MS/MS system. The analytes were separated using an isocratic elution of injected samples within 2 min. Kynurenine and tryptophan were detected in the + multiple reaction mode using electrospray ionization MS (ESI) in the positive mode. This method has been previously published (Adu-Gyamfi et al., 2017).

Data analysis

Bland-Altman's test and Spearman correlation curves were used to determine agreement between MS and ELISA. Parametric data were obtained as means with standard deviations (SDs). Nonparametric data were expressed as median values with interquartile ranges (IQRs). Categorical data were analyzed using Fisher's exact test. The Mann-Whitney test was used for unpaired non-parametric groups, and the Wilcoxon test was used for paired groups. The Kruskal-Wallis test was used for multiple nonparametric groups. Additionally, factors associated with the K/T ratio were fitted using

multiple linear regression. The covariates included body mass index (BMI), HIV status, and presence of active TB disease.

Diagnostic sensitivity, specificity, and positive and negative predictive values (PPVs and NPVs) were calculated. We used receiver operating characteristic (ROC) curves to evaluate the most suitable cutoffs and determined the areas under the curves (AUCs). All analyses were performed using GraphPad Prism 7 (San Diego, CA, USA) and SAS Enterprise Guide 7.15 (SAS Institute Inc., Cary, NC, USA).

Ethics

The MRC-SHIP study protocols were approved by the Human Research Ethics committee of the Faculty of Health Sciences at the University of Cape Town (HREC 126/2006). The Lung Cohort Study was approved by the Johns Hopkins Medicine Institutional Review Board and the University of the Witwatersrand's Human Research Ethics Committee. This sub-study using stored samples from both cohorts was approved by the University of the Witwatersrand's Human Research Ethics Committee (M10336).

Table 1

Demographic characteristics of patients from the MRC-SHIP cohort.

	Active TB cases (n = 45)	Latent TB infection (n = 45)	P-value
Age (years)[a]	36 11	38 9	0.1997
Women(n, %)	17 (38%)	30 (66%)	0.0016
BMI[b]	20 (19-23)	26 (22-33)	<0.0001

Bold mean significant value. a Means and standard deviations. b Medians and interquartile ranges.

Results

Demographic characteristics

Table 1 shows the demographic data for our samples from the MRC-SHIP study. There was no difference in mean age between individuals with active disease and those with LTBI. Among the TB cases, there were more males than females. Patients with active TB had a significantly lower BMI than individuals with LTBI (Table 1). The demographic characteristics of the lung cohort study samples used for the comparison of method is shown in Supplementary Table 1.

Analyzing the K/T ratio by gender in the MRC-SHIP samples, we found no significant difference between males and females. BMI was negatively correlated with the K/T ratio (rho = 0.249, P = 0.0180). However, in the subgroups (HIV-infected with or without TB; HIV-negative with or without TB), there were no significant correlations between BMI and the K/T ratio, although a trend was seen in the patients with active TB (Supplementary Figure 1).

In univariate analysis (Table 2), age, BMI, HIV, and active TB were significantly associated with the K/T ratio. Furthermore, in multivariate analysis (Table 2), older age (P = 0.0445), HIV status (P < 0.0001), and having active TB disease (P < 0.0001) remained significantly associated with higher K/T ratios.

Comparison of ELISA to MS

To assess the agreement between ELISA and MS as the gold standard method, we performed a Bland-Altman's test and correlation plots for the plasma K/T ratio to determine the limits of agreement with the previously published MS results (Figure 1). For kynurenine, the mean bias between ELISA and MS was 0.01 mmol/L, with an SD of 1.4 mmol/L and a limit of agreement from 2.8 mmol/L to 2.8 mmol/L (Supplementary Figure 2). For tryptophan, the mean bias between ELISA and MS was 7.0 mmol/L, with an SD of 14.5 mmol/L and limits of agreement from 21.4 mmol/L to 35.5 mmol/L (Supplementary Figure 2). For K/T ratio, there was a mean bias of 0.01, with an SD of 0.04 and limits of agreement from 0.6 to 0.10 between the two methods (Figure 1). We further explored the correlation of the difference between the two methods and the average values (Supplementary Figure 5). For kynurenine, the difference between ELISA and MS showed outliers at high kynurenine values, although there was no significant correlation overall between the

Table 2

Multiple linear regression of factors associated with KT ratio.

Variable	Univariate		Multivariate	
	Est. (SE)	P-value	Est. (SE)	P-value
Age (years)	0.0004 (0.0005)	0.0014	0.0008 (0.0004)	0.0445
Sex: Female vs. Male	0.017 (0.010)	0.0988	–	–
HIV status: HIV+ vs. HIV TB status: TB+ vs. TB BMI	0.040 (0.010)	<0.0001	0.043 (0.008)	<0.0001
	0.053 (0.009)	<0.0001	0.056 (0.008)	<0.0001
	0.002 (0.001)	0.0276	–	–

HIV positive (HIV+), HIV negative (HIV), active TB (TB+), latent TB infection (TB), body mass index (BMI), estimate (Est), standard error (SE).

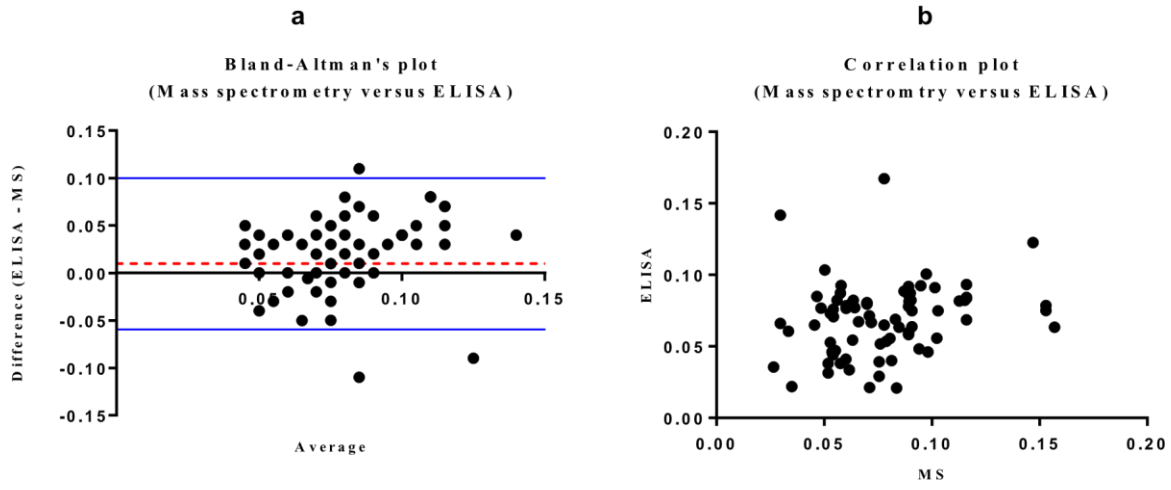

Figure 1. Comparison of K/T ratio measured by mass spectrometry (MS) and ELISA using samples from the Lung cohort study. Bland-Altman's plot (a): we plotted the difference between ELISA and MS versus the average between the two tests, n = 70. There was a good agreement between the methods with a mean bias of 0.01 (SD 0.04), 95% limit of agreement from -0.06 to 0.10. The red dotted line represents the mean bias whilst the blue lines indicate upper and lower limits of agreement. In a Spearman's correlation (b), there was a significant association between K/T ratio measured by ELISA and MS (r = 0.29, P = 0.0122, n = 70). All units are in (umol/l)/(umol/l).

difference in method and the average value (r = 0.0424, P = 0.7083). Tryptophan showed a significant positive correlation between the difference in method and the average value. For the K/T ratio, there was a weak positive correlation (r = 0.2983, P = 0.0093) between the difference in method and the average value, with outliers at high values of the K/T ratio.

We also evaluated intra and inter-run reproducibility of the ELISA assay, using six stored samples from the Lung cohort study over a 3-day period. For intra-run reproducibility, each sample was tested in triplicate in the same run to determine the SD and

percentage co-efficient (%CV). Of the six samples, kynurenine produced a mean SD of 0.2 umol/L with an average %CV of 6%. Tryptophan showed a mean SD of 4

umol/L with an average %CV of 8%. The K/T ratio showed a mean SD of 0.005 with an average intraassay %CV of 8%. For inter-run reproducibility, each sample was tested in duplicate on 3 different days. Duplicate results were averaged to give three readings per sample. For each sample, we calculated the SD and percentage coefficient of variation (%CV) of the three readings. Kynurenine gave a mean SD of 0.21 umol/L with an average %CV of 6%, and tryptophan gave a mean SD of 2 umol/L with an average %CV of 4%. The K/T ratio gave a mean SD of 0.005 with average of %CV of 5%.

Patients with active TB had higher K/T ratio than individuals with LTBI

Using the ELISA method, we compared patients with active TB to LTBI as controls in the MRC-SHIP cohort. Where HIV positive and negative patients were analyzed together, the K/T ratio was markedly elevated in patients with active TB compared to those with LTBI (Figure 2). Patients with active TB had significantly higher K/T ratios than patients with LTBI (median, 0.090 [IQR 0.063–0.105] versus 0.030 [IQR 0.020–0.056], $P < 0.0001$, Figure 3a). In a ROC curve analysis, plasma K/T ratio had an AUC of 0.89 ($P < 0.0001$, Figure 3a). Supplementary Table 2 shows the diagnostic significance of the plasma K/T ratio in patients with active TB at various cutoffs. At a cutoff of 0.040, the K/T ratio had a diagnostic sensitivity of 90% [CI 76–96%], a specificity of 70% [CI 53–81%], a PPV of 74%, and an NPV of 90%.

In HIV-uninfected patients alone, those with active TB had significantly higher K/T ratios than those with LTBI (median, 0.064

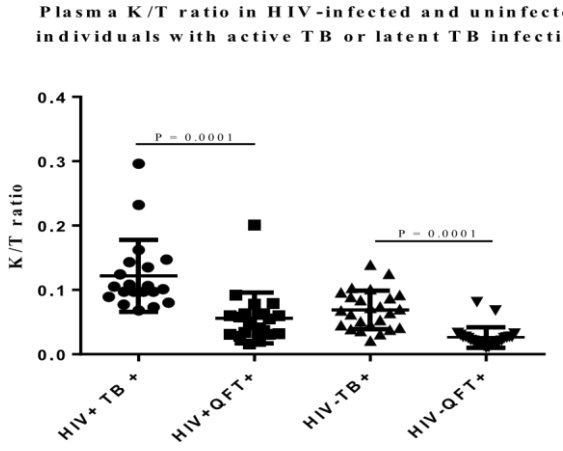

Figure 2. Plasma kynurenine/tryptophan (K/T) ratio measured using ELISA, in HIVinfected active TB patients ($n = 20$), HIV-infected individuals with latent TB ($n = 20$), HIV-uninfected active TB patients ($n = 25$) and HIV-uninfected individuals with latent TB ($n = 25$) from the MRC-SHIP cohort. Patients with active TB had significantly higher K/T ratio than individuals with latent TB (Mann Whitney test, P values shown, Kruskal-Wallis test overall P-value < 0.0001). The upper and lower horizontal lines indicate 25th and 75th percentiles, respectively, with the median is indicated.

[IQR 0.040–0.088] versus 0.022 [IQR 0.016–0.027], $P < 0.0001$, Figure 3b). A ROC analysis gave an AUC of 0.93 (95% CI 0.85–1.00, $P < 0.0001$, Figure 3b). At a cutoff of 0.040, the plasma K/T ratio showed a diagnostic sensitivity of 85% (95% CI 59–93%), a specificity of 92% (95% CI 73–99%), Pa PV 91%, and an NPV 84% (Supplementary Table 3).

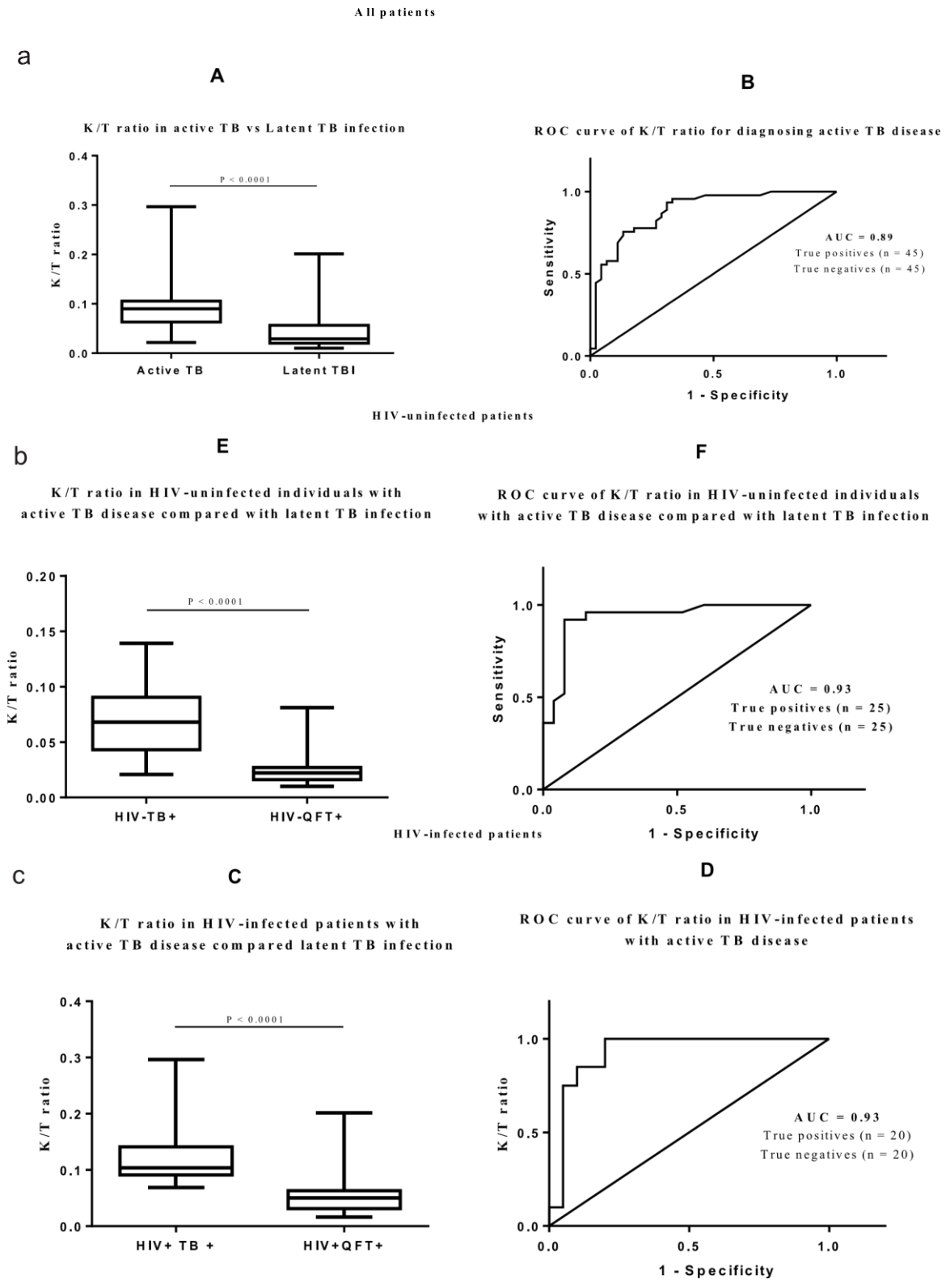

Figure 3. a: In the MRC-SHIP cohort: A; Plasma K/T ratio in active TB patients compared with patients with latent TB infection (controls). Both cases and control groups included patients with and without HIV-infection. Patients with active TB had significantly elevated K/T ratio compared with patients with latent TB (P < 0.0001). Using a cutoff of 0.040, plasma K/T ratio had a diagnostic sensitivity of 90% (CI 76–96%), specificity 70% (CI 53–81%), PPV 74% and NPV of 90%. B; A ROC had an area under the curve of 0.89 (CI 0.81-0.95). b: In the MRC-SHIP cohort: E; Plasma K/T ratio in HIV-uninfected individuals with active TB disease compared with HIV-uninfected individuals with latent TB infection

(controls). HIV-uninfected patients with active TB disease had significantly higher K/T ratio compared with HIV-uninfected patients with latent TB infection (($P < 0.0001). At a cut-off of 0.040, plasma K/T ratio had a diagnostic sensitivity of 85% (CI 59–93%), specificity 92% (CI 73–99%), PPV 91% and 84%. F; Area under the ROC curve was 0.93 (CI 0.85–1.0). c. In the MRC-SHIP cohort: C; Plasma K/T ratio in HIV-infected patients with active TB disease compared with HIV-infected patients with latent TB patients (controls). HIV-infected patients with active TB disease had significantly higher K/T ratio ($P < 0.0001) than HIV-infected patients with latent TB infection. At a cut-off of 0.080, plasma IDO activity had a diagnostic sensitivity of 90% (CI 62–96%), specificity 80% (CI 68–98%), PPV 82% and 90%. D; A ROC curve had an area under the curve of 0.93 (CI 0.83–1.0).

To investigate whether the same cutoff would be appropriate for HIV-positive samples, we evaluated the K/T ratio in the HIV-infected group. HIV-infected patients with active TB disease had a significantly higher K/T ratio than HIV-infected patients with LTBI (median, 0.101 [IQR 0.091–0.140] versus 0.061 [IQR 0.034–0.077], $P < 0.0001, Figure 3c). An ROC curve showed an AUC of 0.93, $P < 0.0001. At a cutoff of 0.080, the K/T ratio had a diagnostic sensitivity of 90% [95% CI 62–96%], a specificity of 80% [95% CI 68– 98%], a PPV of 82%, and an NPV of 90% (Supplementary Table 4).

Neither kynurenine nor tryptophan alone could be as useful as their ratio was for distinguishing active TB from LTBI (Supplementary Figures 3 and 4).

Discussion

We evaluated the plasma K/T ratio as a blood-based diagnostic biomarker for active TB disease in HIV-infected and uninfected individuals. Immunoassays such as ELISA are low-cost, relatively high-throughput methods that could be used in peripheral laboratories and could be further automated. ELISA requires small samples volume and can be completed within a relatively short period of time. MS is the gold standard method for the detection of kynurenine and tryptophan concentrations for calculation of the K/ T ratio, but this technology is not widely available. Furthermore, the cost factor and the expertise required exclude MS from frontline clinical diagnosis.

Our data showed strong reproducibility and good agreement between the values of tryptophan measured by both methods. For kynurenine, there was a more moderate concordance, with some outliers. One possible reason for this may be interference by kynurenine derivatives appearing in the ELISA method that were not present in MS. The difference between the results for ELISA and MS showed outliers for high values of kynurenine, although there was no significant correlation between the difference in method and the average value ($r = 0.0424, $P = 0.7083). For tryptophan there was a significant positive correlation between the difference in method and the average value, again with outliers at high concentrations of tryptophan. In ill patients with other lung diseases, we anticipate higher kynurenine but lower tryptophan values, so further studies are needed to confirm the discriminatory power of the K/T ratio when patients with other lung diseases are included. For the K/T ratio, there was a weak positive correlation ($r = 0.2983, $P = 0.0093) between difference in method and average K/T values, with outliers at high K/T values. Thus, agreement between MS and ELISA should be further assessed in patients with active TB in those with other lung diseases. More importantly, the utility of ELISA to distinguish patients with TB from those with other lung diseases warrants further study. The calculation of the most appropriate K/T ratio cutoff for ELISA found differs from that previously reported, determined using MS (Weiner et al., 2012).

Using the ELISA method, we analyzed patients with active TB disease compared to individuals with LTBI, defined as QFT-positive individuals. In the HIV-uninfected group, patients with active TB had significantly elevated K/T ratios compared to individuals with LTBI. Additionally, HIV-infected patients

with active TB disease also had significantly higher K/T ratios than HIV-infected individuals with LTBI, confirming previous findings (Adu-Gyamfi et al., 2017). A cutoff ratio of 0.040 for HIV-uninfected patients gave a sensitivity of 85%, a specificity of 92%, a PPV o 91%, and an NPV of 84% for the diagnosis of active TB.

For HIV-infected patients, raising the cutoff value to 0.080, the plasma K/T ratio gave a diagnostic sensitivity of 90% and specificity of 80%, with a high PPV of 82% and an NPV of 90% for the diagnosis of active TB. If the HIV status was unknown, a cutoff 0.040 would detect 90% of all active TB patients, with a specificity of 70%, a PPV of 74%, and an NPV of 90%. Thus, if used as an initial diagnostic test, at a cutoff of 0.040, the K/T ratio would detect 100% of HIV-infected and 85% of HIV-uninfected individuals who would have appropriate confirmatory TB testing.

These findings agree with previous reports showing that the K/T ratio is elevated in active TB patients at the time of TB diagnosis (Almeida et al., 2009; Adu-Gyamfi et al., 2017; Suzuki et al., 2013; Suzuki et al., 2012). Furthermore, HIV-infected patients have a higher plasma K/T ratio than HIV-uninfected individuals. The size of the HIV viral reservoir impacts the plasma K/T ratio (Chen et al., 2019; Adu-Gyamfi et al., 2019). Broad evidence indicates that the K/ T ratio is mildly increased in HIV infection and highly increased in active TB (Suchard et al., 2020; Adu-Gyamfi et al., 2019). Separate cutoffs for HIV-uninfected and infected individuals appear to be appropriate.

The K/T ratio is thus a promising diagnostic TB biomarker, according to the WHO TPP guidelines (World Health Organization, 2014). It meets 36 out of 37 TPP criteria for a non-sputum-based test for detecting active pulmonary TB (World Health Organization, 2014). The only disadvantage for the ELISA assay is the overnight (16 h) incubation period required for sample processing.

IDO is the initial and rate-limiting enzyme in tryptophan degradation to kynurenines along the pathway of the de novo biosynthesis of nicotinamide (Katz et al., 2008). IDO is induced by pro-inflammatory cytokines, including Interferon-g and tumor necrosis factor-α. The overexpression of IDO promotes the generation of peripheral immune tolerance to pathogens through starvation of tryptophan in T cells, which is a key nutrient for T cell proliferation (Yeung et al., 2015). Toxic byproducts of IDO catabolism accumulate in the microenvironment and may induce regulatory T cells, which downregulate other immune cell populations (Desvignes and Ernst, 2009; Mezrich et al., 2010a, b). Both Mtb and HIV have been shown to induce IDO activity in vitro and in vivo (Blumenthal et al., 2012; Gautam et al., 2018; Terness et al., 2002). Unlike its host, Mtb can synthesize tryptophan, a feature that protects it in a tryptophan-deprived environment (Williams and Dunbar, 2014).

In animal models, increased IDO activity is associated with unsuccessful immune response to Mtb (Gautam et al., 2018). Nicotinamide improves clinical TB symptoms, and Isoniazid, an anti-TB agent, is a nicotinamide analogue (Murray, 2003b). Elevated IDO appears to be associated with the switch from LTBI to active TB, although whether the enzyme causes or responds to mycobacterial growth is unclear (Suchard et al., 2020). The tryptophan-kynurenine-nicotinamide pathway deserves scrutiny for its possible key role in TB pathogenesis and holds promise for TB biomarker development.

To evaluate the assay as a TB diagnostic, the inclusion of participants with LTBI as our control group reflects the realities of using a blood-based Mtb assay for diagnosis in a TB-endemic area and is a strength of our study.

A diagnostic test with a sensitivity of 90% and an NPV of 90% in HIV-infected patients holds great potential to improve patient care. A blood-based tool has the potential to detect patients who have

difficulty producing sputum, potentially including children and extra-pulmonary TB cases, although this aspect requires further investigation. Additionally, a blood-based tool could improve infection control practices, alleviating the need for sputum collection. Other conditions, including pregnancy or malignancy, should be investigated as potential confounders of the K/T ratio, as they are conditions in which IDO activity is reportedly upregulated (Obayashi et al., 2016).

Interestingly, we noted an inverse correlation between plasma K/T ratio and BMI, which was particularly apparent in TB patients (Supplementary Figure 3), however; this correlation was not significant in subgroup or multivariable regression analysis. Loss of weight is a cardinal TB symptom, so any TB biomarker could be expected to show an inverse correlation with BMI. Multiple regression analyses showed that older age and positive HIV status were associated with higher K/T ratio, but the strongest association was with active TB.

One limitation of our study is that we did not include individuals with other lung diseases, although we have previously reported the good discriminatory power of the K/T ratio even when pneumonia patients were included (Adu-Gyamfi et al., 2017). Similarly, other studies have shown that is one of the most increased metabolites during active TB when compared with other lung diseases (Suzuki et al., 2012; Feng et al., 2015; Weiner et al., 2012; Zhou et al., 2015). Also, our reported PPVs may be overestimated due to our case-control study design, in which the prevalence of the disease is approximately half. Furthermore, the related enzymes tryptophan 2,3-dioxygenase (TDO), IDO-2, and other enzymes encoded by gut-resident bacteria also convert tryptophan to kynurenine. Therefore, the elevated K/T ratio reflects not only IDO-1 activity but a composite picture of host, microbial, and mycobacterial tryptophan and kynurenine metabolism. The measurement of the K/T ratio remains the preferred clinical approach to the quantitative detection of shifts in IDO activity. The CD4 count and HIV viral load data were not available because they were not recorded in the MRC-SHIP study. Nevertheless, the plasma K/T ratio shows a strong potential advantage over conventional diagnostic methods for high-burden TB settings. Furthermore, it is relevant that the plasma K/T ratio can be measured with high thorough-put, low-cost, ELISA methodology.

Conclusion

In summary, we showed that the K/T ratio is a promising plasma-based tool for identifying active TB disease in HIV-infected or uninfected adults. K/T ratio results above a cutoff of 0.040, when HIV status is unknown, could be used to triage patients requiring confirmatory TB testing. We propose that the K/T ratio measured by ELISA assays for kynurenine and tryptophan should be evaluated in large, well-designed prospective studies as a bloodbased screening test for active TB.

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Author contributions

CGAG performed the literature search, conducted experiments, analyzed the data, produced the figures, wrote and revised the manuscript. CGAG takes responsibility for the integrity of the data and the accuracy of the data analysis. LM conducted the laboratory experiments and revised the

manuscript. KO assisted with the statistical analysis and reviewed the manuscript. TS and JAG supervised the laboratory work, assisted with the data interpretation, and edited the manuscript. DS performed the data mining, assisted with the data interpretation, and edited the manuscript. FD, APN, and MF assisted with the patient recruitment and edited the manuscript. TJS, NM, CH, and RC assisted with trial design and patient recruitment and edited the manuscript. MSS, the senior author, designed the hypothesis, supervised the data analysis and interpretation, and edited the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2020.08.028.

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Chapter 5

Evaluation of K/T ELISA as a diagnostic test for Tuberculosis in HIV-infected pregnant women

For a pregnancy to succeed, two immunologically and genetically distinct tissues must co-exist. During pregnancy, the mother's immune system modulates responses to paternal antigens in the foetus. Elevated indoleamine 2,3-dioxygenase (IDO) activity during pregnancy has been reported by several researchers. IDO activity results in T cell hypofunction (1). IDO plays a critical role in immune tolerance from conception to labour. IDO activity has two-fold functions; first, by depleting local tryptophan and secondly, by increasing kynurenine metabolites in the microenvironment (2, 3). These actions have both a direct and indirect effect on T cell maturity and functions (4).

During pregnancy, IDO is highly expressed at the placenta and in the blood of pregnant women (5-7). Women with dysfunctional expression or activity of IDO have been reported to experience unexplained spontaneous abortions (8, 9). In both human and animal models, several studies have demonstrated inhibition of IDO activity during pregnancy leads to fetal-maternal rejection and poor pregnancy outcomes (5, 7, 10).

In pregnant women, TB diagnosis is more difficult. This is because typical signs such as weight loss, night sweat, mild fever, shortness of breath and fatigue, which are classically found in active TB disease are often masked by pregnancy symptoms. The presence of HIV-infection in the pregnant woman presents a further complication to TB diagnosis. A recent study from South Africa and Swaziland reported a poor case detection rate among pregnant women

screened for active TB (11, 12). A maternal audit from the past ten years reports TB as the highest cause of death among pregnant women in South Africa (13, 14).

In this study, we performed a secondary analysis of residual plasma samples from the Tshepiso HIV-infected pregnant women cohort. We aimed to determine whether pregnancy is a confounder to the use of IDO activity as a TB diagnostic biomarker. Our specific objectives were to investigate the diagnostic utility of plasma kynurenine/tryptophan (K/T) ratio as a TB biomarker in pregnant women living with HIV/AIDS and to determine whether the most suitable cut-off value for diagnosing active TB in pregnant women was the same as that in non-pregnant women.

The Tshepiso pregnant women cohort

The Tshepiso pregnancy cohort was established by the Perinatal HIV Research Unit, Johannesburg. Tshepiso was a non-interventional prospective cohort study of HIV-infected pregnant women with active TB disease (cases) and without TB (controls) in Soweto, South Africa.

Pregnant women had been recruited at the time of presentation for antenatal care at a local antenatal clinic or obstetric ward. Eligible women were 18 years or older, gestational age ≥ 13 weeks with a confirmed HIV diagnosis. Participants (cases and controls) had been evaluated for active TB disease at enrolment with the WHO symptom screen (15), and a sputum specimen taken for mycobacterial liquid culture (BACTEC™ MGIT™ 960 System, Becton Dickenson, CA).

Cases had been defined as those with positive *Mycobacterium tuberculosis* (M.Tb) culture result at baseline, whilst a probable/possible TB case was a patient who was smear-positive for acid-fast bacilli (AFB) with a negative or unknown culture result. For each case enrolled, two HIV-infected pregnant women without TB with similar maternal age, gestational age, and whose delivery was planned to be at the same institution as the woman with TB, had been enrolled as controls. Women were enrolled at ten prenatal clinics around the study site. Participants were followed-up to one-year post-partum.

In total, the Tshepiso study had enrolled eighty-three (83) pregnant women with active TB disease and one hundred and sixty (160) control subjects (16). Tshepiso was a non-interventional study so all women received appropriate care in public sector clinics in and around Soweto according to South African antiretroviral (ART) and TB treatment guidelines. All pregnant women who were not initiated on Isoziad preventive treatment (IPT) before enrolment were offered IPT by the study clinician. Plasma samples were stored at -70^0C at the Clinical Laboratory Services in Johannesburg until testing.

For this secondary analysis, we retrieved clinical records and blood samples of all pregnant women with active TB disease. Six active TB cases yielded non-tuberculous mycobacteria on culture, while five had TB diagnosis before or after pregnancy, and were excluded from our analysis. In total, we analysed 72 active TB cases. Due to sample availability, we analysed one hundred and seventeen (117) women as controls. Plasma samples were tested at the centre for Vaccines and Immunology, the National Institute for Communicable Diseases in Johannesburg.

We found that plasma K/T ratio was significantly elevated during pregnancy compared with non-pregnant periods ($p < 0.0001$). At the time of TB diagnosis, plasma K/T ratio was markedly elevated in pregnant patients with active TB disease compared to individuals without TB ($p < 0{\cdot}0001$). Applying a previously optimized cut-off value of 0.080, plasma K/T ratio gave a diagnostic sensitivity of 96%, specificity of 86%, a PPV of 77% and an NPV of 99%. A ROC gave an area under the curve of 0.95. The optimal cut-off in HIV-infected pregnant women was, however, calculated as 0.100 with a sensitivity of 94%, specificity of 90%, a PPV of 85% and an NPV of 98%. Thus, we assert that the higher cut-off is more appropriate in pregnancy.

A novel finding from this work was that in our control group, pregnant women who received isoniazid preventative treatment (IPT) had lower plasma K/T ratio than those who did not receive IPT, suggesting that isoniazid alone decreases IDO activity.

In conclusion, our results demonstrated that plasma K/T ratio is a robust screening test for patients with active TB disease. Plasma K/T ratio showed good sensitivity with high specificity, excellent positive and negative predictive values for diagnosis of active TB disease among pregnant women. This work has been accepted for publication in *Clinical Infectious Diseases* (CID) and follows hereafter.

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Manuscript 3

Plasma kynurenine-to-tryptophan ratio, a highly sensitive blood-based diagnostic tool for Tuberculosis in HIV-infected pregnant women

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Key words

IDO; kynurenine/tryptophan ratio; sensitivity; specificity; blood-based; biomarker; TB; HIV; Target product profile.

Abstract

Background: For pregnant women living with human immunodeficiency virus (HIV), concurrent active Tuberculosis (TB) disease increases the risk of maternal mortality and poor pregnancy outcomes. Plasma indoleamine 2,3-dioxygenase (IDO) activity, measured by kynurenine-to-tryptophan (K/T) ratio has been proposed as a blood-based TB biomarker. We investigated whether plasma K/T ratio could be used to diagnose active TB among HIV-infected pregnant women.

Methods: Using an enzyme-linked immunosorbent assay (ELISA), we measured K/T ratio in 72 HIV-infected pregnant women with active TB and compared to 117 HIV-infected pregnant women without TB, matched by age and gestational age.

Results: Plasma K/T ratio was significantly elevated during pregnancy compared to sampling done after pregnancy ($p < 0.0001$). Pregnant women who had received isoniazid preventive therapy (IPT) before enrolment had decreased plasma K/T ratio compared to those who had not received IPT ($p = 0.0174$). Plasma K/T ratio was elevated in women with active TB at time of diagnosis compared to those without TB ($p < 0{\cdot}0001$). Using a cut-off of 0.100, plasma K/T ratio gave a diagnostic sensitivity of 94% (CI 82-95), specificity of 90% (CI 80-91), PPV 85% and NPV 98%. A receiver operating characteristic curve (ROC) gave an area under the curve of 0.95 (CI 0.92-0.97, $p < 0.0001$).

In conclusion, plasma K/T ratio is a sensitive blood-based diagnostic test for active TB disease in pregnant women living with HIV. Plasma K/T ratio should be further evaluated as an initial TB diagnostic test to determine its impact on patient care.

1.0 Introduction

Despite global efforts to end tuberculosis (TB), TB remains a global health threat, associated with more than a million deaths annually (1). Active TB in pregnancy is risky for both mothers and infants. TB diagnosis has challenges, particularly using methods which rely on sputum samples. Sputum is difficult to obtain in severely ill patients and is not useful in patients with extra-pulmonary TB (2). As a result, there is a gap between the number with active TB and the number diagnosed. Undiagnosed cases fuel TB transmission (3) and cause maternal and foetal morbidity and mortality. The World Health Organisation (WHO) has highlighted the urgent need for a rapid, quantitative, non-sputum based marker that does not depend on mycobacterial direct detection and can indicate pulmonary or extra-pulmonary TB, as a key intervention to attain TB control targets (4).

A significant burden of TB occurs in women of reproductive age (15-45 years) (1). Pregnant women are at an increased risk of developing active TB disease (5-7). Concurrent TB-HIV infection in pregnancy is a significant cause of mortality in settings of high HIV prevalence (6, 8). In Johannesburg, Black *et al* reported that about 70% of deaths in HIV-infected pregnant women were due to TB infection (9).

Indoleamine 2,3-dioxygenase (IDO) breaks down tryptophan to kynurenines (10). IDO is the first and rate-limiting enzyme in the *de novo* biosynthesis of nicotinamide adenine dinucleotide (NAD) (11, 12). IDO activity suppresses T cell function, resulting in generation of regulatory T cells and apoptosis of other T cells (13, 14). IDO is highly expressed at the placenta, and declines to non-pregnancy levels after delivery (15, 16).

Recently, we reported that IDO activity measured by product-to-substrate ratio (kynurenine/tryptophan [K/T]) is a suitable blood-based TB biomarker in HIV-infected and uninfected patients (17, 18). In this study, we investigated whether pregnancy acts as a potential confounder of IDO activity as a screening tool for active TB disease.

2.0 Materials and methods

2.1 Study Subjects

This was a nested case-control study. Study samples were from the Tshepiso cohort recruited from 2011 to 2014 (19). Tshepiso was a prospective cohort study of HIV-infected pregnant women with active TB disease (cases) and without TB (controls) in Soweto, South Africa (19). Pregnant women were recruited at the time of presentation for antenatal care. Eligible women were 18 years or older and had gestational age ≥13 weeks with a confirmed HIV diagnosis. Participants (cases and controls) were evaluated for active TB disease at enrolment with the WHO symptom screen (20) and a sputum specimen for mycobacterial liquid culture (BACTEC™ MGIT™ 960 System, Becton Dickenson, CA). For each case enrolled, two HIV-infected pregnant women without TB with similar maternal age, gestational age, and whose delivery was planned to be at the same institution as the woman with TB, were enrolled as controls. Cases were defined as those with a positive *Mycobacterium tuberculosis* (M.Tb) culture result, whilst a probable TB case was a patient who was smear positive for acid-fast bacilli (AFB) with a negative or unknown culture result and a possible TB case was a patient with clinical symptoms of active TB but negative or unconfirmed sputum smear and culture result. Participants were followed-up to one-year post-partum.

In total, Tshepiso enrolled eighty-three (83) pregnant women with active TB and one-hundred and fifty-five (155) women without TB (21). Tshepiso was a non-interventional study and women received care in public sector clinics according to South African guidelines. Standard of care from 2011 to 2013 depended on CD4 count. For CD4 counts above 350 cells/ml women were given zidovudine (AZT) monotherapy with single dose nevirapine during labour, intrapartum AZT and one post-partum dose of tenofovir and emtricitabine (Option A). Women with CD4 counts below 350 cells/ml received WHO option B (zidovudine, lamivudine and efavirenz triple therapy). In 2013 guidelines changed to lamivudine or emtricitabine with tenofovir and efavirenz for those with CD4 counts below 350 cells/ml. At enrolment, the study clinician offered isonaizid preventive therapy (IPT) to participants who had not

previously received IPT. Plasma samples were stored at -70^{0}C at the Clinical Laboratory Services in Johannesburg until testing.

After study completion, we accessed plasma samples and clinical data of participants from enrolment to one year post-partum. For this secondary analysis, we excluded six women because non-tuberculous mycobacteria were identified on culture and five women diagnosed either prior to or after pregnancy. All active TB cases were diagnosed at the time of recruitment but at varying weeks of gestation. We combined probable and possible TB cases from the Tshepiso study as "probable/possible" TB in this analysis. In total, we analysed 72 women with active TB that occurred during pregnancy and compared to one hundred and seventeen (117) control specimens available in the repository.

2.2 ELISA for plasma K/T ratio

Plasma samples were tested at the National Institute for Communicable Diseases in Johannesburg. EDTA-anticoagulated plasma was used to measure plasma kynurenine and tryptophan by competitive ELISA using a commercial kit (ImmuSmol, Inc. France). Briefly, acylated or derivatised plasma kynurenine or tryptophan competed with solid phase bound kynurenine or tryptophan for antibody binding over a 15-hour incubation period (overnight). Free antigen and free antigen-antibody complexes were removed by washing. The antibody bound to the solid phase was detected by an anti-rabbit IgG-peroxidase conjugate using 3,3',5,5'-tetramethylbenzidine as a substrate. The reaction was monitored at 450 nm using a microplate reader. Quantification of unknown samples was achieved by comparing their absorbance with a reference curve prepared with known standards provided with the kit. IDO activity was calculated as the ratio of kynurenine (µmol/l)/tryptophan (µmol/l). Investigators were blind to TB status of samples during analysis.

2.3 Statistical Analysis

We used GraphPad Prism 6 software (GraphPad Software, USA) and SAS Enterprise Guide 7.15 (SAS Institute., USA) for analysis. Normally distributed data were shown as mean ± standard deviation (SD)

whereas non-normally distributed data were expressed as median with interquartile range [IQR]. Wilcoxon and Man-Whitney tests were used to determine significant differences between two non-parametric paired and unpaired groups respectively. We used the Kruskal-Wallis test to compare multiple non-parametric groups.

Sensitivity, specificity, positive and negative predictive values were calculated. We derived receiver operating characteristic (ROC) curves to evaluate the most suitable diagnostic cut-off value for K/T ratio to discriminate women with active TB from those without TB. For controls in the ROC curve analysis, we included multiple time-points, excluding labor, from each subject; that is for controls, we included all other time-points. For cases, we included all other time-points except for enrolment as a control time-point during which the woman did not have active TB. We used this approach because each person could visit the clinic multiple times and require a TB test, thus each time-point is a valid time-point for inclusion as a control. We excluded the labor time-point from the analysis. Labor has many physiological alterations which may affect the IDO activity and labor, however, is not a typical TB screening time-point for a patient. We repeated the ROC curve analysis using only the enrolment time-point for both cases and controls in our supplementary analysis.

We assessed association between K/T ratio and clinical parameters such as body mass index (BMI), CD4 count and viral load using Spearman's correlation. Using baseline data, we modelled factors associated with K/T ratio by running univariate and multivariate linear regression to determine the parameter estimate and standard error (SE). The covariates included study arm (TB cases versus controls), age, CD4 count, BMI, gestational age (in weeks) and viral load (in $\log_{10}$).

The Tshepiso cohort study was approved by the John Hopkins University Institutional Review Board. The University of the Witwatersrand Human Research Ethics Committee approved the Tshepiso cohort study (M10336) and this nested sub-study (M170476).

3.0 Results

3.1 Baseline clinical parameters

Bacteriologically confirmed, probable and possible TB were combined as active TB cases. Of the 72 women with active TB, 35 (49%) were bacteriologically confirmed by culture (Table 1). Furthermore, 66 (92%) had pulmonary TB while six (6) patients had extra-pulmonary TB (pleural, peritoneal, pericardial and disseminated TB). One woman was diagnosed with drug-resistant TB. At baseline, 70 (97%) women with active TB and 110 (94%) of the controls were receiving antiretroviral treatment (ARVs). There was no difference in the proportion of women with active TB and controls on ART, however, controls had been on ART longer than cases (Table 1).

Regarding isoniazid preventive therapy (IPT), 58% of controls had received IPT for an average of 63 days before baseline sampling. No patient with active TB disease had received IPT prior to enrolment into the study. Lower body mass index and CD4 cell count were apparent in TB patients at baseline compared with controls. There was a trend towards higher viral loads in TB patients compared to controls (Table 1).

3.2 Plasma K/T ratio is elevated during pregnancy

First, we investigated whether plasma K/T ratio was increased during pregnancy using only specimens from the control group. The median K/T ratio at enrolment was 0.075 [IQR 0.050-0.12], significantly higher than at six weeks after delivery (0.061 [IQR 0.045-0.082], $p < 0.0001$, Figure 1).

Next, we analysed intra-individual variability over time using only non-pregnant time-points (six weeks, six months and one year post-partum) in controls. The mean plasma K/T ratio was 0.050 with SD of 0.022 and a coefficient of variation (CV) of 31% (Supplementary figure 1)

We further evaluated whether plasma K/T ratio was elevated in women with active TB. In women with active TB, median K/T ratio was 0.222 [IQR 0.151-0.391] compared to 0.075 [IQR 0.050-0.121] in controls at enrolment, $p < 0.0001$ (Figure 1).

3.3 Effect of TB treatment on plasma K/T ratio We determined the effect of TB treatment on plasma K/T ratio using forty-four (44) women who had samples available from enrolment, 6-weeks post-partum and 6-month post-partum. At 6 weeks post-partum, TB patients had received anti-TB therapy for a median of 16 (12-19) weeks. We observed that plasma K/T ratio declined significantly in all patients 16 (12-19) weeks after starting TB therapy and remained at low level even 6 months post-partum. The median K/T ratio at enrolment (time of TB) was 0.300 [IQR 0.164-0.440] compared to 0.042 [0.031-0.057] and 0.033 [0.027-0.050] at 6 weeks post-partum and 6 months post-partum respectively, with overall $P < 0.0001$, Figure 2.

3.4 Diagnostic value of plasma K/T ratio

We evaluated the diagnostic significance of plasma K/T ratio to detect TB disease. We included all time-points from enrolment to one-year post-partum, except the labour time point. We calculated sensitivity, specificity, positive and negative predictive values (PPV and NPV) to determine the presence of active TB (Table 2). We used a receiver operating characteristics (ROC) curve to determine the optimal cut-off. At a cut-off of 0.100, plasma K/T ratio gave an optimal sensitivity of 94% (CI 82-95), specificity of 90% (CI 80-91), PPV 85% and NPV 98% to diagnose active TB (table 2). A ROC gave AUC of 0.95 (CI 0.92-0.97, $p < 0.0001$) (Figure 3). Even when using a lower cut-off of 0.080, previously optimized in non-pregnant HIV-infected patients, K/T ratio gave a sensitivity of 96% (CI 88-98), a specificity of 86% (CI 73-85), PPV 77% and NPV of 99% for diagnosis of TB (table 2). We repeated the analysis using K/T ratio measurements from enrolment only (supplementary figure 2).

3.5 Pulmonary TB compared to extra-pulmonary TB

We evaluated whether plasma K/T ratio differed by site of TB. We found no significant difference in K/T ratio between patients with pulmonary TB (median 0.230 [IQR 0.145-0.382]) and those with extra-pulmonary TB (0.205 [IQR 0.155-0.445], $p = 0.956$, Supplementary figure 3A).

Next, we compared plasma K/T ratio in patients with bacteriologically confirmed TB to possible/probable TB patients. There was no significance difference between bacteriologically confirmed TB cases (median 0.222 [IQR 0.160-0.385]) compared to possible/probable TB cases (median 0.185 [0.098-0.383], p = 0.375, Supplementary figure 3B).

3.6 Association of K/T ratio with body mass index, CD4 count and HIV viral load

We analysed association of plasma K/T ratio with clinical parameters at the baseline visit. Plasma K/T ratio was inversely correlated with body mass index (BMI) in patients with active TB, while in controls there was no correlation (Figure 4). In controls but not in TB patients, plasma K/T ratio showed a weak correlation with viral load. For CD4 count, there was no correlation with plasma K/T ratio in either group (Figure 4).

In a univariate regression, factors significantly associated with plasma K/T ratio were BMI, CD4 and presence of active TB disease (Table 3). In the multivariate analysis, women with active TB had a K/T ratio of 0.153 higher than controls ($p < 0.0001$).

3.7 The effect of IPT

We explored the effect of IPT on plasma K/T ratio in the control group. Of the 117 controls, 68 (58%) had used IPT while 49 (52%) had not used IPT before enrolment. The median plasma K/T ratio of women who had received IPT before enrolment was 0.068 [IQR 0.047-0.103], significantly lower than in IPT naïve women (median 0.092 [0.050-0.155], p = 0.0174, Figure 5A). Among 68 pregnant women who had taken IPT for an average of 63 (±114) days, we found no correlation between number of days on IPT and plasma K/T ratio (r = -0.117, p = 0.9451, Figure 5B). Of the 49 women who were IPT naïve, 35 were initiated on IPT at enrolment while 14 were not. We compared plasma K/T ratio at enrolment to 8 (IQR 2-11) weeks after IPT use. In a paired analysis, the median plasma K/T ratio declined from 0.091 [IQR 0.042-0.139] to 0.039 [IQR 0.030-0.058] ($p < 0.0001$). Conversely, in 14 controls who never took IPT, median plasma K/T ratio at enrolment increased from 0.097 [IQR 0.052-0.213] to 0.101 [IQR 0.053-0.263] at the 32 week visit (p = 0.0042, Figure 5C).

3.8 The effect of ART

We investigated whether ART affected K/T ratio. In the control subjects, median plasma K/T ratio of those who were on ART was 0.070 [IQR 0.050-0.120], significantly lower than those not on ART (0.130 [IQR 0.065-0.307], p=0.0331, supplementary figure 4A). We found no significant difference between individuals on AZT-based options compared to late regimen B (p = 0.2252, supplementary figure 4B). Furthermore, there was no significant correlation between length of time on ART and K/T ratio in patients or controls (Figure 6).

In TB patients at enrolment, we found no significant difference in K/T ratio between patients who had been on ART compared to those who had not been on ART (p = 0.6445, data not shown). Additionally, there was no significant difference in K/T ratio among TB patients who were given AZT-based options compared to late option B (p = 0.2252).

4.0 Discussion

The lack of a reliable TB biomarker is an obstacle to controlling TB. Recently, we and others reported that increased IDO activity detected by plasma K/T ratio was a promising TB biomarker in HIV-infected and uninfected patients (18, 22, 23). In this study, we evaluated the potential of plasma K/T ratio to serve as a host-derived TB biomarker in HIV-infected pregnant women.

Our results demonstrate minimal intra-individual fluctuation of plasma K/T ratio over a one-year period with CV of 31%. Pregnant women had higher plasma K/T ratio than non-pregnant women. An increase IDO activity during pregnancy ties in with similar findings of others (24-27). IDO expression and activity plays a critical role in maintaining pregnancy to term (24).

Most importantly, pregnant women with TB could be distinguished from pregnant women without active TB using a K/T ratio cut-off of 0.100. We found that HIV-infected pregnant patients with active TB had higher plasma K/T ratio than those without active TB disease. At a cut-off value of 0.100, K/T

ratio showed a sensitivity of 94%, a specificity of 90% and NPV of 98%. This suggests that plasma K/T ratio could be used as a rule-out first diagnostic test for active TB disease in HIV-infected women. We suggest this higher cut-off value of 0.100 is more suitable for pregnant patients.

Additionally, in patients with active TB, we noticed that K/T ratio rapidly declined concurrent with starting anti-tuberculous treatment. The finding of K/T ratio decreasing after initiation of TB treatment is consistent with our previous work and that of others (18, 22, 23, 28). A similar difference was also observed in controls taking IPT compared to those never initiated on IPT. We conclude that administration of isoniazid alone decreases K/T ratio. The finding of K/T ratio decreasing after isoniazid preventive therapy is novel. Isoniazid was originally developed as a nicotinamide analogue (29). Thus, isoniazid administration may bypass the need for endogenous synthesis of nicotinamide through the IDO-catalysed pathway. Notably, no patients in the TB group had been taking IPT prior to enrolment, which leads us to speculate that one of the ways Isonaizid prevents development of active TB is by bypassing the need for endogenous nicotinamide synthesis (30).

Plasma K/T ratio showed a significant inverse correlation with BMI in TB patients but not in controls. The relation between K/T ratio and BMI in TB patients is consistent with our previous findings and those of others in non-pregnant populations (31, 32). As weight loss is cardinal sign of TB, an appropriate TB biomarker should be expected to show inverse correlation with BMI in TB patients. There was a weak association between plasma K/T ratio and viral load in controls; in keeping with literature (33), however, the relation was not observed in TB patients. Controls on ART had lower K/T ratios than those who were ART-naïve, but in TB patients there was no difference in K/T ratio between those on ART and those who were ART-naïve. There was no association of plasma K/T ratio with CD4 cell count in either group. Thus, high K/T ratio in TB patients is not merely due to correlation of kynurenine or tryptophan with immune activation or HIV progression.

Plasma K/T ratio showed no difference between bacteriologically confirmed and possible/probable TB, nor between pulmonary and extra-pulmonary TB. This finding is in keeping with our previous research

(17). Although our numbers with extrapulmonary disease were small, our results suggest that plasma K/T ratio may be useful for diagnosing extrapulmonary TB.

This study has several strengths. We detected K/T ratio from plasma, which is an easily collected clinical sample. Our patients were from a TB endemic area, implying that many of our controls were likely latently infected with TB, although latency was not tested. Most blood-based diagnostics fail to discriminate active disease from previous infection or latent TB and perform poorly in HIV-infection. We further used an ELISA method which does not require complicated equipment and is simple to perform.

Study limitations include the lack of inclusion of patients with pneumonia, or other pulmonary pathology. Our reported positive predictive values may be affected due to the case-control study design in which prevalence of the disease is pre-determined. Our assay should be assessed in non-pregnant women with symptoms suggestive of TB.

These findings, together with our previous work (17, 32) , suggest that plasma K/T ratio can be used to diagnose active TB disease in HIV infected individuals, including pregnant women. We report here that elevated K/T ratio could detect almost all active TB cases among pregnant women. In those who tested negative for K/T ratio (below a cut-off of 0.100), 99% did not have TB. Our results suggest that K/T ratio has the potential to be used in pregnancy with an adjusted cut-off value of 0.100. The PPV of 85% would be of great clinical value, and ideally could be confirmed by a second testing method. A negative result would, however, confidently exclude TB. An advantage of this assay over other existing diagnostics is its applicability to plasma as a sample type, avoiding the need for sputum collection.

In conclusion, our findings suggest that plasma K/T ratio, measured by ELISA with a cut-off of 0.100 may serve as a sensitive TB diagnostic tool in HIV-infected pregnant women. TB treatment decreased plasma K/T ratio, suggesting that plasma K/T ratio may prove a potential tool for monitoring TB

therapy. Additionally, IPT decreased K/T ratio, suggesting that prospective monitoring of K/T ratio in latently infected individuals may be predictive of risk of progression to active TB disease.

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Conflict of interest

The researchers declare no competing interests.

Author contributions

CAG performed the literature search, conducted experiments, analyzed data, produced the figures, wrote and revised the manuscript.

LM conducted laboratory experiments and revised the manuscript.

KO assisted with statistical analysis and reviewed manuscript.

JAG assisted with data interpretation, and edited the manuscript.

DS performed data mining, assisted with data interpretation, and edited the manuscript.

NSA, NM & RC assisted with trial design and patient recruitment and edited the manuscript.

MSS, senior author, designed the hypothesis, supervised data analysis and interpretation, and edited the manuscript.

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Figures and Figure legends

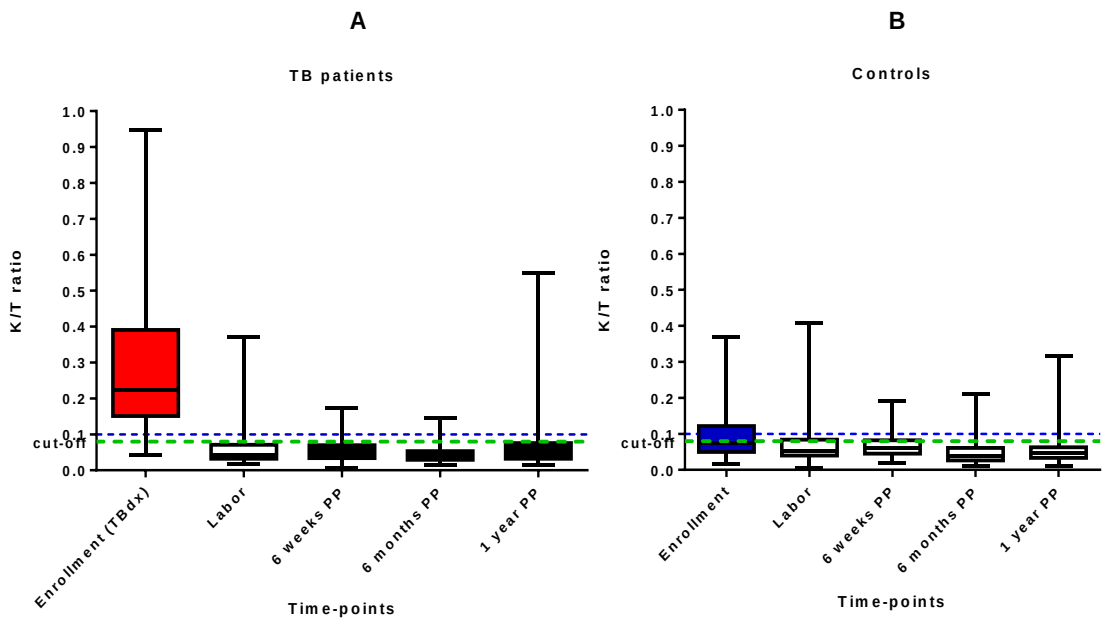


Figure 1: *Box plot of plasma kynurenine/tryptophan ratio measured by ELISA. A: HIV-infected pregnant women at the time of TB diagnosis during pregnancy (enrolment n=71), labour (n= 55), six weeks post-partum (n= 66), six months post-partum (n= 57) and one year post-partum (n=50). K/T ratio was significantly elevated at the time of TB diagnosis (red-box and whisker) (median 0.220 [IQR 0.160-0.400]). B: HIV-infected pregnant women with no active TB (n=117) over the same period as TB cases. The median K/T ratio at time of enrolment (blue-box whisker box) was 0.080 [IQR 0.050-0.114]. The green dotted line indicates previously optimized cut-off in non-pregnant HIV-infected individuals, while the blue dotted line indicates new cut-off for HIV-infected pregnant women. The upper and lower horizontal lines of the boxes indicate 25th and 75th percentiles, respectively, with the median shown. Whiskers show the range.*

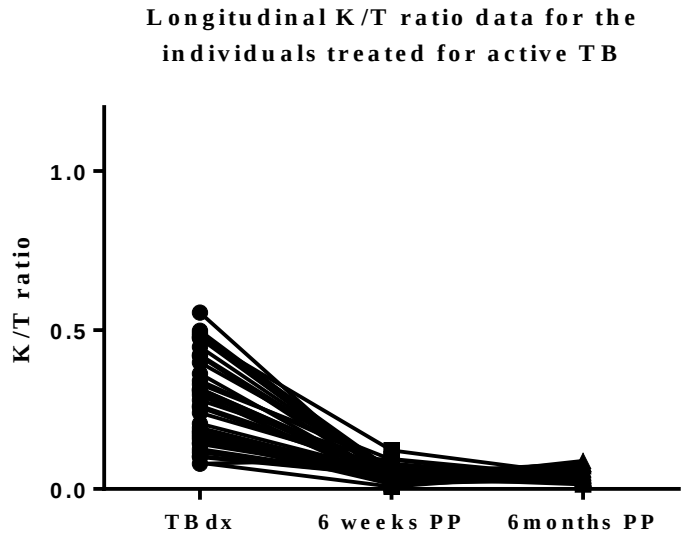


Figure 2: *Longitudinal plasma K/T ratio of HIV-infected women treated for active TB. Forty-four (44) out of the 72 women with samples available from enrolment, 6 weeks post-partum and 6 month post-partum. Plasma K/T ratio declined significantly in all patients by median 16 (IQR 12-19) weeks. The median plasma K/T ratio at time of TB was 0.300 [IQR 0.164-0.440] compared to 0.042 [0.031-0.057] and 0.033 [0.027-0.050] at 6 weeks post-partum and 6 months post-partum respectively. In a paired Friedman test, the overall P < 0.0001. TBdx (time of active TB), 6 weeks PP (6 weeks post-partum) and 6 months PP (6 months post-partum).*

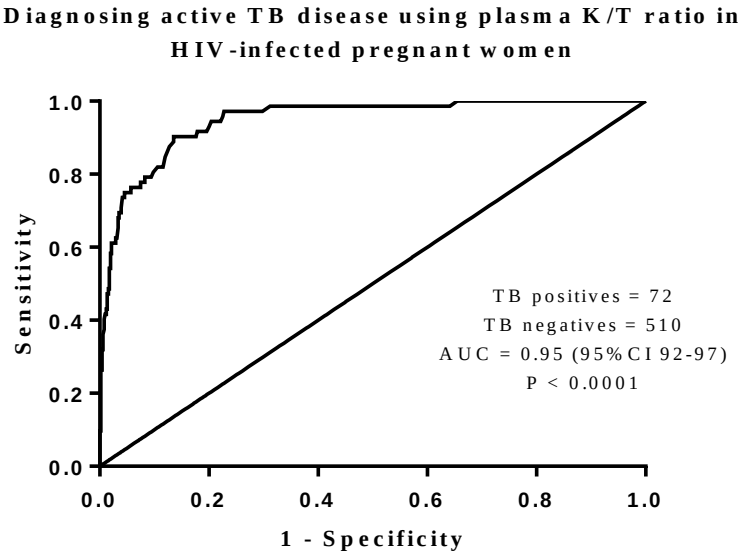


Figure 3: *Receiver operating characteristic curve (ROC) of plasma K/T ratio for detecting the presence of active TB disease among HIV-infected pregnant patients. At a cut-off value of 0.100, plasma K/T ratio gave a sensitivity of* 94% (95%CI 82-95), specificity of 90% (95%CI 80-91), PPV 85% and NPV 98% *with AUC of 0.95 (95%CI 92-97).*

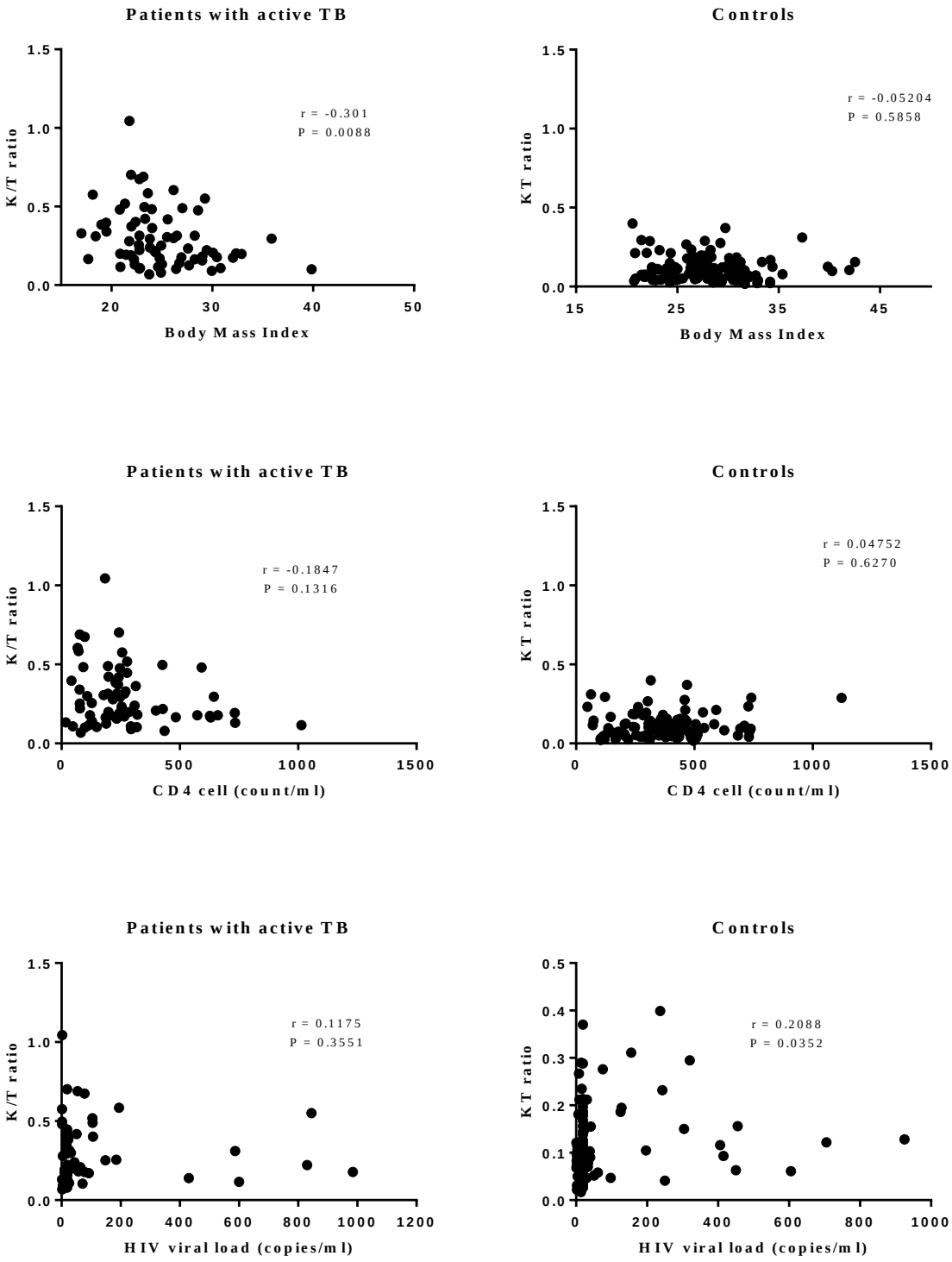


Figure 4: *Association of plasma K/T ratio and body mass index, CD4 cell count and HIV viral at enrolment. Plasma K/T ratio was negatively correlated with body mass index in TB patients, but not in controls. There was no correlation between K/T ratio and CD4 count in patients with active TB or controls. Plasma K/T ratio showed a weak correlation with HIV viral load in controls while there was no correlation found in TB patients.*

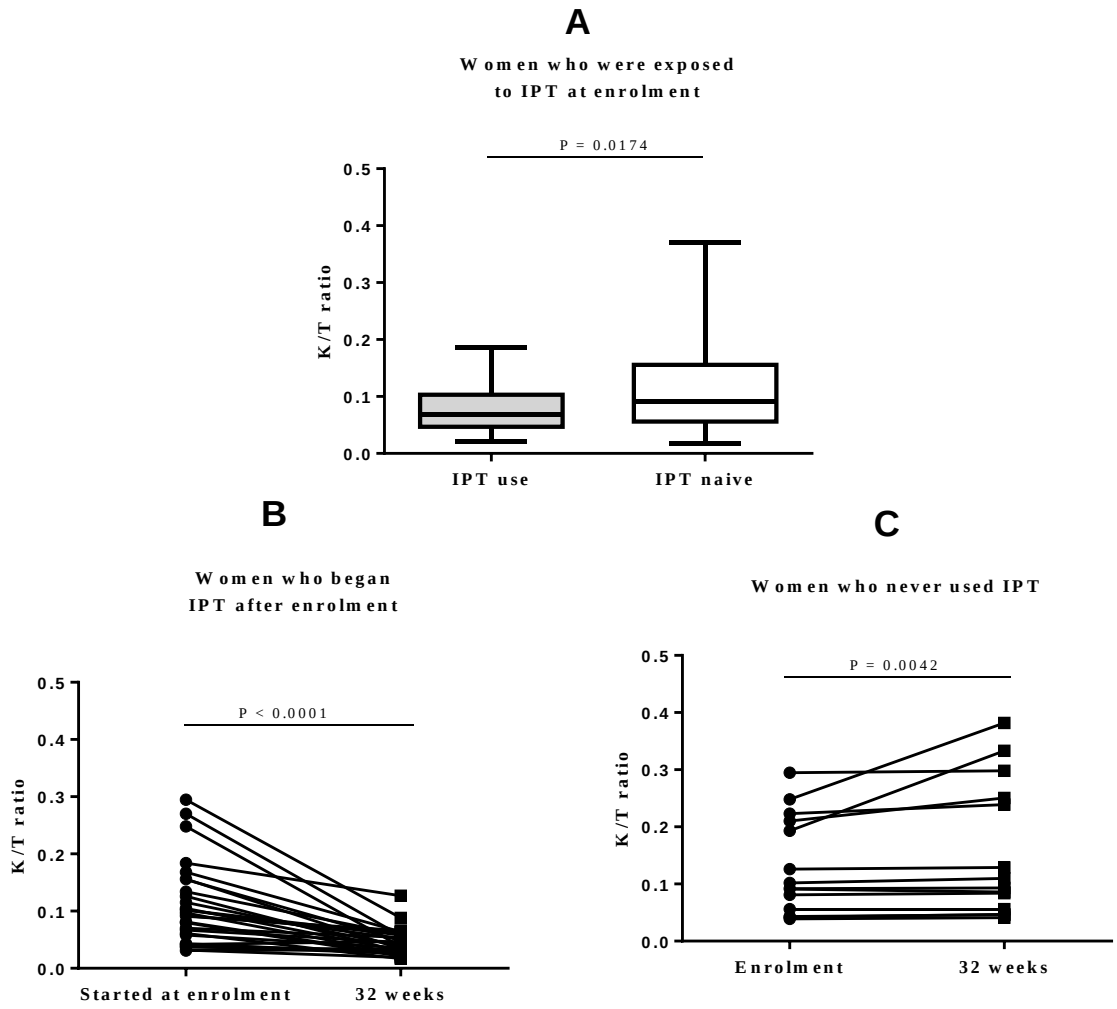


Figure 5: *The effect of Isoniazid preventative treatment (IPT) use on plasma K/T ratio in HIV-infected pregnant women. Sixty-eight (68) of the 117 controls had used IPT while 49 were IPT naïve at time of study enrolment. A: At enrolment, there was a significant difference between those who had used IPT and those who were IPT naïve (p = 0.0174). The median plasma K/T ratio of pregnant women on IPT was 0.068 [IQR 0.047-0.103] versus 0.091 [0.050-0.155] in IPT naïve women. B: Of the 49 IPT naïve HIV-infected pregnant women, thirty-five were given IPT at enrolment. After 8 (IQR 2-11) weeks, plasma K/T ratio significantly decreased from a median of 0.091 [IQR 0.042-0.013] to 0.039 [IQR 0.026-0.057] (p = 0.0001). C: In a paired analysis of fourteen individuals who never*

took IPT, we compared their plasma K/T ratio at enrolment to 3 (IQR 2-5) weeks after enrolment. The median plasma K/T ratio was 0.097 [IQR 0.052-0.213] at enrolment compared to 0.101 [IQR 0.053-0.263] at 32 weeks (p = 0.0042).

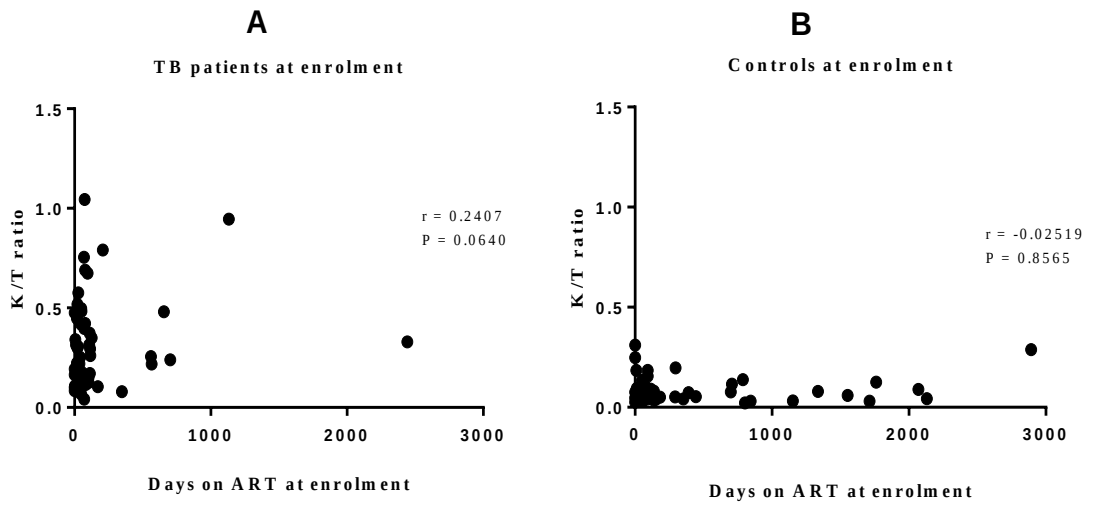


Figure 6: *Correlation between plasma K/T ratio and number of days on ART at enrolment. A: HIV-infected women with active TB. B: HIV-infected controls. There was no significant correlation between K/T ratio at enrolment and number of days on ART both in TB cases and controls.*

Tables

Table 1: Subjects' demographics and baseline clinical data

Maternal characteristics at baseline			
	TB cases (n = 72)	**Controls (n = 117)**	**p value**
Age (years)[A]	36 ± 4	36 ± 4	0.9838
Gestational age (Weeks)[B]	29 (25-34)	30 (26-35)	0.3031
Body Mass Index (BMI)[B]	24 (22-28)	27 (24-30)	< **0.0001**
CD4 cell (count/ml)[B]	243 (128-314)	374 (248-483)	< **0.0001**
HIV Viral load (copies/ml)[B]	19 (19-77)	19 (17-29)	0.0836
Classification of TB disease			
Confirmed TB	35 (49%)	-	
Possible/Probable TB	37 (51%)	-	
Site of TB location			
Pulmonary TB	66 (92%)	-	
Extra pulmonary TB	6 (8%)	-	
ART regimen at enrolment			
Proportion of individuals on/off ART	70/2	110/7	0.4865
Median number of days on ART (IQR)	48 (20-106)	90 (41-430)	**0.0023**
Isoniazid preventive treatment			
Number of individuals on/off IPT	0/72	68/49	< **0.0001**
Number of days on IPT (days)[A]	0	63±100	**-**

[A]*Mean ± SD, Interquartile ranges(IQR), IPT – Isoniazid Preventive Therapy, ART – Antiretroviral therapy, HAART – Highly active antiretroviral therapy*

Table 2: Diagnostic significance of K/T ratio in active TB disease

TB positives: All active TB cases at time of enrolment (n = 72)

TB negatives: TB cases at time-points other than TB diagnosis or labour, and controls at all times excluding labour (n = 510 specimens from HIV-infected women with no TB)

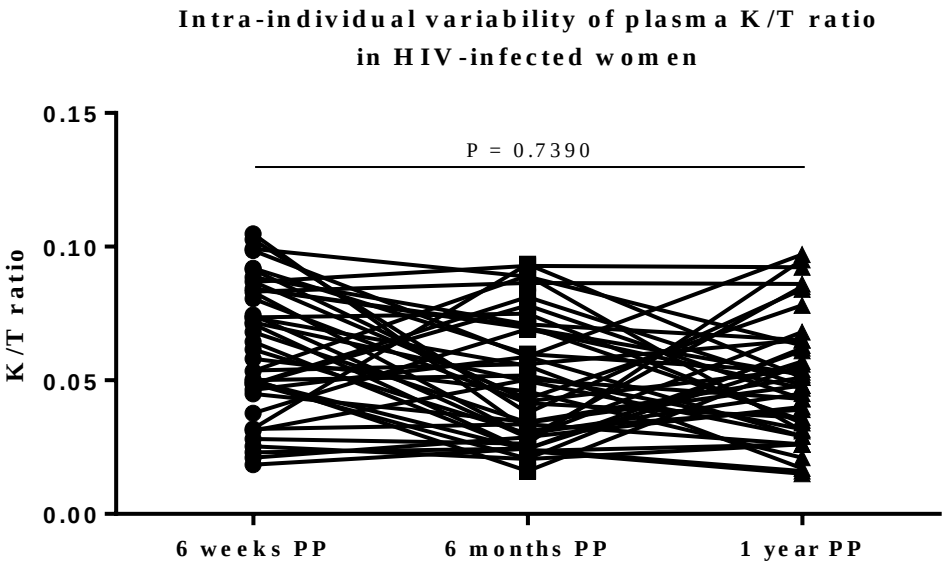
IDO activity for diagnosing active TB disease

Cut-off	Sensitivity (%)	95% CI	Specificity (%)	95% CI	PPV (%)	NPV (%)
0.010	100	95-100	30	14-24	22	100
0.020	100	95-100	37	21-31	27	100
0.030	100	95-100	39	24-33	30	100
0.040	100	95-100	42	31-39	32	100
0,050	99	92-99	49	43-51	35	100
0,060	98	92-99	62	57-66	45	99
0,070	97	90-99	77	66-74	58	99
0,080	96	88-98	86	73-85	77	99
0,090	95	85-96	90	78-85	79	98
0,100	**94**	**82-95**	**90**	**80-91**	**85**	**98**
0,110	90	70-90	92	85-90	86	97
0,120	87	67-87	93	88-92	87	97
0,130	82	66-86	94	89-94	90	96
0,140	80	64-85	95	90-95	92	96
0,150	79	63-84	96	92-96	94	95

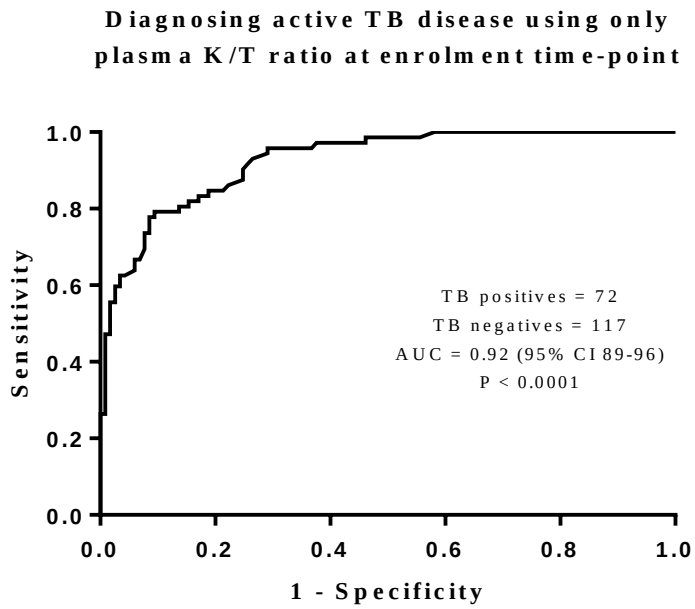
Table 3: Regression analysis for factors associated with KT ratio

	Univariate		**Multivariate**	
Variables arm	**Estimate (SE)**	**p-value**	**Estimate (SE)**	**p-value**
Active TB vs. Control	0.188 (0.019)	< 0.0001	0.153 (0.023)	**< 0.0001**
Age	-0.001 (0.003)	0.689	-	-
CD4 Count	-0.018 (0.006)	0.003	-0.01(0.005)	0.057
BMI*	-0.011 (0.002)	< 0.0001	-0.006 (0.002)	**0.006**
Gestational age (weeks)	0.001 (0.002)	0.595	-	-
Log_{10} Viral Load	0.011 (0.010)	0.266	-	-
**Body mass index (BMI), Standard error (SE)*				

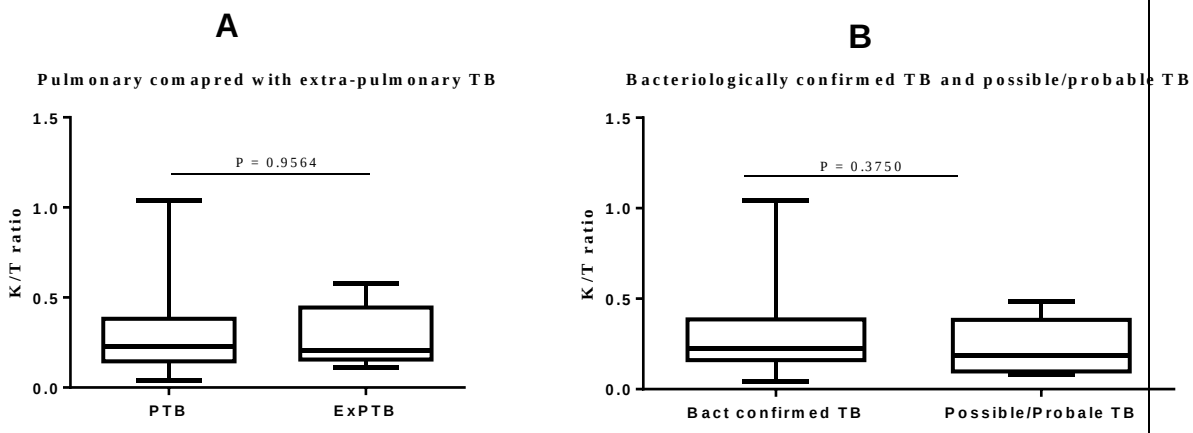
Supplementary figures



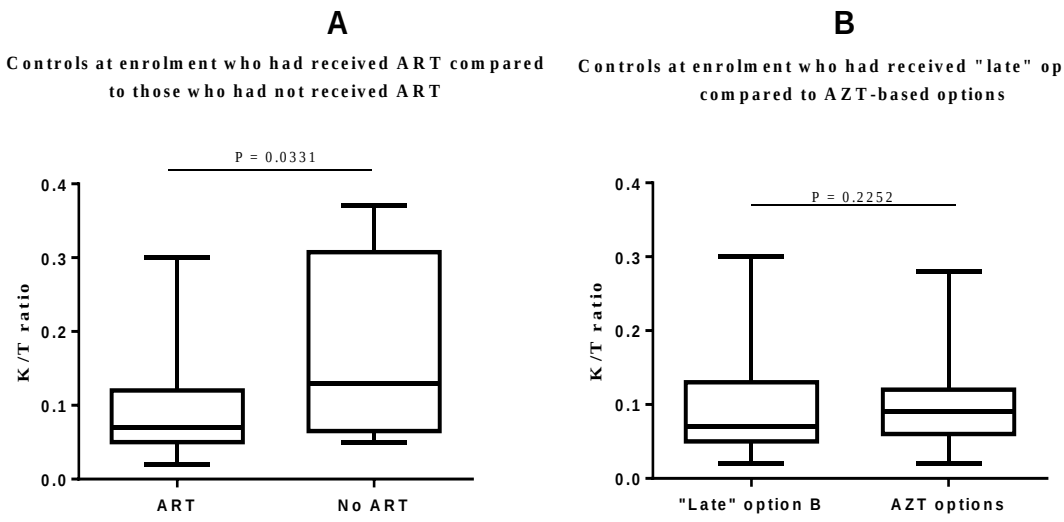
Supplementary figure 1*: Longitudinal plot of intra-individual variability of plasma K/T ratio in HIV-infected women without TB (controls) at non-pregnancy time-points (6 weeks post-partum [6 weeks PP], 6 months post-partum [6 months PP] and 1 year post-partum 1 year PP]). The mean plasma K/T ratio was 0.050 with SD of 0.022 and CV of 31%.*



Supplementary figure 2: *Receiver operating characteristic curve (ROC) of plasma K/T ratio for detecting the presence of active TB disease among HIV-infected pregnant patients. At a cut-off value of 0.100, plasma K/T ratio gave a sensitivity of* 94% (CI 86-98), specificity of 70% (CI 79-91), PPV 68% and NPV 94% *with AUC of 0.92 (CI 89-96).*



Supplementary figure 3: *Plasma K/T ratio in HIV-infected pregnant women according to site of TB (A) and mode of TB diagnosis (B). A: There was no significant difference between patients with pulmonary TB (n=66, median 0.230 [IQR 0.145-0.382]) and extra-pulmonary TB (n= 6, median 0.205 [IQR 0.155-0.445]), P = 0.9564). B: There was no significant difference between bacteriologically confirmed cases (n=35, median 0.297 [IQR 0.194-0.300]) compared to possible/probable TB (n=37, median 0.185 [IQR 0.098-0.383]), P = 0.3750). PTB: pulmonary Tuberculosis, ExPTB: extrapulmonary Tuberculosis, Bact confirmed TB: Bacteriologically confirmed Tuberculosis.*



Supplementary figure 4: *Plasma K/T ratio in HIV-infected pregnant who were on antiretroviral therapy (ART) (A) and ART option (B) at enrolment. A: There was significant difference in plasma K/T ratio of individuals who were on ART at the time of enrolment compared to those who had not been on ART (P = 0.0331). The median plasma K/T ratio of those who were on ART was 0.070 [IQR 0.050-0.120] compared to 0.130 [IQR 0.065-0.307] of those who were not on ART.). B: There was no significant difference between individuals on "late" option B and AZT-based options (P = 0.2252). The median K/T ratio was 0.070 [IQR 0.050-0.130] versus 0.090 [IQR 0.060-0.120] in individuals on AZT option and late option B ART. AZT: Zidovudine.*

Chapter 6

Discussion and conclusion

6.1 Discussion

We evaluated plasma IDO activity measured as kynurenine/tryptophan (K/T) ratio as a candidate biomarker for active TB disease in a TB/HIV endemic setting. We reviewed the literature dating from the early HIV era and summarized evidence that elevated plasma IDO activity (high K/T ratio) is associated with the severity of HIV disease and with active TB. We highlighted that IDO-mediated tryptophan catabolism via the kynurenine pathway represents a common axis upregulated in HIV infection and TB disease. Even though not uniquely elevated in TB, the cumulative elevation of IDO activity in HIV and TB may explain the extraordinarily high rate of active TB disease among people living with HIV. Elevated IDO activity may thus hold promise as a marker of active TB disease in both HIV-infected and uninfected patients.

Presently, the gold standard test for quantitating plasma kynurenine and tryptophan concentration is high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS), which is known for its excellent analytical sensitivity and specificity. Mass spectrometry, however, is not a frontline diagnostic tool, even in highly resourced centres. The running cost as well as the required management expertise exclude it from primary health care centres. In this work, we showed that ELISA, a widely available method has strong reproducibility and good agreement with HPLC-MS for measuring plasma kynurenine and tryptophan concentration. From our results, we showed that the commercial ELISA had good intra- and inter-run reproducibility and can accurately replace mass spectrometry for measuring plasma kynurenine and tryptophan concentration for IDO activity quantitation.

From the World Health Organization (WHO) target product profile (TPP) guidelines, the minimum required sensitivity for a non-sputum-based biomarker test for smear-positive, culture-positive PTB should be equal or greater than 65% and with a specificity greater than 98%. The goal of a non-sputum based biomarker test for the diagnosis of PTB and extrapulmonary TB is to facilitate starting TB treatment in the same clinic encounter of an active case. The test should be easy to perform and implemented at lower health care facilities without requiring massive laboratory infrastructure.

For a triage test, the goal is to identify individuals with symptoms suggestive of active TB or risk of developing active TB who may require a confirmatory TB test. The recommended minimum sensitivity for a triage test should be greater than 90% with a specificity above 70%. A triage test should be point-of-care testing device (small device) usable by auxiliary health personnel at a lower health facility or in the community. A triage test should not require any sophisticated instrument.

Using the ELISA, we evaluated the utility of plasma K/T ratio as a TB biomarker in two nested case-control studies, the MRC-SHIP study and the Tshepiso pregnant women cohort. From our results, plasma K/T ratio was a robust diagnostic tool for active TB disease in both HIV-infected and uninfected patients. In HIV-infected patients with active TB disease, plasma K/T ratio gave a diagnostic sensitivity of 90%, a specificity 80%, PPV 82% and 90% at a cut-off value of 0.080. In HIV-uninfected patients with active TB disease, plasma K/T ratio gave a sensitivity of 85%, specificity 92%, PPV 91% and 84% using a cut-off of 0.040. Even though

patient characteristic such as pregnancy affected plasma K/T ratio, the diagnostic cut-off could be adjusted for applicability to pregnant women living with HIV.

According to the WHO TPP, a new non-sputum-based test for detecting TB should have a minimum sensitivity greater than 65% (but should be greater than 98% among patients with smear-positive culture positive PTB) with near absolute specificity (1). Comparing to the WHO's TPPs, we have demonstrated from two TB/HIV cohorts with varying patient characteristics that plasma K/T ratio, meets the minimum test characteristics required for a new screening or diagnostic test for active TB (1). Plasma K/T ratio has excellent diagnostic sensitivity and specificity in both HIV-infected and uninfected patients, including pregnant women. Furthermore, plasma K/T ratio has high positive and negative predictive values either as a rule-in or rule-out test for active TB disease. Except for time to results, determination of plasma K/T ratio by ELISA meet all the targets for new non-sputum-based TB test.

Plasma K/T ratio was detected from unstimulated blood by a simple, inexpensive ELISA, which did not require specialized expertise to interpret results. Additionally, plasma K/T ratio at different cut-off value could differentiate latently infected individuals from active TB in the presence or absence of HIV-infection.

We confirmed the clinical utility of elevated plasma K/T ratio as TB biomarker in pregnant patients living with HIV. Our results showed that plasma K/T ratio was elevated during pregnancy compared to the non-pregnant state in HIV-infected women. This finding is in keeping with other published literature (2, 3). Most importantly, plasma K/T ratio was significantly higher in pregnant patients with active TB disease compared to pregnant women

who did not have TB. Our result suggests that, although IDO activity plays a critical role in a successful pregnancy, pregnancy was not a confounder to use of K/T ratio as a TB diagnostic marker. Plasma K/T ratio showed strong diagnostic potential in detecting active TB disease even in the presence of HIV-infection. In those who tested negative, 98% did not have active TB, thus the assay would be a good rule-out test for screening high numbers of women suspected of active TB disease, particularly in TB/HIV endemic settings. The high NPV suggests that plasma K/T ratio above the proposed cut-off value is an excellent initial blood-based TB test. We suggest that plasma K/T ratio should be evaluated in an expanded prospective cohort as an initial diagnostic test for active TB. We did not evaluate utility of K/T ratio in HIV-uninfected pregnant women, in whom further studies are required.

Regarding plasma K/T ratio being used to monitor anti-TB therapy, our results suggest that plasma K/T ratio decreased among pregnant women receiving TB treatment as well as in pregnant women who did not have active TB disease but received isoniazid prophylactic treatment (IPT). The decrease in plasma K/T ratio in response to isoniazid, one of the main anti-TB drugs, suggests that isoniazid directly impacts IDO activity. Whether K/T ratio can be used to monitor TB therapy awaits further study of patients who are cured compared with those with poor treatment outcomes.

Comparing plasma K/T ratio to weight loss measured as body mass index (BMI), we observed an interesting association. Plasma K/T ratio showed an inverse correlation with BMI in patients with active TB disease but not in controls. A decline in BMI is a cardinal sign of progression to active TB. It would be expected that an appropriate TB biomarker should show an inverse correlation with BMI in TB patients. Lack of correlation between BMI and K/T in controls

shows that elevated plasma K/T ratio during TB is not merely a more general association with low weight. Our finding suggests that plasma K/T ratio is an appropriate candidate biomarker for active TB disease.

6.2 Study strengths and limitations

Our work has particular strengths as well as limitations. As strengths, we have evaluated the plasma K/T ratio from TB patients in a TB/HIV endemic area. In the SATVI study, inclusion criteria for controls included positive interferon gamma release assays, and there was likely a high prevalence of latent TB in our Tshepiso control group although latency had not been tested. Inclusion of controls with prior TB exposure is appropriate for evaluation of a biomarker for active TB disease that should distinguish active from latent TB. Additionally, we have evaluated the plasma K/T ratio from blood. Blood as a sample type is easier to obtain and does not have infectious hazards associated with sputum. While most blood-based diagnostics fail to discriminate active disease from the previous infection, plasma K/T seems a promising blood-based tool.

Limitations of our work include the nested case-control nature of our studies. As a result, our reported positive and negative predictive values may be affected due to the study design in which the prevalence of the disease is pre-determined. Also, in this work, we have evaluated only homogenous TB positive samples. The presence of other co-morbidities such as pneumonia or lung cancer await further study. We only evaluated HIV-uninfected patients in a single cohort and validation in further cohorts awaits further study. It is not clear whether our results apply to HIV-uninfected pregnant women or paediatric TB cases.

6.3 Way forward

We have shown from the various works submitted in this thesis, striking evidence that plasma IDO activity measured as K/T ratio is a promising candidate TB biomarker; however, much work remains to be done before proposing it for inclusion in the South African TB diagnostic algorithm. Most proposed biomarkers fail repeat validation exercises and independent confirmation processes. To be able to recommend plasma K/T ratio as a TB biomarker requires further evaluations in different patient cohorts.

Future studies should evaluate the association between IDO activity and IDO gene expression. IDO mRNA is post-translationally modified, hence IDO gene expression may give differing results from K/T ratio. Furthermore, additional sample types may prove useful. IDO mRNA and activity has been detectable in blood, pleural fluid, cerebrospinal fluid and urine, although very few studies have used these samples for purposes of TB diagnosis.

Currently, the major drawbacks of the ELISA assay are that it is laboratory-based and it has a prolonged sample preparation time. However, the future potential of the assay is strong as it could be fully automated. The ELISA method could also be developed into a lateral flow assay in future, presenting a true point of care option. Recently, dried-blood spot (DBS), a form of sampling where blood is blotted and dried on filter paper, has become a preferred alternative sample type for molecular and serological tests. DBS samples are gaining increased acceptability for clinical diagnostic use due to relatively easy to collect, store and transport samples to the testing centre. DBS samples should be evaluated for IDO mRNA expression and activity as an alternate sample type to plasma for TB diagnosis. Blood spot or “finger/heel prick” and saliva samples would offer a great advantage in children.

Also, as a diagnostic test for active TB, future works should prospectively compare plasma K/T ratio to molecular assays such as the new Xpert Ultra (Cepheid, Inc, USA) for TB diagnosis. Molecular TB diagnostics were not in routine practice at the time of enrollment of our parent studies. Additionally, future works should evaluate plasma K/T ratio in patients presenting with chronic lung diseases other than TB with active TB disease. Furthermore, plasma K/T ratio should be investigated in children with active TB disease. In all the study presented in this thesis, our findings have come from adult TB patients. We cannot confirm if these finding would be the same in children with active TB, with or without HIV-infection.

Additionally, we found plasma K/T ratio to be elevated at pre-IPT administration compared with post IPT samples in pregnant women living with HIV. This may suggest that Plasma K/T ratio may be a possible marker of treatment adherence with the use of IPT in pregnancy. Future studies will compare plasma K/T ratio in a prospective cohort of pregnant women taking IPT. Cerebrospinal fluid (CSF) testing is also important particularly in patients with Tuberculous meningitis (TBM) as has been explored by others (4).

Finally, further studies should investigate whether induction of IDO is causally related to TB pathogenesis, rather than merely a biomarker of active TB disease. In support, some authors have proposed that the human host and the mycobacterium may compete for nicotinamide. Elevated IDO activity may be the adaptive response of the cell to raise nicotinamide levels in times of nicotinamide shortage (5, 6). Broad evidence on TB pathogenesis particularly in patients living with HIV points to increased IDO activity (elevated K/T ratio). Interestingly, isoniazid treatment alone was associated with K/T decline in controls without active TB. This

novel finding may be due to direct interaction of isoniazid, a nicotinamide analogue, with the kynurenine-tryptophan-nicotinamide pathway. An interpretation of this finding could be that therapeutic manipulation of IDO activity using isoniazid can cure active TB or, in the form of isoniazid preventive therapy (IPT), decrease the likelihood of progression to active TB. We can hypothesize that the IDO axis may be causally related to TB pathogenesis, as reviewed elsewhere (4). The role of IDO activity and nicotinamide pathways in TB pathogenesis warrants attention. Applicability of K/T ratio to monitoring of TB treatment requires evaluation of cohorts of successful compared with unsuccessful TB treatment.

6.4 Conclusions

In conclusion, we have demonstrated in this thesis that ELISA could replace mass spectrometry for measuring kynurenine and tryptophan concentration for the quantitative determination of IDO activity. Furthermore, IDO activity measured as plasma K/T ratio is a suitable candidate non-sputum-based TB diagnostic tool in both HIV-infected and uninfected individuals. At a cut-off value of 0.040 in HIV-uninfected, 0.080 in HIV-infected participants and 0.100 in pregnant patients was most appropriate to diagnose active TB disease. Plasma K/T ratio satisfies the TPP criteria for a non-sputum-based TB diagnostic test. Interestingly, isoniazid caused a fall in plasma K/T ratio, both in patients with active TB and in HIV-infected patients without active TB who were on IPT. This finding will be relevant in future studies investigating K/T ratio to monitor TB treatment.

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Appendix 1

Ethics certificate

R14/48 Adu-Gyamfi Clement

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170476

NAME: (Principal Investigator)	Adu-Gyamfi Clement
DEPARTMENT:	Chemical Pathology Centre for Vaccines and Immunology National Communicable Diseases
PROJECT TITLE:	Evaluation of Indoleamine 2, 3-Dioxygrnse Activity, a Potential Biomarker for Active Tuberculosis Disease in South Africa
DATE CONSIDERED:	05/05/2017
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Melinda Suchard
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	14/06/2017

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 Research Office Secretary in Room 10004,10th floor, Senate House/3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the conditions under which I am/we are authorised 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 to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially review April and will therefore be due in the month of April each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix 2

Tables for chapter 4 & manuscript 2

Table 4B: Diagnostic significance of plasma K/T ratio in active TB disease

Gold standard positives: All confirmed active TB (n = 45)

True negatives: All latent TB infection QFT+ (n = 45)

K/T ratio for diagnosing active TB disease

Cut-off	**Sensitivity (%)**	**95% CI**	**Specificity (%)**	**95% CI**	**PPV (%)**	**NPV (%)**
0,020	100	92-100	33	20-49	60	100
0,025	98	88-99	38	23-53	61	94
0,030	98	89-99	40	36-66	62	95
0,035	96	84-99	66	51-80	60	90
0,040	**90**	**76-96**	**70**	**53-81**	**74**	**90**
0,045	87	70-93	70	55-84	74	84
0,050	80	67-92	71	58-85	73	86
0,055	78	62-89	75	60-87	73	76
0,060	76	63-89	76	63-88	72	74
0,065	74	60-87	87	73-94	70	68
0,070	70	53-85	90	73-94	75	70
0,075	60	44-74	90	75-96	75	65
0,080	56	53-70	94	81-98	78	66
0,085	53	37-68	95	84-99	83	66
0,090	51	32-62	93	84-99	88	66
0,095	40	27-57	97	88-94	90	61
0,100	36	20-49	98	88-99	89	60

Table 4C: Diagnostic significance of plasma K/T ratio in HIV-uninfected patients

Gold standard positives: All HIV-negative with active TB (n = 25) True negatives: All HIV-negative with latent TB infection (QFT+) (n = 25)						
Plasma K/T ratio for diagnosing active TB disease						
Cut-off	Sensitivity (%)	**95% CI**	Specificity (%)	**95% CI**	PPV (%)	NPV (%)
0,020	100	86-100	40	21-61	61	100
0,025	96	79-99	60	38-78	67	93
0,030	96	79-99	84	63-95	83	95
0,035	92	73-99	92	73-99	92	92
0,040	**85**	**59-93**	**92**	**73-99**	**91**	**84**
0,045	76	54-90	92	73-99	90	79
0,050	68	46-85	92	73-99	89	82
0,055	60	38-78	92	73-99	88	70
0,060	60	38-78	92	73-99	88	70
0,065	52	31-72	92	73-99	87	66
0,070	48	27-68	96	79-99	92	65
0,075	40	21-61	96	79-99	90	91
0,080	36	20-57	96	79-99	90	90
0,085	32	19-53	100	86-100	100	60
0,090	28	17-51	100	86-100	100	57
0,095	20	15-49	100	86-100	100	56
0,100	19	15-48	100	86-100	100	54

Table 4D: Diagnostic significance of plasma K/T ratio in HIV-infected patients

Gold standard positives: HIV-positive with confirmed active TB (n = 20) True negatives: HIV-positive with latent TB infection QFT+ (n = 20)						
Plasma K/T ratio for diagnosing active TB disease in HIV-infected patients						
Cut-off	Sensitivity (%)	**95% CI**	Specificity (%)	**95% CI**	PPV (%)	NPV (%)
0,020	100	83-100	5	12-33	51	100
0,025	100	83-100	5	12-33	51	100
0,030	100	83-100	10	32-37	53	100
0,035	100	83-100	25	15-60	57	100
0,040	**100**	**83-100**	**30**	**19-63**	**59**	**100**
0,045	100	83-100	35	23-68	61	100
0,050	100	83-100	35	27-72	61	100
0,055	100	83-100	35	31-76	61	100
0,060	100	83-100	40	45-88	63	100
0,065	100	83-100	55	56-94	69	100
0,070	95	75-99	60	56-94	70	92
0,075	90	68-98	65	56-94	72	91
0,080	**90**	**62-96**	**80**	**68-98**	**82**	**90**
0,085	80	56-94	80	68-98	80	80
0,090	75	50-91	85	68-98	83	77
0,095	75	50-91	90	75-99	88	78
0,100	55	31-76	90	75-99	85	67

Supplementary tables of manuscript 2

Supplementary table 2: Diagnostic significance of K/T ratio in active TB disease						
Gold standard positives: All confirmed active TB (n = 45) True negatives: All latent TB infection QFT+ (n = 45)						
K/T ratio for diagnosing active TB disease						
Cut-off	**Sensitivity (%)**	**95% CI**	**Specificity (%)**	**95% CI**	**PPV (%)**	**NPV (%)**
0,020	100	92-100	33	20-49	60	100
0,025	98	88-99	38	23-53	61	94
0,030	98	89-99	40	36-66	62	95
0,035	96	84-99	66	51-80	60	90
0,040	90	76-96	70	53-81	74	90
0,045	87	70-93	70	55-84	74	84
0,050	80	67-92	71	58-85	73	86
0,055	78	62-89	75	60-87	73	76
0,060	76	63-89	76	63-88	72	74
0,065	74	60-87	87	73-94	70	68
0,070	70	53-85	90	73-94	75	70
0,075	60	44-74	90	75-96	75	65
0,080	56	53-70	94	81-98	78	66
0,085	53	37-68	95	84-99	83	66
0,090	51	32-62	93	84-99	88	66
0,095	40	27-57	97	88-94	90	61
0,100	36	20-49	98	88-99	89	60

Supplementary table 3: Diagnostic significance of K/T ratio in HIV-uninfected patients

Gold standard positives: All HIV-negative with active TB (n = 25) True negatives: All HIV-negative with latent TB infection (QFT+) (n = 25)						
K/T ratio for diagnosing active TB disease						
Cut-off	Sensitivity (%)	**95% CI**	Specificity (%)	**95% CI**	PPV (%)	NPV (%)
0,020	100	86-100	40	21-61	61	100
0,025	96	79-99	60	38-78	67	93
0,030	96	79-99	84	63-95	83	95
0,035	92	73-99	92	73-99	92	92
0,040	85	59-93	92	73-99	91	84
0,045	76	54-90	92	73-99	90	79
0,050	68	46-85	92	73-99	89	82
0,055	60	38-78	92	73-99	88	70
0,060	60	38-78	92	73-99	88	70
0,065	52	31-72	92	73-99	87	66
0,070	48	27-68	96	79-99	92	65
0,075	40	21-61	96	79-99	90	91
0,080	36	20-57	96	79-99	90	90
0,085	32	19-53	100	86-100	100	60
0,090	28	17-51	100	86-100	100	57
0,095	20	15-49	100	86-100	100	56
0,100	19	15-48	100	86-100	100	54

Supplementary table 4: Diagnostic significance of K/T ratio in HIV-infected patients

Gold standard positives: HIV-positive with confirmed active TB (n = 20)

True negatives: HIV-positive with latent TB infection QFT+ (n = 20)

K/T ratio for diagnosing active TB disease in HIV-infected patients

Cut-off	Sensitivity (%)	**95% CI**	Specificity (%)	**95% CI**	PPV (%)	NPV (%)
0,020	100	83-100	5	12-33	51	100
0,025	100	83-100	5	12-33	51	100
0,030	100	83-100	10	32-37	53	100
0,035	100	83-100	25	15-60	57	100
0,040	100	83-100	30	19-63	59	100
0,045	100	83-100	35	23-68	61	100
0,050	100	83-100	35	27-72	61	100
0,055	100	83-100	35	31-76	61	100
0,060	100	83-100	40	45-88	63	100
0,065	100	83-100	55	56-94	69	100
0,070	95	75-99	60	56-94	70	92
0,075	90	68-98	65	56-94	72	91
0,080	90	62-96	80	68-98	82	90
0,085	80	56-94	80	68-98	80	80
0,090	75	50-91	85	68-98	83	77
0,095	75	50-91	90	75-99	88	78
0,100	55	31-76	90	75-99	85	67