

https://dspace.org/

Evaluation of novel assay formats for indoleamine 2, 3 dioxygenase as a tb biomarker

Tsolo, Semakaleng Theresia

2023

University of the Witwatersrand, Johannesburg

https://hdl.handle.net/10539/43144

Tsolo, Semakaleng Theresia. (2024). Evaluation of novel assay formats for indoleamine 2, 3 dioxygenase as a tb biomarker [Master's dissertation, University of the Witwatersrand, Johannesburg]. WireDSpace.

Downloaded from a DSpace repository. https://dspace.org/

UNIVERSITY OF WITWATERSRAND, JOHANNESBURG
FACULTY OF HEALTH SCIENCES
SCHOOL OF PATHOLOGY



EVALUATION OF NOVEL ASSAY FORMATS FOR INDOLEAMINE 2, 3 DIOXYGENASE AS A TB BIOMARKER

Semakaleng Theresia Tsolo

Student number: 2496186

Supervisor : Dr Heena Ranchod

Co-Supervisor: Associate Professor Melinda Suchard

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Science (Medicine) Vaccinology.

Johannesburg, 2023

DECLARATION

I, Semakaleng Theresia Tsolo declare that this Research Report is my own, unaided work. It is being submitted for the Degree of Masters of Science (Medicine) Vaccinology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

02 day of November 2023 in Polokwane

This research project is dedicated to my mothers, my husband, my son and daughter.

PRESENTATIONS

S.T. Tsolo, M. Suchard, H. Ranchod. *Evaluation of Novel Assay Formats for Indoleamine 2, 3 Dioxygenase as a Tb Biomarker*. Poster presentation, IXth Conference of the South African Immunology Society, 2 - 4 October 2022.

Table of Contents

DECLARATION ii
ABSTRACT vii
ACKNOWLEDGEMENTS viii
LIST OF FIGURES ix
LIST OF TABLES x
NOMENCLATURE/ ABBREVIATIONS xi
1 BACKGROUND 1
1.1 Mycobacterium tuberculosis 2
1.1.1 Diagnosis 3
1.2 Indoleamine 2, 3 dioxygenase 4
1.2.1 Indoleamine Dioxygenase 2, 3 as TB Biomarker 5
1.3 Dried Blood Spots 6
1.4 AIM 9
1.4.1 OBJECTIVES 9
2 METHODS 10
2.1 Ethical Approval 10
2.2 Method Optimization 10
2.3 Study Population 13
2.4 Sample Processing 13
2.5 ELISA-based determination of IDO activity using the Kyn/Tryp Ratio 13
2.5.1 Sample Preparation for ELISA 14
2.5.2 Kynurenine measurement 14
2.5.3 Tryptophan measurement 15
2.5.4 Kyn/tryp Ratio Calculation 16
2.6 Determination of IDO mRNA gene expression levels by real-time PCR 16
2.6.1 Sample Preparation 16
2.6.2 RNA Extraction 16
2.6.3 Real-time PCR 17
2.7 DATA ANALYSIS 17
3 RESULTS 18
3.1 Optimisation results 19
3.2 Determination of Kyn concentration in Plasma and DBS 24
3.3 Tryp concentration in Plasma and DBS 24
3.4 Comparison of kyn concentration in Plasma and DBS 24
3.5 Comparison of Tryp ELISA results between plasma and DBS 25
26
3.6 Comparison of kyn/tryp ratio between plasma and DBS 26
3.7 IDO mRNA gene expression in Whole Blood and DBS 27

3.8 Comparison of ΔCt (GAPDH) in whole blood and DBS 28
3.9 Comparison of ΔCt (BACT) in whole blood and DBS 29
3.10 Correlation of plasma IDO activity and IDO mRNA gene expression in whole blood 29
3.11 Correlation of IDO activity and IDO mRNA gene expression in DBS 30
4 DISCUSSION 32
5 CONCLUSION 35
6 REFERENCES 36

ABSTRACT

INTRODUCTION

The World Health Organization has prioritized the development of non-sputum-based assays that are capable of detecting active Tuberculosis (TB). Tryptophan (tryp) is converted to kynurenine (kyn) by the rate-limiting enzyme indoleamine 2, 3-dioxygenase (IDO). IDO activity may serve as a biomarker for active TB. Dried blood spots (DBS) can be collected outside of medical institutions and are simple to transport. We wanted to explore the use of DBS as an alternative sample type to measure the kyn/tryp ratio and IDO mRNA gene expression in healthy people.

METHODS

We optimised methods for elution of dried blood spots, exploring various elution buffers. Following method optimisation, we enrolled 40 healthy participants, and collected whole blood and DBS samples. Kyn and tryp concentrations were measured using ELISA (ImmuSmol, France). IDO mRNA gene expression was determined by real-time PCR using two housekeeping genes GAPDH and BACT. Statistical analysis was performed to determine the correlation and agreement between peripheral blood samples and DBS.

RESULTS

For IDO activity, tryp showed good agreement between plasma and DBS with a median percentage similarity of 91.1%. In contrast, no agreement was observed for kyn with a median percentage similarity of 56.6%. The kyn/tryp ratio performed poorly due to poor detection of kyn in DBS. Percentage similarity between whole blood and DBS IDO mRNA expression using GAPDH 87.1%, while using BACT was 84.6%. Using either traditional sample types or DBS, there was no correlation between IDO gene expression and kyn/tryp ratio.

CONCLUSION

We showed that tryp was measurable in DBS. Tryp in DBS was 91.1% similar to values in plasma. Despite method optimization, there was poor agreement between DBS and plasma for kyn. Although IDO mRNA gene expression was detectable in DBS, method agreement with whole blood was unsatisfactory. Alternative methods for the stabilization of kyn in DBS should be explored in future studies. IDO mRNA expression should be measured from whole blood in future studies.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my primary supervisors, Dr Heena Ranchod of the Department of Chemical Pathology, Faculty of Health Sciences, University of the Witwatersrand, and Dr Melinda Shelley Suchard of the Department of Chemical Pathology, Faculty of Health Sciences, University of the Witwatersrand. Dr Ranchod excellently guided me during the method optimization, validation, and testing of samples. Dr Suchard built my understanding of various concepts in immunology and statistics and my research skills.

I would also like to show our gratitude to the African Leadership in Vaccinology Expertise (Alive), School of Pathology, Faculty of Health Science, University of the Witwatersrand, who partially financed the research project.

My gratitude also goes to Mrs. Lillian Makhathini, Centre for Vaccines and Immunology, NICD, for sharing her knowledge and skills with me during the course of this research. Thank you for your time and assistance.

I would like to acknowledge all the staff at the Centre for Vaccines and Immunology and the National Institute for Communicable Diseases (NICD) for their assistance and pleasant welcomes.

I would like to thank all my colleagues and friends who gave me support and encouraged me.

LIST OF FIGURES

Figure 1 Stages of DBS processing: (A) DBS filter card filled with blood, (B) punched out DBS dispersed in elution buffer. (C) Eluate from DBS 18

Figure 2 Kyn concentration in DBS compared with plasma. (A) Passing and Bablok regression analysis for method agreement. (B) Bland Altman plot for plasma and DBS differences vs average 25

Figure 3 Tryp concentration in DBS compared with plasma. (A) Passing and Bablok regression analysis. (B) Bland Altman plot. 26

Figure 4 IDO activity comparison between Plasma and DBS. (A) Passing and Bablok regression analysis and (B) Bland Altman plot for method agreement. 27

Figure 5 Comparison of IDO/GAPDH in whole blood and DBS for IDO mRNA gene expression. (A) Passing and Bablok regression analysis. (B) Bland Altman plot 29

Figure 6 Whole blood and DBS IDO mRNA gene expression comparison using housekeeping gene BACT. (A) Passing and Bablok regression analysis. (B) Bland Altman plot 29

Figure 7 Correlation between IDO activity in plasma and IDO mRNA gene expression in whole blood. (A) using housekeeping gene GAPDH. (B) using housekeeping gene BACT 30

Figure 8 Correlation IDO activity and IDO mRNA gene expression in DBS. (A) using housekeeping gene BACT. (B) using housekeeping gene GAPDH 31

LIST OF TABLES

Table 1 Optimisation buffers for Kyn and Tryp measurement using ELISA.....10

Table 2 Optimisation buffers and conditions for IDO mRNA gene expression 12

Table 3 Kyn Standards.....15

Table 4 Tryp Standards.....15

Table 5 Demographic characteristics of participants.....18

Table 6 ELISA DBS elution buffer optimisation result 20

Table 7 Real-Time PCR DBS elution buffer optimisation results.....23

Table 8 Kyn concentration in Plasma and DBS.....24

Table 9 Tryp concentration in Plasma and DBS.....24

Table 10 Comparison of whole blood and DBS ΔCt (GAPDH).....27

Table 11 ΔCt IDO (BACT) in whole blood and DBS 28

NOMENCLATURE/ ABBREVIATIONS

Conc - Concentration

DBS – Dried Blood Spot

M.tb – Mycobacterium Tuberculosis

IDO – Indoleamine 2,3-dioxygenase

WB – Whole Blood

Kyn – Kynurenine

Tryp – Tryptophan

TB – Tuberculosis

HIV – Human Immunodeficiency Virus

HBV – Hepatitis B virus

HCV – Hepatitis C virus

RNA – Ribonucleic acid

WHO – World Health Organisation

Ct – Cycle Threshold

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

BACT –Beta Actin

TDO – Tryptophan 2,3-dioxygenase

LTBI – Latent Tuberculosis Infection

ELISA – Enzyme-linked immunosorbent assay

PCR – Polymerase chain reaction

% similarity – percentage similarity

mRNA – Messenger Ribonucleic acid

1 BACKGROUND

The WHO designated tuberculosis (TB) as a global health emergency in 1993 (Sakamoto *et al.*, 2019). One of the United Nations' Sustainable Development Goals (SDGs) targets a 90% reduction in TB-related mortality and an 80% reduction in TB incidence rates by 2030 (WHO, 2021). A target of 35% fewer TB deaths and 20% lower TB incidence rate was set for 2020 (Sakamoto *et al.*, 2019). Only a 9.2% reduction in TB deaths and an 11% decrease in TB-related new infections had been accomplished by 2020 (WHO, 2021). Various interventions are used to control and end the TB epidemic and they are not adequate. Currently, the only licensed vaccination for TB is the BCG vaccine, which was developed more than 70 years ago. *Mycobacterium tuberculosis* (*M. tb*) spread is unaffected by the BCG vaccine. Additionally, BCG protects against severe tuberculosis in children but only provides limited protection against adult pulmonary tuberculosis (Sakamoto *et al.*, 2019).

Diagnostics in low-income countries are inadequate, with only half of all estimated TB cases being correctly diagnosed (WHO, 2021). Prolonged TB treatment regimens lead to poor compliance and termination of treatment, with the possibility of drug resistance. Although drug-resistant TB strains are treatable with second line drugs, their presence renders efforts to eradicate TB futile (Singh and Chibale, 2021). There is also a lack of reliable biomarkers to monitor the success of drug therapy. It is vital to understand the interaction between the human immune response and the pathogen *M. tb* (Gengenbacher and Kaufmann, 2012).

In children, TB is particularly difficult to diagnose and has become a neglected area of the TB epidemic (Esposito, Tagliabue and Bosis, 2013). Many TB cases in children are undiagnosed and inadequately treated. In 2016, only 43% of the projected 1 million paediatric TB cases were reported to national TB programs (Sakamoto *et al.*, 2019). The presentation of TB disease in children varies and is usually smear-negative with non-specific clinical and radiological signs. A timely diagnosis is imperative. Active TB in infants and young children can result in severe forms of TB due to children’s underdeveloped immune systems (Esposito, Tagliabue and Bosis, 2013). New diagnostic tests and optimal treatment for children are urgently needed. Also required is development of child-friendly sampling techniques such as dried blood spots (DBS), which is a minimally invasive specimen collection technique that uses a finger prick or heel prick. (Esposito, Tagliabue and Bosis, 2013).

1.1 Mycobacterium tuberculosis

Mycobacterium tuberculosis (M. tb), which causes TB infection and disease in humans, was discovered by Robert Koch in 1882 (Koch, 1882). Infection with TB is contracted by inhaling aerosolized droplets containing *M. tb* from an infected person (Mwandumba *et al.*, 2004). The *M. tb* travels to the lungs, where it encounters highly differentiated mononuclear cells, known as alveolar macrophages. Alveolar macrophages are able to internalise particulate material and deliver it to acidic, degrading lysosomes (Zhai *et al.*, 2019). Alveolar macrophages are the first professional phagocytes to engulf *M. tb* (Mwandumba *et al.*, 2004). The phagocytosed *M. tb* resides in the macrophages and blocks maturation, lysosomal fusion, and acidification, as well as preventing the exposure of phagosomes containing *M. tb* to inducible nitric oxide synthase (iNOS) (Upadhyay, Mittal and Philips, 2018). The macrophages that engulf *M. tb* provide a protective and hospitable environment for *M. tb* to thrive. Different outcomes may occur following exposure to *M. tb*: 1) exposure to *M. tb* but no infection acquired, 2) infection acquired with a rapid immune response and clearance of the infection, 3) containment of the infection with the absence of symptomatic disease (latent tuberculosis infection (LTBI)), and 4) development of active TB disease (Goletti *et al.*, 2016).

The formation of granulomas is the hallmark of TB. Granulomas are aggregates of immune cells that ensure containment of *M. tb* by preventing its dissemination in the host in early TB infection (Ulrichs and Kaufmann, 2006). In 90% of infected people, *M. tb* within the granuloma will remain quiescent, leading to an equilibrium state between the bacteria and the host called latency or dormancy (Barac *et al.*, 2019). The metabolic states and effector molecules characterize alveolar macrophages into classically activated (M1) or alternatively activated (M2). These two alveolar macrophage subtypes regulate the homeostatic state. iNOS-expressing macrophages are classified as M1, and arginine-expressing macrophages are known as M2 (Shaun Lott, 2020). During the latency state, a symbiotic relationship is maintained between the host and *M. tb,* leading to bacterial persistence (Suchard *et al.*, 2020). M1 macrophages are stimulated by T-helper-1 immune responses that assist in suppressing TB disease progression through macrophage antibacterial products. The macrophage antibacterial products include reactive oxygen species

(ROS), nitric oxide (NO), and the production of pro-inflammatory cytokines (Briken *et al.*, 2018).

M2 macrophages are critical for the anti-inflammatory impact because they promote inflammation resolution and prevent an intensified, persistent inflammatory state. Therefore M2 macrophages balance the function of the M1 macrophages. M2 macrophages support the T-helper-2 immune response and have immunosuppressive and healing effects (Briken *et al.*, 2018). The metabolic dispositions that favour M2 macrophages lead to a tryptophan (tryp) catabolic pathway and the formation of kynurenine (kyn). The enzyme indoleamine 2, 3 dioxygenase catabolizes tryp to kyn (Lee *et al.*, 2014).

1.1.1 Diagnosis

When diagnosing TB, the history of exposure is also taken into consideration, symptom monitoring, examination, radiographic examination and special investigations. The detection of bacteria via a number of methods from various sample types such as sputum, pleural fluid and cerebrospinal fluid assist in diagnosis of TB. Identification of acid-fast bacilli in sputum or other fluids indicates an *M .tb* infection (Turner *et al.*, 2020). The WHO has recommended the use of nucleic acid amplification-based tests such as Xpert MTB/RIF (Cepheid) for TB diagnosis and detection of TB drug resistance (WHO, 2021).

TB diagnostic tests that target the host-response include the tuberculin skin test (TST) and interferon gamma release assays (IGRAs). The cytokine IFN-gamma, produced by specific T cells that recognize M.tb, is measured in IGRAs (Sia and Rengarajan, 2019). The TST is a form of hypersensitivity reaction test; however, positive results are relatively non-specific for *M. tb* (Turner *et al.*, 2020). One of the greatest limitations of the TST and interferon-release assays is that they cannot differentiate LTBI from active TB disease (Barac *et al.*, 2019). Another limitation of TST is cross-reactivity with the Bacillus Calmette–Guérin (BCG) vaccine and non-tuberculous mycobacterial infections (Rakotosamimanana *et al.*, 2015), necessitating adjustment of cut-offs based on local epidemiology.

Simple, rapid biomarkers that can detect active TB disease can lead to early diagnosis and treatment, with better chances of completely curing TB in diagnosed cases. New tests should be based on easily accessible sample types other than

sputum and should have utility in areas with high TB burdens (Gengenbacher and Kaufmann, 2012). New approaches to controlling the spread of TB are needed, including tools to predict the development of an active TB from LTBI (Esposito, Tagliabue and Bosis, 2013). The development of a non-sputum-based diagnostic assay can help alleviate the challenges encountered with sputum collection, especially in infants, young children, and frail people (Turner *et al.*, 2020).

1.2 Indoleamine 2, 3 dioxygenase

Indoleamine 2, 3-dioxygenase (IDO) is an inducible enzyme that converts tryp into kyn (Cao *et al.*, 2022). IDO is found predominantly in the lungs, placentas and macrophages while a related enzyme, tryptophan dioxygenase (TDO) is found in the liver (Tanner *et al.*, 2014). In plasma, 5–10% of tryp is free, while the rest is usually bound to albumin (Badawy and Guillemin, 2019). In normal physiological states, 90% of dietary tryp is catabolized in the liver through the kyn pathway. The extra-hepatic kyn pathway accounts for 2% of tryp degradation (Badawy and Guillemin, 2019). In the liver TDO is upregulated when tryp is in excess and converts tryp to N-formylkynurenine. The cytokine interferon gamma induces IDO expression, which highlights the immunologic significance of this enzyme (Badawy and Guillemin, 2019).

Tryp is an essential amino acid that humans obtain from their diet (Borghi *et al.*, 2020). Amino acids are not only protein building blocks, but they are also essential in the regulation of cellular responses via nutrient sensor signalling. Amino acid concentration adjustments regulate immune cells response to infection (Cao *et al.*, 2022). Following infection, amino acid metabolism is usually affected (Crowther and Qualls, 2021). Interferon gamma-induced depletion of cellular tryp is one of the innate immune system's responses to microbial infection (Shaun Lott, 2020). Metabolite changes present potential diagnostic tools as *M. tb* biomarkers (Moreira *et al.*, 2021).

Tryp is essential for bacterial growth, yet pathogenic microorganisms are unable to synthesize tryp (Shaun Lott, 2020). Tryp deprivation affects bacteria with respect to their microbial energy consumption and metabolic activity, leading to autophagy and the elimination of intracellular bacteria (Sia and Rengarajan, 2019). However, M. tb is able to synthesize its own tryp which may suggest why tryp deprivation does not kill

M. tb (Zhang *et al*., 2014). Interferon-gamma-induced IDO activity and tryp deprivation fail to exert an anti-mycobacterial effect on *M. tb* (Sakamoto, 2012).

The tryp-kyn pathway leads to immunosuppression and tolerance due to the potent immunoregulatory effect on T-cells induced by upregulated IDO (Adu-Gyamfi *et al.*, 2020). The mechanism of how tolerance is induced by IDO is not elucidated, however, a theory has been postulated: T-cells are starved of micronutrients through the depletion of tryp, affecting the T-cells' ability to proliferate and function (Ravishankar *et al.*, 2012). The tryp metabolites such as kyn metabolites bind to receptors on T-cells or other immune cells, resulting in suppression of the T-cell response (Mellor *et al.*, 2017). Kyn further promotes T-cell stress, anergy, and cell death (Roy *et al.*, 2019).

1.2.1 Indoleamine Dioxygenase 2, 3 as TB Biomarker

IDO plays a crucial role in pregnancy by maintaining maternal-foetal tolerance through immunoregulatory effects (Tanner *et al.*, 2014). IDO is also involved in various diseases through the regulation of immune responses. For example, IDO has been used as a prognostic marker for cancer. In certain cancers, elevated IDO activity was associated with a poor prognosis (Suzuki *et al.*, 2012).

IDO activity, measured as the kyn/tryp ratio, was suggested to be a potential biomarker for TB diagnosis. IDO may play a crucial role in the pathogenesis of TB disease (Suchard *et al.*, 2020). Decreased tryp and increased levels of kyn were observed in the sera and urine of individuals with active TB disease compared to healthy controls (Weiss and Schaible, 2015). Additional studies observed low levels of tryp and high concentrations of kyn in the sera (Vrieling *et al.*, 2019) and pleural fluid (Yuzo Suzuki et al., 2013) of TB patients.

In the literature, an increase in IDO activity was observed in MDR-TB patients compared to drug-sensitive TB patients. The IDO activity increase was associated with lung cavity lesions in patients with TB (Shi *et al.*, 2019). This study concluded that plasma IDO activity may be used as a complementary tool for diagnosis and prognosis (Shi *et al.*, 2019). In other work, tryp and kyn were the most significantly altered metabolites between people with and without active TB (Collins *et al.*, 2020). A decrease in the kyn/tryp ratio was observed in people undergoing TB treatment

(Collins *et al.*, 2020). The study concluded that accurate classification of TB disease may be performed using the kyn/tryp ratio.

IDO-1 and IDO-2 are closely related enzymes that catabolize tryp to kyn. However, they differ in immunomodulatory functions with IDO-1 observed in immune-tolerance and suppression whilst IDO-2 was observed to have proinflammatory effect (Merlo L.M.F. and Mandik-Nayak L., 2016). Using whole blood transcriptomics to evaluate expression of IDO-1 and IDO-2 in people with active TB and people with LTBI, IDO-1 transcripts were significantly increased in active TB. IDO-1 expression was heterogeneous in people with LTBI (Collins et al., 2020). In children, no significant differences in the expression of IDO-1 were observed between children with active TB and LTBI. However, expression of IDO-1 was significantly increased in children with active TB or LTBI compared to controls without active TB (Collins *et al.*, 2020).

1.3 Dried Blood Spots

Blood biomarkers that represent the TB bacterial burden or immune response to *M. tb* are needed to monitor and tailor treatment for people who have had a TB exposure (McNerney *et al.*, 2012). The biomarkers can be used to improve current diagnostic tests by detecting the early progression of tuberculosis infection in cases where *M. tb* has not been found or in people with suspected tuberculosis but a negative sputum result.

A dried blood spot (DBS) is a minimally invasive specimen collection technique that uses a finger prick or heel prick. The collected blood is dried on a filter paper card (Malsagova *et al.*, 2020). DBS can be used in areas where transportation of samples is a challenge, or where there are no facilities or a lack of skilled professionals to take a venous blood sample. The use of microsamples such as DBS is commonly observed in newborn screening programs for metabolic disorders. Robert Guthrie was the first to screen neonates for phenylketonuria in 1963 (Neesgaard et al., 2018). Since then, the use of DBS has expanded to include serological tests such as enzyme-linked immunosorbent assay (ELISA), molecular tests such as polymerase chain reaction (PCR) and immunological studies (Gupta *et al.*, 2020). Molecular assays for viral genes are the gold standard for diagnosis of infectious diseases however use of serological assays is important in mass population testing and response to outbreaks and pandemics. In serological testing, DBS has been used to detect infectious diseases such as hepatitis B virus (HBV), syphilis, hepatitis C virus

(HCV), human immunodeficiency virus (HIV), rubella, and measles (Freeman et al., 2018). DBS samples have shown good sensitivity and specificity in the diagnosis of HIV, HBV and HCV in infants (Tuaillon *et al.*, 2020).

DBS has been successfully used to quantify the concentration of HCV-RNA in DBS samples, dried at 22^{o}C - 24^{o}C and sent by post. Although the concentrations of HCV-RNA from DBS correlated with plasma concentrations, the concentrations of DBS HCV-RNA were lower compared to plasma (Neesgaard *et al.*, 2018). The WHO has endorsed the use of DBS for the improvement of diagnostics for HIV as well as HCV and HBV in hard-to-reach populations (Tuaillon *et al.*, 2020). The Centres for Disease Control and Prevention (CDC) concluded that DBS samples have shown the same level of precision and reproducibility as conventional venous samples (Whittaker *et al.*, 2021).

The COVID-19 pandemic sped up the use of home sample collection and testing. Increasing interest in DBS samples during the pandemic resulted in a rise in studies that detected SARS-CoV-2 antibodies using DBS (Meyers *et al.*, 2021). Various surveys and pilot studies have been conducted during the COVID-19 pandemic. In one study, DBS showed specificity of 97.1% to 98.8% and sensitivity of 95% to 97.1% for detection of SARS-CoV-2 IgG antibodies in nursing home staff and residents. This makes DBS an appropriate sample type for the elderly (Meyers *et al.*, 2021). DBS samples can also be used to measure biomarkers for non-infectious diseases, demonstrating their versatile use within a clinical setting. In a study conducted in China, a total of 12 compounds were screened for their ability to identify cardiovascular disease. The results from the DBS samples for the detection of 12 biomarkers showed sensitivity of 77.0% and assisted in characterizing different types of cardiovascular disease (Liu *et al.*, 2020).

The finger-prick sampling method reduces the risk of infection posed by infectious samples to laboratory personnel (Malsagova *et al.*, 2020). DBS requires only a small volume of samples and, in some studies, improves analyte stability (Lim, 2018). In large epidemiological studies, the use of DBS has mitigated challenges such as obtaining and transporting biological samples. DBS can be used for self-sampling. In a study conducted by Sakhi et al., 2015, 90% of self-sampled DBS samples were considered valid for analysis. DBS has the potential to be used as an appropriate sampling method for various blood analytes, including TB blood biomarkers.

DBS samples that are self-sampled or collected outside healthcare facilities have been described as an alternative to rapid diagnostic tests (RDTs) (Tuaillon *et al.*, 2020). RDTs are commonly used in Africa for HIV screening due to underdeveloped laboratories in geographically inaccessible areas. Some reported disadvantages associated with RDTs are poor sensitivity with unsatisfactory positive predictive values in low incidence areas and a lack of screening for other co-infections transmitted through similar routes as HIV, such as HBV and HCV (Kania *et al.*, 2013). The use of DBS can mitigate the limitations of RDTs because they can easily be collected, stored, and transported to more advanced laboratories where specialized confirmatory tests can be conducted (Björkesten *et al.*, 2017).

Testing performed on DBS may be less expensive compared to venous blood or other invasive sample types. Studies have shown that switching to DBS home sampling in two paediatric groups showed a significant cost reduction of 43% for haemato-oncology patients and 61% for nephrology patients of the total costs per blood draw (Martial *et al.*, 2016). DBS sampling eliminates the need for specialized sample transportation and storage facilities (Kania *et al.*, 2013). DBS can have stable measurements after decades depending on the storage temperature, with a shelf half-life of 10 years observed for some blood proteins (Björkesten *et al.*, 2017).

The ease of collection makes DBS a preferred sampling method for screening new-borns for viral infections such as congenital cytomegalovirus (Dollard *et al.*, 2021). Incorporation of screening TB biomarkers on DBS collected from children exposed to TB in households as well as people in remote areas would be of tremendous value in controlling TB, as LTBI infection could be identified and monitored early.

We hypothesized that using DBS to measure the kyn and tryp ratios may be an effective sampling method for measuring IDO activity. The development of the method may play a useful role in TB diagnostics if validated by clinical studies.

We compared samples of venous blood and DBS to explore agreement between sample types for measurement of kyn and tryp by ELISA and measurement of IDO mRNA by real-time PCR.

1.4 AIM

This research project aimed to investigate the use of dried blood spots for determining IDO activity and IDO mRNA expression levels in healthy individuals. The specific focus was on investigating whether IDO measured from fresh peripheral blood samples was comparable to dried blood spots.

1.4.1 OBJECTIVES

1) To measure and compare tryp concentrations in plasma and DBS using ELISA.
2) To measure and compare kyn concentrations in plasma and DBS using ELISA.
3) To measure and compare the kyn/tryp ratio in plasma and DBS
4) To measure and compare IDO mRNA expression levels by real-time PCR in whole blood and DBS.
5) To investigate whether IDO mRNA expression levels measured by real-time PCR correlate with IDO activity levels measured by ELISA in whole blood and DBS.

2 METHODS

IDO activity can be measured through the kyn/tryp ratio in serum or plasma. Alternatively, IDO mRNA expression can be measured in nucleic acid isolated from whole blood or PBMCs (Mancuso *et al.*, 2015).

ELISA is an immunoassay technique used for detecting and quantifying the presence of antigens, antibodies, proteins or hormones in biological specimens. In this study, a competitive ELISA was used for the determination of kyn and tryp concentrations. The competitive ELISA relies on the competitive binding of the primary antibody between the target antigen in a sample and the solid phase-bound antigen on the microtiter plate (Alhajj and Farhana, 2022).

In this study, we compared IDO activity in plasma and dried blood spots by measuring kyn and tryp concentrations using ELISA. We measured IDO mRNA expression in whole blood and dried blood spots using quantitative real-time PCR.

2.1 Ethical Approval

Ethical clearance for the proposed study was obtained from the Human Research Ethics Committee of the University of the Witwatersrand Medical School, Johannesburg (Ethics clearance certificate: **M211186, Appendix A)**.

2.2 Method Optimization

Due to the lack of available studies using DBS for tryp and kyn measurement and IDO mRNA gene expression, analytical optimization was performed. We optimized storage of DBS at various temperatures and explored various types and volumes of elution buffers. The components of the elution buffers included proteins Tween 20 and Bovine Serum Albumin (BSA), which are known to improve assay signal by stabilizing proteins and blocking non-specific binding sites (Grüner, Stambouli and Ross, 2015). (See table 1).

Table 1 Optimisation buffers for Kyn and Tryp measurement using ELISA

Elution Buffer Name	**DBS filter card Storage Conditions**	**Elution Buffer Volume And Spots**	**Incubation Time**

Elution Buffer Name	DBS filter card Storage Conditions	Elution Buffer Volume And Spots	Incubation Time
PBS	Stored in a foil bag with a desiccant at room temperature	1 spot and 250µl elution buffer	15-18hrs at 600rpm at room temperature.
25ml PBS+0.05% Tween 20+0.08% Sodium Azide, **25ml PBS** + 0.05% Tween 20	Stored in a foil bag with a desiccant at room temperature	1 spot and 250µl elution buffer	15-18hrs at 600rpm at room temperature.
PBS volume was 25ml in all elution buffer prep **PBS,** **PBS**+0.1% Tween 20, **PBS+**0,05% Tween 20, **PBS**+0.05%Tween20+ 2%BSA, **PBS**+0.05% Tween 20+ 0.08% Sodium Azide, **PBS**+0.05% Tween 20 +1%BSA+ 0.1% Triton X-100	Stored in a foil bag with a desiccant at room temperature	1 spot and 250µl elution buffer	15-18hrs at 600rpm at room temperature.
PBS volume was 25ml in all elution buffer prep **PBS,** **PBS**+0.1% Tween 20, **PBS**+0,05% Tween 20, **PBS**+0.05% Tween20+2%BSA, **PBS**+0.05% Tween 20+ 0.08% Sodium Azide, **PBS**+0.05% Tween 20 +1%BSA+ 0.1% Triton X-100,	Stored in a foil bag with a desiccant at room temperature	1 spot and 100µl elution buffer. 2 spots and 200µl PBS elution buffer	15-18hrs at 600rpm at room temperature
PBS, **PBS**+0.1% Tween 20, **PBS**+0,05% Tween 20, **PBS**+0.05% Tween20+2%BSA, **PBS**+0.05% Tween 20+ 0.08% Sodium Azide, **PBS**+0.05% Tween 20 +1%BSA+ 0.1% Triton X-100,	Stored in a foil bag with a desiccant at room temperature	2 spots and 200µl elution buffer	15-18hrs at 600rpm at room temperature
PBS+0.05% Tween 20, **PBS**+ 0.05% Tween 20, 1% BSA, 0.01% Triton X-100.	Stored in a foil bag with a desiccant at room temperature	2 spots and 200µl elution buffer 1 spot and 120µl elution buffer	15-18hrs at 600rpm at room temperature
PBS+ 0.05% Tween 20, 1% BSA, 0.01% Triton X-100.	Stored in a foil bag with a desiccant at room temperature for 1 week	1 spot and 120µl elution buffer	2hrs 4hrs 15-18hrs All at 600 rpm at room temperature
PBS+ 0.05% Tween 20, 1% BSA,	Stored in a foil	1 spot and	2hrs

Elution Buffer Name	DBS filter card Storage Conditions	Elution Buffer Volume And Spots	Incubation Time
0.01% Triton X-100.	bag with a desiccant at 4^{o}C fridge for 1 week.	120µl elution buffer	4hrs 15-18hrs All at 600 rpm at room temperature
PBS+ 0.05% Tween 20, 1% BSA, 0.01% Triton X-100.	Stored in a foil bag with a desiccant at minus (-) 20^{o}C freezer for 1 week.	1 spot and 120µl elution buffer	2hrs 4hrs 15-18hrs All at 600 rpm at room temperature

For PCR we explored various volumes and types of elution buffers (See table 2).

Table 2 Optimisation buffers and conditions for IDO mRNA gene expression

DBS card (Whatman no. 903) conditions	Elution Buffer name	Elution buffer volume and spots	Incubation Time and conditions
none	PBS	250µl and 1 spot	1 hr at 600rpm at room temperature 15-18hrs at 600rpm at room temperature
none	PBS	250µl and 1 spot	15-18hrs at 600rpm at room temperature
1. No RNALater added (normal spot). 2. RNALater added on empty spot 24hrs before spotting blood. 3. RNALater added immediately after spotting blood on card. 4. RNALater added 4hrs later after spotting blood on card.	PBS	250µl and 1 spot	1hr at 600rpm shaker at room temperature
1. No RNALater added (normal spot). 2. RNALater added on empty spot 24hrs before spotting blood. 3. RNALater added immediately after spotting blood on card. 4. RNALater added 4hrs	PBS	250µl and 1 spot	15-18hrs at 600rpm at room temperature

later after spotting blood on card			
1. No RNALater added (normal spot). 2. RNALater added on empty spot 24hrs before spotting blood. 3. RNALater added immediately after spotting blood on card. 4. RNALater added 4hrs later after spotting blood on card	PBS Lysis solution and sodium acetate from RiboPure RNA extraction kit	250µl and 1 spot 800µl lysis solution+50µl sodium acetate and 1 spot.	15-18hrs at 600rpm at room temperature

2.3 Study Population

The study group included 40 healthy adults (>18 years), both males and females. A questionnaire was used to determine whether to exclude potential participants. Individuals with recent TB exposure, people living with HIV, and pregnant women were excluded from the study. Patient recruitment, sample collection, and experimental procedures were conducted at the Centre for Vaccines and Immunology, National Institute for Communicable Diseases, Johannesburg.

2.4 Sample Processing

Capillary blood from a finger puncture was spotted onto a perforated Whatman 903 Protein Saver filter card (a dried blood spot). The spotted blood was dried in a biosafety class II cabinet for a minimum of 4 hours, then stored at room temperature (22°C –24°C) in a foil zipper bag containing a desiccant.

From the 5 mL EDTA tube with whole blood, 500 µl of whole blood was aliquoted into 2 x 1.5 mL microtubes. A volume of 1 ml of RiboPure^{TM} RNA Later (Thermofisher) was added to two microtubes containing whole blood to inactivate RNAse and preserve RNA and protein integrity for downstream analysis. The mixture was vortexed for 15 seconds. Microtubes were stored overnight at 4°C and transferred to the -70°C freezer the next day. The remaining blood was centrifuged for 10 minutes at 3000 g. The plasma (top clear layer) was collected and divided between 2 x 1.5 mL labelled microtubes. The plasma was frozen at -70°C until further use.

2.5 ELISA-based determination of IDO activity using the Kyn/Tryp Ratio

We used a commercial ELISA kits (ImmuSmol, Inc., France) to determine the concentrations of kyn and tryp in plasma samples.

2.5.1 Sample Preparation for ELISA

Frozen plasma specimens were thawed at room temperature. A dried blood spot was eluted using a freshly prepared elution buffer. Various elution buffers were investigated during the optimization step. For the participant DBS samples, the elution buffer was prepared using final concentrations of 0.05% Tween 20, 0.1% Triton X-100, and 1% Bovine Serum Albumin (BSA), in 25 ml of phosphate buffered saline (PBS). This solution was left in the refrigerator until the BSA had dissolved. The dried blood spot was punched out along the perforated line. A volume of 120 µl of elution buffer was added to one punched-out, dried blood spot in a microtube. The dried blood spot with the elution buffer was incubated at room temperature on a 600 rpm thermo shaker incubator (Allsheng, China) for 17 hours.

After incubation, the filter paper was removed from the microtube using forceps, removing any excess eluate absorbed by the filter paper. An eluate of 50–75µl was obtained following the elution of the dried blood spot. The eluted sample was centrifuged at 3000 g for 10 minutes to obtain the final sample to be used for the ELISA.

2.5.2 Kynurenine measurement

Plasma and DBS samples were acylated as per the manufacturer's instructions. Acylation allows improved detection of small analytes and improved stability (Orata, 2012). Acylated plasma and DBS samples and rabbit anti-kyn antibody are added to antigen precoated kyn microwell plate. Acylated samples and the solid-phase-bound analyte compete for a fixed number of antibody binding sites on the rabbit anti-kyn antibody over a 17-hours (overnight) incubation period at 2–8 °C. Free antigen and free antigen-antibody complexes were removed by washing four times with the wash buffer. The antibody bound to the solid phase was detected with a goat anti-rabbit IgG-peroxidase conjugate using tetramethylbenzidine (TMB) as substrate for a chromogenic signal. The reaction was measured at 450 nm using BioTek 800TS microplate reader (Agilent, United States). Unknown samples were quantified by comparing their absorbance to a standard curve prepared using known standards, see table 3 below.

Table 3 Kyn Standards

Standards (Kyn)	Concentrations (ng/ml)
Standard A	0
Standard B	100
Standard C	300
Standard D	1000
Standard E	3000
Standard F	10000

2.5.3 Tryptophan measurement

Samples from plasma and DBS were derivatized per manufacturer instructions. A derivatization treatment was required as part of the ELISA process to allow the tryp to be recognized by the commercial antibody (Orata, 2012). Derivatized plasma and DBS samples and rabbit anti-tryp antibody are added to antigen precoated tryp microwell plate. The derivatized samples and the solid-phase-bound analyte competed for a fixed number of antibody binding sites on the rabbit anti-tryp antibody over a 17-hours (overnight) incubation period at 2–8 °C. Free antigens and free antigen-antibody complexes were washed off three times using the wash buffer. The antibody bound to the solid phase was then detected with a goat anti-rabbit IgG-peroxidase conjugate using TMB as substrate for a chromogenic signal. The reaction was measured at 450 nm using a BioTek 800TS microplate reader (Agilent, United States). Unknown samples were quantified by comparing their absorbance to a standard curve prepared using known standards, see table 4 below.

Table 4 Tryp Standards

Standards (Tryp)	Concentrations (ng/ml)
Standard A	0
Standard B	2500
Standard C	7500

Standard D	25000
Standard E	75000
Standard F	250000

2.5.4 Kyn/tryp Ratio Calculation

Plasma IDO activity was calculated as the ratio of kyn (the product) to tryp (the substrate). The kyn and tryp concentrations were converted to the same unit of measurement (ng/ml) to facilitate calculation of the kyn/tryp ratio; therefore, the plasma kyn/tryp ratio was written with no unit, per the formula below.

Kyn/tryp ratio = kyn (ng/ml)/tryp (ng/ml)

2.6 Determination of IDO mRNA gene expression levels by real-time PCR

2.6.1 Sample Preparation

For the participant samples, one punched-out dried blood spot was eluted using 800µl of lysis solution and 50µl of sodium acetate and incubated at 600 rpm on a thermo shaker incubator (Allsheng, China) for approximately 16 hours (overnight). Frozen whole blood samples were thawed at room temperature on the day of extraction.

2.6.2 RNA Extraction

RNA was extracted from whole blood and DBS eluate using the RiboPure™ RNA Purification Kit (Thermofisher Scientific's, United States). The amount of extracted RNA and water to be added to the total volume of 20µl per tube was determined to ensure a standard quantity of 100ng of RNA was added for cDNA synthesis. Invitrogen™ SuperScript™ IV First-Strand Synthesis System (Thermofisher Scientific, United States) reagent mixtures one and two were added to the mixture of RNA extract and water according to the manufacturer's instructions. The mixture was run on the GeneAmp 9700 PCR System Thermocycler (Applied Biosystems) at an initial temperature of 23 °C for 10 minutes, then at 50 °C for 10 minutes, and at 80 °C for 10 minutes. A volume of 2µl RNAseH was added to the samples, which were then incubated at 37°C for 20 min. The cDNA was used for real-time PCR.

2.6.3 Real-time PCR

Real-time PCR experiments were performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystems) using pre-designed primers (Applied Biosystems) (Mancuso *et al.*, 2015). Two housekeeping genes with IDO (assay ID: Hs00984148_m1) were used as targets. Each microtiter plate well contained cDNA, Gene Expression Master Mix (Applied Biosystem), FAM-labelled IDO probe, either VIC-labelled Actin beta (BACT) or Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping genes, and water. The PCR cycles were as follows: 10 minutes at 95°C are followed by 40 cycles of 15°C at 95°C and 1 minute at 60°C. All reactions were performed in triplicate.

Delta Cycle Threshold (ΔCt) Calculation

The Ct is the number of cycles required for the fluorescent signal to cross the threshold. For each sample, the relative gene expression of IDO mRNA was calculated, relative to an endogenous reference gene.

Formula:
Ct IDO - Ct GAPDH = Δ Ct (GAPDH)
OR Ct IDO - Ct BACT = Δ Ct (BACT)

2.7 DATA ANALYSIS

Statistical analysis was performed using GraphPad Prism 8 and Excel (Microsoft). The Bland-Altman plot and analysis was used to evaluate the bias (mean difference) between the two sampling techniques (DBS and plasma). Passing and Bablok regression was used to evaluate the relationship and agreement between the two sampling methods. The percentage (%) similarity was calculated using Excel (Microsoft). The % similarity was calculated by dividing the average of the DBS value and the plasma or whole blood value by the plasma or whole blood value. The result was multiplied by 100 to obtain a percentage. Any % similarity greater than 100% indicated that the DBS value was larger than the plasma or whole blood value while % similarity less than 100% meant the DBS value was lower than the plasma or whole blood value.

% Similarity formula: [({A+B}/2)/A]x100

Where: A = plasma value/whole blood value

B = DBS value

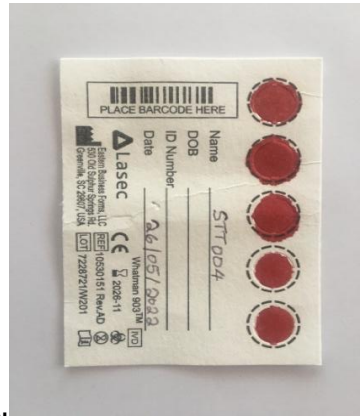
3 RESULTS

Forty participants were recruited to evaluate the use of dried blood spot for determining IDO activity in healthy individuals. We investigated whether IDO measurement from fresh peripheral blood samples was comparable to dried blood spots. The demographic characteristics of the participants are summarised in Table 5.

Table 5 Demographic characteristics of participants

CHARACTERISTICS				
Race	***Female (n)***	***Male (n)***	***Median Age***	***Total***
Asian	4	0	30,5	**4 *(10%)***
African	14	9	33	**23 *(58%)***
Coloured	1	0	32	**1 *(2%)***
Caucasian	9	3	31,5	**12 *(30%)***
Median Age	32,5	30		

The DBS samples were collected, stored, and processed before the ELISAs were performed. Figure 1 illustrates the different phases of DBS processing.

A. 

B. 
C. 

Figure 1 Stages of DBS processing: (A) DBS filter card filled with blood, (B) punched out DBS dispersed in elution buffer. (C) Eluate from DBS

3.1 Optimisation results

For storage of DBS, the sample stored at room temperature showed the best results compared with 4^{o}C or -20^{o}C. For the ELISA for tryp and kyn measurements, DBS samples eluted with 120µl elution buffer (PBS, 0.05% Tween 20, 1% BSA, 0.01% Triton X-100) and incubated for 17-hours on a shaker showed the best results. Table 6 demonstrates results of the various elution buffers explored for this study.

Table 6 ELISA DBS elution buffer optimisation result

Elution Buffer Name	**DBS filter card Storage Conditions**	**Elution Buffer Volume And Spots**	**Incubation Time**	**Kyn Result**	**Tryp Result**
PBS	Stored in a foil bag with a desiccant at room temperature	1 spot and 250µl elution buffer	15-18hrs at 600rpm at room temperature.	Too low to be detectable on DBS samples	DBS, serum and Plasma were in agreement.
25ml PBS+0.05% Tween 20+0.08% Sodium Azide, **25ml PBS** + 0.05% Tween 20	Stored in a foil bag with a desiccant at room temperature	1 spot and 250µl elution buffer	15-18hrs at 600rpm at room temperature.	Too low to be detectable on DBS samples	DBS, serum and Plasma were in agreement
PBS volume was 25ml in all elution buffer prep **PBS,** **PBS**+0.1% Tween 20, **PBS**+0,05% Tween 20, **PBS**+0.05% Tween20+2%BSA, **PBS**+0.05% Tween 20+ 0.08% Sodium Azide, **PBS**+0.05% Tween 20 +1%BSA+ 0.1% Triton X-100,	Stored in a foil bag with a desiccant at room temperature	1 spot and 100µl elution buffer. 2 spots and 200µl PBS elution buffer	15-18hrs at 600rpm at room temperature	Kyn conc was measured in some samples. However in some the conc was too low to be detected due to some eluted samples not being enough for both kyn and tryp ELISA. Kyn results were better as kyn was measurable in 2 spots with 200µl PBS as elution buffer.	Tryp conc was measured in all DBS samples. Tryp conc was higher in 2 spots with 200µl PBS as elution buffer
PBS, **PBS**+0.1% Tween 20, **PBS**+0,05% Tween 20, **PBS**+0.05% Tween20+2%BSA, **PBS**+0.05% Tween 20+ 0.08% Sodium Azide, **PBS**+0.05% Tween 20 +1%BSA+ 0.1% Triton X-100,	Stored in a foil bag with a desiccant at room temperature	2 spots and 200µl elution buffer	15-18hrs at 600rpm at room temperature	Kyn conc measurable in spots eluted with elution buffers; PBS+0.05% Tween 20 and PBS+0.05% Tween 20+ 2% BSA + 0.01% Triton X-100	Tryp conc was measurable in all DBS samples
PBS+0.05% Tween 20,	Stored in a foil bag	2 spots and	15-18hrs at 600rpm	kyn conc was	Tryp conc was

Elution Buffer Name	DBS filter card Storage Conditions	Elution Buffer Volume And Spots	Incubation Time	Kyn Result	Tryp Result
PBS+ 0.05% Tween 20, 1% BSA, 0.01% Triton X-100.	with a desiccant at room temperature	200µl elution buffer 1 spot and 120µl elution buffer	at room temperature	measurable in samples eluted with both 120µl elution buffer with 1 spot and 200µl elution buffer with 2 spots.	measurable in both elution buffers.
PBS+ 0.05% Tween 20, 1% BSA, 0.01% Triton X-100.	Stored in a foil bag with a desiccant at room temperature for 1 week	1 spot and 120µl elution buffer	2hrs 4hrs 15-18hrs All at 600 rpm at room temperature	Kyn conc was measurable in all samples incubated at varying times with the elution buffer. 17-hrs incubation on a shaker showed better results than all the different storage and incubation times.	Tryp conc was measurable in all samples. Sample incubated for 17-hrs showed better results.
PBS+ 0.05% Tween 20, 1% BSA, 0.01% Triton X-100.	Stored in a foil bag with a desiccant at 4^{o}C fridge for 1 week.	1 spot and 120µl elution buffer	2hrs 4hrs 15-18hrs All at 600 rpm at room temperature	kyn conc was measurable in all samples incubated at varying times with the elution buffer. 17 hrs incubation on shaker showed better results	Tryp conc was measurable in all samples
PBS+ 0.05% Tween 20, 1% BSA, 0.01% Triton X-100.	Stored in a foil bag with a desiccant at minus (-) 20^{o}C freezer for 1 week.	1 spot and 120µl elution buffer	2hrs 4hrs 15-18hrs All at 600 rpm at room temperature	kyn conc was measurable in all samples incubated at varying times with the elution buffer. 17 hrs incubation on shaker showed better results	Tryp conc was measurable in all samples

For RNA extraction and PCR, the best results were observed from the room temperature stored DBS sample, eluted with 800µl lysis solution and 50µl sodium acetate from the RiboPure^{TM} RNA Purification Kit (Thermofisher Scientific, United States) and incubated for 17-hours on a shaker. The various elution buffers explored for real-time PCR DBS optimization results are illustrated in Table 7.

Table 7 Real-Time PCR DBS elution buffer optimisation results

DBS card (Whatman no. 903) conditions	Elution Buffer name	Elution buffer volume and spots	Incubation Time and conditions	Results
none	PBS	250µl and 1 spot	1 hr at 600rpm at room temperature 15-18hrs at 600rpm at room temperature	Signal detected in overnight incubated DBS.
none	PBS	250µl and 1 spot	15-18hrs at 600rpm at room temperature	no signal detected in all samples
1. No RNALater added (normal spot). 2. RNALater added on empty spot 24hrs before spotting blood. 3. RNALater added immediately after spotting blood on card. 4. RNALater added 4hrs later after spotting blood on card.	PBS	250µl and 1 spot	1hr at 600rpm shaker at room temperature	no signal detected in all samples
1. No RNALater added (normal spot). 2. RNALater added on empty spot 24hrs before spotting blood. 3. RNALater added immediately after spotting blood on card. 4. RNALater added 4hrs later after spotting blood on card	PBS	250µl and 1 spot	15-18hrs at 600rpm at room temperature	no signal detected in all samples
1. No RNALater added (normal spot). 2. RNALater added on empty spot 24hrs before spotting blood. 3. RNALater added immediately after spotting blood on card. 4. RNALater added 4hrs later after spotting blood on card	PBS Lysis solution and sodium acetate from RiboPure RNA extraction kit	250µl and 1 spot 800µl lysis solution+50µl sodium acetate and 1 spot.	15-18hrs at 600rpm at room temperature	Signal was detected only in DBS samples eluted with lysis solution and sodium acetate buffer.

3.2 Determination of Kyn concentration in Plasma and DBS

Table 8 represents the kyn results between the two samples. Kyn raw data and percentage similarity for each sample are shown in appendix **B.** The percentage similarity of kyn concentration in the two sample types was calculated. The median percentage similarity was 56.6%.

Table 8 Kyn concentration in Plasma and DBS

KYN			
	PLASMA (ng/ml)	***DRIED BLOOD SPOT (ng/ml)***	***% SIMILARITY***
MINIMUM	162.9	0.0	50.0
MEDIAN	442.5	48.8	56.6
MAXIMUM	961.9	379.2	88.3

3.3 Tryp concentration in Plasma and DBS

Table 9 represents the tryp results between the two samples. Tryp raw data and percentage similarity for each sample are shown in appendix **B.** The median percentage similarity for tryp concentration in plasma and DBS was calculated. The median percentage similarity was 91.1%.

Table 9 Tryp concentration in Plasma and DBS

TRYP			
	PLASMA (ng/ml)	***DRIED BLOOD SPOT (ng/ml)***	***% SIMILARITY***
MINIMUM	4818.5	2812.5	64.2
MEDIAN	9071.5	7462.5	91.1
MAXIMUM	20703	12618	152.4

3.4 Comparison of kyn concentration in Plasma and DBS

For kyn, Spearman's rank correlation showed a significant positive association between plasma and DBS kyn ($r = 0.559$, $P = 0.0002$). To evaluate the method agreement between plasma and DBS we used the Passing and Bablok regression and Bland- Altman analyses.

Passing and Bablok regression shows the scatter diagram with regression line and the grey area between the dashed lines which represents confidence intervals for regression line (**Figure 2A**). The regression equation was y= -87.514 + 0.334 x. The 95% confidence intervals for intercept A (95% CI; -144.273 to -42.542), for slope B (95% CI 0.216 to 0.463). Proportional and constant bias were observed between plasma and DBS. There was no significant deviation from linearity

The difference between Plasma and DBS kyn versus the average between the two sample types was plotted on a Bland-Altman plot (**Figure 2B**). The mean bias was 388.3 ng/ml with SD of 148.3 ng/ml, and 95% limits of agreement from 97.7 to 679.0 ng/ml. There was a proportional bias between the two sampling types, the difference between the plasma and DBS values increased as the average kyn value increased.

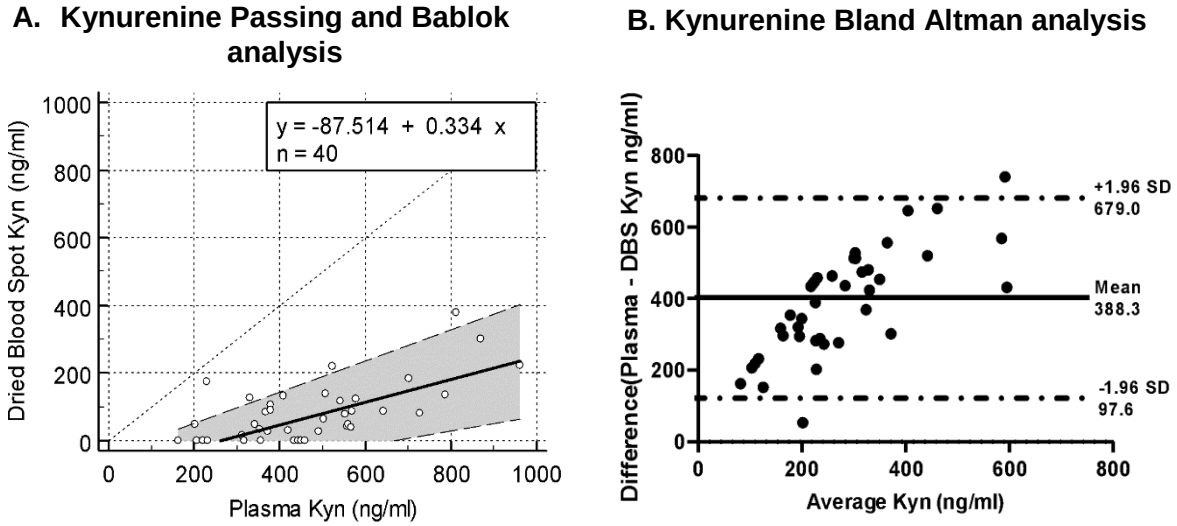


Figure 2 Kyn concentration in DBS compared with plasma. (A) Passing and Bablok regression analysis for method agreement. (B) Bland Altman plot for plasma and DBS differences vs average

3.5 Comparison of Tryp ELISA results between plasma and DBS

For tryp, there was no significant correlation between the two sample types. The Passing and Bablok regression analysis of two sampling methods for tryp concentration was performed to determine method agreement.

Passing and Bablok regression shows the scatter diagram with regression line and the grey area between the dashed lines which represents confidence intervals for regression line (**Figure 3A**). The regression line equation was y= 1800.181 + 0.648x, the 95% CI for intercept A is -2414.887 to 4786.449 and the slope for B was 0.322 to 1.105 with no significant deviation from linearity.

The difference between Plasma and DBS tryp versus the average between the two sample types was plotted on a Bland-Altman plot (**Figure 3B**). The mean bias was 1792 ng/ml with SD of 4204 ng/ml, and 95% limits of agreement from -6448 to 10032 ng/ml.

A. Tryptophan Passing and Bablok analysis **B. Tryptophan Bland Altman analysis**

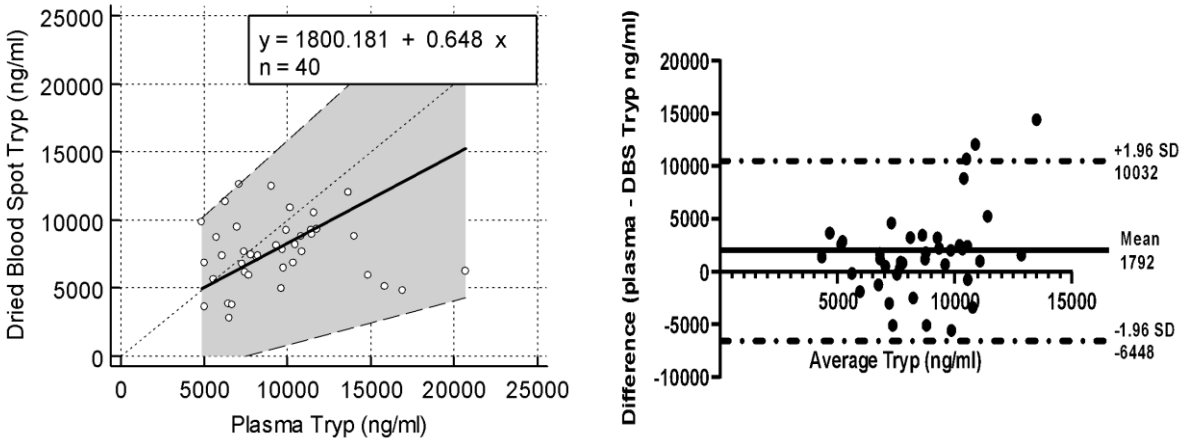


Figure 3 Tryp concentration in DBS compared with plasma. (A) Passing and Bablok regression analysis. (B) Bland Altman plot.

3.6 Comparison of kyn/tryp ratio between plasma and DBS

Spearman correlation was used to assess the strength and correlation between DBS and plasma kyn/tryp ratio. Spearman's rank correlation showed a significant positive association between kyn/tryp ratio measured in plasma and DBS (r = 0.419, P = 0.0071).

Passing and Bablok regression shows the scatter diagram with regression line and the grey area between the dashed lines which represents confidence intervals for regression line (**Figure 4A**). the regression equation of kyn/tryp ratio for plasma and DBS sampling methods was y = -0.004 + 0.255 x. 95% CI for intercept A was -0.004 (95% CI: -0.011 to 0.001) and the slope B was 0.255 (95% CI: 0.129 to 0.4107). Constant bias was observed.

The difference between Plasma and DBS tryp/kyn ratio versus the average between the two sample types was plotted on a Bland-Altman plot (**Figure 4B**). The mean bias was 0.045 with SD of 0.025, and 95% limits of agreement from -0.004 to 0.094.

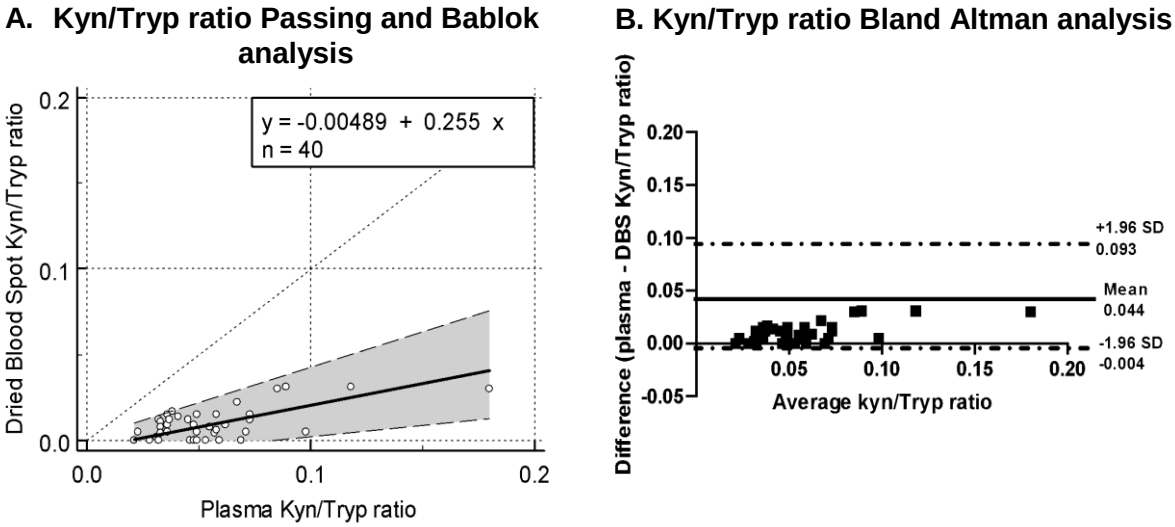


Figure 4 IDO activity comparison between Plasma and DBS. (A) Passing and Bablok regression analysis and (B) Bland Altman plot for method agreement.

3.7 IDO mRNA gene expression in Whole Blood and DBS

Next, we measured expression of the IDO gene through real-time PCR. We investigated and compared DBS to whole blood using two housekeeping genes, GAPDH and BACT. The delta Ct (ΔCt) is calculated by finding the difference between the IDO Ct value and the housekeeping gene Ct value.

$\Delta Ct = Ct_{IDO} - Ct_{Housekeeping\ gene}$

The median percentage similarity for ΔCt (GAPDH) was 87.1%. The range of percentage similarity for ΔCt (GAPDH) was 0.0 to 200.4%. See appendix **C**. Table 10 shows the ΔCt (GAPDH) comparison between whole blood and DBS.

Table 10 Comparison of whole blood and DBS ΔCt (GAPDH)

ΔCT (GAPDH)			
	WHOLE BLOOD	**DRIED BLOOD SPOT**	**% SIMILARITY**
MINIMUM	0.0	0.0	0.0
MEDIAN	3.6	2.7	87.1
MAXIMUM	7.7	5.7	200.4

The median percentage similarity for ΔCt (BACT) was 84.6% which is slightly lower than ΔCt (GAPDH). The range of percentage similarity for ΔCt (BACT) was 0.0 to 237.9%. See appendix **C**. Table 11 illustrates the ΔCt (BACT) in whole blood and DBS.

Table 11 ΔCt IDO (BACT) in whole blood and DBS

Δ CT (BACT)			
	WHOLE BLOOD	**DRIED BLOOD SPOT**	**% SIMILARITY**
MINIMUM	0.0	0.0	0.0
MEDIAN	6.9	5.3	84.6
MAXIMUM	10.7	8.1	237.9

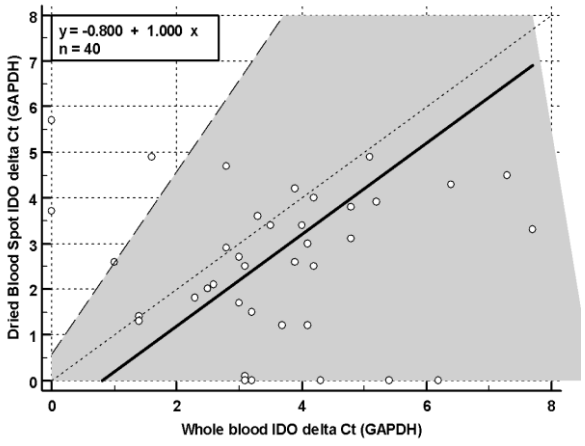
3.8 Comparison of ΔCt (GAPDH) in whole blood and DBS

Spearman's correlation between whole blood and dried blood spot showed a positive association (r = 0.612) but this was not significant. For evaluation of method agreement between whole blood and DBS ΔCt (GAPDH), Passing and Bablok regression analysis and Bland Altman analysis were performed.

Passing and Bablok regression shows the scatter diagram with regression line and the grey area between the dashed lines which represents confidence intervals for regression line (**Figure 5A**). The regression equation was y= -0.800 + 1.000 x. The 95% confidence for intercept A (95% CI; -5.050 to 0.575), for slope B (95% CI; 0.583 to 2.000). No proportional and constant bias observed.

The difference between whole blood and DBS ΔCt (GAPDH) versus the average between the two sample types was plotted on a Bland-Altman plot (**Figure 5B**). The mean bias of ΔCt values was 1.055 with SD of 2,355, and 95% limits of agreement from -3.560 to 5.670.

A. IDO delta Ct (GAPDH) Passing and Bablok analysis



B. IDO delta Ct (GAPDH) Bland Altman analysis

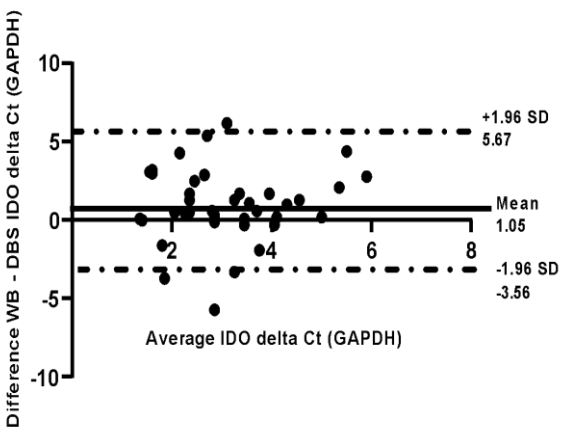


Figure 5 Comparison of IDO/GAPDH in whole blood and DBS for IDO mRNA gene expression. (A) Passing and Bablok regression analysis. (B) Bland Altman plot

3.9 Comparison of ΔCt (BACT) in whole blood and DBS

For ΔCt (BACT) the Spearman's correlation showed a weak correlation between the two sample types (r = 0.339) which was not significant.

Passing and Bablok regression shows the scatter diagram with regression line and the grey area between the dashed lines which represents confidence intervals for regression line (**Figure 6A**). The regression equation was y= -3.987 + 1.226 x. The 95 % CI for the intercept A (-10.418 to 0.526) and the slope B (0.655 to 2.091) includes zero and one, respectively. There was no constant and proportional bias between the two sampling methods indicating method agreement.

The difference between whole blood and DBS ΔCt (BACT) versus the average between the two sample types was plotted on a Bland-Altman plot (**Figure 6B**). The mean bias of ΔCt values was 2.068 with SD of 3.407, and 95% limits of agreement from -4.611 to 8.746

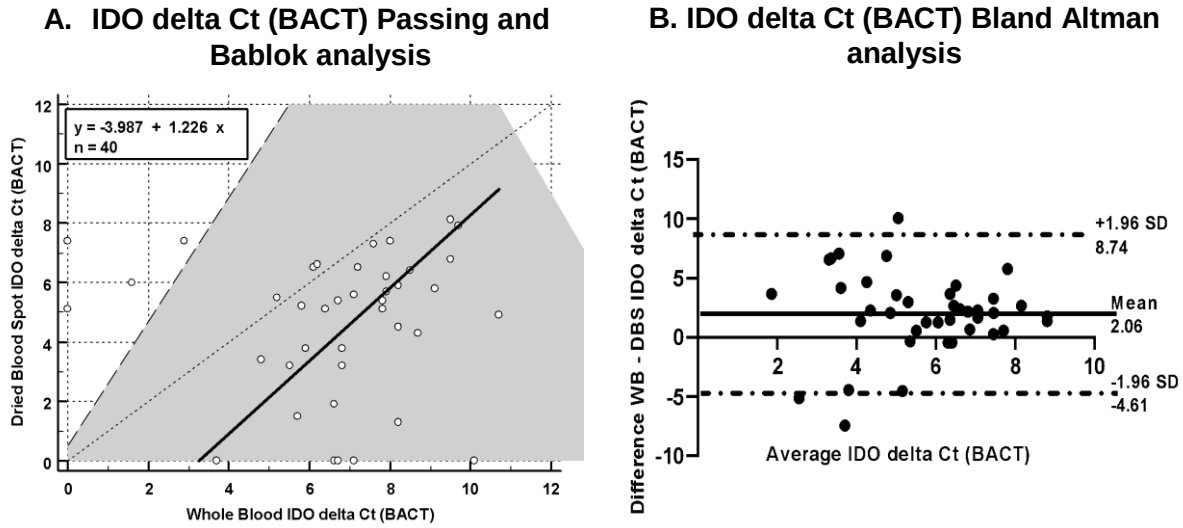


Figure 6 Whole blood and DBS IDO mRNA gene expression comparison using housekeeping gene BACT. (A) Passing and Bablok regression analysis. (B) Bland Altman plot

3.10 Correlation of plasma IDO activity and IDO mRNA gene expression in whole blood

Using the traditional blood sample types, whole blood for PCR and plasma for ELISA, we investigated the relationship between IDO gene expression and IDO activity. Using the GAPDH housekeeping gene, Spearman's correlation between ELISA

plasma kyn/tryp ratio and whole blood real-time PCR for IDO using GAPDH, figure 7(A) showed a negative weak association between the two IDO determination methods (r = -0.1108) which was not significant.

Using the BACT housekeeping gene, a Spearman's correlation between ELISA plasma kyn/tryp ratio and whole blood real-time PCR for IDO, figure 7(B) showed a negative weak association between the two methods (r = -0.2468) which was not significant.

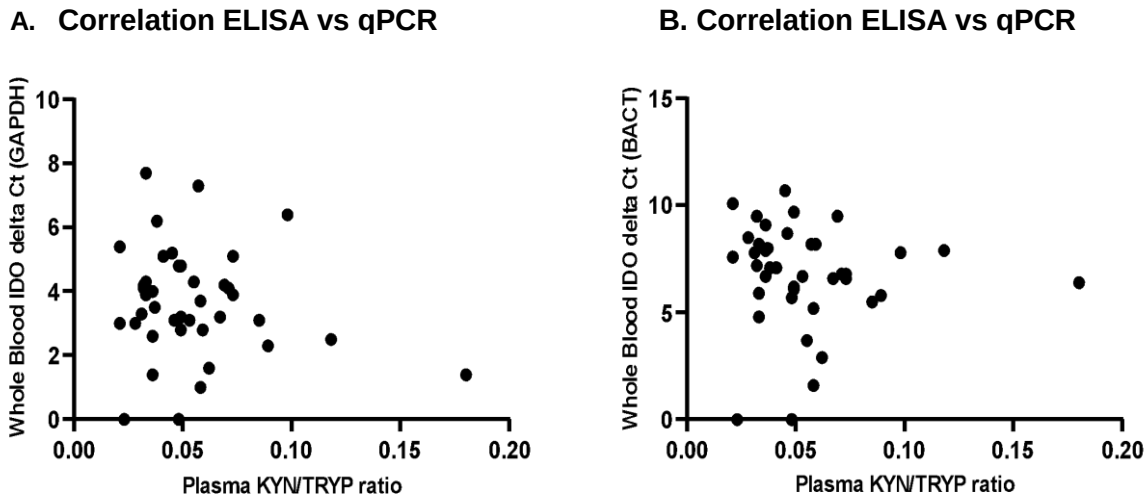


Figure 7 Correlation between IDO activity in plasma and IDO mRNA gene expression in whole blood. (A) using housekeeping gene GAPDH. (B) using housekeeping gene BACT

3.11 Correlation of IDO activity and IDO mRNA gene expression in DBS

In DBS samples, we also explored the relationship between IDO activity, expressed as kyn/tryp ratio, and IDO gene expression. Using GAPDH as a housekeeping gene, Spearman's correlation between IDO activity in dried blood spot and dried blood spot IDO mRNA expression showed no association between the two methods (r = -0.0552) which was not significant, Figure 8(A).

Using BACT housekeeping gene, Spearman's correlation between IDO activity measured by ELISA in DBS and DBS IDO gene expression by real-time PCR, figure 8(B) showed a weak negative association between the two methods (r = -0.1219) which was not significant.

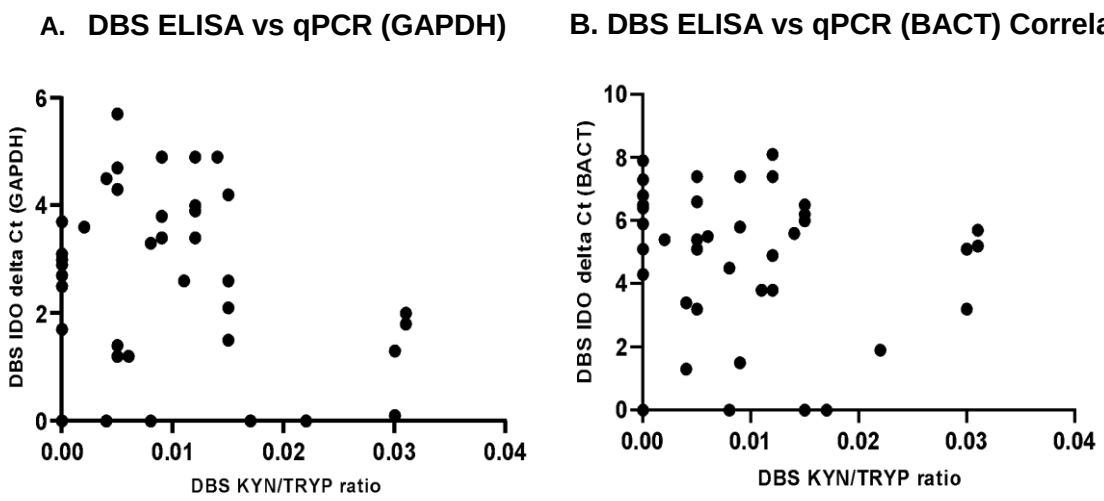


Figure 8 Correlation IDO activity and IDO mRNA gene expression in DBS. (A) using housekeeping gene BACT. (B) using housekeeping gene GAPDH

4 DISCUSSION

Our study investigated the use of DBS for determining IDO activity in healthy individuals. We compared IDO measurement from fresh peripheral blood samples to DBS samples. DBS has been used for neonatal screening since 1960, and many other clinical analytes have been successfully analysed using the DBS sample (Bloom, Ditewig Meyers and Bennett, 2016). DBS samples are compatible with most analytical methods. The potential for DBS to be used as a sample for IDO measurement, and a TB biomarker, could assist in the diagnosis of TB in areas with less specialized laboratory equipment and clinical personnel. Before DBS can be trialled as a TB biomarker, it was necessary to optimize the methods for detection of kyn, tryp and IDO mRNA from DBS.

Using healthy donors, we found that tryp concentrations in plasma and DBS were comparable, with good method agreement. However, kyn concentrations did not show good agreement, with a constant and proportional bias observed between plasma and DBS. The IDO activity measured by the kyn/tryp ratio also showed no agreement between DBS and plasma, likely due to the difficulties with kyn measurement. Plasma kyn/tryp ratio was always higher in plasma compared to DBS.

For kyn measurement in plasma and DBS, no method agreement was observed between the two sample types. Some participants' kyn concentrations were below the limit of detection, which resulted in some concentrations reading as 0 ng/ml. Limits of detection for dilute samples can affect measurement of analytes in DBS. This could have been due to the dilution from the elution buffer. The stability of kyn may have been compromised and degraded during storage and processing of the DBS sample, contributing to the low kyn values obtained. The drying process of DBS may also denature some proteins and alter some enzyme activity in blood proteins (Lehmann *et al.*, 2013). Thus DBS samples may require more optimization for kyn detection.

We found good agreement of tryp concentration between plasma and DBS. In a study by Geisler et al., (2015) reference values for tryp, kyn, and other immunological biomarkers were investigated and established in a cohort of healthy blood donors. Their study reported the tryp concentration ranged from 57.2 umol/l to 77.6 umol/l. Our findings for tryp concentration range from 23.6 umol/l (4818.50 ng/ml) to 101.2

umol/l (20703 ng/ml) in plasma and 13.7 umol/l (2812.50 ng/ml) to 61.7 umol/l (12618 ng/ml) in DBS in healthy participants.

Our results showed that tryp in DBS and plasma samples were comparable, with a mean percentage similarity of 91.1%. In the Bland-Altman analysis, the values were evenly distributed along the identity line; suggesting agreement was observed with only 3 outliers. There was no evidence of constant or proportional bias. The plasma and DBS tryp concentrations were in good agreement therefore DBS may be acceptable for measurement of tryp. Another study that compared amino acid concentrations in DBS and plasma found comparable results, with some amino acids having higher concentrations in DBS (Aslan and CanpolatErkan, 2022).

For kyn/tryp ratio, we found a significant correlation between plasma and DBS. However, proportional bias was identified, and the plasma kyn/tryp ratio was always higher in plasma compared to DBS. The difference can be attributed to the challenges in measuring kyn in DBS.

The use of DBS in the measurement of DNA and RNA has been demonstrated in various studies and yielded comparable results to plasma and whole blood samples (Lehmann *et al.*, 2013). Nucleic acids have been reported to be stable in DBS for over 10 years with ambient storage conditions. Reliable gene expression from DBS using RNA sequencing was reported by Bybjerg-Grauholm et al., (2017). We investigated the use of different sample types for IDO mRNA expression. Two housekeeping genes, GAPDH and BACT, were used. In a study by Reust et al., (2018) the authors demonstrated a positive correlation between the sequenced RNA transcriptomes using $\log_2$-adjusted gene counts in paired whole blood and DBS samples. In our study, the evaluation of IDO delta Ct using either housekeeping gene GAPDH or BACT showed fair agreement between whole blood and DBS. We found that in some DBS samples the IDO delta Ct results were higher than those in whole blood samples. This makes the DBS a potential sample type for the measurement of IDO mRNA gene expression. Freeman et al., (2018) reported improved stability of DBS which makes it suitable for measurement of various analytes.

We evaluated correlation between IDO gene expression and kyn/tryp ratio, first using traditional sample types and then using DBS. In traditional sample types (plasma for IDO activity and whole blood for IDO mRNA gene expression), there was no

significant correlation detected. In DBS, no correlation was observed between kyn/tryp ratio and IDO mRNA gene expression. In a study by Okamoto et al., (2007) they used the IDO inhibitor, 1-methyl-tryptophan which demonstrated IDO activity and IDO gene expression are independent variables. The IDO inhibitor 1-methyl-tryptophan affects the kyn/tryp ratio but has no effect on IDO gene expression (Okamoto *et al.*, 2007). This was also demonstrated in a study by O'Connor et al., (2009), where in mice treated with BCG the kyn/tryp ratio was decreased and IDO gene expression unaffected in the presence of 1-methyl-tryptophan. The gut flora is another factor which may lead to no correlation between IDO activity and IDO gene expression. The gut flora plays a role in tryp metabolism which may affect the levels of circulating tryp (Badawy and Guillemin, 2019). In normal physiological state kyn is rapidly cleared from circulating blood and excreted by the kidneys. This rapid clearance may also affect levels of kyn in blood (Badawy and Guillemin, 2019). Post-transcriptional regulation of the IDO gene expression through factors such as tumour necrosis factor- alpha and IFN-y regulate the IDO gene expression leading to no correlation between IDO gene expression and IDO enzymatic activity (Robinson, Shirey and Carlin, 2003). Therefore increased IDO gene expression does not correlate to increased enzyme functional activity.

Limitations of our study were time and resource limitations to explore various methods for kyn stabilisation. Strength was that the study was able to evaluate of both enzyme activity and IDO gene expression with peripheral blood as a reference material. We performed sufficient optimization to reliably detect tryp from DBS, which may be of benefit for future applications related to TB or other diseases.

5 CONCLUSION

In this study we successfully measured tryp and IDO mRNA in DBS samples stored at room temperature. Kyn concentrations determined in DBS were lower than the corresponding plasma values, which is probably due to the drying of the DBS which may affect the kyn concentration. Although tryp was largely comparable in DBS to plasma values, the large confidence intervals suggest precision error.

This study showed that DBS has the potential to be an alternative sample for tryp measurement but has insufficient utility for IDO activity measurement using the current method due to poor detection of kyn. There was reasonable method agreement between DBS and whole blood samples in IDO mRNA gene expression. Further studies can use DBS as an alternative sample for measurement of IDO mRNA gene expression.

As we found no significant correlation between IDO mRNA expression in whole blood and kyn/tryp ratio in plasma even using peripheral blood as a traditional sample type, further studies exploring IDO as a TB biomarker should consider analysing both measures as each gives different information.

6 REFERENCES

1. Adu-Gyamfi, C.G. *et al.* (2020) 'Diagnostic accuracy of plasma kynurenine/tryptophan ratio, measured by enzyme-linked immunosorbent assay, for pulmonary tuberculosis', *International Journal of Infectious Diseases*, 99, pp. 441–448. Available at: https://doi.org/10.1016/j.ijid.2020.08.028.
2. Aslan, O. and Canpolat Erkan, R.E. (2022) 'Comparison of Amino Acid Concentrations in Plasma and Dried Blood Spot Samples Using LC-MS/MS', *Iranian Journal of Pediatrics*, 32(3). Available at: https://doi.org/10.5812/ijp-117073.
3. Badawy, A.A.B. and Guillemin, G. (2019) 'The Plasma [Kynurenine]/[Tryptophan] Ratio and Indoleamine 2,3-Dioxygenase: Time for Appraisal', *International Journal of Tryptophan Research*, 12. Available at: https://doi.org/10.1177/1178646919868978.
4. Barac, A. *et al.* (2019) 'Immune Biomarkers for Diagnosis and Treatment Monitoring of Tuberculosis: Current Developments and Future Prospects'. Available at: https://doi.org/10.3389/fmicb.2019.02789.
5. Björkesten, J. *et al.* (2017) 'Stability of proteins in dried blood spot biobanks', *Molecular and Cellular Proteomics*, 16(7), pp. 1286–1296. Available at: https://doi.org/10.1074/mcp.RA117.000015.
6. Bloom, K., Ditewig Meyers, G. and Bennett, M.J. (2016) 'A Quantitative Method for the Measurement of Dried Blood Spot Amino Acids Using Ultra-Performance Liquid Chromatography', *The Journal of Applied Laboratory Medicine*, 1(3), pp. 271–279. Available at: https://doi.org/10.1373/jalm.2016.020289.
7. Borghi, M. *et al.* (2020) 'Tryptophan as a Central Hub for Host/Microbial Symbiosis', *International Journal of Tryptophan Research*, 13. Available at: https://doi.org/10.1177/1178646920919755.
8. Briken, V. *et al.* (2018) 'Mycobacterium tuberculosis: An Adaptable Pathogen Associated with Multiple Human Diseases', *Frontiers in Cellular and Infection Microbiology* | *www.frontiersin.org*, 1, p. 158. Available at: https://doi.org/10.3389/fcimb.2018.00158.
9. Bybjerg-Grauholm, J. *et al.* (2017) 'RNA sequencing of archived neonatal dried blood spots', *Molecular Genetics and Metabolism Reports*, 10, pp. 33–37. Available at: https://doi.org/10.1016/j.ymgmr.2016.12.004.
10. Cao, T. *et al.* (2022) 'Single-nucleotide polymorphisms and activities of indoleamine 2,3-dioxygenase isoforms, IDO1 and IDO2, in tuberculosis patients', *Hereditas*, 159(1), pp. 1–8. Available at: https://doi.org/10.1186/s41065-022-00219-y.
11. Collins, J.M. *et al.* (2020) 'Tryptophan catabolism reflects disease activity in human tuberculosis', *JCI Insight*, 5(10), pp. 1–16. Available at: https://doi.org/10.1172/JCI.INSIGHT.137131.
12. Crowther, R.R. and Qualls, J.E. (2021) 'Metabolic Regulation of Immune Responses

to Mycobacterium tuberculosis: A Spotlight on L-Arginine and L-Tryptophan Metabolism', *Frontiers in Immunology*, 11(February), pp. 1–16. Available at: https://doi.org/10.3389/fimmu.2020.628432.

13. Dollard, S.C. *et al.* (2021) 'Sensitivity of Dried Blood Spot Testing for Detection of Congenital Cytomegalovirus Infection', *JAMA Pediatrics*, 175(3). Available at: https://doi.org/10.1001/jamapediatrics.2020.5441.
14. Esposito, S., Tagliabue, C. and Bosis, S. (2013) 'Tuberculosis in children', *Mediterranean Journal of Hematology and Infectious Diseases*, 5(1). Available at: https://doi.org/10.4084/mjhid.2013.064.
15. Freeman, J.D. *et al.* (2018) 'State of the science in dried blood spots', *Clinical Chemistry*, 64(4), pp. 656–679. Available at: https://doi.org/10.1373/clinchem.2017.275966.
16. Geisler, S. *et al.* (2015) 'Serum tryptophan, kynurenine, phenylalanine, tyrosine and neopterin concentrations in 100 healthy blood donors', *Pteridines*, 26(1), pp. 31–36. Available at: https://doi.org/10.1515/pterid-2014-0015.
17. Gengenbacher, M. and Kaufmann, S.H.E. (2012) 'Mycobacterium tuberculosis: Success through dormancy', *FEMS Microbiology Reviews*, 36(3), pp. 514–532. Available at: https://doi.org/10.1111/j.1574-6976.2012.00331.x.
18. Goletti, D. *et al.* (2016) 'Tuberculosis biomarkers: from diagnosis to protection', *Infectious Disease Reports*, 8. Available at: https://doi.org/10.4081/idr.2016.6568.
19. Grüner, N., Stambouli, O., Ross, R.S. Dried Blood Spots - Preparing and Processing for Use in Immunoassays and in Molecular Techniques. *J. Vis. Exp.* (97), e52619, doi:10.3791/52619 (2015).
20. Gupta, M. *et al.* (2020) 'The need for COVID-19 research in low- and middle-income countries.
21. Kania, D. *et al.* (2013) 'Combining rapid diagnostic tests and dried blood spot assays for point-of-care testing of human immunodeficiency virus, hepatitis B and hepatitis C infections in burkina faso, west africa', *Clinical Microbiology and Infection*, 19(12), pp. 12–18. Available at: https://doi.org/10.1111/1469-0691.12292.
22. Koch, R. (1882) *Die Ätiologie der Tuberkulose. 1) (Nach, einem in der Physiologischen Gesellschaft zu Berlin am 24. März 1882 gehaltenen Vortrage.)*.
23. Lauren M.F. Merlo and Laura Mandik-Nayak. IDO2: A Pathogenic Mediator of Inflammatory Autoimmunity. Clinical Medicine Insights: Pathology. 2016 10.4137/CPath.S39930
24. Lee, Y.-K. *et al.* (2014) 'Heme-binding-mediated negative regulation of the tryptophan metabolic enzyme indoleamine 2,3-dioxygenase 1 (IDO1) by IDO2', *Experimental & Molecular Medicine*, 46, p. 121. Available at: https://doi.org/10.1038/emm.2014.69.

25. Lehmann, S. *et al.* (2013) 'Current and future use of "dried blood spot" analyses in clinical chemistry', *Clinical Chemistry and Laboratory Medicine*, pp. 1897–1909. Available at: https://doi.org/10.1515/cclm-2013-0228.
26. Lim, M.D. (2018) 'Dried Blood Spots for Global Health Diagnostics and Surveillance: Opportunities and Challenges', *Am. J. Trop. Med. Hyg*, 99(2), pp. 256–265. Available at: https://doi.org/10.4269/ajtmh.17-0889.
27. Liu, L. *et al.* (2020) 'A Novel Dried Blood Spot Detection Strategy for Characterizing Cardiovascular Diseases', *Frontiers in Cardiovascular Medicine*, 7(October), pp. 1–10. Available at: https://doi.org/10.3389/fcvm.2020.542519.
28. Malsagova, K. *et al.* (2020) 'diagnostics Dried Blood Spot in Laboratory: Directions and Prospects', *Diagnostics*, (10), p. 248. Available at: https://doi.org/10.3390/diagnostics10040248.
29. Mancuso, R. *et al.* (2015) 'Indoleamine 2,3 dioxygenase (IDO) expression and activity in relapsing-remitting multiple sclerosis', *PLoS ONE*, 10(6). Available at: https://doi.org/10.1371/journal.pone.0130715.
30. Martial, L.C. *et al.* (2016) 'Cost evaluation of dried blood spot home sampling as compared to conventional sampling for therapeutic drug monitoring in children', *PLoS ONE*, 11(12). Available at: https://doi.org/10.1371/journal.pone.0167433.
31. McNerney, R. *et al.* (2012) 'Tuberculosis Diagnostics and Biomarkers: Needs, Challenges, Recent Advances, and Opportunities Tuberculosis Diagnostics: Challenges and Needs d JID 2012:205 (Suppl 2) d S147', *The Journal of Infectious Diseases*, 18, pp. 147–58. Available at: https://doi.org/10.1093/infdis/jir860.
32. Mellor, A.L. *et al.* (2017) 'Indoleamine 2,3-Dioxygenase and Tolerance: Where Are We Now?', *Frontiers in Immunology*, 8(October), pp. 1–6. Available at: https://doi.org/10.3389/fimmu.2017.01360.
33. Meyers, E. *et al.* (2021) 'Comparison of Dried Blood Spots and Venous Blood for the Detection of SARS-CoV-2 Antibodies in a Population of Nursing Home Residents', *Microbiology Spectrum*, 9(2). Available at: https://doi.org/10.1128/spectrum.00178-21.
34. Moreira, F.M.F. *et al.* (2021) 'Blood-based host biomarker diagnostics in active case finding for pulmonary tuberculosis: A diagnostic case-control study', *EClinicalMedicine*, 33, pp. 1–8. Available at: https://doi.org/10.1016/j.eclinm.2021.100776.
35. Mwandumba, H.C. *et al.* (2004) 'Mycobacterium tuberculosis Resides in Nonacidified Vacuoles in Endocytically Competent Alveolar Macrophages from Patients with Tuberculosis and HIV Infection', *The Journal of Immunology*, 172(7), pp. 4592–4598. Available at: https://doi.org/10.4049/jimmunol.172.7.4592.
36. Neesgaard, B. *et al.* (2018) 'Determination of anti-HCV and quantification of HCV-RNA and IP-10 from dried blood spots sent by regular mail', *PLoS ONE*, 13(7), pp. 1–

13. Available at: https://doi.org/10.1371/journal.pone.0201629.
37. O'Connor, J.C. *et al.* (2009) 'Induction of IDO by Bacille Calmette-Guérin Is Responsible for Development of Murine Depressive-Like Behavior', *The Journal of Immunology*, 182(5), pp. 3202–3212. Available at: https://doi.org/10.4049/jimmunol.0802722.
38. Okamoto, T. *et al.* (2007) 'Transcriptional regulation of indoleamine 2,3-dioxygenase (IDO) by tryptophan and its analogue: Down-regulation of the indoleamine 2,3-dioxygenase (IDO) transcription by tryptophan and its analogue', *Cytotechnology*, 54(2), pp. 107–113. Available at: https://doi.org/10.1007/s10616-007-9081-4.
39. Orata, F. (2012) *5 Derivatization Reactions and Reagents for Gas Chromatography Analysis*. Available at: www.intechopen.com.
40. Rakotosamimanana, N. *et al.* (2015) 'Biomarkers for risk of developing active tuberculosis in contacts of TB patients: A prospective cohort study', *European Respiratory Journal*, 46(4), pp. 1095–1103. Available at: https://doi.org/10.1183/13993003.00263-2015.
41. Ravishankar, B. *et al.* (2012) 'Tolerance to apoptotic cells is regulated by indoleamine 2,3-dioxygenase', *Proceedings of the National Academy of Sciences of the United States of America*, 109(10), pp. 3909–3914. Available at: https://doi.org/10.1073/pnas.1117736109.
42. Reust, M.J. *et al.* (2018) 'Dried blood spot RNA transcriptomes correlate with transcriptomes derived from whole blood RNA', *American Journal of Tropical Medicine and Hygiene*, 98(5), pp. 1541–1546. Available at: https://doi.org/10.4269/ajtmh.17-0653.
43. Robinson, C.M., Shirey, K.A. and Carlin, J.M. (2003) *Synergistic Transcriptional Activation of Indoleamine Dioxygenase by IFN-γ and Tumor Necrosis Factor-α*.
44. Roy, C.J. *et al.* (2019) 'Indoleamine 2, 3-Dioxygenase-Mediated Tryptophan Catabolism: A Leading Star or Supporting Act in the Tuberculosis and HIV Pas-de-Deux?', *Frontiers in Cellular and Infection Microbiology | www.frontiersin.org*, 9, p. 372. Available at: https://doi.org/10.3389/fcimb.2019.00372.
45. Sakamoto, H. *et al.* (2019) 'Challenges and opportunities for eliminating tuberculosis - Leveraging political momentum of the un high-level meeting on tuberculosis', *BMC Public Health*, 19(1), pp. 1–7. Available at: https://doi.org/10.1186/s12889-019-6399-8.
46. Sakamoto, K. (2012) 'The Pathology of Mycobacterium tuberculosis Infection', *Veterinary Pathology*, 49(3), pp. 423–439. Available at: https://doi.org/10.1177/0300985811429313.
47. Sakhi, A.K. *et al.* (2015) 'Feasibility of self-sampled dried blood spot and saliva samples sent by mail in a population-based study', *BMC Cancer*, 15(1), pp. 1–9.

Available at: https://doi.org/10.1186/s12885-015-1275-0.

48. Shaun Lott, J. (2020) 'The tryptophan biosynthetic pathway is essential for Mycobacterium tuberculosis to cause disease', *Biochemical Society Transactions*, 48(5), pp. 2029–2037. Available at: https://doi.org/10.1042/BST20200194.
49. Shi, W. *et al.* (2019) 'Plasma indoleamine 2,3-dioxygenase activity as a potential biomarker for early diagnosis of multidrug-resistant tuberculosis in tuberculosis patients', *Infection and Drug Resistance*, 12, pp. 1265–1276. Available at: https://doi.org/10.2147/IDR.S202369.
50. Sia, J.K. and Rengarajan, J. (2019) 'Immunology of mycobacterium tuberculosis infections', *Gram-Positive Pathogens*, 7(4), pp. 1056–1086. Available at: https://doi.org/10.1128/9781683670131.ch64.
51. Singh, V. and Chibale, K. (2021) 'Strategies to Combat Multi-Drug Resistance in Tuberculosis', *Accounts of Chemical Research*, 54(10), pp. 2361–2376. Available at: https://doi.org/10.1021/acs.accounts.0c00878.
52. Suchard, Melinda S *et al.* (2020) 'Evolutionary Views of Tuberculosis: Indoleamine 2,3-Dioxygenase Catalyzed Nicotinamide Synthesis Reflects Shifts in Macrophage Metabolism Indoleamine 2,3-Dioxygenase Reflects Altered Macrophage Metabolism During Tuberculosis Pathogenesis'. Available at: https://doi.org/10.1002/bies.201900220.
53. Suzuki, Y. *et al.* (2012) 'Serum indoleamine 2,3-dioxygenase activity predicts prognosis of pulmonary tuberculosis', *Clinical and Vaccine Immunology*, 19(3), pp. 436–442. Available at: https://doi.org/10.1128/CVI.05402-11.
54. Suzuki, Y. *et al.* (2013) 'Indoleamine 2,3-dioxygenase in the pathogenesis of tuberculous pleurisy', *International Journal of Tuberculosis and Lung Disease*, 17(11), pp. 1501–1506. Available at: https://doi.org/10.5588/ijtld.13.0082.
55. Tanner, R. *et al.* (2014) 'Serum indoleamine 2,3-dioxygenase activity is associated with reduced immunogenicity following vaccination with MVA85A', *BMC Infectious Diseases*, 14(1), pp. 1–10. Available at: https://doi.org/10.1186/s12879-014-0660-7.
56. Tuaillon, E. *et al.* (2020) 'Dried Blood Spot Tests for the Diagnosis and Therapeutic Monitoring of HIV and Viral Hepatitis B and C', *Frontiers in Microbiology*, 11(March), pp. 1–7. Available at: https://doi.org/10.3389/fmicb.2020.00373.
57. Turner, C.T. *et al.* (2020) 'Blood transcriptional biomarkers for active pulmonary tuberculosis in a high-burden setting: a prospective, observational, diagnostic accuracy study', *The Lancet Respiratory Medicine*, 8(4), pp. 407–419. Available at: https://doi.org/10.1016/S2213-2600(19)30469-2.
58. Ulrichs, T. and Kaufmann, S.H. (2006), New insights into the function of granulomas in human tuberculosis. J. Pathol., 208: 261-269. https://doi.org/10.1002/path.1906
59. Upadhyay, S., Mittal, E. and Philips, J.A. (2018) 'Tuberculosis and the art of

macrophage manipulation', *Pathogens and Disease*, 76(4), pp. 1–12. Available at: https://doi.org/10.1093/femspd/fty037.

60. Vrieling, F. *et al.* (2019) 'Plasma metabolomics in tuberculosis patients with and without concurrent type 2 diabetes at diagnosis and during antibiotic treatment', *Scientific Reports*, 9(1), pp. 1–12. Available at: https://doi.org/10.1038/s41598-019-54983-5.
61. Weiss, G. and Schaible, U.E. (2015) 'Macrophage defense mechanisms against intracellular bacteria', *Immunological Reviews*, 264(1), pp. 182–203. Available at: https://doi.org/10.1111/imr.12266.
62. Whittaker, K. *et al.* (2021) 'Dried blood sample analysis by antibody array across the total testing process', *Scientific Reports*, 11(1), pp. 1–13. Available at: https://doi.org/10.1038/s41598-021-99911-8.
63. Zhai, W. *et al.* (2019) 'The immune escape mechanisms of Mycobacterium Tuberculosis', *International Journal of Molecular Sciences*, 20(2). Available at: https://doi.org/10.3390/ijms20020340.
64. World Health Organization. Global tuberculosis report 2021. Geneva: WHO; 2021 https://www.who.int/tb/publications/global_report/en/.

Appendix A

Ethics clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Innovation)

TO:	Ms S Tsolo School of Pathology Department of Immunology Medical School University E-mail: semakalengtsholo@gmail.com
CC:	Supervisor: Drs H Ranchod and M Suchard <heenar@nicd.ac.za> and <HREC-Medical Research Office@wits.ac.za>
FROM:	Mr Iain Burns Human Research Ethics Committee (Medical) Tel: 011 717 1252 E-mail: Iain.Burns@wits.ac.za
DATE:	2022/02/14
REF:	R14/49
PROTOCOL NO:	**M211186** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)
PROJECT TITLE:	*Evaluation of novel assay formats for Indoleamine 2, 3 dioxygenase as a TB biomarker*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

Appendix B
Study results for ELISA

Tryp and Kyn measurement with ELISA

	Plasma Results			Dried Blood Spot			% Similarity (Plasma vs DBS)		
Participant ID	Kyn(ng/ml)	Tryp(ng/ml)	Kyn/Tryp	Kyn(ng/ml)	Tryp(ng/ml)	Kyn/Tryp	Kyn(%)	Tryp(%)	Kyn/Tryp(%)
STT001	541,7	9338,5	0,058	117,7	8115	0,015	60,9	93,4	62.9
STT002	559,5	5711,5	0,098	46,7	8674,5	0,005	54,2	125,9	52.6
STT003	568,3	7795,5	0,073	87,3	7462,5	0,012	57,7	97,9	58.2
STT004	702,3	10440,5	0,067	181,7	8209	0,022	62,9	89,3	66.4
STT005	508,0	13627	0,037	138,5	12027	0,012	63,6	94,1	66.2
STT006	522,6	6116,5	0,085	220,2	7343	0,030	71,1	110,0	67.6
STT007	354,5	6655,5	0,053	0,0	3748	0,000	50,0	78,2	50.0
STT008	556,7	9670	0,058	43,2	7836,5	0,006	53,9	90,5	55.2
STT009	489,9	14811,5	0,033	26,0	5942	0,004	52,7	70,1	56.1
STT010	379,3	11403	0,033	105,4	9230	0,011	63,9	90,5	66.7
STT011	787,0	10841,5	0,073	134,8	8785,5	0,015	58,6	90,5	60.3
STT012	642,6	11587	0,055	86,0	10546,5	0,008	56,7	95,5	57.3
STT013	727,2	11754,5	0,062	81,0	9311	0,009	55,6	89,6	57.3
STT014	553,3	11459,5	0,048	78,3	8929	0,009	57,1	89,0	59.4

	Plasma Results			Dried Blood Spot			% Similarity (Plasma vs DBS)		
Participant ID	**Kyn(ng/ml)**	**Tryp(ng/ml)**	**Kyn/Tryp**	**Kyn(ng/ml)**	**Tryp(ng/ml)**	**Kyn/Tryp**	**Kyn(%)**	**Tryp(%)**	**Kyn/Tryp(%)**
STT015	311,7	9941	0,031	15,0	9222	0,002	52,4	96,4	53.2
STT016	501,3	7075,5	0,071	64,4	12618	0,005	56,4	139,2	53.5
STT017	420,3	7409,5	0,057	31,0	7625,5	0,004	53,7	101,5	53.5
STT018	408,9	10861,5	0,038	131,9	7629	0,017	66,1	85,1	72.4
STT019	566,5	16922,5	0,033	38,3	4819,5	0,008	53,4	64,2	62.1
STT020	371,6	15848,5	0,023	26,9	5135	0,005	53,6	66,2	60.9
STT021	458,7	9606,5	0,048	0,0	4960,5	0,000	50,0	75,8	50.0
STT022	576,9	14034,0	0,041	122,4	8748	0,014	60,6	81,2	67.1
STT023	206,7	7298,0	0,028	0,0	6752,5	0,000	50,0	96,3	50.0
STT024	329,2	10179,5	0,032	126,3	10902,5	0,012	69,2	103,6	68.8
STT025	869,2	4818,5	0,180	300,4	9865	0,030	67,3	152,4	58.3
STT026	442,5	20703,0	0,021	0,0	6252	0,000	50,0	65,1	50.0
STT027	448,6	6503,0	0,069	0,0	2812,5	0,000	50,0	71,6	50.0
STT028	201,1	5547,5	0,036	48,8	5664,5	0,009	62,1	101,1	62.5
STT029	434,6	7425,0	0,059	0,0	6178,5	0,000	50,0	91,6	50.0
STT030	317,1	6448,0	0,049	0,0	3830	0,000	50,0	79,7	50.0
STT031	367,9	8192,5	0,045	85,2	7331,5	0,012	61,6	94,7	63.3

	Plasma Results			Dried Blood Spot			% Similarity (Plasma vs DBS)		
Participant ID	**Kyn(ng/ml)**	**Tryp(ng/ml)**	**Kyn/Tryp**	**Kyn(ng/ml)**	**Tryp(ng/ml)**	**Kyn/Tryp**	**Kyn(%)**	**Tryp(%)**	**Kyn/Tryp(%)**
STT032	228,3	6261,0	0,036	174,7	11308	0,015	88,3	140,3	70.8
STT033	961,9	8149,0	0,118	220,9	7200	0,031	61,5	94,2	63.1
STT034	352,7	9726,5	0,036	32,0	6458,5	0,005	54,5	83,2	56.9
STT035	232,6	5018,5	0,046	0,0	6868	0,000	50,0	118,4	50.0
STT036	342,4	6985,5	0,049	47,8	9442	0,005	57,0	117,6	55.1
STT037	811,0	9071,5	0,089	379,2	12429	0,031	73,4	118,5	67.4
STT038	378,8	7672,0	0,049	90,3	5906,5	0,015	61,9	88,5	65.3
STT039	219,6	10352,5	0,021	0,0	6864	0,000	50,0	83,2	50.0
STT040	162,9	5020,0	0,032	0,0	3606	0,000	50,0	85,9	50.0
MINIMUM	162,9	4818,5	0,021	0,0	2812,5	0,000	50.0	64.2	50.0
MAXIMUM	961,9	20703,0	0,180	379,2	12618,0	0,031	88.3	152.4	72.4
MEAN	468,6	9460,1	0,054	81,1	7653,2	0,009	58.0	95.0	58.5
MEDIAN	442,5	9071,5	0,048	48,8	7462,5	0,008	56.6	91.1	57.7

**Kyn = kynurenine (ng/ml) Tryp = Tryptophan (ng/ml) Kyn/Tryp = IDO activity*

Appendix C
Study Results for PCR

Delta Ct (GAPDH)

	Whole Blood			Dried Blood Spot			% Similarity
Participant ID	**GAPDH**	**IDO**	**Delta Ct**	**GAPDH**	**IDO**	**Delta Ct**	**Delta Ct IDO (GAPDH) WB vs DBS**
STT001	34,2	35,2	1,0	33,5	36,1	2,6	177.6
STT002	29,4	35,8	6,4	31,9	36,2	4,3	83.9
STT003	29,3	34,4	5,1	31,8	36,7	4,9	97.5
STT004	28,6	31,8	3,2	36,7	0,0	0,0	50.0
STT005	30,0	33,5	3,5	32,3	35,7	3,4	98.2
STT006	29,3	32,4	3,1	37,6	37,7	0,1	51.3
STT007	28,3	31,4	3,1	37,6	0,0	0,0	50.0
STT008	31,0	34,7	3,7	35,8	37,0	1,2	66.7
STT009	29,9	34,2	4,3	36,3	0,0	0,0	50.0
STT010	29,1	33,0	3,9	34,3	37,0	2,6	83.9
STT011	32,2	36,1	3,9	33,1	37,3	4,2	104.0
STT012	29,9	34,2	4,3	37,3	0,0	0,0	50.0
STT013	28,5	30,1	1,6	29,1	34,0	4,9	200.4
STT014	27,1	31,9	4,8	32,8	36,6	3,8	89.2
STT015	31,2	34,4	3,3	32,4	36,0	3,6	104.6
STT016	29,5	33,6	4,1	36,7	37,9	1,2	64.6
STT017	27,2	34,5	7,3	32,7	37,2	4,5	81.2
STT018	27,9	34,1	6,2	36,3	0,0	0,0	50.0
STT019	28,3	36,0	7,7	34,8	38,1	3,3	71.4
STT020	34,6	0,0	0,0	30,7	36,4	5,7	0.0
STT021	0,0	0,0	0,0	33,9	37,6	3,7	0.0
STT022	27,0	32,1	5,1	31,8	36,7	4,9	98.5
STT023	27,6	30,6	3,0	31,7	34,4	2,7	94.4

	Whole Blood			Dried Blood Spot			% Similarity
Participant ID	**GAPDH**	**IDO**	**Delta Ct**	**GAPDH**	**IDO**	**Delta Ct**	**Delta Ct IDO (GAPDH) WB vs DBS**
STT024	29,5	33,7	4,2	31,5	35,5	4,0	97.9
STT025	30,2	31,6	1,4	33,9	35,2	1,3	98.1
STT026	28,4	33,8	5,4	35,4	0,0	0,0	50.0
STT027	29,1	33,3	4,2	34,2	36,7	2,5	79.7
STT028	28,5	32,5	4,0	33,8	37,2	3,4	92.9
STT029	29,3	32,1	2,8	33,9	36,8	2,9	102.1
STT030	28,5	33,3	4,8	34,0	37,2	3,1	82.5
STT031	27,0	32,3	5,2	33,4	37,3	3,9	87.3
STT032	28,7	31,3	2,6	31,4	33,5	2,1	91.0
STT033	27,4	30,0	2,5	33,2	35,2	2,0	88.9
STT034	27,8	29,2	1,4	31,2	32,7	1,4	98.8
STT035	27,3	30,4	3,1	33,9	36,5	2,5	91.0
STT036	29,0	31,8	2,8	32,6	37,2	4,7	133.2
STT037	28,1	30,4	2,3	31,6	33,4	1,8	89.3
STT038	28,4	31,6	3,2	35,2	36,7	1,5	73.0
STT039	29,5	32,5	3,0	34,1	35,8	1,7	78.6
STT040	31,5	35,6	4,1	33,2	36,2	3,0	86.9
MINIMUM	0,0	0,0	0,0	29,1	0,0	0,0	0.0
MAXIMUM	34,6	36,1	7,7	37,6	38,1	5,7	200.4
MEAN	28,5	31,2	3,6	33,7	30,8	2,6	83.5
MEDIAN	28,9	32,5	3,6	33,7	36,3	2,7	87.1

Delta Ct (BACT)

	Whole Blood			Dried Blood Spot			% Similarity
Participant ID	**BACT**	**IDO**	**Delta Ct**	**BACT**	**IDO**	**Delta Ct**	**Delta Ct IDO (BACT) WB vs DBS**
STT001	31,8	33,4	1,6	30,7	36,7	6,0	237.9
STT002	26,3	34,1	7,8	31,9	37,0	5,1	82.6
STT003	26,1	32,8	6,8	29,6	33,4	3,8	78.0
STT004	25,1	31,6	6,6	34,5	36,4	1,9	64.7
STT005	26,2	34,2	8,0	28,0	35,3	7,4	96.0
STT006	25,8	31,3	5,5	34,4	37,6	3,2	79.3
STT007	24,5	31,2	6,7	34,8	0,0	0,0	50.0
STT008	27,7	32,8	5,2	31,4	37,0	5,5	103.8
STT009	27,4	32,2	4,8	34,0	37,3	3,4	84.8
STT010	26,4	32,3	5,9	33,0	36,8	3,8	82.0
STT011	29,1	35,6	6,6	30,1	0,0	0,0	50.0
STT012	27,3	31,0	3,7	33,5	33,5	0,0	50.0
STT013	25,5	28,4	2,9	26,0	33,4	7,4	175.8
STT014	25,1	30,8	5,7	33,4	34,9	1,5	63.0
STT015	28,3	36,1	7,8	29,5	34,9	5,4	84.4
STT016	28,2	35,0	6,8	34,8	37,9	3,2	73.3
STT017	26,8	35,1	8,2	32,7	34,0	1,3	57.7
STT018	26,6	33,7	7,1	37,1	0,0	0,0	50.0
STT019	26,3	34,5	8,2	33,0	37,5	4,5	77.5
STT020	31,4	0,0	0,0	29,6	37,0	7,4	0.0
STT021	0,0	0,0	0,0	31,9	37,0	5,1	0.0
STT022	24,8	31,9	7,1	31,5	37,1	5,6	89.7
STT023	22,3	30,7	8,5	28,0	34,4	6,4	87.8
STT024	24,4	33,9	9,5	27,3	35,4	8,1	92.6

	Whole Blood			Dried Blood Spot			% Similarity
Participant ID	**BACT**	**IDO**	**Delta Ct**	**BACT**	**IDO**	**Delta Ct**	**Delta Ct IDO (BACT) WB vs DBS**
STT025	25,6	32,0	6,4	30,2	35,3	5,1	89.8
STT026	23,7	33,8	10,1	31,6	0,0	0,0	50.0
STT027	23,9	33,4	9,5	30,1	37,0	6,8	85.9
STT028	23,7	32,8	9,1	31,6	37,4	5,8	81.9
STT029	24,3	32,5	8,2	31,4	37,3	5,9	86.0
STT030	23,8	33,6	9,7	29,9	37,7	7,9	90.4
STT031	21,8	32,5	10,7	29,8	34,7	4,9	72.6
STT032	23,8	31,6	7,9	27,6	33,8	6,2	89.7
STT033	22,2	30,1	7,9	29,6	35,4	5,7	86.5
STT034	23,2	29,9	6,7	27,7	33,1	5,4	90.0
STT035	22,2	30,9	8,7	30,7	35,0	4,3	74.6
STT036	24,7	31,0	6,2	30,4	37,0	6,6	103.0
STT037	24,6	30,4	5,8	28,3	33,5	5,2	94.7
STT038	24,6	30,7	6,1	30,7	37,3	6,5	103.5
STT039	25,7	33,2	7,6	30,9	38,2	7,3	98.1
STT040	27,5	34,8	7,2	29,6	36,1	6,5	95.2
MINIMUM	0,0	0,0	0,0	26,0	0,0	0,0	0.0
MAXIMUM	31,8	36,1	10,7	37,1	38,2	8,1	237.9
MEAN	25,0	30,9	6,7	31,0	32,3	4,6	82.6
MEDIAN	25,3	32,4	6,9	30,7	35,8	5,3	84.6