Dysregulation of tryptophan metabolism in a sub-Saharan HIV/AIDS population

by

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Submitted in partial fulfilment of the requirements for the degree

Philosophiae Doctor **(PhD) in Human Physiology**

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2015

Declaration

I, Priyesh Bipath, hereby declare this thesis has not been plagiarised. All of the experiments and sample analyses included for this study were conducted by me, except for the results obtained from patient files and reports.

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P Bipath **Date**

Acknowledgements

- Prof M Viljoen for her supervision, mentorship, advice and encouragement
- Prof PF Levay for his assistance with the sourcing of patients and for doing the clinical examinations
- Prof DH van Papendorp for allowing the study to take place at the Department of Physiology
- The personnel of the departments of Chemical Pathology and Pharmacology for the use of the GC-MS instrumentation and support
- The library and support services of the University of Pretoria
- The Medical Research Council of South Africa and the South African Sugar Association for the research grants
- The SANBS blood bank and blood donors for allowing the collection of control bloods
- My parents and brothers for their love and support throughout my studies

Summary

Title: Dysregulation of tryptophan metabolism in a sub-Saharan HIV/AIDS population

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The essential amino acid tryptophan is an important substrate for the synthesis of serotonin, melatonin, tryptamine, proteins and the kynurenines. The aim of this study was to investigate tryptophan metabolism along the kynurenine pathway in a low income sub-Saharan HIV/AIDS patient population from the Gauteng Province of South Africa.

The first objective was to develop and validate a novel gas chromatography mass spectrometry method to enable reliable quantification of tryptophan and metabolites of the kynurenine pathway in plasma. Validation parameters for the detection of tryptophan, kynurenine, quinolinic acid and nicotinamide conformed to international criteria for newly developed methods. The next objective of the study was to find an appropriate biomarker against which to express the results. Several substances previously described as indicators were assessed and compared, including plasma neopterin, procalcitonin, C-reactive protein, the cytokines IL-2, IL-4, IL-6, IL-10, TNF, and IFN-gamma, as well as factors routinely measured and elsewhere described as biomarkers in HIV, i.e., albumin, the albumin/globulin ratio, haemoglobin and red cell distribution width. Neopterin was shown to be superior as indicator of pro-inflammatory status, as indicator of the degree of immune deficiency, to predict disease progression, to distinguish between patients with and without tuberculosis co-infection and to reflect the success of highly active antiretroviral treatment (HAART).

In the analyses of the kynurenine pathway metabolites, tryptophan levels were seen to be significantly lower (24.36 ± 4.14 vs. 43.57 ± 11.85 µmol/l; $p<0.0001$), while the activity of the enzyme, indoleamine 2,3 dioxygenase (IDO), (K/T:136.03 vs. 52.18; $p<0.001$), as well as kynurenine (3.21 ± 1.33 vs. 2.14 ± 0.45 µmol/l; $p<0.001$) and quinolinic acid (4.46 ± 2.32 vs. 0.25 ± 0.058 µmol/l; $p<0.001$) levels were significantly higher in the total patient group (n=105) than in the control group (n=60). Patients on HAART showed not only significantly

higher CD4 counts (296.21 ± 195.50 vs. 170.05 ± 167.26 cells/µl; p=0.003), but also lower inflammatory activity (neopterin: 35.51 ± 35.70 vs. 66.63 ± 40.73 nmol/l; p<0.001 and IL-6: 9.56 ± 12.54 vs. 15.04 ± 19.34 pg/ml; p<0.05), lower IFN-γ (41.43 ± 14.14 vs. 53.68±34.39 pg/ml; p<0.05), higher tryptophan levels (25.13 ± 3.80 vs. 22.04 ± 4.32 µmol/l; p=0.033), lower kynurenine levels (3.08 ± 1.28 vs. 3.58 ± 1.42 µmol/l; p=0.144) and lower quinolinic acid levels (4.03 ± 2.04 vs. 5.77 ± 2.65µmol/l; p=0.072) than patients not on HAART.

Tryptophan depletion and IDO activity, as well as the levels of kynurenine and quinolinic acid, were generally greater than in populations from developed countries. Indications are that this can be ascribed to higher levels of inflammatory activity at comparable levels of immune deficiency in the disadvantaged population of this study. The degree of tryptophan depletion and quinolinic acid accumulation found could negatively impact on the physical and neuropsychiatric wellness of the population. Correlations between quinolinic acid, and nicotinamide levels showed a significant contribution of kynurenine pathway metabolism to the plasma levels of nicotinamide. This *de novo* synthesis of nicotinamide could offer protection against niacin deficiency and NAD depletion in populations with inadequate dietary intake. This is the first study to assess plasma tryptophan, kynurenine, quinolinic acid and nicotinamide levels, as well as IDO activity, pro-inflammatory status and IFN-γ levels, simultaneously in one population and to compare it to that of HIV/AIDS patients in developed countries.

Keywords: tryptophan, kynurenine, HIV/AIDS, neopterin, cytokines, immune activity, gas chromatography-mass spectrometry

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List of Abbreviations:

A/G	Albumin to globulin ratio
ACMS	2-amino-3-carboxymuconate semialdehyde
ACMSD	Aminocarboxymuconate semialdehye decarboxylase
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral treatment
AUROC	Area under the ROC curve
BMI	Body mass index
CBA	Cytometric bead array
CD4	Cluster of differentiation 4
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CV	Coefficient of variance
EI	Electron impact ionisation
ELISA	Enzyme-linked immunosorbent assay
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
HAART	Highly active antiretroviral treatment
Hb	Haemoglobin
HIV	Human immunodeficiency virus
IDO	Indoleamine 2,3 dioxygenase
IFN-γ	Interferon gamma
IL	Interleukin
K/T	Kynurenine to tryptophan ratio
MS	Mass spectrometry
NAD+	Nicotinamide adenine dinucleotide
NHLS	National Health Laboratory Service
NMDA	N-methyl-D-aspartate
NPT	Neopterin
OD	Optical density

PCT	Procalcitonin
PFPA	Pentafluoropropionic anhydride
PFPOH	Pentafluoropropanol
QA	Quinolinic acid
QPRT	Quinolinic acid phosphoribosyltransferase
RDW	Red cell distribution width
ROS	Reactive oxygen species
TB	Tuberculosis
TDO	Tryptophan 2,3 dioxygenase
TNF	Tumor necrosis factor

Chapter 1

Introduction

1.1 Epidemiology and phases of HIV infection

Human immunodeficiency virus (HIV) infection is still highly prevalent despite the first cases of acquired immunodeficiency syndrome (AIDS) being reported over twenty five years ago and despite international efforts to curb the spread of the disease [1,2]. This global pandemic constitutes one of the major public health problems. There is an increasing rate of HIV infection together with the high number of infected individuals in southern Africa, most parts of Asia and Eastern Europe [3,4]. South Africa is reported to have the highest number of HIV infected people in the world and sub-Saharan Africa remains the most affected by the burden of disease [5]. The rapid increase in the number of HIV infected individuals on the sub-Saharan African continent exacerbates the problem of the current lack of resources and overburdened public healthcare systems [6]. In Africa tuberculosis is the leading cause of death among HIV positive patients who have developed AIDS [7]. However, there is also a high prevalence of other secondary bacterial opportunistic infections in this patient population [5,6].

Within a few weeks of HIV infection, i.e., during the acute phase of infection there is a high rate of HIV replication in the blood with a concurrent decline in CD4+ T lymphocyte counts [8]. After the acute phase there is usually a decline in the HIV viral load and the CD4+ T-cell numbers level off to near normal. This is due to the host immune response directed towards HIV, whereby the cytotoxic T lymphocytes and the lesser antibody response restrict viral replication [8,9]. During this period, i.e. the chronic, asymptomatic phase of infection, the infected individual may be clinically well for many years. However up to a billion HIV particles and up to two billion CD4+ T-cells can be destroyed and produced daily. As this asymptomatic phase of infection progresses the virus continues to replicate resulting in a systematic decline in CD4+ T-cell numbers [8]. The individual eventually becomes susceptible to various opportunistic infections due to the drop in CD4+ numbers (immune deficiency). This brings about the onset of the AIDS, during which the host immune function deteriorates as viral load increases [8,9]. Measurements of CD4 cell count and viral load are considered to be the gold standard, not only for examining the extent of viral replication,

immune deficiency and disease progression, but also for identifying the stage for initiation of antiretroviral treatment (ART) [8,10]. Further clinical, immunological and biochemical assessments may be required to investigate underling subclinical and secondary infections as part of the treatment response. This may be of primary importance, especially in sub-Saharan African patients of low socioeconomic status, where malnutrition, lack of resources and prevalence of infections are considered to reduce the period of progression to full blown AIDS [5,10]. Unfortunately, coupled to the resource limited health care settings, shortcomings in the control and management of HIV infection may sometimes lead to less than optimal care of the HIV patient [10,11].

1.2 Tryptophan metabolism

Tryptophan is classified as one of the essential amino acids - "essential" meaning that it cannot be synthesised by the body and needs to be provided by the diet in order to sustain normal levels for optimum physiological functioning [12-15]. Tryptophan is absorbed from the gut into the blood stream after normal dietary intake and digestion. The majority of tryptophan is transported in the blood in one of two forms, i.e., as a protein bound complex, namely tryptophan bound to albumin [12,16], and as unconjugated tryptophan which constitutes the free tryptophan [12,16]. Owing to the instability of the binding of tryptophan to albumin, cerebral uptake of tryptophan across the blood-brain barrier is not hindered by protein binding [12,17]. Tryptophan is transported via the blood to tissues where it may follow several metabolic pathways under specific enzymatic induction. One of the most important functions of tryptophan is its utilisation as substrate for the synthesis of various proteins [12,18]. About 3,5 grams of tryptophan contributes to the daily protein production. According to Peters [18] this tissue protein production accounts for the most significant utilisation of tryptophan. However, tryptophan does not only serve as a nutritional entity but, as will be discussed below, forms an important substrate for alternative metabolic pathways.

Beyond its role in protein synthesis, tryptophan metabolism follows two major metabolic pathways. The first of the two pathways, of which about 1% dietary tryptophan is used as the substrate, is the synthesis of 5-hydroxytryptamine (serotonin) in the nervous system and

other body cells such as the enterochromaffin cells in the gut and blood platelets, and as substrate for the production of melatonin in the pineal gland [12,15,19]. The second or alternative pathway for the metabolism of tryptophan is the kynurenine pathway [12,20]. This pathway takes place in the liver, as well as in extra-hepatic tissue, is under the influence of relevant enzymes, and occurs under specific induction criteria.

The kynurenine pathway is schematically depicted in Figure 1. The initial step for the oxidation of tryptophan metabolism along the kynurenine pathway can occur under one of two enzymes, tryptophan 2,3 dioxygnase (TDO) or indoleamine 2,3 dioxygenase (IDO) [21]. Kynurenine is the first stable metabolite formed in the kynurenine pathway when tryptophan is oxidized under influence of either TDO or IDO. TDO, the liver specific, rate limiting enzyme, is stimulated by tryptophan and corticosteroids. Excess tryptophan, i.e., at levels above the requirement for protein and serotonin synthesis, is oxidized in the liver under influence of TDO, with subsequent metabolism to the end products ATP, CO_2 and water [21]. The IDO enzyme occurs mainly in extra-hepatic tissue such as the spleen, kidneys, lungs, and immune cells and is said not to be limited by a decrease in tryptophan levels [21]. IDO is stimulated by inflammation, mainly by the pro-inflammatory cytokine interferon gamma (IFN-γ) in the periphery, and by interleukin 6 (IL-6) in the central nervous system, but other pro-inflammatory cytokines as well as the HIV tat and nef proteins may also have an influence [22,23]. Two iso-forms of the IDO enzyme have been identified, i.e, IDO1 and IDO2. These have similar structural and enzymatic activities, however their expression patterns differ [21,24].

Following the conversion of tryptophan to kynurenine, a number of enzymatic steps occur along the kynurenine pathway (Figure 1). Kynurenine is converted to 3-hydroxykynurenine, followed by the conversion to 3-hydroxyanthranillic acid and thereafter to 2-amino-3- carboxymuconate semialdehyde (ACMS). Under influence of the enzyme aminocarboxymuconate semialdehye decarboxylase (ACMSD), ACMS is metabolised to picolinic acid, or can be completely oxidised to ATP, CO_2 and water [21,25,26]. Alternatively ACMS may non-enzymatically be converted to quinolinic acid. Quinolinic acid, under influence of the enzyme quinolinate phosphoribosyl transferase (QPRT), is further metabolised along a pathway in which NAD+ and precursors of NAD+ are synthesised [21].

Other metabolic products of the kynurenine pathway include kynurenic acid and anthranilic acid formed from kynurenine, and xanthurenic acid from 3-hydroxykynurenine.

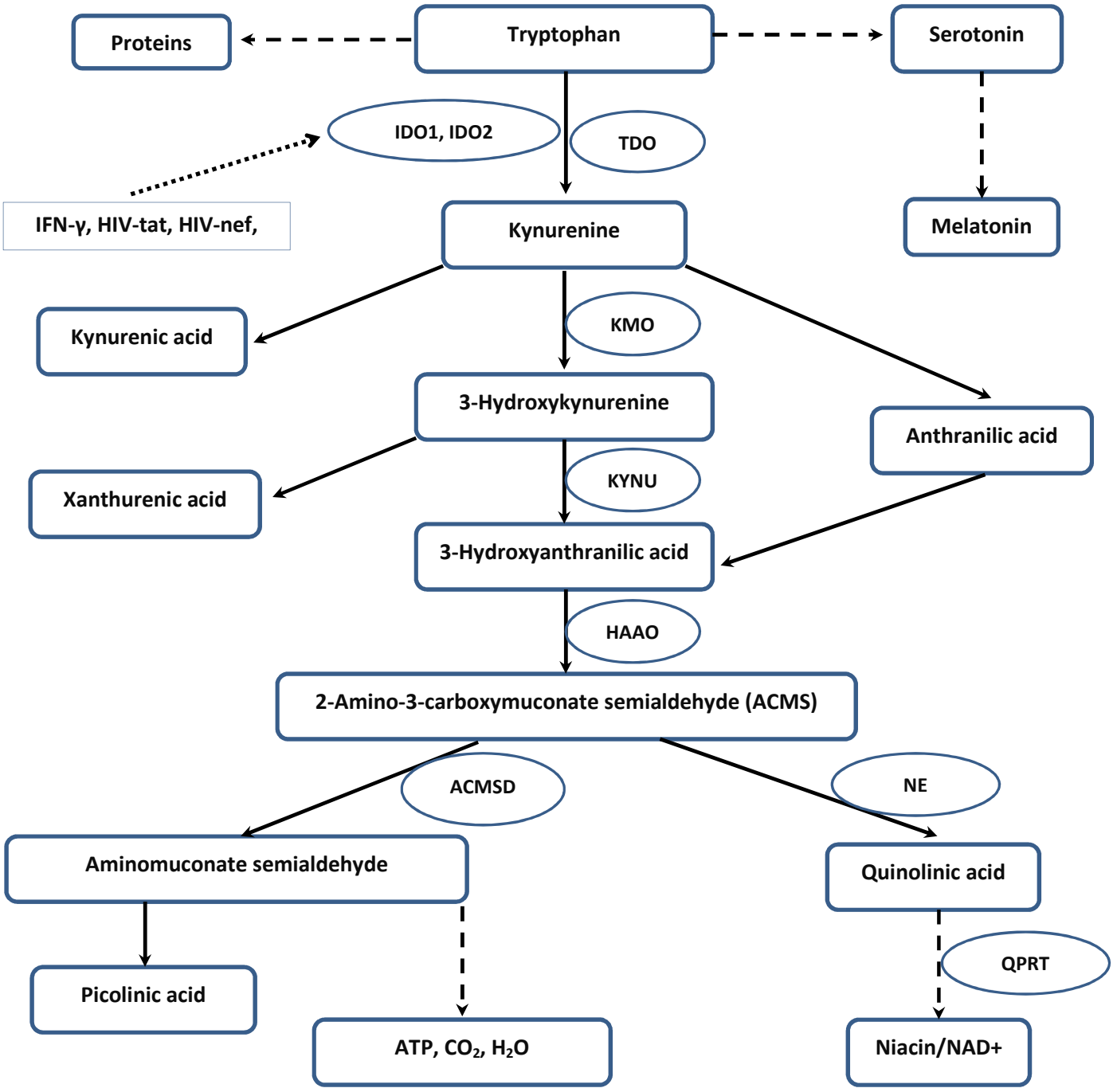

Figure 1 Schematic overview of tryptophan metabolism along the kynurenine pathway. IDO= indoleamine 2,3 dioxygenase, TDO=tryptophan 2,3 dioxygenase, KMO=kynurenine 3-monoxygenase, KYNU=kynureninase, HAAO=3-hydroxyanthranilate 3,4-dioxygenase ACMSD=ACMS dicarboxygenase, NE= non enzymatic conversion, QPRT= quinolinate phosphoribosyl transferase. Adapted from [21,26,27]

Quinolinic acid, a major metabolite of the tryptophan/kynurenine pathway, is considered to be a neurotoxin and, under certain pathophysiological conditions, accumulates in the brain and cerebrospinal fluid (CSF) [28,29]. As a neurotoxin quinolinic acid is an agonist of the N-methyl-D-aspartate (NMDA) receptor subgroup and when present in high concentrations, will exert excitatory effects on these receptors. Another reason for the metabolite's neurotoxicity is situated in its prevention of glutamate uptake into the astrocytes, thus causing an increase in glutamate and thereby overstimulation of the receptors [28,30]. It is in this regard that quinolinic acid has been implicated in number of inflammation-associated neurodegenerative diseases [30-32]. In contrast to quinolinic acid, kynurenic acid is said to have neuroprotective capabilities [33]. Apart from the neurological effects, certain other pathophysiological consequences such as the inhibition of gluconeogenesis [34], erythropoiesis, drug binding and lymphocyte blast formation [35] were noted with the accumulation of quinolinic acid. In addition, quinolinic acid is known to cause lipid peroxidation, to increase the production of reactive oxygen species (ROS) and to promote apoptosis [36-38]. Other metabolites of the kynurenine pathway, such as 3-hyroxykynurenine, xanthurenic and anthranilic acid are reported to also have implications in health and disease [39], but as their levels were not measured in the present study, it is not considered necessary to discuss them.

1.3 Enzyme activity for the conversion of tryptophan to kynurenine

There are certain factors that influence the induction or inhibition, as well as the rate, of TDO enzyme activity. The adrenal stress hormone cortisol is known to induce hepatic TDO activity [21,40]. TDO is also substrate (tryptophan) concentration dependent; hence an increase in tryptophan intake will cause an increase in the normal oxidative breakdown via the kynurenine pathway under influence of TDO [40]. In contrast, there are certain agents that completely inhibit or alter the enzyme activity of TDO. Some pharmacological agents such as non-steroidal anti-inflammatory agents such as tolmetin and sulindac (Sigma Chemicals Company, USA) have been demonstrated to be inhibitors of liver TDO activity [41]. These agents have been shown to be beneficial to patients with depression as they are also known to inadvertently increase brain serotonin levels. This may in part be attributed to increased tryptophan availability for the serotonin pathway as a result of TDO inhibition

and redirection along the tryptophan metabolic pathways. Other inhibitors of TDO, the so-called novel 3-(2-pyridylethenyl) indoles [42], display effects similar to that of the non-steroidal anti-inflammatory agents and also increase brain tryptophan and serotonin availability.

The enzyme activity of IDO is relatively low during normal non-pathological conditions. Amongst one of the major factors that can induce IDO activity, in a number of tissues, is an inflammatory condition where pro-inflammatory cytokines are expressed to a significant extent [21,23]. As IDO is not substrate (tryptophan concentration) dependent, an increase in the induction of IDO may result in reduced tryptophan levels due to increased oxidation/degradation.

As tryptophan is the substrate, not only for protein synthesis, but also for the serotonin pathway, tryptophan depletion via the kynurenine pathway may have a negative effect on general well-being. Although, at first glance, the process of kynurenine pathway IDO activity seems to be only disadvantageous, it may have beneficial effects. Tryptophan is essential for protein synthesis, not only for the host but also for the growth of bacteria, for uncontrolled cell proliferation (cancer cells) and for the intracellular replication of viruses [43,44]. Tryptophan degradation via IFN-γ induction of IDO would therefore form part of the antimicrobial and anti-proliferating characteristics of the immune response [44]. However, this, within our current knowledge, would appear to be a double edge sword as some studies have demonstrated T-cell proliferation to be inhibited by IFN-γ induced IDO-mediated tryptophan depletion - taking into account that tryptophan is essential for the differentiation of T-cells [45,46]. The latter process may contribute to a state of "immunodeficiency" as is the case with HIV infected patients [27]. The main reason probably lies in the fact that tryptophan is also required for the proliferation, differentiation and maturation of T-cells [45]. Thus, under conditions of chronic immune activation and T-cell response, the resulting sustained IDO induced tryptophan catabolism (via the release of pro-inflammatory cytokines, mainly IFN-γ) and subsequent depletion of tryptophan may suppress proliferation of the Th1- type (cellular) immune response [45,46]. This may contribute to the classic profile of conditions such as HIV, whereby an immune activation occurs as a result of the infection, but an immune suppression is also evident. This potential

immune suppression may contribute to the susceptibility and vulnerability of the host to contract various other infections, adding to the burden of the host's already compromised immunity [27].

1.4 Tryptophan depletion and HIV infection

Given the importance of tryptophan for the synthesis of serotonin, melatonin and proteins, as well as its utilisation for other biological and immunological functions [12-15,21,46], systemic depletion of tryptophan is likely to have negative physiologic consequences. With tryptophan depletion due to dietary insufficiencies tryptophan supplementation may in theory be used to correct the depletion and thus restore the substrate availability for the synthesis pool. However, despite some controversy on the matter, a number of physical side-effects have been reported with tryptophan supplementation which may include fatigue, nerve and muscle pain, rash and skin changes, confusion, hallucinations, tachycardia, fever and the eosinophilia-myalgia syndrome [47-49]. In addition, if tryptophan depletion is immune driven via the induction of IDO, tryptophan supplementation may give rise to increased downstream metabolites such as kynurenine and the neurotoxin quinolinic acid [21,46].

The Th1-type immune response is typical for the active phase of virus replication during HIV [8,9], and indications are that the cytokine balance may be skewed towards pro-inflammatory throughout the disease [9]. Viral infection may thus be accompanied by increases in IFN-γ and HIV tat and nef proteins that could induce the enzyme IDO. A number of papers have indeed shown tryptophan depletion in HIV populations from developed countries [50-53], and isolated reports point towards an increase in quinolinic acid levels [54,55]. In sub-Saharan Africa it is feasible to expect that tryptophan metabolism in HIV/AIDS may be compromised by more than the presence of the immunodeficiency virus – e.g. due to malnutrition.

1.5 Niacin and HIV

Niacin is the generic name for two vitamin B3 compounds nicotinic acid and nicotinamide [56]. The main function for niacin is that it plays an important role for enabling the release of energy from carbohydrates and fats and by helping to metabolise proteins [56]. Supplementation with niacin has been shown to be beneficial in reducing cardiovascular events and the progression of artherosclerosis [57]. Oral and intravenous vitamin B3 have been used in conditions such as multiple sclerosis, dysmenorrhea, high triglycerides and cardiovascular disease [57-60]. It can be assumed that some of the effects are mediated by NAD+ as both NAD+ levels are dependent on circulating levels of niacin.

The literature is somewhat confusing with regard to the terminology as it seldom distinguishes between nicotinamide and nicotinic acid and even the values for normal plasma levels are expressed for niacin and not for nicotinamide or nicotinic acid [61,62]. Nevertheless, nicotinamide is said to be the most prevalent and biologically active form of niacin in the circulation [56,63]. The supply of niacin (nicotinamide) to the body occurs via two routes, namely the dietary intake of niacin and through the *de novo* synthesis along the tryptophan-kynurenine-nicotinamide pathway of tryptophan metabolism [64]. Quinolinic acid is the main precursor of niacin (please refer to Figure 1), whereby the enzyme QPRT is involved in this conversion along the *de novo* pathway [21,64]. The rate of conversion, or niacin turnover, is dependent on the demand for the synthesis of nicotinamide adenine nucleotide (NAD+) and its derivatives from niacin [64]. Under normal conditions, this *de novo* synthesis appears not to be influenced by the dietary intake of niacin [56,64]. Therefore quinolinic acid levels may not be influenced by changes in dietary niacin intake in patients where no increased pro-inflammatory activity is present.

A deficiency in niacin and/or tryptophan is known to contribute to pellagra [65]. The symptoms include diarrhea, dermatitis and dementia and death may eventually occur from severe forms of pellagra [65]. Depletion in niacin can result from reduced dietary intake of vitamin B3 or niacin rich foods, or from depletion of the essential amino acid tryptophan - which can bring about a reduced *de novo* synthesis. Although supplementation of niacin is known to be beneficial, large doses have been reported to be intolerable [66]. Cutaneous flushing and hepatic toxicity, due to incorrect formulations, were reported with excess

niacin [66,67]. Short-term niacin trials have also resulted in insulin resistance in some participants [66].

Niacin or NAD+ status in HIV patients is still somewhat controversial. Many studies still refer to a niacin or NAD+ deficiency in HIV/AIDS [27,63,68-70]. However, no clinical study has proved niacin deficiency in HIV patients without pellagra.

The present study

The study presented here investigated disturbances along the kynurenine pathway of tryptophan metabolism in a black sub-Saharan population from the Gauteng Province of South Africa. The thesis is organised into the following sections:

Chapter 2: Deals with the background and demographic information of the patients and controls, ethical clearance for the study, informed consent, patient recruitment, sample collection and processing, and the materials and methods used for the determination of the markers of immune activity.

Chapter 3: Deals with the development and validation a novel gas chromatography mass spectrometry (GC-MS) method to enable simultaneous reliable quantification of the plasma levels of tryptophan and three tryptophan metabolites of the kynurenine pathway.

Chapter 4: Presents the first phase in the search for an appropriate biomarker against which to express the results. The value of procalcitonin, C-reactive protein (CRP) and neopterin as indicators of immune deficiency, co-infection, efficacy of treatment, and disease progression, was investigated for patients with advanced HIV-1 infection.

Chapter 5: In view of the results described in the previous chapter the potential of neopterin as non-specific biomarker in patients with advanced HIV/AIDS was examined further. The special focus of this chapter was on neopterin as pro-inflammatory indicator. Neopterin was validated against cytokines and other indicators of immune activity and health.

Chapter 6: Presents results on tryptophan levels in this sub-Saharan HIV/AIDS population and compared it to levels reported for developed countries. Tryptophan depletion is further examined in context of the general nutritional and inflammatory status.

Chapter 7: This chapter investigated whether higher levels of inflammatory activity, especially IFN-γ, contributes to higher levels of indoleamine 2,3 dioxygenase (IDO)-induced tryptophan degradation in resource-limited populations than in populations from developed countries. The study further examined the levels of quinolinic acid and nicotinamide and deliberated on the contribution of *de novo* synthesis of nicotinamide in the kynurenine pathway of patients with HIV/AIDS.

Chapter 8: Presents a final summation including shortcomings and suggestions for future studies.

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Chapter 2

Materials and methods

2.1 Abstract

This chapter deals with the background and demographic information of the patients and controls, ethical clearance for the study, informed consent and patient recruitment. It further describes the sample collection and processing and the materials and methods used for the determination of the markers of immune activity.

2.2 Patients

This cross-sectional study received approval from the Faculty of Health Sciences Research and Ethics Committee (Clearance Number 107/2008) of the University of Pretoria and from the hospital superintendent of Kalafong Hospital. Informed consent was obtained from all participants. The immunology clinic at the Kalafong secondary hospital in Pretoria, Gauteng province of South Africa, was used as the research site for the recruitment of HIV positive patients. The immunology clinic provides health services to HIV positive patients from areas west of Pretoria as well as the surrounding township. Patients are mostly of low socioeconomic status while many are unemployed or survive on a single grant or pension. An estimated 30% of patients, attending the clinic, are foreigners from surrounding sub-Saharan African countries.

A total patient group of one hundred and five adult (>18 years of age) HIV positive patients were voluntarily recruited at random during their scheduled visit to the clinic. HIV status was confirmed by the clinic which utilises testing performed by the National Health Laboratory Service (NHLS) at Kalafong. Of the total patient group 30 patients were not yet on highly active antiretroviral treatment (HAART), hereafter referred to as the HAART-naïve group. The cohort included 75 patients who were already receiving HAART, hereafter referred to as the HAART group. The patient demographics are provided in Table 2.1.

Table 2.1 Patient demographic information according to antiretroviral treatment

	HAART	HAART-naïve
n	75	30
Females	48 (64%)	18 (60%)
Age (years)	37.86 ± 8.86	37.13 ± 10.24
Ethnicity	75 Black	30 Black
Married	21 (28%)	11 (37%)
Employed	33 (44%)	13 (43%)
Smoker (≥ 1 cigarette per day)	13 (17%)	7 (23%)
Alcohol consumer (≥ 1 drink per week)	7 (9%)	5 (17%)
Body mass index - BMI (kg/m^2)	23.83 ± 6.31	20.96 ± 3.62
Average months on HAART	15.86 ± 16.49	-
Tuberculosis co-infection	14 (19%)	10 (33%)

Data expressed as mean ± SD. Percentages given in parentheses where necessary

There were no differences in demographics between the male and female patients, apart from females having a lower mean age (35.97 ± 9.58 vs. 40.59 ± 7.92 years; p=0.07) and higher BMI (23.97 ± 6.74 vs. 21.83 ± 3.94 kg/m^2; p=0.097).

Triple therapy was given to patients in the HAART group, mostly efavirenz (EFV), nevarapine (NVP), lamuvidine (3TC), and stavudine (D4T). Patients are entitled to go on HAART when their CD4 counts are below 200 cells/µl. Presently the clinic is converting to the FDC (fixed drug combination: emtricitabine, tenofovir, lamuvidine), single dose with apparently better patient compliance. At the time of sample collection 22.9% of the total HIV patient group were confirmed by sputum smears as TB positive. TB positive patients were treated with isoniazid (INH), pyrazinamide (PZA), rifampicin (RIF), and ethambutol (ETH) for at least 6 months pending the sensitivity of the TB organism. Vitamin B complex (B.CO containing 10 mg nicotinamide per tablet; prescribed as two tablets per day) supplements were made available to patients, irrespective of their CD4 counts. However, medical as well as hospital pharmacy staff had reservations about patient compliance and intake could therefore not be accounted for. Although patients are initially educated as to a well-balanced healthy diet and their weights monitored, they do not have regular one-on-one counselling with a dietician.

2.2.1 Patient inclusion and exclusion criteria:

The study cohort had to be HIV positive patients with confirmation thereof. In the case of the HAART group, patients should have been on treatment for at least 1 month. Previous HAART defaulters and those deemed not fit to benefit from HAART were excluded from the study. In the case of the HAART-naïve group, patients who were previously on HAART were excluded. Any of the patients with signs of severe secondary infections, or with clinical assessments that showed other major disorders in addition to HIV, were excluded. As all TB positive patients were on anti-TB treatment they were not excluded. Only patients who gave informed consent voluntarily were included.

2.3 Controls

Controls were recruited from the South African National Blood service (SANBS) satellite site based at Pretoria West. These were healthy volunteers who donate blood regularly (more than once) for the blood bank. Volunteers are pre-screened for HIV at the donor site and confirmation testing is also performed by an external laboratory. A total of 60 HIV negative controls (63% female) with a mean age of 31.18 ± 8 .09 years and BMI of 21.96 ± 4.81 kg/m^2 were randomly recruited for this study. Ethical clearance and approval were also received from the SANBS Human Research Council (Clearance Number 2010/03) and written informed consent was obtained from all of the participants.

2.4 Sample collection and processing

Trained phlebotomists collected blood samples into EDTA tubes. The procedure was performed with the application of a tourniquet and by venous puncture of an ante-cubital vein. A 70% isopropyl alcohol saturated cotton swab was used to disinfect the needle puncture site. Although participants were not instructed to fast overnight, samples were collected in the morning during the patients' or controls' visit to the clinic or blood bank donor site, respectively. In order to prevent haemolysis and analyte degradation, the blood samples for the determination of the immunology and chromatographic variables were processed at the recruitment site. Whole blood EDTA samples were centrifuged at 4000 revolutions per minute for 10 minutes to enable separation of the plasma. Plasma

supernatants were immediately aliquoted into 1.5 ml Eppendorf tubes and placed in an ice container for temporary storage. Light protection measures, with the use of aluminium foil, were maintained throughout the sample processing steps. Sample aliquots were, at arrival at the laboratory, stored at -70^{0}C until analysis. Whole blood EDTA/heparin/gel tubes for plasma or serum samples were also collected and sent to the NHLS for blood chemistry measurements as per request by the clinician involved in this study. Although the latter formed part of the routine analyses for HIV patients some of the results such as albumin, full blood counts and other were used in the study.

2.5 Patient clinical evaluation and blood chemistry

Patients underwent additional clinical examinations and assessments aligned with the purpose and inclusion/exclusion criteria of this study. These were conducted, by the clinician involved in this study, prior to obtaining blood samples. The following indices were evaluated/noted: previous hospital admissions and/or surgery, patient and family history for diabetes mellitus, hypertension and rheumatic fever. Symptoms of fatigue, dyspnoea, headache, oedema, photosensitivity, confusion, neck stiffness, severe weight loss, nausea and vomiting were checked. Signs for blood pressure and pulse abnormalities, as well as temperature, respiration rate, Glasgow-coma scale (GCS), Kernig's and Brudzinski's sign, anaemia, cachexia, opportunistic infections and other signs were also assessed. In terms of the heart the following were evaluated: apex position, character, size, parasternal, jugular venous pressure, palpable murmurs, abdominojugular test (AJR), S1, S2, S3, S4, rub, other murmur and abnormal signs. The lungs, abdomen, musculoskeletal and neurological system were assessed for any abnormalities. Patients were also checked for symptoms of pellagra (diarrhoea, dermatitis and dementia). Even though maize meal is the staple diet of the patient population, it was unfortunately not possible to assess complete dietary intake specifics for each patient.

Some of the routine and other blood chemistry measurements for the total patient group, as performed by the NHLS, are presented in Table 2.2. Additional information was also captured from the patients' files.

Table 2.2 Routine blood chemistry and other results for the total patient group (n=105)

Measurement	Mean results of the patient group	Laboratory reference range
CD4 count (µmol/l)	257.97 ± 193.06	500 – 2010
Viral load (log_{10} copies/ml)	2.75 ± 1.36	0
C-reactive protein (CRP) (mg/l)	25.93 ± 51.22	0.0 – 10.0
Sodium (mmol/l)	136.6 ± 4.04	135 – 147
Potassium (mmol/l)	4.13 ± 0.57	3.3 – 5.3
Chloride (mmol/l)	102.45 ± 4.12	99 – 113
Carbon dioxide (mmol/l)	23.89 ± 3.67	18 – 29
Urea (mmol/l)	4.23 ± 2.88	2.6 – 7.0
Creatinine (µmol/l)	73.56 ± 23.91	60 – 100
Bilirubin (µmol/l)	4.02 ± 3.63	0 – 21
Total Protein (g/l)	86.43 ± 8.68	60 – 85
Albumin (g/l)	33.40 ± 7.55	35 – 52
ɣ-Glutamyl Transferase (GGT) (U/l)	82.87 ± 109.87	0 – 35
Alanine Transaminase (ALT) (U/l)	30.45 ± 19.03	5 – 40
Aspartate Transaminase (AST) (U/l)	36.02 ± 20.79	5 – 40
Alkaline Phosphatase (U/l)	126.53 ± 66.19	40 – 120
Red Cell Count (x 10^{12}/l)	3.67 ± 0.63	4.13 – 5.67
Haemoglobin (g/dl)	11.83 ± 2.33	12.1 – 16.3
Haematocrit (l/l)	0.35 ± 0.07	0.370 – 0.490
Red Cell Distribution Width (%)	17.45 ± 4.35	11.6 – 14.0
Platelets (x 10^{9}/l)	288.34 ± 110.61	178 – 400
White Cell Count (x 10^{9}/l)	5.48 ± 2.54	4.00 – 10.00
Vitamin B12 (pmol/l)	303.58 ± 129.83	142 – 725

Results expressed as mean ± SD. Reference ranges determined by Kalafong NHLS Laboratory

2.6 Neopterin assay

The enzyme immunoassay (Neopterin ELISA IB29125) kit manufactured by Immuno-Biological Laboratories (IBL, Inc. Minnesota, USA) was used for determination of neopterin levels in the plasma samples. The assay was performed according to the manufacturer's protocol. Briefly, ready-to-use standards and quality controls were added to the appropriate wells of the microtiter plates which were pre-coated with anti-mouse IgG Fc (Goat, polycolonal). Unknown patient and control samples were also added to the wells alongside the standards. This step was followed by addition of the ready-to-use solutions containing the neopterin monoclonal antibody and peroxidase enzyme conjugate. After a 90 minute incubation in the dark, the wells were washed 3 times with the wash buffer. The ready-to-use tetramethylbenzidine (TMB) substrate solution was thereafter added to each well

followed by another 15 minute incubation. The substrate reaction was stopped by addition of the stop solution (1N HCl) and the optical density (OD) at 450 nm was immediately measured with a microplate reader. Results were calculated from a standard curve (OD versus neopterin concentration). It was decided for this particular assay to process the samples in duplicate and to use the average OD to calculate the final result. Prior to the actual sample analyses the reproducibility of the kit was tested for our laboratory. Repeat controls measurements were performed and the individual values were all less than 15% from the corresponding mean values. The kit has a sensitivity of 0.7 nmol/l with intra- and inter-assay coefficient of variances (CV) less than 10%.

2.7 Procalcitonin assay

The human procalcitonin ELISA kit (ELH-PROCALC-001) manufactured by Ray Biotech (RayBio, Inc. USA) was used for the determination of procalcitonin levels from plasma samples. The assay procedure was carried out according to the manufacturer's kit protocol. The preparation steps were similar to that of neopterin. However, calibration standard dilutions had to be prepared, the main incubation time was 2.5 hours and two wash cycle steps were required. Absorbance was read at an OD of 450 nm. The sensitivity of the kit is typically less than 30 pg/ml and has an intra-assay reproducibility of CV<10% and inter-assay CV<12%.

2.8 Cytokine assays and flow cytometry

The cytometric bead array (CBA) human Th1/Th2 cytokine kit (Cat no. 551809) was used for the simultaneous determination of the cytokines. The kit is manufactured by Becton, Dickinson and Company (BD Biosciences, Inc.) and is specific for the following cytokines: the interleukins IL-2, IL4, IL6 and IL-10, tumor necrosis factor (TNF) and interferon-gamma (IFN-γ). Flow cytometry with FCAP Array software (Softflow, Inc.) was used for the measurement and calculation of the mean fluorescent intensities. The assay was conducted according to the protocol with minor adjustments made to the staining and incubation times as determined at our laboratory. The kit makes use of capture analyte beads, of known size

and fluorescence, conjugated with the specific cytokine antibodies. Cytokine bead populations with the sandwich complexes created a CBA which was resolved on the red channel of the flow cytometer (BD FACSArray ^{TM}, Inc.). Samples were prepared in single tubes by addition of the antibodies and detection reagents followed by a 3.5 hour incubation and addition of the wash buffer. The stained samples were transferred to BD 96 well plates prior to flow cytometry acquisition. Cytometer setup beads were used to calibrate the flow cytometer and detector. The kit has a sensitivity typically less than 10 pg/ml and has an intra-assay reproducibility of CV<10% and inter-assay CV<12%, with differences in variability between the individual cytokines at different concentrations.

This concludes the chapter on the materials and methods. The next chapter deals with development and validation of the gas chromatography mass spectrometry method used for the quantification of the tryptophan and kynurenine pathway metabolites.

Chapter 3

Development and validation of a novel gas chromatography mass spectrometry method for the determination of tryptophan and metabolites of the kynurenine pathway

3.1 Abstract

This chapter describes the development and validation of a novel gas chromatography mass spectrometry (GC-MS) method to enable simultaneous reliable quantification of the plasma levels of tryptophan and three tryptophan metabolites of the kynurenine pathway. The developed method was sensitive and selective to separate the analyte peaks within a single chromatographic run. Validation outcomes were acceptable for newly developed methods thereby ensuring reliable quantification of the said metabolites from the patients and controls.

3.2 Introduction

Analytical methods for the determination of tryptophan and kynurenine pathway metabolites are not readily available. The methods used in this study had to be newly developed. Furthermore, assessment of tryptophan and its metabolites is complicated by technical difficulties, especially when performed in a laboratory not aligned to do it on a routine basis.

A number of analytical techniques have elsewhere been used to measure one or more tryptophan and kynurenine pathway metabolites from biological specimens [1-8]. Chromatography instrumentation such as high performance liquid chromatography (HPLC), gas chromatography, gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) have on occasion been used to separate and quantify the said metabolites. Mass spectrometry, although regarded to be expensive and not widely available, is considered to be superior and to have selectivity and sensitivity that could enable identification and separation of co-eluting metabolite peaks in a single analytical run [1,7,8].

The main objective, therefore, was to develop and validate a novel gas chromatography mass spectrometry (GC-MS) method to enable simultaneous and reliable quantification of the required metabolites in plasma.

3.3 Method

A GC-MS method was developed for the simultaneous determination of major kynurenine pathway metabolites, i.e., tryptophan, kynurenine, quinolinic acid and nicotinamide. The method was newly developed in our laboratory in accordance with availability of specific instrumentation. A number of trial and error steps were involved, until eventual arrival at a suitable method. Furthermore, the difficulty of a chromatographic analytical technique escalates with every additional metabolite peak concurrently measured within acceptable differentiation of the retention times. For this study the main method was refined and optimised before undergoing validation to confirm suitability and reliability of the sample analyses.

3.3.1 Instrumentation and reagents

Two GC-MS instruments were used for purposes of this study. The first was a Hewlett Packard (HP, Inc.) GC 6890 gas chromatographer coupled to a MS 5973 series mass spectrometer. The initial phase of the method development and validation was conducted on this instrument. However, due to lengthy problems with the vacuum pump down time, pressure and equilibration irregularities, it was decided to complete the final phase of the metabolite quantification on a newer GC-MS instrument. Quality control and established validation parameters were ensured and carried over to the method on the newer instrument. This instrument was a Thermo Scientific (TS, Inc.) Trace 1300 Series GC coupled to a single quadropole ISQ mass spectrometer. Thermo Xcalibur (TS, Inc.) software was used for the method setup, instrument parameters, analyser tuning and sample data acquisition. A DB-5-MS capillary GC column and an electron impact (EI) MS ionisation source were used for the final sample analyses.

Reagents with analytical grade greater than 98% purity were purchased pre-packaged as dry weighed powder. Structural chemistry and integrity of the reagents were crucial for the mass spectral analysis of the standards and unknown samples. The following analytical reagents were used to make up stock solutions for the dilutions of the calibration standards and sample internal standards:

- L-tryptophan: $C_{11}H_{12}N_2O_2$ (204.23 g/mol)
  CAS No. 72-22-3, reagent grade NT > 98% (Sigma-Aldrich)

- L-kynurenine: $C_{10}H_{12}N_2O_3$ (208.21 g/mol)
  CAS No. 2922-83-0 (Sigma-Aldrich)

- Quinolinic Acid: $C_7H_5NO_4$ (167.2g/mol) (FLUKA)
  CAS No. 89-00-9 (2,3-pyridinedicarboxylic acid)

- 3-nitro-L-tyrosine: $O_2NC_6H_3$-4-(OH)$CH_2CH(NH_2)CO_2H$ (226.19g/mol)
  CAS No. 621-44-3 (surrogate internal standard for kynurenine) (Sigma-Aldrich)

-2,6-pyridinecarboxylic acid: $C_7H_5NO_4$ (167.2g/mol) (FLUKA)
  CAS No. 499-83-2 (surrogate internal standard: quinolinic acid, nicotinic acid, nicotinamide)

- Nicotinic acid: $C_6NH_5O_2$ (123.11 g/mol)
  CAS No. 59-67-6 (≥ 98% Sigma-Aldrich, Vitamin B3, Niacin) (Sigma-Aldrich)

- Nicotinamide: $C_6H_6N_2O$ (122.12 g/mol)
  CAS No. 98-92-0 (≥ 98% Sigma-Aldrich, Vitamin B3, Niacinamide) (Sigma-Aldrich)

- 615862 L-Tryptophan-2',4',5',6',7'-D_5 (INDOLE-D_5): $C_{11}D_5H_7N_2O_2$ (209.26 g/mol)
  PubChem ID 24881953 (Deuterated labelled tryptophan internal standard)

In order to prepare the samples suitable for GC-MS analysis selected chemical reagents were used for the analyte derivatization. Reasons for the selection of the following regents will be discussed later.

- Pentafluoropropionic anhydride: $(CF_3CF_2CO)_2O$ (310.05 g/mol)
  CAS No. 356-42-3 (PFPA for GC derivatization, 99%) (Sigma-Aldrich)

- Pentafluoropropanol: $CF_3CF_2CH_2OH$ (150.05 g/mol)
  CAS No. 422-05-9 (PFPOH for GC derivatization, 99%) (Sigma-Aldrich)

The following reagent was also used during the GC-MS sample preparation procedure:

- Trichloroacetic acid: $C_2HCl_3O_2$ (163.39 g/mol)
  CAS No. 76-03-9 (TCA protein precipitation, ≥99%) (Sigma-Aldrich)

Double distilled water (de-ionised water) was used and methanol and other reagents used were all of chromatographic grade.

Volumetric micropipettes were used for dilution of calibration standards and for the preparation of samples. Adjustable pipettes with volume ranges of 20µl, 100µl, 200ul and 1000µl were used. All pipettes were calibrated and checked for correctness prior to and during the sample preparation stages. A calibrated micro-scale (mg) was used to accurately weigh out the analytes for preparation of the stock solutions.

3.3.2 Sample preparation and derivatisation

The use of gas chromatography (GC) requires samples to be derivatised. This entails chemical modification of the analyte to increase volatility of otherwise non-detectable compounds. In addition, the polarity of water soluble functional groups such as COOH, OH, NH and SH should be changed to prevent adsorption to the stationery phase of the GC capillary column. The chromatographic behaviour of the compound of interest is thus improved upon. A number of derivatising reagents were tested during the method development stages. These included silylation, alkylation and acylation reagents. Owing to variation in the functional groups of the analytes measured in this study, it was decided to use a combination of alkylating reagents which were well suited to target all of the analytes. For the purpose of this study the fluorinated anhydride pentafluoropropionic anhydride (PFPA) was used. This derivatising reagent reacts with alcohols, amines and phenols. Pentafluoropropanol (PFPOH) was used in conjunction with PFPA because it was shown, when tested, to be ideal for derivatisation of the remaining functional groups not targeted by PFPA. This combination enabled the simultaneous analysis of all the required metabolites from a single sample.

Stock solutions of the individual analytes and internal standards were prepared with chromatography grade de-ionised water. At first 200 µl serial dilutions of stock solutions were extracted with 800 µl of ethyl acetate, by thorough vortexing and centrifuging at 4000 rpm for 5 minutes. Thereafter 500 µl of the supernatant was transferred to GC amber vials and evaporated to dryness. The dried extracted samples were incubated with 50 µl of PFPA and 20 µl of PFPOH at a temperature of 80^0C for 30 minutes. The derivatised samples were reconstituted in 120 µl of ethyl acetate, transferred to GC auto-sampler vials with inserts and injected for GC-MS analysis. Pooled plasma, from a batch of control plasma samples, was also used to simulate the matrix during preparation of the calibration standards as discussed below. For the plasma based samples 200 µl were precipitated with 50 µl of trichloro acetic acid after addition of the internal standards. The plasma samples were vortexed and centrifuged at 6000 rpm for 8 minutes. The deproteinated supernatant was extracted similarly with ethyl acetate.

3.3.3 Mass spectral identification of the metabolites

Each of the analytes was at first analysed separately so as to identify the individual fragmentation pattern of the mass spectrum under electron impact ionisation conditions. The analyte samples were run parallel to matrix blank and solvent blank samples to differentiate between derivative responses on the chromatogram. Under normal GC-MS operating conditions the analyser was set at -70 eV in scan mode to obtain ions at a range of 50 to 600 mass to charge (m/z) units. The largest most prominent ion for each analyte was chosen for selective ion monitoring (SIM) mode to be used as a qualifier for identification. The most abundant mass ions 286, 266, 254 and 255 for tryptophan, kynurenine, quinolinic acid and nicotinamide (or nicotinic acid) respectively were selected for quantification.

3.3.4 GC-MS setup and method optimisation

The GC was operated under optimised conditions once the method was developed and refined. Optimisation mainly included GC temperature programing, SIM scan times and mass spectrometry (MS) tuning. Furthermore, the MS transfer line temperature and ion source temperature were set at 280^0C and 250^0C respectively. The flow rate of the helium

carrier gas was 1.0 ml/min, while the inlet temperate was set at 200^{0}C. A 1µl volume of sample was injected by the auto-sampler injector and the inlet was set at splitless mode.

For chromatographic separation of the peaks of interest a 30 m DB-5MS GC capillary column, with an internal diameter of 250 µm and a film thickness of 0.10 µm, was found to be ideal. In order to achieve the best peak separation within acceptable retention times the GC oven was programmed according to the rate and run time as indicated in Table 3.1.

Table 3.1 Optimised GC temperature programming

Ramp	Rate (^{0}C/min)	Temperature (^{0}C)	Hold time (min)
Initial	-	80.0	1.20
1	20.0	180.0	5.00
2	10.0	280.0	3.00

Column Max temp: 325^{0}C Equilibration time: 0.50 min Run time: 24 min MS SIM scan time: 4 to 18 min

Although nicotinic acid could be analysed individually by the developed method, it was decided to quantify nicotinamide only because the retention times of nicotinic acid met with interference from the solvent elution when separating multiple peaks at varied retention times. Therefore the temperature programming and MS scan time (Table 3.1) was moderated without consideration of the nicotinic acid peak. Figures 3.1 and 3.2 represent a typical total ion chromatogram illustrating the retention times of the analyte peaks from a spiked plasma sample.

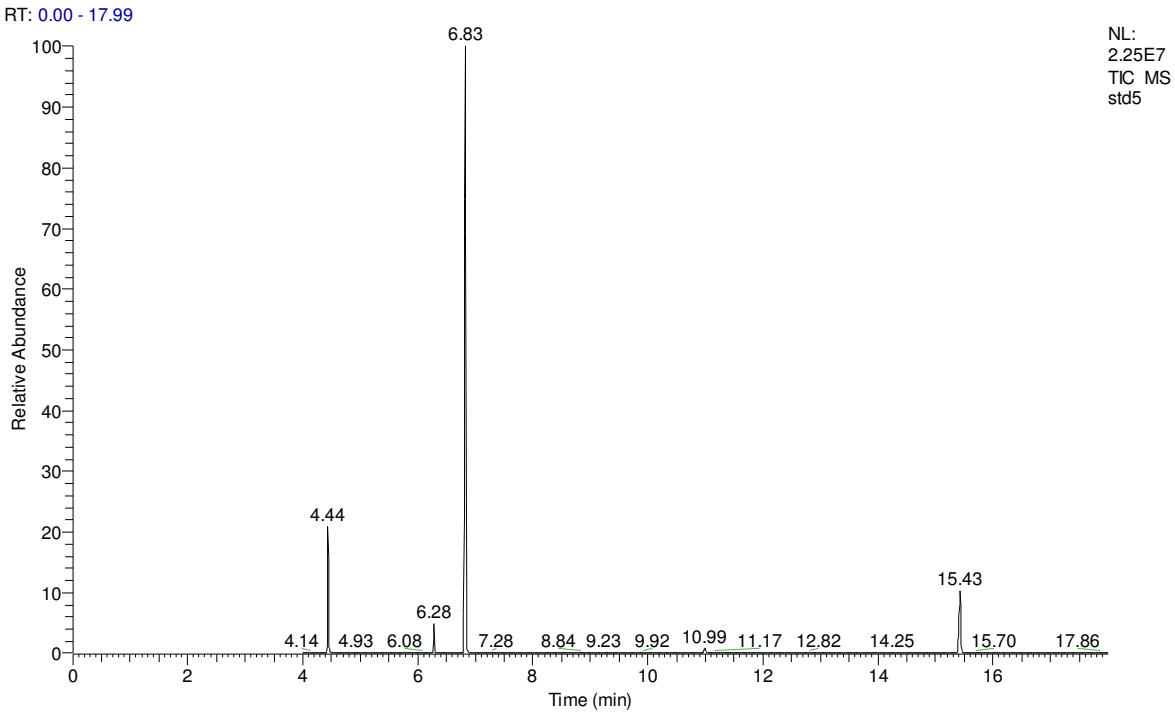

Figure 3.1 A typical chromatogram illustrating the analyte peaks from a spiked plasma sample. The retention times were as follows 4.44 min (nicotinamide), 6.28 min (quinolinic acid), 6.83 min (2,6-pyridinedicarboxyclic acid) and 15.43 (tryptophan and tryptophan-indole-D5).

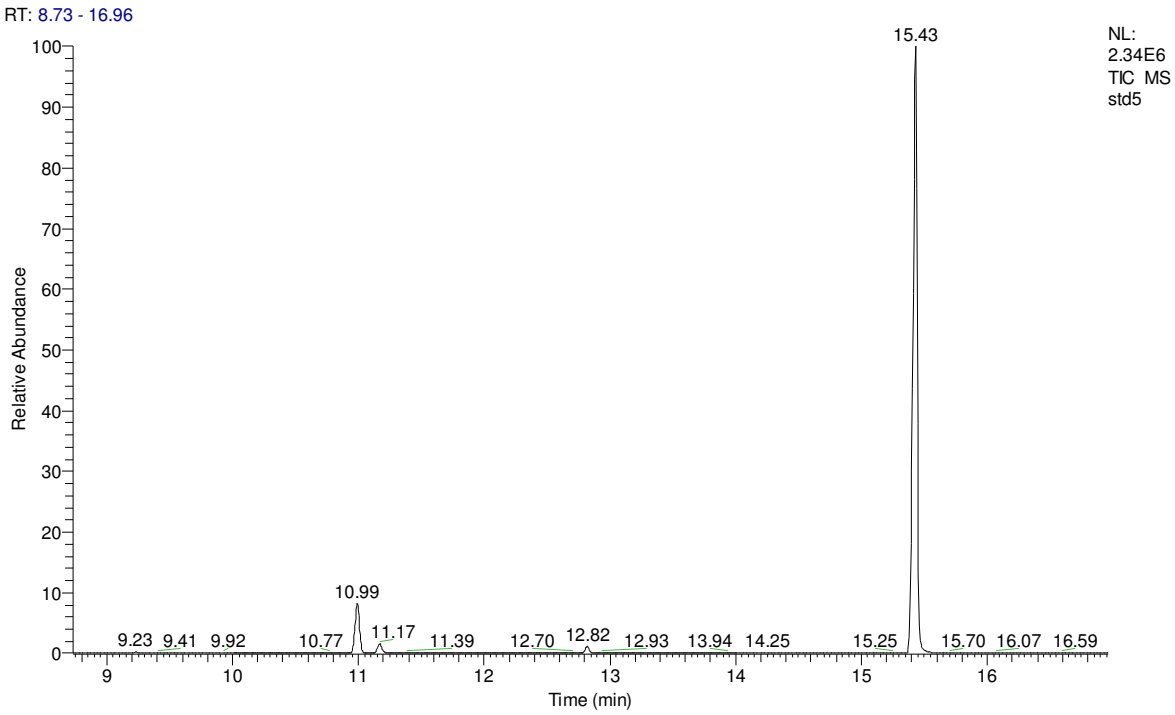

Figure 3.2 Illustration of the zoomed-in chromatogram as represented by figure 3.1. Kynurenine is represented at retention time 10.99 min followed by tryptophan at 15.43 min.

3.4 Method validation

Method validation experiments were conducted according to the optimised GC-MS method. The following validation parameters in terms of sample preparation as well as instrumentation sensitivity, selectivity and reliability were investigated, i.e., linearity, variance, sample stability, recovery and limits of detection. The main validation procedures and criteria are given.

3.4.1 Linearity and quantification

Calibration standards were first prepared by proportionally diluting the stock solution with de-ionised water. Linearity was determined by spiking a matrix (plasma based) pool with the standards in order to simulate actual samples. Six calibration standards, including a blank, for each of the metabolites were prepared. The concentration of 25 µmol/l and a 50 µl volume addition of the relevant internal standards remained the same for each set of standards and blank samples. The preparation of the calibration standards was as follows. First the predetermined concentration of stock solution, containing all of the analytes, was diluted with water in a 1:1 ratio which served as the highest standard. Thereafter for the fourth standard 200µl of the highest standard was diluted with 200µl of water. The third standard consisted of 200µl of the fourth diluted with 200µl of water. The same consecutive procedure was followed for each of the remaining standards. Calibration standards were subjected to the same sample preparation procedure and GC-MS analysis as all other samples. With the use of Excel or X-calibur integration software the calibration curves for each metabolite were constructed using the relative response (analyte divided by internal standard peak intensity) on y-axis versus the concentration value on the x-axis. A set of n=5 individual calibration curves served to provide the average linear standard curve ($y=mx+c$ and r^2 value > 0.98). Any peak response from the matrix blank was subtracted from the respective standard peak responses such that the calibration blank intercepted zero on the axis. High and low quality control standards were also prepared by diluting the highest standard according to 1:1.25 and 1:10 ratios respectively. The values of the quality controls were calculated from the relevant calibration curves. Quality control standards were also used during the other validation experiments as well as actual sample analyses. A new

calibration curve was constructed on each day of the patient and control sample analyses. The five calibration standard concentration points for tryptophan and nicotinamide were 100, 50, 25, 12.5 and 6.25 µmol/l. The high and low quality control concentrations were 80 and 10 µmol/l respectively. For kynurenine and quinolinic acid the calibration points were 25, 12.5, 6.25, 3.13 and 1.56 µmol/l and the high and low quality controls were 20 and 2.5 µmol/l.

3.4.2 Within- and between-day variations

Individual sets of high and low quality controls were determined over 5 consecutive days. The coefficient of variance (CV), calculated as the standard deviation divided by the mean, were examined and the average CV over the 5 days served as the between-day variation. Quality controls were chromatographed under regular sample preparation and laboratory conditions. CVs were determined from n=6 sets each of high and low quality controls as analysed on each consecutive day. For determination of within-day CV n=20 sets of high and low quality controls were determined over the course of the day. Within-day CV of less than 10% and between-day CV less than 15% were considered acceptable for validation.

3.4.3 Stability

Pre-analytical sample stability was tested using n=6 sets of matrix spiked high and low quality controls which were freshly prepared and analysed on the first day. Portions of the same batch of freshly prepared quality controls were frozen at -70^{0}C and thereafter reanalysed on a weekly basis over 4 consecutive weeks. For each of these analysis periods n=2 high and low quality controls were determined. Stability at each of the weekly periods was expressed as a percentage of the mean values obtained on the first day. Percentages less than 75% were considered as unreliable according to validation criteria. In terms of processed sample (auto-sampler) stability n=3 sets of high and low quality controls were analysed in the morning and thereafter reanalysed after 8 hours of standing on the instrument auto-sampler. Stability of these samples was expressed as a percentage of the values observed in the morning.

3.4.4 Recovery

Recovery from spiked plasma samples was determined according the regular sample preparation conditions. For the recovery experiment stock solutions were diluted 1:3 and 1:9 to provide 2 concentration levels which were analysed in groups of n=6 samples. The average recovery for each metabolite was calculated from the 2 concentration points. Proportional error was calculated as the percentage difference of the recovery value from 100 and indicates the relative error in the method for the recovery of a particular analyte.

3.4.5 Limits of detection

Serial dilutions of the lowest concentration standard for each analyte were prepared and analysed in triplicate. The lowest detectable concentration was determined as the peak intensity differentiated from the background noise (baseline) corresponding to a signal to noise ratio of 3:1 or more. The signal to noise ratio of 10:1 was used to calculate the average limit of quantification for each of the analytes. All samples were chromatographed according to regular sample preparation and instrumentation conditions. Peak integration software was also used to subtract the background noise during SIM mode.

3.5 Validation results

The results obtained from the main method validation experiments are given. In terms of linearity the calibration standards were linear over all of the concentration ranges for each of the analytes. Furthermore the R^2 for each analyte calibration curve was always greater than 0.98 throughout the validation experiments and sample analyses.

Table 3.2.1 Typical calibration results for tryptophan

Concentration (µmol/l)	Analyte Peak Area	IS Peak Area	Relative Response
0	0	26153	0
6.25	54174	88324	0.61
12.5	157136	104058	1.51
25	539619	183749	2.94
50	1018242	160205	6.36
100	2334466	191202	12.21

IS: Internal Standard Relative response = analyte peak area divided by IS peak area

Table 3.2.2 Typical calibration results for kynurenine

Concentration (µmol/l)	Analyte Peak Area	IS Peak Area	Relative Response
0	0	278520	0
1.5625	537111	108326767	0.005
3.125	1618412	101059929	0.016
6.25	4624113	126108915	0.037
12.5	10619514	160133827	0.066
25	29269615	220596576	0.133

Table 3.2.3 Typical calibration results for quinolinic acid

Concentration (µmol/l)	Analyte Peak Area	IS Peak Area	Relative Response
0	0	278520	0
1.5625	144171	6604866	0.022
3.125	498081	5089503	0.098
6.25	1669481	6073152	0.275
12.5	4299032	6083620	0.707
25	7661242	5442979	1.408

Table 3.2.4 Typical calibration results for nicotinamide

Concentration (µmol/l)	Analyte Peak Area	IS Peak Area	Relative Response
0	0	278520	0
6.25	72409	6604866	0.011
12.5	144818	5089503	0.028
25	289636	6073152	0.048
50	579272	6083620	0.095
100	1158544	5442979	0.213

Tables 3.2.1 to 3.2.4 provide the typical calibration results for tryptophan, kynurenine, quinolinic acid and nicotinamide. The corresponding curves are illustrated by Figures 3.3.1 to 3.3.4 which were constructed on the last day of validation testing. Concentrations of quality controls and samples were calculated from the equation y=mx + c as determined from the particular curve where y is the relative response, m is the gradient, x is the concentration and c is the intercept.

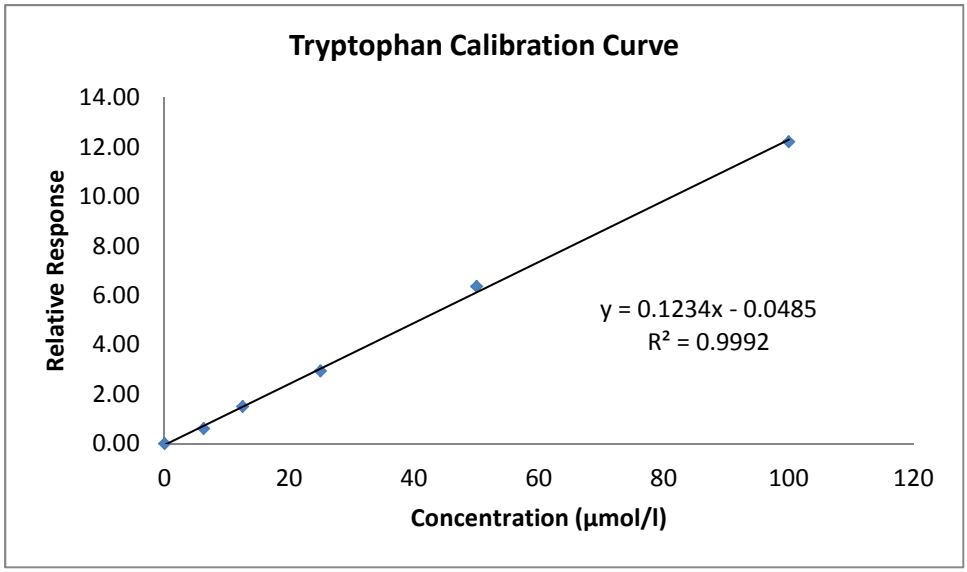

Figure 3.3.1 Typical calibration curve for tryptophan.

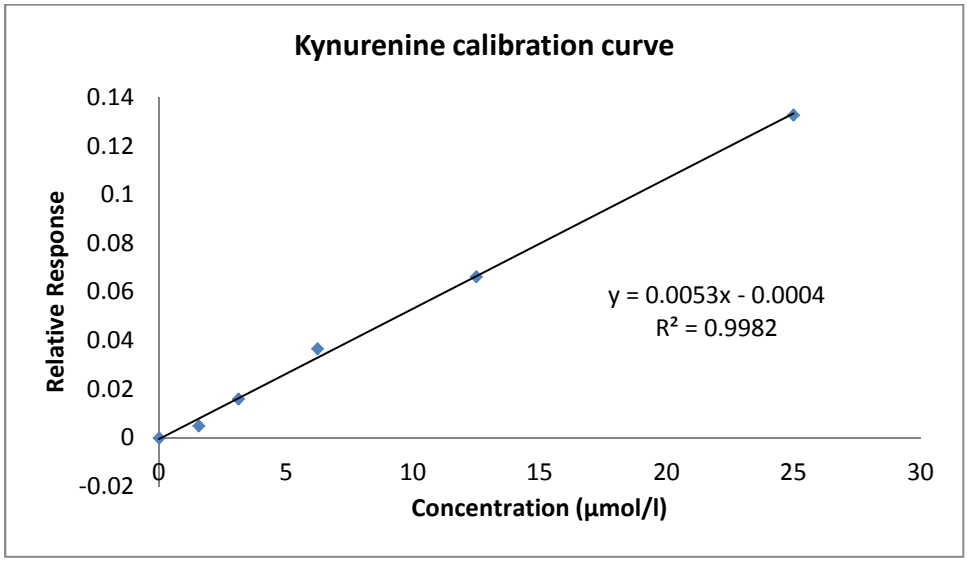

Figure 3.3.2 Typical calibration curve for kynurenine.

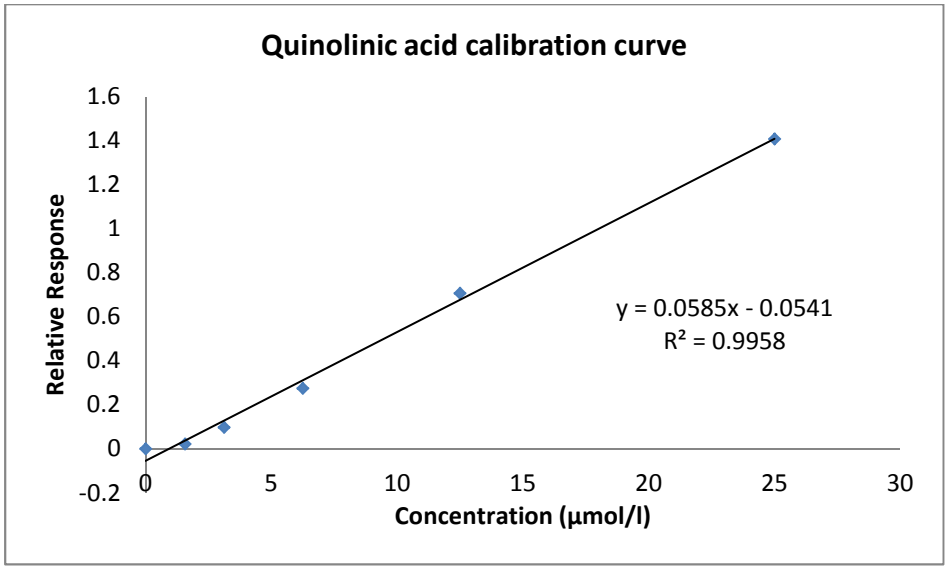

Figure 3.3.3 Typical calibration curve for quinolinic acid.

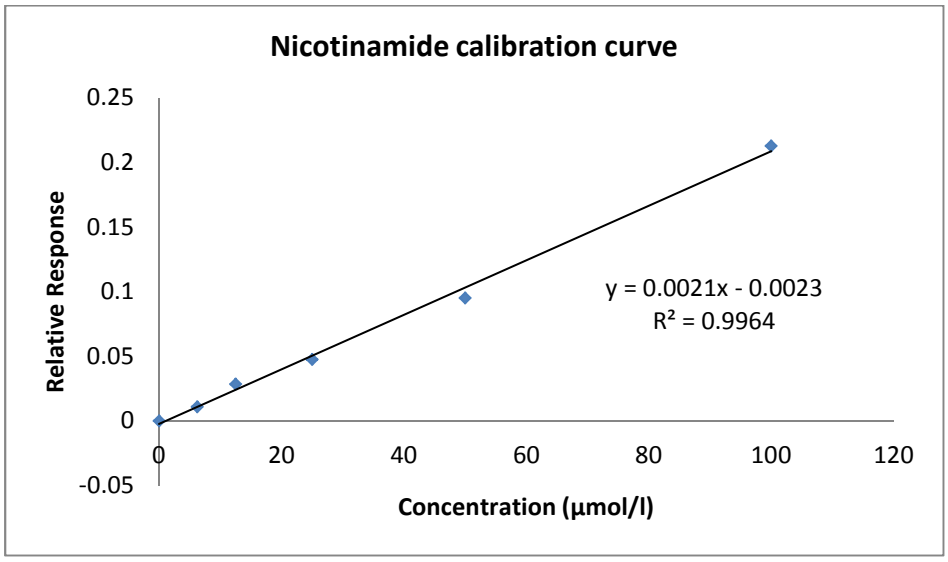

Figure 3.3.4 Typical calibration curve for nicotinamide.

Regarding variance, the average (n=6) between-day CV results for each analyte are given in Table 3.3. The average CV results for the within-day variance (n=20) was <10 % for all of the analytes.

Table 3.3 The average between-day coefficient of variance results obtained for each analyte

		Day 1	Day 2	Day 3	Day 4	Day 5	Average
Tryptophan	HC	3.33	4.80	3.21	3.98	4.23	**3.91**
	LC	3.19	6.68	6.36	6.18	5.88	**5.66**
Kynurenine	HC	5.89	6.66	5.82	5.79	7.84	**6.40**
	LC	7.72	8.24	6.70	6.19	5.40	**6.85**
Quinolinic Acid	HC	3.40	5.91	5.52	5.42	5.17	**5.08**
	LC	8.22	6.39	7.68	5.01	6.17	**6.69**
Nicotinamide	HC	3.64	4.94	3.50	4.14	3.92	**4.03**
	LC	7.03	4.15	5.41	6.96	5.01	**5.71**

Data for each day expressed as the average of n=6 quality control repeats. CV = standard deviation divided by mean expressed as a percentage. HC: High quality control; LC: Low quality control

Samples which were analysed over the course of four weeks were stable with >75% stability for all of the analytes investigated. The stability results are presented in Table 3.4. In terms of processed sample stability, samples were >98% stable for each analyte when analysed after standing for 8 hours on the instrument auto-sampler.

Table 3.4 Stability results for analytes determined over the course of four weeks

		Week 1	Week 2	Week 3	Week 4
Tryptophan	HC	104.58	99.35	93.34	101.23
	LC	97.97	98.97	100.77	101.39
Kynurenine	HC	104.29	92.18	93.50	107.10
	LC	84.45	79.24	79.79	84.85
Quinolinic Acid	HC	98.20	103.12	101.24	96.55
	LC	101.07	104.09	94.46	91.88
Nicotinamide	HC	101.53	95.05	106.06	97.05
	LC	100.92	102.04	97.69	101.55

Data expressed as a percentage of results observed on first day of analysis. LC: Low quality control; HC: High quality control

The recovery results of tryptophan and kynurenine metabolites from human plasma are given in Table 3.5. The average recovery of the analytes for the first level concentration was >85% and for the second level >75%. The systemic proportional errors were less than 25%.

Table 3.5 Average recovery and proportional error results of tryptophan and kynurenine metabolites from human plasma

	Recovery		Proportional Error	
	Concentration 1	Concentration 2	Concentration 1	Concentration 2
Tryptophan	98.94	88.16	1.06	11.84
Kynurenine	87.12	76.30	12.88	23.70
Quinolinic Acid	97.04	85.80	2.96	14.20
Nicotinamide	98.46	85.49	1.54	14.51

Data expressed as average percentage of n=6 repeats. Proportional error is the difference between 100 and % recovery.

The limits of detection from plasma spiked samples were determined to be 0.043, 0.084, 0.026 and 0.071 µmol/l for tryptophan, kynurenine, quinolinic acid and nicotinamide respectively.

3.6 Summary

The analysis of tryptophan and kynurenine pathway metabolites is challenging with many technical difficulties [1] and most rapid techniques require the use of mass spectrometry for optimised runtimes [7]. Even though we were fortunate to use mass spectrometry for this study, this method was not designed with fast throughput routine analyses in mind and optimisation, therefore, was focussed on the reliable separation of the analyte peaks without the intention of reducing analysis runtimes. Tryptophan, kynurenine, quinolinic acid and nicotinamide could successfully and simultaneously be determined from a single plasma sample within 18 minutes using a total chromatographic runtime of 24 minutes per sample. Validation parameters were within acceptable limits for newly developed methods [9,10]. All four analytes showed good linearity across the concentration ranges while variations of sample analyses were below 10% CV. Samples were stable when tested over four weeks and processed samples were stable prior to GC-MS analysis during auto-sampler acquisition. More than 75% of the specific analytes could be recovered from plasma. The developed and validated method was thus sensitive and selective enough to simultaneously measure tryptophan and the kynurenine metabolites from plasma. The main objective of this phase of the study was met, thereby ensuring the reliable quantification of the said metabolites from the patient and control samples.

3.7 References

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Chapter 4

Levels of procalcitonin, C-reactive protein and neopterin in patients with advanced HIV-1 infection

4.1 Abstract

This chapter deals with the first phase of the search for an appropriate biomarker against which to express the results of the tryptophan-kynurenine pathway. The chapter compares the values of procalcitonin, C-reactive protein (CRP) and neopterin as indicators of immune deficiency, co-infection, efficacy of treatment, and disease progression, in patients with advanced HIV-1 infection. The results showed neopterin to be superior to procalcitonin and CRP. Negative associations were found between neopterin levels and the degree of immunodeficiency and positive associations were found between neopterin and co-infection with TB. Neopterin levels were significantly lower in patients on antiretroviral treatment than those not on antiretrovirals. Higher neopterin levels at baseline were associated with a decline in CD4 counts over the ensuing 6 month period, and patients with higher baseline neopterin levels developed more complications over the 6 month period – pointing to a prognostic value for neopterin.

4.2 Introduction

Procalcitonin, C-reactive protein (CRP) and neopterin are three of the markers most commonly used, with varying degrees of success, as diagnostic or prognostic indicators to monitor disease progression and to estimate the efficacy of therapeutic interventions in infectious diseases and non-infectious inflammatory conditions. All three are, to a lesser or greater extent, used among HIV-positive patients.

Procalcitonin is the pro-hormone of calcitonin. In normal conditions, transcription of the procalcitonin gene occurs in the C-cells of the thyroid under conditions of hypercalcaemia and neoplastic disease [1]. However, in the presence of bacterial infection or endotoxins, virtually all cells produce calcitonin precursors [1]. Recent indications are that, in infectious or inflammatory conditions, procalcitonin may in fact be considered an acute phase reactant, with the liver being the major source of procalcitonin [2]. Procalcitonin levels

increase in certain pro-inflammatory conditions, especially bacterial infections, but are thought not to show significant increases with viral and non-infectious inflammatory conditions [3]. The levels are often used to differentiate between patients with sepsis and those with systemic inflammatory response syndrome (SIRS) [4]. Procalcitonin levels have been recommended for distinguishing between bacterial and non-bacterial infections, and therefore as a guideline in the prescription of antibiotics [5,6]. One disadvantage in the use of procalcitonin is that the levels in healthy individuals are below the reliable detection limit (10pg/ml) of most clinical assays.

C-reactive protein is an acute-phase protein, and its levels are upregulated in viral, bacterial and fungal infections, as well as in non-infectious inflammatory conditions. The cytokine profile found with raised CRP levels is predominantly pro-inflammatory, and CRP levels are often used as a non-specific indicator of inflammatory activity, irrespective of the cause [7]. The levels of CRP in bacterial and viral infections differ, and high levels (e.g. >100 mg/l) can be found with bacterial infections, while lower levels (usually <10 mg/l) are more commonly associated with viral infections [8]. As an acute-phase reactant, macrophage- and perhaps adipocyte-derived IL-6 is a major stimulant for the production of CRP, and liver failure is the major cause for a decline in CRP synthesis [9,10].

Neopterin (6-D-erythro-hydroxy propyl pteridine) is a catabolic product of the purine nucleotide guanosine triphosphate. Neopterin is produced in macrophages from guanosine 5'-triphosphate (GTP) which is cleaved by GTP-cyclohydrolase 1 to 7,8-dihydroneopterin triphosphate, followed by conversion of 7,8-dihydroneopterin triphosphate to neopterin and 7,8-dihydroneopterin under the influence of phosphatases [11]. GTP-cyclohydrolase 1 is stimulated, predominantly, by T-helper cell type-1 derived interferon-γ, but co-stimulation by tumour necrosis factor alpha may contribute [11]. Neopterin is used as indicator of both macrophage function and cell-mediated immunity. When cell-mediated immunity dominates, circulating neopterin levels are usually high and, when humoral immunity dominates, neopterin levels are low [11]. Increased neopterin levels are found with viral infections, intracellular bacterial infections, intracellular parasites, a number of auto-immune diseases, malignancies, rheumatoid arthritis, systemic lupus erythromatosus, acute cellular graft rejection or graft-v.-host disease, and in almost every condition where cellular

immunity dominates [12,13]. In HIV-1 infection, serum neopterin has been described as an immune activation marker and predictor of disease progression [14].

In HIV/AIDS, plasma HIV-1 RNA concentration reveals the degree of viral replication, and CD4 counts reflect the degree of immune deficiency and, it is speculated, end-organ damage. The outcome is, however, largely influenced by the co-existence of other complications, especially co-infection with TB. Although viral load and CD4 counts are considered the diagnostic gold standards for HIV, soluble markers may add valuable information about immune activation status and prognosis. In addition, cost-effective reliable serum markers would be of benefit in resource-limited settings where restrictions are placed on the frequency of laboratory investigations such as viral loads. The aim of this investigation was to compare the associations of procalcitonin, C-reactive protein and neopterin and measures of HIV disease status and co-infection with TB.

4.3 Methods

HIV-positive outpatients were randomly recruited from the Immunology Clinic at the Kalafong Hospital, Pretoria. The study took place during 2010 – 2011, and patients were followed-up 6 months after baseline, wherever possible.

Informed consent was obtained from 82 adult patients who were attending the clinic on a Friday, who freely gave informed consent to take part, and who were not ruled out by the exclusion criteria. Exclusion criteria included patients <18 years of age, patients with CD4 counts >400 cells/µl, patients on highly active antiretroviral treatment (HAART) for <2 months, treatment defaulters from the HAART group and, for the HAART-naïve group, patients previously on any HAART. Ethical approval was obtained from the Faculty of Health Sciences Research and Ethics Committee, University of Pretoria.

The patients were firstly divided into a group on active HAART (n=57) and a group not on HAART (HAART-naïve; n=25). The HAART group was further subdivided into groups depending on their time on treatment prior to baseline investigation (2 months - 1 year; 1 - 2 years, and >2 years). At the 6-month follow-up, patients were subdivided into 2 groups according to baseline neopterin levels, and the groups were compared in terms of the CD4

counts and development of complications diagnosed by the attending physician and confirmed by the specialist involved in the study. Neopterin and procalcitonin levels were, however, only determined at baseline and not after 6 months follow up.Blood specimens collected at baseline were centrifuged on site; plasma aliquots were stored at -70^{o}C until analysis. Procalcitonin (RayBiotech Inc., USA) and neopterin (Immuno-Biological Laboratories Inc., USA) were measured by commercial enzyme-linked immune-absorbent assay (ELISA) kits. CRP and other routine blood investigations (CD4 count, WBC count, haemoglobin etc.) at baseline were determined according to standard procedures of the National Health Laboratory Service (NHLS), and results were extracted from the laboratory reports and patient files.

Student's t-test and nonparametric Mann-Whitney U-test were used to determine group differences. Kruskal-Wallis one-Way ANOVA indicated variance across multiple groups. Correlations were determined by regression analysis and Spearman rank correlation co-efficient. Statistical analysis was performed using NCSS/PASS (Hintze J 2001) software, and all testing was done at a significance level <0.05 unless otherwise specified.

4.4 Results

The demographic profiles for the patient groups are presented in Table 4.1. The two groups were comparable in age, body mass index (BMI), gender distribution, race and employment status.

Table 4.1 Patient demographic information at baseline according to antiretroviral treatment

	HAART	HAART-naïve
n	57	25
Females	35 (61.4%)	15 (60%)
Age (years)	36.6 ± 8.2	36.8 ± 10.8
Race	57 black	25 black
Body mass index – BMI (kg/m^2)	22.6±5.0	21.2 ± 3.5
Married	10 (17.5%)	7 (28%)
Employed	22 (38.6%)	12 (48%)
Alcohol consumer (≥ 1 drink per week)	3 (5.3%)	3 (12%)
Smoker (≥ 1 cigarette per day)	9 (15.8%)	5 (20%)
Average months on treatment	13.6 ± 16.2 (2 - 63)	-
Tuberculosis positive at baseline	10 (17.5%)	8 (32%)

Data expressed as mean ± SD. Percentages given in parentheses where necessary. No significant differences noted.

Results of the baseline blood measurements and the comparison between the HAART and HAART-naïve groups are presented in Table 4.2. No significant differences were noted between males and females for the measurements as given in Table 4.2. Neopterin levels were significantly higher (p=0.0096) in the HAART-naïve group than in the HAART group. Negative correlations were found between neopterin and CD4 counts for the total group of patients (r=-0.482; p<.0001; n=82), as well as for the HAART group (r=-0.451; p=0.0045; n=57). Neopterin also correlated negatively with haemoglobin levels for the total patient group (p=-0.597; p<0.0001; n=82). No such correlations were found for CRP and PCT.

Table 4.2 Comparison of baseline blood measurements for the two groups

	HAART	HAART-naïve	p value
n	57	25	-
Procalcitonin (pg/ml)	13.2 ± 3.3	12.9 ± 1.5	0.767
Neopterin (nmol/l)	39.5 ± 38.9	64.4 ± 39.4	0.001*
C-reactive protein - CRP (mg/l)	25.3 ± 38.5	34.9 ± 82.9	0.567
CD4 count (cells/µl)	288.2 ± 196.4	157.5 ± 181.9	0.027*
Viral load (log_{10} copies/ml)	2.4 ± 0.9	3.6 ± 1.7	0.005*
Red cell count ($x10^{12}$/l)	3.6 ± 0.5	3.9 ± 0.7	0.048*
Haemoglobin (g/dl)	14.2 ± 15.2	11.1 ± 2.0	0.345
White cell count ($x10^{9}$/l)	4.9 ± 1.5	5.7 ± 2.8	0.107
Neutrophils ($x10^{9}$/l)	2.7 ± 1.2	3.8 ± 2.7	0.026*
Lymphocytes ($x10^{9}$/l)	1.6 ± 0.8	1.4 ± 0.8	0.211
CD4 % of lymphocytes	17.4 ± 7.6	9.2 ± 7.3	0.0006*

Note: Viral load measured within 2 months of baseline (*p<0.05; results expressed as mean ± SD).

Six months after the baseline measurements, 47 of the original 82 patients were still available and could be followed up with regard to CD4 counts and the development of complications. A comparison between patients with complications and those without complications, at baseline and at follow-up, is shown in Table 4.3. Additional complications at follow-up consisted of TB (n=6, two of whom had extrapulmonary disease); pneumonia (n=5); severe lymphadenopathy (n=4); cardiac/renal disease (n=4) and haematological complications such as anaemia, thrombocytosis or neutrophilia (n=10).

Table 4.3 Comparisons for patients who were followed up after 6 months

	Complications after 6 months	No complications after 6 months	p value
n	29 (61.7%)	18 (38.3%)	-
HAART	12 (41.4%)	15 (83.3%)	-
Baseline CD4 count (cells/µl)	237.0 ± 213.61	327.7 ± 212.96	0.358
6 month CD4 count (cells/µl)	232.5 ± 230.95	325.1 ± 210.22	0.280
Baseline viral load (log_{10} copies/ml)	2.34 ± 0.9	2.3 ± 1.0	0.807
Baseline C-reactive protein - CRP (mg/l)	17.233 ± 25.48	9.5 ± 0.7	0.265
Baseline neopterin (nmol/l)	53.9 ± 33.9	10.8 ± 7.6	0.010*
Baseline procalcitonin - PCT (pg/ml)	13.7 ± 4.5	12.6 ± 0.43	0.313

Data expressed as mean ± SD. *p < 0.05

The relationship between neopterin and CD4 counts over the 6-month period following the baseline assessments was examined. Patients who developed additional complications, stopped taking anti-retroviral drugs or HAART-naïve patients who started HAART during this period were excluded. Seven patients stopped HAART over this period; the reasons included non-compliance and drug side-effects. This cessation resulted in a drastic decline in sample sizes, i.e. 11 patients (8 on HAART) had a decrease, and 9 (all on HAART) had an increase in CD4 over the period. Mean baseline neopterin was significantly higher in the patients whose CD4 counts were decreased at follow-up (35.09 vs. 10.82 nmol/l; p=0.035). In the group whose CD4 counts decreased over the 6-month period, baseline neopterin levels correlated negatively with both baseline CD4 count (r=-0.68; p=0.03) and follow-up CD4 count (r=-0.58; p=0.07).

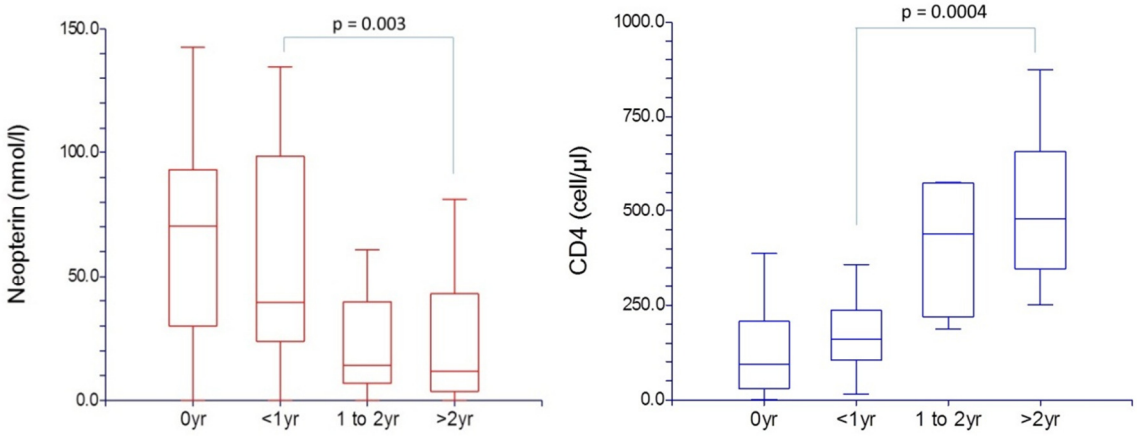

Figure 4.1 Box plots illustrating neopterin and CD4 levels for patients after 0 yr (n=25), <1 yr (n=30), 1 - 2 yr (n=10) and >2yr (n=10) on HAART

As shown in Figure 4.1, the patients were subdivided into groups according to the period of time they had been on treatment prior to the baseline investigations. Analysis of variance showed that neopterin levels were significantly ($p<0.01$) lower and CD4 counts significantly higher ($p<0.001$) in the patients who had been on treatment >1 year.

4.5 Discussion

This study examined the associations of 3 laboratory markers of disease in HIV-positive patients. The key findings are that neopterin is more strongly associated with the degree of immunodeficiency and of co-infection with TB than CRP or procalcitonin. Higher neopterin levels at baseline were inversely related with CD4 counts and positively associated with the development of more complications over the ensuing 6-month period. Limitations of this study include the fact that not all patients could be traced for the 6-months follow-up, that the groups became progressively smaller as patients who had a change in treatment over this period were excluded from the statistical comparisons, and that disease progression could only be estimated from CD4 counts and not viral loads.

The results of this study suggest that CRP levels are not specifically associated with immune deficiency, the effects of HAART, or disease progression. These results are in agreement with those of a study in India, in which CRP measurement in HIV-positive patients was found neither to be of value as diagnostic aid nor as prognostic marker in HIV/AIDS [15]. CRP levels in HIV-positive individuals are generally significantly lower in viral than in bacterial infection [8]. However, significantly raised levels of CRP could be an indication to investigate for a possible co-infection, keeping in mind that other conditions marked by a pronounced pro-inflammatory response can also lead to increases in the levels of CRP. This finding is in line with the results of a South African study by Wilson *et al.* who showed that normal CRP levels, in combination with clinical evaluation, could be useful to rule out TB in populations with a high prevalence of HIV [16].

Procalcitonin (PCT) is known for its increase in bacterial infections and is used by some to differentiate between viral and bacterial infections [17]. One explanation as to why procalcitonin levels remain low in purely viral infections is based on the fact that the production of PCT is primarily stimulated by tumour necrosis factor. It is suggested that

increases in procalcitonin do not occur with viral infections because alpha interferon, synthesised as a result of viral infections, inhibits synthesis of tumour necrosis factor [1]. Should this be true, the question remains whether procalcitonin would be of much use for the detection of bacterial co-infection in HIV-positive patients. In developing countries such as South Africa, co-infections with TB and other bacterial infections in HIV-positive individuals are common – even major sources of morbidity and mortality – especially at CD4 counts <200 cells/µl. The level of circulating PCT in normal healthy individuals is generally below the limit of detection (10 pg/ml) of most clinical assays [18]. According to sensitive research assays, the normal level for plasma/serum PCT is 33±3 pg/ml [1]. The analytical sensitivity for the assay of this study was typically below 30 pg/ml and, from linear extrapolation, individual PCT levels were all >10 pg/ml. However, the mean PCT levels for the total group of patients were normal, with no significant difference between the HAART and HAART-naïve groups, and no significant correlations between PCT and CD4 counts or viral loads. Although the value of PCT as a reliable marker of active TB has on occasion been questioned [19], the overriding assumption is that PCT is indeed a valuable marker of *Mycobacterium tuberculosis* in non-immunocompromised patients [20]. The procalcitonin findings of this study are in line with studies that showed suppression of the procalcitonin response in HIV-positive individuals [20,21]. Although some diagnostic and prognostic value for the measurement of PCT in HIV/TB-co-infection has been described in a South African study, only 58% of their HIV-positive patients with TB had PCT levels marginally above 100 pg/ml [22]. This is, in view of better performing markers, not adequate for clinical use in individual patients. Although procalcitonin induction in HIV-positive individuals is known to occur in sepsis, and reports exist of significant increases in procalcitonin in pneumococcal and a number of other non-viral infections [23], it would appear that secondary infections in HIV-positive patients do not in general trigger overt increases in procalcitonin synthesis [21,23], provided that the infections are localised or organ-related without systemic inflammation.

Neopterin levels were increased above normal (10 nmol/l) in 92% of the HAART-naïve group and in 75% of the HAART group. The levels were significantly higher (p<0.01) in the HAART-naïve group and were inversely associated with CD4 counts. These results confirm the value of neopterin levels as a reflection of the degree of immunodeficiency. Figure 4.1 shows the

increase in CD4 counts that occurred over the same periods on HAART as the decrease in neopterin. This implication (that neopterin may be an indicator of the efficacy of HAART) warrants further investigation.

Among the 18 patients (>26% of the study population; 50% on HAART) in whom active TB-co-infection was confirmed at the baseline investigations, neopterin levels were significantly higher ($p<0.001$), and CD4 counts significantly lower ($p=0.028$), than among the patients without TB co-infection. These results are in agreement with previous indications that neopterin levels are significantly higher in HIV-positive patients with TB-co-infections and that, although neopterin levels may decrease with anti-TB therapy, high levels of neopterin persist with progression of the immune deficiency and a poor prognosis [24].

As neopterin levels reflect the degree of immune deficiency in HIV-positive patients, and perhaps the response to HAART, the question was asked whether neopterin has indeed, as claimed elsewhere, prognostic value concerning disease progression [25]. Baseline neopterin was significantly higher ($p<0.01$) in the group of patients in whom other complications were present 6 months after baseline investigations, than patients who progressed well (53.9±39.9 vs. 10.8±7.6 nmol/l; $p<0.01$). When all patients who stopped HAART over the 6-month period were excluded, the mean neopterin levels were significantly higher in the group with complications than in the group without complications (59.29 vs. 30.9 nmol/l; $p=0.018$). When those patients who did not change antiretroviral status were split into groups, the mean neopterin levels were significantly higher in the group that developed complications than those who did not, both for the HAART (45.9 vs. 24.13 nmol/l; $p=0.04$) and the HAART-naïve (75.02 vs. 30.99 nmol/l; $p=0.001$) groups. The possibility that neopterin levels could perhaps be predictive of disease progression was further examined by looking at the changes in CD4 counts. The baseline neopterin values were compared between patients whose CD4 counts decreased and those that increased over the 6-month period following baseline assessments. To minimise the number of confounding factors, any patient who had additional complications or a change in HAART during the 6 months was excluded. This resulted in a drastic decline in sample sizes, i.e. 11 patients (8 on HAART) had a decrease, and 9 (all on HAART) had an increase in CD4 counts over the period. Mean baseline neopterin levels were significantly higher in patients whose CD4 counts decreased, and significantly lower in patients whose CD4 counts increased. In the group whose CD4 count

decreased over the 6-month period, baseline neopterin levels correlated with both baseline CD4 counts (r=-0.68; p=0.03) and follow-up CD4 counts (r=-0.58; p=0.07). Although the group divisions, owing to the exclusion criteria, were small, the association of neopterin levels with CD4 counts is nonetheless seen. These results warrant further investigation into the value of neopterin as a possible predictor of disease progression.

In view of the stimulatory role of IFN-γ in neopterin synthesis [11], the link between chronic elevation of IFN-γ and HIV-1 progression, as well as the active role of neopterin in the disease [25], the value of neopterin is not surprising. Neopterin has previously been described as one of the better immunological markers in patients with HIV-1 infections [14,25]. It has even been said that neopterin levels increase before other markers of HIV infections have risen [25]. In the present study, 40% of HAART, and 75% of HAART-naïve, patients had CD4 counts <200 cells/µl, and all had CD4 counts <400 cells/µl. Therefore, with regard to patients in the advanced stages of the disease, the results of this study support the notion of neopterin as an inexpensive indicator of CD4 status and as an indicator of bacterial co-infection.

The results of this chapter warranted further investigation of neopterin as an appropriate marker against which to express the results of the tryptophan-kynurenine pathway.

4.6 References

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Chapter 5

Neopterin as pro-inflammatory indicator and as non-specific biomarker in HIV/AIDS

5.1 Abstract

In this chapter neopterin is examined further as biomarker. Pro-inflammatory activity is seen as the major inducer of increased activity in the kynurenine pathway. The primary objective, therefore, was to evaluate neopterin as a marker of inflammatory activity – compared to that of markers such as CRP and pro-inflammatory cytokines. Significant correlations between neopterin and other indicators of inflammation showed neopterin to be a good indicator of active inflammatory status and of the effect of HAART on the immune system. Neopterin was found to be superior to C-reactive protein and to individual cytokines as indicator of immune deficiency. The discriminatory power between patients with and those without tuberculosis co-infection was higher for neopterin than that for other immunological factors. Increased neopterin levels were associated with a decline in albumin, haemoglobin and the albumin/globulin ratio, and with an increase in red cell distribution width.

5.2 Introduction

The search for a suitable biomarker in HIV/AIDS is ongoing. Evermore markers are being evaluated as indicators of immune suppression and disease progression and to better understand the immunopathogenesis of the disease [1-3]. In contrast to the need for biomarkers for specific purposes, a general non-specific marker of disease that could alert the clinician to further investigate the patient would be of value, especially in resource limited environments. Such a tool should be relatively inexpensive and facilities for its determination readily available. In view of the role of inflammatory activity, not only in HIV/AIDS, but in many physical and mental disturbances, it appears feasible to predict a good marker of inflammation perhaps to be the best non-specific marker of general well-being.

The previous chapter showed neopterin to be superior to C-reactive protein and to procalcitonin. In this part of the study the value of neopterin as non-specific indicator was examined in terms of its potential as indicator of inflammatory status, as indicator of immune deficiency or dysfunction, the effects of anti-retroviral treatment and as indicator of TB co-infection. In these assessments the efficacy of neopterin was validated by comparison with immune-related factors such as the acute phase protein C-reactive protein (CRP), CD4 counts, as well as pro- and anti-inflammatory cytokines. In addition, neopterin levels were compared to that of a number of factors routinely measured and elsewhere described as biomarkers.

5.3 Methods

This was a cross-sectional, non-intervention study comprising of 105 HIV positive adult patients recruited from the Kalafong secondary hospital in Pretoria, South Africa, and a control group of 60 HIV negative blood donors from the South African National Blood Service. A total of 75 patients were on antiretroviral therapy (HAART group) and 30 patients were HAART-naïve. Ethical clearance, in accordance with the declaration of Helsinki, the National Health Act and the policy of the University, was received from the Faculty of Health Sciences Research and Ethics Committee of the University of Pretoria (Number: 107/2008). The committee also approved the documentation used for obtaining either written or verbal voluntary informed consent prior to the study. Participants who could not read or write were informed by a clinician about the nature and purpose of the study before verbal consent was obtained. Verbal consent was documented under the relevant section on the consent form which was acknowledged and signed by the clinician and investigator.

Neopterin was measured by commercial enzyme linked immune-absorbent assay (Immuno-Biological Laboratories Inc., USA). The cytokines IL-2, IL-4, IL-6, IL-10, TNF and IFN-γ were measured by cytometric bead array (CBA) kit protocol (BD Biosciences, San Jose, CA, USA) using flow cytometry. The CBA cytokines were analysed on a FACS Array Bioanalyzer using FCAP FCS Filter and FCAP Array software (BD Biosciences, USA). Routine blood variables were analysed by the National Health Laboratory Service (NHLS) at Kalafong.

The data was first tested for normality, followed by log transformation as the raw data was not normally distributed. Transformed data were analysed using one way analysis of variance (ANOVA) for comparison between groups. Pairwise comparisons were undertaken by Tukey HSD and Scheffe methods. Spearman rank correlations were computed to determine associations between group variables. Area under the ROC curve (AUROC) values were determined using logistic regression and CD4 cut-offs of less than 200 cells/µL. Analyses were performed at a significance level of $p<0.05$ using STATA statistical analysis software (version 12.1).

5.4 Results

The suitability of neopterin as a general non-specific marker of disease activity in HIV/AIDS patients was examined in a total patient group of 105 HIV/AIDS (HIV-1) patients with a mean viral load of 2.75 ± 1.36 $\log_{10}$ copies/mL and a CD4 count of 257.97 ± 193.06 cells/µL. The demographic information of the study subjects can be seen in Table 5.1.

Table 5.1 Demographic information for the patient and control groups

	HAART	HAART-naïve	Controls
n	75	30	60
Females	48 (64%)	18 (60%)	38 (63%)
Age (years)	37.86±8.86	37.13±10.24	31.18±8.09
Body Mass Index (kg/m^2)	23.83±6.31	20.96±3.62	21.96±4.81
Average months on HAART	15.86±16.49	-	-
TB co-infection at baseline	14 (19%)	10 (33%)	-

CRP, cytokines and CD4 counts were employed in the appraisal of neopterin as non-specific biomarker of the inflammatory status, immune deficiency, the effects of anti-retroviral treatment and TB co-infection. The cytokines included the pro-inflammatory cytokines IL-2, IL-6, TNF and IFN-γ, as well as the anti-inflammatory cytokines IL-4 and IL-10. A comparison between the Total patient group, the group on HAART, the HAART-naïve and the control group levels of immunological factors can be seen in Table 5.2. The validity of neopterin as indicator of immune deficiency was tested against CD4 counts, CRP and cytokine levels. The results can be seen in Table 5.3.

Table 5.2 Comparison of immunological and other variables between the controls and patient groups

Variable	Controls	Total Patients	HAART	HAART-Naïve	p-value			
	C	T	H	N	T vs. C	H vs. C	N vs. C	H vs. N
n	60	105	75	30	-	-	-	-
IL-2 (pg/mL)	9.14±2.26	20.06±8.31	18.95±8.41	22.74±7.53	<0.0001	0.0003	<0.0001	0.076
IL-4 (pg/mL)	8.07±2.01	11.96±4.01	11.65±4.16	12.70±3.40	<0.0001	<0.0001	<0.0001	0.198
IL-6 (pg/mL)	0.69±1.62	11.16±14.95	9.56±12.54	15.04±19.34	0.0001	0.035	0.001	0.010*
IL-10 (pg/mL)	1.45±1.32	14.61±12.53	12.44±12.38	19.82±11.51	<0.0001	<0.0001	<0.0001	0.026*
TNF (pg/mL)	1.71±1.78	5.74±3.68	5.65±3.89	5.95±3.19	<0.0001	0.0002	<0.0001	0.473
IFN-γ (pg/mL)	24.85±2.96	44.00 ± 22.55	41.43±14.14	53.68±34.39	<0.0001	0.0003	<0.0001	0.017*
NPT (nmol/L)	8.23±5.71	45.57±41.82	34.51±35.70	66.63±40.73	<0.0001	<0.0001	<0.0001	0.0001*
NPT/IL-4	1.04±0.75	3.81±3.74	3.04±3.34	5.63±4.05	0.001	0.02	0.0001	0.002*
IL-2/IL-4	1.15±0.26	1.68±0.76	1.59±0.64	1.90±0.97	<0.0001	0.001	0.0007	0.058
IL-6/IL-4	0.06±0.14	0.86±1.17	0.74±1.05	1.16±1.39	<0.0001	0.001	0.0065	0.102
IFN-γ/IL-4	3.25±1.02	3.97±3.01	3.59±1.26	4.87±5.12	0.030	0.019	0.009	0.050
CRP (mg/L)	-	25.93±51.23	22.88±36.13	34.08±75.78	-	-	-	0.839
CD4 (cells/µL)	-	257.97±193.06	296.21±195.50	170.05±167.26	-	-	-	0.003*
VL (log_{10} copies/mL)	-	2.75±1.36	2.48±1.12	3.57±1.71	-	-	-	0.014*
Albumin (g/L)	-	33.40±7.55	34.94±6.67	29.50±8.37	-	-	-	0.027*
A/G ratio	-	0.68±0.25	0.73±0.24	0.53±0.21	-	-	-	0.004*
Haemoglobin (g/dL)	-	11.83±2.36	12.16±2.36	10.97±2.05	-	-	-	0.125
RDW (%)	-	17.45±4.35	17.52±4.50	17.28±4.01	-	-	-	0.334

Data expressed as mean±SD; IL = Interleukin; TNF = tumor necrosis factor; IFN = interferon; NPT = neopterin; VL = HIV viral load; A/G = albumin to globulin ratio; RDW = red cell distribution width. The 3 patient groups are compared individually to the control group. Comparisons performed between HAART and HAART-naïve groups.
Data given as mean ± SD. *p<0.05 for HAART vs. HAART-naïve

Table 5.3 Correlations of neopterin with CD4 counts, CRP, IL-6, albumin, A/G ratio, haemoglobin and red cell distribution width

	Total Patients		HAART		HAART-Naïve	
Neopterin with:	Rho	p-value	Rho	p-value	Rho	p-value
CD4	-0.484	0.0001*	-0.43	0.001*	-0.503	0.02*
IL-6	0.371	0.0010*	0.490	0.00001*	0.128	0.510
CRP	0.355	0.0006*	0.610	0.00001*	0.180	0.597
IFN-γ	0.301	0.002*	0.277	0.017*	0.216	0.260
Albumin	-0.547	0.0001*	-0.447	0.0002*	-0.457	0.014*
A/G ratio	-0.489	0.00001*	-0.423	0.0004*	-0.373	0.061
Haemoglobin	-0.597	0.00001*	-0.555	0.00001*	-0.33	0.093
RDW	0.342	0.001*	0.472	0.0001*	0.112	0.577

*Spearman Rho rank correlation statistically significant, p<0.05

The power of discrimination, in terms of area under the ROC curve (AUROC), of neopterin, CRP and IL-6 was also tested for the total patient group in relation to CD4 counts of less

than 200 using logistic regression. The discriminatory power of neopterin (AUROC = 0.803) was found to be higher than that for CRP (AUROC = 0.658), and for IL-6 (AUROC = 0.753).

Twenty four of the total HIV patient group were confirmed as having TB co-infection (sputa smears). Neopterin (p=0.008) and CRP (p=0.004) levels were both significantly higher in the HIV/TB positive group (neopterin: 65.73±48.94 nmol/L; CRP: 47.35±57.12 mg/L) than in the HIV/TB negative group (neopterin: 37.37±34.48 nmol/L; CRP: 18.99±47.56 mg/L).

The discriminatory power for neopterin (AUROC = 0.898) was higher than that for CRP (AUROC = 0.6252) for the TB-co-infection group. Likewise neopterin (AUROC = 0.7367) showed to be a better discriminator than CRP (AUROC = 0.5945) for the TB-negative patients.

Substances routinely measured in the clinic and previously described as biomarkers, i.e., albumin, the albumin/globulin (A/G) ratio, haemoglobin and red cell distribution width (RDW) were evaluated as indicators of immune deficiency by comparing their levels to CD4 counts. The results can be seen in Table 5.4.

Table 5.4 Correlations for CD4 with blood variables of the different groups

	Total Patients		**HAART**		**Naïve**	
CD4 with:	**Rho**	**p-value**	**Rho**	**p-value**	**Rho**	**p-value**
Neopterin	-0.484	0.0001*	-0.43	0.001*	-0.503	0.02*
IL-6	-0.431	0.0001*	-0.553	0.009*	-0.285	0.193
CRP	-0.328	0.007*	-0.410	0.005*	-0.250	0.287
Albumin	0.491	0.00001*	0.497	0.0004*	0.142	0.551
A/G ratio	0.486	0.00001*	0.505	0.0003*	0.113	0.636
Haemoglobin	0.420	0.0003*	0.392	0.004*	0.256	0.276
RDW	-0.370	0.010*	-0.470	0.0004*	0.017	0.942

*Spearman Rho rank correlation statistically significant, p<0.05

Logistic regression results in a comparison between neopterin, albumin, the AG/ratio, haemoglobin and RDW as indicators of immune deficiency were: neopterin (AUROC = 0.803), albumin (AUROC = 0.487), the A/G ratio (AUROC = 0.504), haemoglobin (AUROC = 0.334) and RDW (AUROC = 0.589).

5.5 Discussion

The value of neopterin as non-specific indicator was examined in terms of its potential as indicator of inflammatory status, the effects of anti-retroviral treatment, as indicator of immune deficiency or dysfunction and as indicator of TB co-infection. The validity of neopterin as non-specific biomarker in HIV/AIDS was compared to that of CRP, cytokines and to a number of substances routinely measured in the clinic.

5.5.1 Neopterin as indicator of inflammatory (cellular immunity) status in HIV/AIDS

Advanced HIV/AIDS is characterised by chronic immune activation and a concomitant immune deficiency. Although the levels of pro-inflammatory, as well as anti-inflammatory, cytokines may be raised [5], advanced HIV/AIDS is predominantly associated with increased inflammatory activity [6.7]. The inflammatory process is implicated as a major contributor to the pathogenesis of many physical disorders, and is likely to play a role in the cardiovascular, renal, liver, metabolic, haematological and skeletal abnormalities, as well as the premature systemic aging associated with HIV infection [6,7]. Increased inflammatory activity is similarly linked to neuropsychological impairments [8], including that found in HIV/AIDS [9,10].

Neopterin levels are generally considered as an indication of both macrophage function and cell mediated immunity. When cell-mediated immunity dominates, circulating neopterin levels are usually high and when humoral immunity dominates, neopterin levels are low [3-11]. Abundant evidence exists for neopterin levels to be increased in disturbances marked by inflammatory activity and raised neopterin levels and increases with disease progression, have previously been described [12]. However inflammatory activity, as indicated by raised neopterin levels, have also been ascribed for several other substances.

In the present study the value of neopterin as general marker of the inflammatory status was examined by comparing neopterin levels to that of two other recognised biomarkers of inflammation, i.e., CRP and IL-6 [6, 11-13]. As seen in Table 5.2, the mean neopterin levels were significantly higher than that of the control group for both the HAART-naïve ($p<0.001$) and the HAART ($p<0.001$) groups. IL-6 levels were also significantly higher than that of the

control group for the HAART naïve (p=0.001) and the HAART groups (p=0.035). CRP and IL-6 levels correlated positively with that of neopterin for the total patient group (CRP: r=0.355, p=0.0006; IL-6: r=0.371, p=0.001) and for the HAART group (CRP: r=0.61, p<0.00001; IL-6: r=0.49, p<0.0001) (Table 5.3). These results validated neopterin as marker of inflammatory activity.

However, although higher pro-inflammatory activity was confirmed in the patients, higher than control levels (p<0.05) were also seen in the levels of the anti-inflammatory cytokines (Table 5.2). Nevertheless, when the pro- versus anti-inflammatory activity was investigated it was found that the ratio neopterin/IL-4 was indeed higher in the HAART-naïve (p=0.0001), as well as in the HAART patients (p=0.02), than in the controls. This shift towards inflammation in HIV/AIDS was confirmed by the ratios of the individual pro-inflammatory cytokines to that of the anti-inflammatory cytokine IL-4. Significantly higher than control ratios were seen for IL-2/IL4 (HAART: p=0.001; HAART-naïve: p=0.0007), IL-6/IL4 (HAART: p=0.001; HAART-naïve: p=0.0065), as well as for IFN/IL4 (HAART: p=0.019; HAART-naïve: p=0.009).

Pro-inflammatory dominance with cell-mediated immunity in ascendancy was thus shown in the group on anti-retroviral therapy, as well as in the group not yet on anti-retroviral therapy. Such inflammatory activity, demonstrated here by neopterin levels and the results confirmed by cytokine results, is implicated in the pathogenesis of HIV/AIDS [6,7]

5.5.2 Effect of HAART on the pro-inflammatory/anti-inflammatory (cellular/humoral immune) status

The mean neopterin level in the HAART group was 48% lower (p=0.0001) than that of the HAART-naïve group, the mean IL-6 level was 43% lower (p=0.01), the mean IFN-γ was 22.8% lower (p=0.017), the mean IL-2 was 16.7% lower (p=0.076) and the CRP level, although not statistically significant, was 33% lower. However, the HAART group still showed significantly higher inflammatory activity than the control group. In contrast, the levels of the anti-inflammatory cytokine, IL-4, were not significantly different between the HAART and HAART-naïve groups (Table 5.2). These results, which demonstrated a down-regulation of neopterin and therefore in inflammatory activity upon treatment with highly active

antiretroviral treatment are in agreement with that of Amirayan-Chevillard *et al* [14]. Amirayan-Chevillard *et al* further showed that neopterin levels again increase with cessation of antiretroviral treatment [14], a finding that implies neopterin to be a useful marker of the efficacy of HAART and perhaps for the assessment of patient compliance.

As an imbalance between pro- and anti-inflammatory activity, in favour of pro-inflammatory, has previously been implicated in the pathogenesis of HIV/AIDS [7], it was of interest to see to what extent this imbalance was corrected by antiretroviral treatment. The ratio between neopterin and the main anti-inflammatory cytokine IL-4 for HAART was significantly lower (3.04 ± 3.34 vs. 5.63 ± 4.05; p=0.002) than that for the HAART-naïve patients – pointing to suppression of cellular (pro-inflammatory) relative to humoral (anti-inflammatory) activity by antiretroviral treatment. However, the ratio in the HAART group was still higher (3.04 ± 3.34 vs. 1.04 ± 0.75; p=0.02) than in the control group, confirming the persistence of a shift towards pro-inflammatory activity despite anti-retroviral treatment (Table 5.2). Although the ratios of all the individual pro-inflammatory cytokines to the level of the anti-inflammatory cytokine IL-4 (IL-2/IL-4, IL-6/IL-4, IFN-γ /IL-4) were also lower in the HAART than in the HAART-naïve group, none were significantly lower.

This persistent, albeit downgraded, presence of inflammatory, and imbalance between pro- and anti-inflammatory activity, despite effective antiretroviral therapy, has previously been reported and is described as a major contributor to the perpetuation of non-AIDS defining co-morbidities and premature systemic aging in patients on HAART [7]. Among the best known contributors to such persistence are said to be clinical or subclinical infections, gastrointestinal microbial translocation, infectious, as well as non-infectious, HIV virions and thymic dysfunction [7].

5.5.3 Neopterin as indicator of immune deficiency

Neopterin has previously been shown as a measure of the degree of immune deficiency in HIV/AIDS patients [2,15,16]. In the present study the potential of neopterin as indicator of immune deficiency was again investigated by comparing neopterin levels to that of CD4 counts. Results with neopterin were then compared to that with CRP and cytokines.

Negative correlations were found between neopterin levels and CD4 counts for the total patient group (r=-0.484, p=0.0001), the HAART group (r=-0.43, p=0.001) and the HAART-naïve group (r=-0.503, p=0.02) (Table 5.3). 93.3% of the HAART-naïve patients and 73.3% of the HAART patients had higher than normal neopterin levels. These results, which suggest neopterin as an indicator of immune deficiency, are in line with that of previous publications [2,15,16].

Neopterin was subsequently compared to CRP and cytokines as immune deficiency indicators. CRP and one of the cytokines investigated, that is, IL-6, showed significant negative correlations with CD4 counts for the total patient group (CRP: r=-0.328, p=0.007; IL-6: r=0.431, p=0.0001) and the HAART group (CRP: r=-0.410, p=0.005; IL-6: r=-0.553, p=0.009), but not for the HAART-naïve group (Table 5.4). In contrast to neopterin, only 49.3% of the HAART-naïve patients and 46.7% of the HAART patients had higher than normal (>10 mg/l) CRP reference levels (0 to 10 mg/l). 83.3% of the HAART-naïve patients and 65.3% of the HAART patients had higher than normal IL-6 levels.

Logistic regression showed the discriminatory power of neopterin (AUROC = 0.803) to be higher than that for CRP (AUROC = 0.658), and for IL-6 (AUROC = 0.753). Neopterin was therefore shown to be superior to CRP and to individual cytokines as indicator of immune deficiency.

5.5.4 Neopterin and HIV/AIDS with TB-co infection

Neopterin levels are known to be significantly higher in individuals with active tuberculosis than in patients with inactive tuberculosis or controls [17]. It would thus seem feasible to expect that HIV/AIDS patients with TB-co-infection would have higher levels of neopterin than HIV patients without TB-co-infection. The ability of neopterin to discriminate between HIV patients with and without active TB was therefore investigated and compared to that of CRP and cytokines.

Neopterin (p=0.008) and CRP (p=0.004) levels were both significantly higher in the 24 HIV/TB positive than in the HIV/TB negative patients. This is in agreement with previous reports [9]. Logistic regression analysis showed the discriminatory power for neopterin

(AUROC = 0.898) to be higher than that for CRP (AUROC = 0.6252). Neopterin therefore seems to be the better indicator of TB-co-infection in patients with HIV/AIDS.

Previous indications that cytokines such as IFN-γ, TNF-alpha, IL-6, IL-10 and the IFN/IL-10 ratio may be of diagnostic/prognostic value in TB [19-24] were not substantiated by the present study. In contrast to our expectations that the balance between pro- and anti-inflammatory activity would be more unfavourable with TB-co-infection, the difference for the neopterin/IL-4 ratio between patients with and without TB co-infection (5.59 ± 4.20 vs. 3.22 ± 3.44; p=0.059) was non-significant when judged at a 0.05 level of significance.

5.5.6 Advantage of neopterin above that of the measurement of individual cytokines

Locally produced cytokines could bind to their target tissues or be neutralized by soluble receptors [25]. Cytokine release may therefore not be accurately reflected by circulating levels. The circulating level of neopterin, on the other hand, is largely a product of the balance between synthesis and renal excretion [15]. It therefore speaks for itself that neopterin could very well be a better biomarker than individual cytokines. In addition, because of the pleiotropic nature of cytokines as well as the multiple interactions and co-operations between cytokines, the measurement of one specific cytokine may not reflect its contribution to inflammation and other immune processes. Neopterin is said to reflect the multiple cooperations between immunocompetent cells [26].

5.5.7 Comparison of neopterin levels with that of factors in the circulation elsewhere described as biomarkers in HIV/AIDS patients

Albumin concentration, the albumin/globulin (A/G) ratio, red cell distribution width (RDW) and haemoglobin (Hb) concentration are routinely measured during clinical investigations. Yet they have elsewhere all been described as biomarkers of disease progression and/or immune deficiency, in general, as well as in HIV/AIDS [27-32].

Previous associations shown between immune deficiency (decreased CD4 counts) with albumin, A/G ratios, haemoglobin and RDW, respectively [27-32], were supported by results on the total patient and HAART groups of the present study (Table 5.4). Positive correlations

were found between albumin and the CD4 counts for the total patient group (r=0.491, p=0.00001) and the group on HAART (r=0.497, p=0.0004). Positive correlations were also found between the A/G ratio and the CD4 counts for the total patient group (r=0.486, p=0.00001) and the group on HAART (r=0.505, p=0.0003). Haemoglobin concentration correlated positively with the CD4 count for the total patient group (r=0.42, p=0.0003) and for the HAART group (r=0.392, p=0.004). RDW correlated negatively with the CD4 count for the total patient group (r=-0.37, p=0.010) and the group on HAART (r=-0.47, p=0.0004).

It is known that the levels of albumin, A/G ratios, haemoglobin and RDW are all adversely influenced by inflammation. With chronic inflammation the levels of albumin, a negative acute-phase protein, decreases as a result of lower synthesis, an increase in fractional metabolic rate, appetite suppression and through an increase in microvascular albumin leakage [33,34]. Chronic inflammatory conditions can influence haemoglobin levels, RDW and other red blood cell parameters in various ways, most notably processes involved in the anaemia of chronic disease [35]. The association between RDW and inflammation is so strong that RDW has been described as indicative of inflammation [36].

In the present study negative correlations between neopterin and albumin concentrations were observed for the total patient group (r=-0.547, p=00001), the group on HAART (r=-0.447, p=0.0002) and the HAART-naïve group (r=-0.475, p=0.014). As for albumin, the A/G ratio declined with increases in neopterin as seen in the negative correlations for the total patient group (r=-0.489, p=0.00001), the HAART group (r=-0.423, p=0.0004) and the HAART-naïve group (r=-0.373, p=0.061). Negative correlation were also found between the haemoglobin and neopterin levels for the total patient group (r=-0.597, p=0.00001) and for the HAART group (r=-0.555, p=0.00001), while for the HAART-naïve group only a weak negative correlation was found (r=-0.33, p=0.093). Significant positive correlations were found between RDW and neopterin for the total patient group (r=0.342, p=0.001) and for the HAART group (r=0.472, p=0.0001). In line with previous publications, the present study thus demonstrated a decline in the levels of albumin, the A/G ratio and haemoglobin concentrations, and an increased RDW, with increases in inflammatory activity, as reflected by neopterin levels.

Although albumin, the A/G ratio, haemoglobin and RDW were seen to have a positive association with the degree of immune deficiency, neopterin (AUROC = 0.803) was shown to be a better indicator of immune deficiency than albumin (AUROC = 0.487), the A/G ratio (AUROC = 0.504), haemoglobin (AUROC = 0.334) and the RDW (AUROC = 0.589).

5.5.8 Additional reasons why neopterin could be a good non-specific biomarker in HIV/AIDS patients

In addition to evidence of neopterin as a good non-specific marker of disease, as shown in our study, various other phenomena support this assumption. The value of neopterin as indicator of disease progression in HIV has been shown by several laboratories [16], and in an earlier paper on HIV/AIDS patients of African ethnicity we showed neopterin to be superior to CRP and procalcitonin as indicator of disease progression [2]. Another connection between neopterin levels and disease is suggested by the correlation between neopterin and IFN-γ (Table 5.3). IFN-γ is the major factor responsible for a shift in tryptophan metabolism towards the kynurenine pathway [37]. Abnormalities in tryptophan metabolism can have a widespread influence on both physical and psychological well-being. In the present study significant positive correlations were found between neopterin levels and the levels of IFN-γ (total patient group: r=0.301, p=0.002; HAART: r=0.277, p=0.017). A further potential link between neopterin levels and disease activity in HIV/AIDS lies in the production of reactive oxygen species (ROS). Excessive ROS is known to contribute to the pathogenesis of HIV/AIDS and correlations have been shown to exist between neopterin levels, increased production of ROS and decreased circulating antioxidants [38-41]. Other associations, not to be discussed here exist, such as that between neopterin and upregulation of the expression of the proto-oncogene c-fos [42], the nuclear factor-κB [41], and the iNOS gene [43]. However, the most important association probably remains that between neopterin and inflammation.

5.6 Summary/Conclusions

The study showed neopterin to be a better indicator of the inflammatory status and as indicator of TB-co-infection in patients with HIV/AIDS than CRP or cytokines. In line with the effects of inflammation on various systems, neopterin levels correlated negatively with the levels of albumin, haemoglobin and the albumin/globulin ratio, but positively with the red

cell distribution width. In addition, neopterin was shown to be superior to CRP, the cytokines measured, as well as to albumin, the AG/G ratio, haemoglobin and RDW as indicator of immune deficiency in patients with HIV AIDS. It is our contention that neopterin levels represent a good non-specific indicator, not only of inflammatory activity, but also of ill-health, especially in HIV/AIDS. This statement is made in view of its associations with inflammation, the effects of HAART, immune deficiency, TB-co-infection, the levels of several plasma proteins and, as previously been reported, disease progression, increased production of ROS, decreased levels of anti-oxidants, and upregulation of the expression of the proto-oncogene c-fos, the nuclear factor-κB, and the iNOS gene. However, in view of the effect of inflammation on all physiological systems, the primary importance of neopterin probably lies in its reflection of the degree of inflammation, a major contributor to the pathogenesis of HIV/AIDS. Neopterin therefore offers a relatively inexpensive non-specific biomarker in resource limited environments to alert the clinician to investigate further. The results of this chapter confirmed neopterin as the most suitable biomarker for the present study.

5.7 References

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Chapter 6

Tryptophan depletion in a sub-Saharan HIV/AIDS population

6.1 Abstract

This chapter deals with the first part of the tryptophan-kynurenine pathway, i.e., the plasma levels of tryptophan. It presents the tryptophan levels in the sub-Saharan HIV/AIDS population of this study and compares it to levels reported for developed countries. Tryptophan depletion is further examined in context of the general nutritional and inflammatory status. It is shown that tryptophan depletion due to immune induced IDO activity forms part of the much wider effect of pro-inflammatory activity on the nutritional profile of HIV/AIDS. It is hypothesized that higher levels of inflammatory activity in low income populations from sub-Saharan countries may contribute to higher levels of IDO-induced tryptophan degradation.

6.2 Introduction

Tryptophan, as an essential amino acid, can't be synthesized in the body and must be acquired through intake and from tryptophan released during protein turnover [1]. A daily nutritional intake at around 20mmol is said to be required to sustain normal plasma levels [2]. The majority of tryptophan is utilised for the synthesis of tissue proteins [3], whereas about 1% of dietary tryptophan serves as a substrate for the biosynthesis of serotonin [2,4]. Tryptophan is also used for the *de novo* synthesis of niacin and is said to play a role in the immune regulation of normal T-cell function [5-7]. Excess tryptophan, i.e., at levels above the requirement for protein and serotonin synthesis, is oxidized in the liver under influence of the liver-specific enzyme L-tryptophan 2,3-dioxygenase (TDO), to ATP, CO_2 and water. In contrast, tryptophan oxidation under influence of the inflammation-inducible enzyme indoleamine 2,3-dioxygenase (IDO) occurs in various cell types and is not limited by a decrease in tryptophan levels [6,7].

Oxidation of tryptophan along the kynurenine pathway, under influence of the rate-limiting enzyme IDO, is considered to be the main cause of tryptophan depletion in HIV AIDS [5,6,8,9]. Tryptophan depletion in HIV/AIDS has been described previously [6,8-20]. However, these results are generally based on populations from developed countries. This study examined tryptophan levels in a black, low income population from the Gauteng Province, South Africa and compared it to that reported for populations from developed countries. The study further examined tryptophan depletion in context of the inflammatory and general nutritional status of HIV/AIDS patients.

6.3 Methods

A group of 105 HIV positive adult (>18 years old) black patients were recruited from the Immunology Clinic at Kalafong Secondary Hospital which provides health services predominantly to communities of Atteridgeville and surrounding areas west of Pretoria. Although the patients lived predominantly in Atteridgeville they were mostly born outside of the township and it is estimated that about one third are immigrants from other African countries. They are generally of low income, with poor housing, and many are unemployed. Some families survive on a single grant or pension. Maize meal is the staple diet of the population, but a tendency to convert to fast foods occurs when their socio-economic status allows. Sixty four percent (64%) of the HAART group, 60% of the HAART naïve group, and 63% of the control group were female. There were no significant differences between the mean ages of the different groups (HAART: 37.86 ± 8.86; Naïve: 37.13 ± 10.24 and Controls: 31.18 ± 8.09 years).

Patients were on antiretroviral treatment for an average period of 15.86 ± 16.49 months. At the time of sample collection, triple therapy was given, mostly efavirenz (EFV) / nevaripine (NVP), lamuvidine (3TC), and stavudine (D4T). Presently the clinic is converting to the FDC (fixed drug combination: emtricitabine, tenofovir, lamuvidine), single dose with apparently better patient compliance. At baseline 22.9% of the total HIV patient group were confirmed by sputum smears as TB positive. TB positive patients were treated with isoniazid (INH), pyrazinamide (PZA), rifampicin (RIF), and ethambutol (ETH) for at least 6 months pending the sensitivity of the TB organism. Although patients are initially educated as to a well-

balanced healthy diet and their weights are monitored, they do not have regular one-on-one counselling with a dietician. The HIV patients were divided into HAART (n=75) and HAART naïve (n=30) groups. Patients were also subdivided according to co-infection with TB (n=24). Sixty HIV negative controls were recruited from the South African National Blood Service (SANBS) satellite site based in Pretoria West.

This was a cross-sectional study that received ethical approval from the Faculty of Health Sciences Research and Ethics Committee of the University of Pretoria (107/2008) and from the SANBS Human Research Committee (2010/03). Written or verbal informed consent, as witnessed and formally recorded, was obtained from all volunteers prior to participation in this study. Although patients were not instructed to fast overnight, bloods were drawn in the morning during the patients' scheduled visit to the clinic. Plasma samples for neopterin, IL-6 and tryptophan were processed on site and stored at -70^{o}C until analysis.

Tryptophan was quantified by gas chromatography mass spectrometry (GC-MS) using the developed method as given in Chapter 3. Briefly, samples were processed and derivatized with pentaflouropropionic anhydride and pentaflouropropanol before analysis. The GC oven was programmed to begin at an initial temperature of 80^{0}C with a ramp at a rate of 20^{o}C up to 180^{o}C followed by a 10^{o}C ramp up to a maximum temperature of 280^{o}C. Sample peaks were eluted on a DB-5MS capillary column within a chromatographic runtime of 18 minutes using a Thermo Scientific Trace 1300 gas chromatographer coupled to an ISQ single quadropole mass spectrometer. Neopterin was measured by sandwich ELISA according to the manufacturer's kit protocol (Immuno-Biological Laboratories Inc., USA). The pro-inflammatory cytokine IL-6 was determined using a cytometric bead array kit (BD Biosciences, San Jose, CA, USA) and flow cytometry. All other laboratory variables were determined by the National Health Laboratory service (NHLS) at Kalafong.

Group comparisons were assessed by ANOVA following data collation testing for normality and log transformations. Because the data did not show a normal distribution, the non-parametric Kruskal-Wallis test was employed for comparisons between subgroups. Actual values are expressed as mean and standard deviation. Non-parametric Spearman rank correlation coefficients were used to determine associations between group variables. All

analyses were performed at a significance level of p<0·05 using Statistical Package for Social Sciences version 22 (SPSS, IBM, Endicott, NY, USA).

6.4 Results

The total HIV/AIDS patient group presented with a mean viral load of 2.75 ± 1.36 log_{10} copies/ml and a CD4 count of 257.97 ± 193.06 cells/µl. The tryptophan level for the total patient group (24.36 ± 4.14 µmol/l) was significantly (p<0.0001) lower than that of the control group (43.57 ± 11.85 µmol/l). Tryptophan levels were thus nearly two fold (44.1%) lower in the patients than in the controls. Patients with a CD4 count of less than 200 cells/µl presented with significantly lower tryptophan levels (23.00 ± 4.23 vs. 26.12 ± 4.14 µmol/l; p=0.03) than patients with CD4 counts greater than 200 cells/µl. The tryptophan levels in the HAART naive group (22.04 ± 4.32 µmol/l) were significantly (p=0.03) lower than that of the HAART group (25.13 ± 3.80 µmol/l). Tryptophan levels correlated with both CD4 count and neopterin values for the total patient (CD4: r=0.341; p=0.004, neopterin: r=-0.399; p=0.0001) and HAART (CD4: r=0.291; p=0.04, neopterin: r=-0.359; p=0.002) groups. Tryptophan levels were not significantly different between the HIV patients with TB (23.87 ± 4.92 µmol/l) and those without TB co-infection (24.50 ± 3.92 µmol/l).

Tryptophan levels were subsequently compared to that found in studies from developed countries (Table 6.1). Although values from developed countries varied, tryptophan levels in patients of our study were in general markedly lower.

Albumin (28.97 ± 7.97 vs. 37.53 ± 3.91 g/l; p<0.001), haemoglobin (10.72 ± 2.43 vs. 12.65 ± 1.95 g/dl; p=0.001), BMI (21.48 ± 5.56 vs. 25.38 ± 7.12 kg/m^2; p=0.02) and albumin/globulin (A/G) ratio (0.52 ± 0.23 vs. 0.74 ± 0.23; p<0.0001) were significantly lower in patients with CD4 counts less than 200 cells/µl. In Table 6.2 correlations for the total group of patients are shown between tryptophan, CD4 counts, neopterin, IL-6, and CRP levels on the one hand, and albumin, globulin, the albumin/globulin (A/G) ratio, haemoglobin, red cell distribution width and BMI and on the other.

Table 6.1 Comparison of tryptophan levels in HIV/AIDS patients of this study and that of developed countries

	A Controls	B Total Group	C ART – naïve	D ART	p-value
[GC] **Present Study**	43.57 (n = 60)	24.36 (n = 105)	22.04 (n = 30)	25.13 (n = 75) (71.% on ART)	<0.001 (A-B) <0.001 (A-C) <0.001 (A-D) 0.033 (C-D)
Developed Countries:					
[H] Werner *et al* 1988 [10] Austria	91.0	44.8 (n = 11)	-	-	<0.0001 (A-C)
[H] Larsson *et al* 1989 [11] Sweden	39.7 (n = 14)	28.4 (n = 24)	-	-	<0.05 (A-B)
[H] Fuchs *et al* 1990 [12] Austria	91.1 (n = 11)	48.8* dementia 70.5	-	-	< 0.05 (A-B)* < 0.01 (A-B)
[H] Fuchs *et al* 1990 [9] Austria	39.7	29.8 (n = 22)	-	-	-
[H] Fuchs *et al* 1991 [13] Austria	91.0	57.0 (n = 42)	(62% Naïve)	(38% on ART)	<0.01 (A-B)
[H] Heyes *et al* 1992 [14] USA	70.9 (n = 20)	40.2 (n =107)	-	-	<0.0001 (A-B)
[H] Gisselen *et al* 1994 [15] Sweden	-	29.4 (n=14)	29.4 (pre-ART)	36.2 (post-ART)	<0.01 (C-D)
[FL] Hortin *et al* 1994 [16] USA	46 (n = 20)	22 (n = 20)	-	(85% on ART)	<0.001 (A-B)
[FL] Laurichesse *et al* 1998 [17] France	59 (n = 8)	51 (n = 7)	-	-	<0.05 (A-B)
[H] Huengsberg *et al* 1998 [18] London	56.3 (n = 72)	50.1 (n = 82)	-	-	<0.01 (A-B)
[GC] Look *et al* 2000 [19] Germany	52.6 (n = 55)	44.6 (n = 17)[$] 37.4 (n = 7) AIDS	44.6 (pre-ART)	53.0 (post-ART))	0.14 (A-B)[$] 0.009 (A-B) 0.039 (C-D)
[H] Murray *et al* 2001 [20] USA	49.3	69.2 (Post Niacin treatment)	-	-	-
[H] Zangerle *et al* 2002 [8] Austria	65.8 (n =40)	44.1 (n=45)	44.1 (pre-ART)	53.2 (post-ART)	<0.001 (A-B) <0.001 (A-D) 0.001 (C-D)
[H] Schroeksnadel *et al* 2008 [6] Austria	-	51.40 (n = 152)	-	(70 % on ART)	-

Analytical methods used: [GC] GC-MS; [FL] Fluorometric procedure; [H] HPLC

Table 6.2 Correlations for the total group of patients between albumin, globulin, the albumin/globulin (A/G) ratio, haemoglobin, red cell distribution width and BMI on the one hand, and tryptophan, CD4 counts, neopterin, IL-6, and CRP levels on the other

	Tryptophan		CD4 count		Neopterin		IL-6		CRP	
	Rho	p-value	Rho	p-value	Rho	p-value	Rho	p-value	Rho	p-value
Albumin (g/l)	0.357*	0.0009	0.491*	0.00002	-0.584*	<0.0001	-0.274*	0.009	-0.442*	<0.0001
Globulin (g/l)	-0.321*	0.0014	-0.271*	0.020	0.351*	0.00036	0.177	0.074	0.378*	0.0002
A/G ratio	0.385*	0.00033	0.486*	0.00003	-0.539*	<0.0001	-0.225*	0.033	-0.468*	<0.0001
Hb (g/dl)	0.378*	0.00028	0.420*	0.00027	-0.576*	<0.0001	-0.193	0.063	-0.250*	0.020
RDW (%)	-0.319*	0.0026	-0.307*	0.0098	0.334*	0.0014	0.044	0.676	0.136	0.212
BMI (kg/m^2)	0.154	0.157	0.368*	0.0036	-0.317*	0.0031	-0.360*	0.001	-0.066	0.573

A/G, Albumin to globulin ratio; Hb, Haemoglobin; RDW, red cell distribution width; BMS, body mass index
Spearman Rho correlation significant, *p<0.05

Patients with CD4 less than 200 cells/µl presented with significantly elevated neopterin (70.48 ± 45.08 nmol/l vs. 24.07 ± 20.93; p<0.0001) as compared to patients with CD4 counts greater than 200 cells/µl. The neopterin levels of HIV patients in the present study were subsequently compared to that reported in developed countries (Table 6.3).

Table 6.3 Neopterin levels in HIV patients compared to that of HIV patients from populations of developed countries.

	Total patient groups		At lower CD4		At higher CD4	
Study	**NPT**	**CD4**	**NPT**	**CD4**	**NPT**	**CD4**
Present Study	45.57	257.97 cells/µl	70.48	<200 cells/µl	24.07	>200 cells/µl
Schroeksnadel 2008 [6]	14.05	404 cells/mm^3	-	-	-	-
Zangerle 2002 [8]	23.4	112 cells/µl	23.4	112 cells/µl	8.0	232 cells/µl
Mildvan 2005 [21]	16.03	75 cells/ml	20.4	50 cells/ml	9.9	200 cells/ml
Hanna 2009 [22]	-	-	24.4	<200 cells/µl	12.5	> 200 cells/µl
Kurz 2009 [23]	25.0	204 cells/mm^3	-	-	-	-
Bogner 1988 [24]	-	-	29.7	264 cells/µl	14.4	487 cells/µl

NPT = neopterin (nmol/l). CD4 counts units given as cells per unit volume as indicated per publication.

6.5 Discussion

Increased oxidation of tryptophan in the kynurenine pathway is considered the major cause of tryptophan depletion in HIV/AIDS. Oxidative metabolism of tryptophan along the kynurenine pathway increases during inflammatory conditions and is regulated by the rate-limiting enzyme indoleamine 2,3-dioxygenase which is stimulated by pro-inflammatory cytokines, predominantly interferon-γ [7,13]. Evidence for decreased tryptophan levels in HIV patients from populations in developed countries has been shown by several studies (Table 6.1). However, a dire lack exists in information on tryptophan levels in HIV/AIDS populations from sub-Saharan regions.

The present study investigated tryptophan levels in a low income, black South African population. The results of this study confirmed the previously shown tryptophan depletion in patients with HIV/AIDS. However, tryptophan levels were markedly lower than that found in developed countries (Table 6.1). In contrast to reports from developed countries that showed tryptophan levels on average to be 18.8% lower than control values (calculated from values in Table 6.1), patient tryptophan levels in the present study were 44.1% lower. The study showed, in line with studies from developed countries (Table 6.1), tryptophan to be significantly lower ($p<0.05$) in the treatment naïve patients than in patients on HAART. No differences were seen between TB positive and TB negative HIV patients. However, TB positive patients were all on anti-TB treatment.

The degree of tryptophan depletion correlated with the decline in the CD4 count ($r=0.341$; $p=0.005$) and therefore with the degree of immune deficiency. Tryptophan levels were subsequently compared to the degree of pro-inflammatory activity as reflected by levels of the pro-inflammatory cytokine IL-6, the acute phase protein CRP and with neopterin. Neopterin, a catabolic product of the purine nucleotide guanosine triphosphate, is produced from guanosine 5'-triphosphate (GTP) that is cleaved by GTP- cyclohydrolase 1 to 7,8-dihydroneopterin triphosphate, followed by conversion of 7,8-dihydroneopterin triphosphate to neopterin and 7,8-dihydroneopterin under the influence of phosphatases [25]. GTP- cyclohydrolase 1 is stimulated, predominantly, by T-helper cell type-1 derived interferon-γ, but co-stimulation by tumour necrosis factor alpha may contribute [25]. Neopterin has been shown, both in our laboratories and in that of others, to be an excellent

indicator of pro-inflammatory activity [13,25,26]. Tryptophan correlated inversely with neopterin (r=-0.399; p=0.0001) and IL-6 (r=-0.230; p=0.026).

The decline in tryptophan levels with increases in inflammatory activity are in agreement with evidence from previous studies [8,9,12]. However, tryptophan levels in the present study were lower. Several factors may contribute to lower tryptophan levels in low income sub-Saharan HIV/AIDS populations when compared to levels found in populations from developed countries, including food insecurity and a higher degree of inflammation.

6.5.1 Food insecurity as a possible reason why tryptophan depletion is more severe than in developed countries

Although tryptophan depletion in HIV/AIDS is generally ascribed to increased oxidation in the kynurenine pathway, tryptophan levels can also be reduced by conditions that influence the general nutritional status of the individual. Malnutrition is, in fact, considered a worldwide phenomenon in HIV, especially where it has progressed to AIDS [27]. Several factors in HIV/AIDS can potentially contribute to this state of malnutrition, including nutrient availability, gastrointestinal problems (such as diarrhoea, dysphagia, odynophagia, nausea, vomiting, gastrointestinal bleeding and neoplasias), suppressed appetite and altered metabolic processes (such as increases in metabolic rate, increased protein catabolism and increases in nutritional requirements [27].

In addition to the conditions mentioned above for HIV/AIDS patients in general, additional factors exist in low income populations of developing countries that may influence the nutritional status and by implication, tryptophan levels in HIV/AIDS patients. Malnutrition, irrespective of HIV status, is a common feature in many populations of Africa [28]. According to the 2012 Global Hunger Index South Africa is ranked 9th in the world for highest hunger levels [29]. Major causes of the widespread malnutrition include limited food or financial resources and poor nutritional value of available food [28]. An additional contributing factor may be dietary changes upon urbanisation. South Africa is in a rural-to-urban transition phase, a phenomenon often associated with a shift towards energy-dense foods poor in proteins and amino acids, and the co-existence of undernutrition and obesity [30,31].

In developed countries the dietary availability of tryptophan would appear not to be a major problem [5]. While the average daily intake of tryptophan in developed countries is estimated to be around 1 gram, the estimated daily requirement is said to be between 175 and 250 mg [5]. This would leave a fair safety margin in populations with adequate nutritional resources. However, the same cannot be said for sub-Saharan populations.

In HIV/AIDS patients in particular, malnutrition is a major concern in sub-Saharan countries to the extent that multiple books, chapters, papers and scientific meetings have been devoted to the subject [32-36]. Reports from different sub-Saharan populations vary, but the overriding consensus appears to be that the prevalence of malnutrition is exacerbated in HIV/AIDS due to factors such as lack of employment income, stigmatisation and other social determinants that contribute to household food insecurity [32-38]. Whether benefits from the academic efforts on malnutrition in HIV patients are filtering through to the HIV/AIDS positive individual remains to be seen. Whatever the case, maize meal represented the staple diet for the majority of patients in the present study - a diet shown in a previous South African study to have a negative impact on the nutritional status of HIV/AIDS patients [39]. In view of the prevalence of malnutrition in the general population and the contribution of HIV/AIDS to household food insecurity it seems feasible to assume that malnutrition may be a contributor to the differences found between tryptophan levels in HIV/AIDS patients of this study and those from developed countries.

6.5.2 Higher inflammatory activity as a contributor to a greater degree of tryptophan depletion in sub-Saharan populations

Another factor that may contribute to lower tryptophan levels in HIV/AIDS patients in low income sub-Saharan populations could be a higher degree of oxidation of tryptophan in the kynurenine pathway. Oxidation in this pathway, under influence of the enzyme IDO, is primarily driven by pro-inflammatory activity [7,13]. When the levels of pro-inflammatory activity, i.e. neopterin, in this study were compared to HIV populations from the developed world it was seen that, at comparable CD4 counts, neopterin levels were much higher in our patients (Table 6.3). These comparisons thus support the notion of higher pro-inflammatory activity, and by implication tryptophan oxidation, at corresponding levels of immune

deficiency. There are two feasible reasons for higher pro-inflammatory activity in low income sub-Saharan, and more specific, low income South African populations, than in populations from the developed world. The first would be the higher prevalence of infections and the second the higher prevalence of malnutrition, coupled to the effect of malnutrition on the immune system.

The high prevalence of infections in sub-Saharan countries are well-known [40]. Factors that may contribute to higher infection-related inflammatory activity in our population include the presence of untreated clinical and subclinical opportunistic infections, higher levels of health-care-associated (nosocomial) infections, as well as lower availability of medical resources and availability and adherence to medications [41-46]. While the physician: patient ratio in the developed world varies around 4 doctors per 1000 patients, the general South African ratio is estimated to be about 0.8 doctors per 1000 patients, with even lower ratios at state hospitals [47,48]. Another factor that may have an influence is the stage of the disease at which HAART is initiated [49]. In contrast to American guidelines which suggest initiation of HAART at CD4 <500 cells/µl, the South African Government guidelines recommended initiation at CD4 cell counts <200 cells/µl [50,51].

The second potential contributor to a higher level of inflammatory activity, in the low income population of this study, is the effect of malnutrition on the immune system. Malnutrition affects all aspects of the immune system and it is often difficult to discern between the effects of malnutrition and that of infections [52]. Malnutrition-induced immune dysfunction has in fact been referred to as Nutrition-Acquired Immune Deficiency Syndrome (NAIDS) [52]. It has been shown that in this multidirectional interaction between infections, the immune system and malnutrition, malnutrition could lead to increases in inflammatory mediators such as pro-inflammatory cytokines and in the levels of the acute phase protein CRP [52]. It is therefore reasonable to suggest increased oxidation of tryptophan in the kynurenine pathway, in nutritional insecure HIV positive populations, to be a function of the immunological alterations induced by HIV/AIDS and other infections, as well as by malnutrition.

6.5.3 Tryptophan depletion as a result of increased oxidation is part of the general immune-induced alteration on the nutritional status of HIV/AIDS

It is important to realize that tryptophan depletion in HIV/AIDS patients forms part of the influence of the altered immune function on the general nutritional status of HIV/AIDS. A multitude of studies have been performed on the nutritional status of patients with HIV/AIDS [27,53]. However, the fact that most factors considered indicators of nutritional status, such as albumin, haemoglobin, transferrin, BMI, lipid profile and wasting, are adversely influenced by the activity of pro-inflammatory cytokines [54-58], are generally overlooked in such studies. The effects of inflammation on nutritional status, i.e., disease-related malnutrition, and the low responsiveness of disease-related malnutrition to nutrient supplementation are well-described in a number of recent publications [54,58-60].

In the present study the previously described effects of inflammation on albumin, globulin, the albumin/globulin (A/G) ratio, haemoglobin, red cell distribution width and BMI [54-58], are supported by the correlations found between their levels and that of the pro-inflammatory markers neopterin, IL-6, and CRP levels (Table 6.2). Similarly, the concept that tryptophan depletion as a result of increased oxidation in the kynurenine pathway forms part of the general immune-induced alterations in the nutritional status of HIV/AIDS is supported by the correlations found between tryptophan levels and the levels of nutritional markers such as albumin, the A/G ratio, haemoglobin and the RDW (Table 6.2).

6.6 Conclusions

The results of this study indicate that tryptophan levels in HIV/AIDS patients are markedly lower in low income, sub-Saharan HIV/AIDS populations than in populations from developed countries. Tryptophan levels were seen to decline in line with the degree of immunodeficiency, as well as with increases in inflammatory activity. However, the levels of inflammatory activity were higher in our low income, black, HIV positive population than in that of developed countries. This could result, not only in lower levels of tryptophan, but also in the levels of substances often considered markers of nutritional status. We contend that tryptophan depletion, due to oxidation in the kynurenine pathway, should be seen as part of the much wider effect of pro-inflammatory activity on the nutritional profile of

HIV/AIDS patients. The possibility that higher levels of IDO-induced tryptophan oxidation could have contributed to the lower levels of tryptophan in the present population than in populations from developed countries are examined in the next chapter.

6.7 References

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Chapter 7

The kynurenine pathway in a sub-Saharan HIV/AIDS population

7.1 Abstract

The previous chapter proposed that, in addition to dietary deficiencies, higher levels of inflammatory activity in HIV/AIDS patients from low income sub-Saharan populations than in patients from developed countries may contribute to lower tryptophan levels. The results of the present chapter support this hypothesis. The chapter further examines the levels of kynurenine, quinolinic acid and nicotinamide. It shows the high kynurenine and quinolinic acid levels, as well as the activity of indoleamine 2,3-dioxygenase, to correlate with inflammatory activity. Indications are presented that nicotonamide levels increase with increases in quinolinic acid up to a quinolinic acid level where saturation of quinolinate phosphoribosyl transferase occurs. The study demonstrated nicotinamide as the exception to the rule where the influence of inflammation on nutritional substances are concerned. While levels of substances like albumin and haemoglobin are adversely affected by pro-inflammatory activity, *de novo* synthesis of nicotinamide in the kynurenine pathway increases due to increased quinolinic acid levels. Further speculations are offered on niacin (nicotinamide and nicotinic acid) status in HIV/AIDS patients.

7.2 Introduction

The essential amino acid tryptophan is important for protein synthesis and serves as substrate for the synthesis of serotonin, melatonin, tryptamine and the kynurenines. After protein synthesis, more than 90% of tryptophan catabolism occurs along the kynurenine pathway [1]. Kynurenine is the first stable metabolite formed in the kynurenine pathway when tryptophan is oxidized under influence of either L-tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO) [2]. Excess tryptophan, i.e., at levels above the requirement for protein and serotonin synthesis, is oxidized in the liver under influence of the liver-specific enzyme TDO, to ATP, CO_2 and water. TDO is stimulated by tryptophan and corticosteroids. In contrast, tryptophan oxidation under influence of the inflammation-

inducible enzyme IDO occurs in various cell types and is said not to be limited by a decrease in tryptophan levels [2]. The main cytokines for the induction of IDO are interferon-gamma (IFN-γ) [3] in the periphery and IL-6 in the CNS, but other pro-inflammatory cytokines as well as the HIV tat and nef proteins may also have an influence [4]. There are two iso-forms of the IDO enzyme with similar structural and enzymatic activities, i.e, IDO1 and IDO2. Both of these are specific for tryptophan and indoles such as 5-hydroxytryptophan and serotonin, however their expression patterns differ [2,5].

After conversion of tryptophan to kynurenine, kynurenine is converted to 3-hydroxy-kynurenine by the enzyme kynurenine 3-monoxygenase; 3-hydroxy-kynurenine is converted under the influence of kynureninase to 3-hydroxy-anthranilic acid and the latter converted under the influence of 3-hydroyxanthranilate 3,4-dioxygenase to 2-amino-3-carboxymuconate semialdehyde (ACMS). ACMS may be completely oxidised to ATP, CO_2 and water, or it may non-enzymatically be converted to quinolinic acid. Quinolinic acid is further metabolised to niacin (nicotinamide and nicotinic acid) under influence of the enzyme quinolinate phosphoribosyl transferase (QPRT). The complete oxidation of ACMS is thought to take place mainly in the liver, while the formation of quinolinic acid and niacin derivatives occurs via the extra-hepatic QPRT pathway [6,7]. Side products of the kynurenine pathway include metabolites such as xanthurenic acid and kynurenic acid [2,6].

Due to the role of tryptophan in cell proliferation and microbial growth, interferon gamma-induced tryptophan depletion in the kynurenine pathway has initially been seen as the major antipathogenic and antitumour functions of the activated pathway [8]. This has become known as the *tryptophan depletion theory* [8]. It has subsequently been shown that other metabolites of the pathway are involved in functions such as immunomodulation, neuromodulation and the *de novo* synthesis of NAD+ [8]. In view of the much wider functional significance of an activated kynurenine pathway Moffet and Namboodiri (2003) suggested the term *tryptophan utilization theory* [8].

Physical disorders such as autoimmune diseases, cancer, AIDS, pellagra, rheumatoid arthritis and cardiovascular abnormalities have been linked to alterations in the kynurenine pathway of tryptophan metabolism [2,4]. While the tryptophan/serotonin/melatonin pathway has long been implicated in neurobehavioural functions, it is now known that kynurenine

pathway metabolites can either act as neuroactive substances or affect neuronal function through their oxidative/reductive properties, or through the supply of adequate NAD+ in conditions of deficient dietary niacin [2,8]. Altered metabolism in the kynurenine pathway, especially increases in quinolinic acid, are implicated in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, as well as in schizophrenia, anxiety, depression and in the AIDS dementia complex [2,8,9].

Alterations in tryptophan metabolism along the kynurenine pathway have previously been shown in HIV/AIDS patients [10-17]. The majority of those studies were on populations from developed countries and primarily dealt with the first segment of the pathway, i.e., conversion of tryptophan to kynurenine. No studies could be found that simultaneously looked at the plasma levels of tryptophan, kynurenine, IDO activity, quinolinic acid, nicotinamide, as well as the relevant immunological factors in HIV/AIDS patients (Table 7.1). The previous chapter described markedly lower plasma tryptophan levels in a low income sub-Saharan HIV/AIDS population than that reported for patients from developed countries. It was postulated that, in addition to dietary deficiencies, higher levels of inflammatory activity in HIV/AIDS patients from low income sub-Saharan populations may contribute to lower tryptophan levels. In the present study this hypothesis, i.e., that higher levels of inflammatory activity, especially IFN-γ, contributes to higher levels of IDO-induced tryptophan degradation and higher levels of kynurenine in resource-limited populations, is tested. The study further examines the levels of quinolinic acid and nicotinamide in the plasma of HIV/AIDS patients and, based on the findings, speculates on the contribution of *de novo* synthesis of nicotinamide in the kynurenine pathway of patients with HIV/AIDS.

7.3 Methods

Tryptophan, kynurenine, quinolinic acid, nicotinamide, neopterin and cytokine levels were determined in the total group of 105 HIV positive patients from the Kalafong Hospital and 60 HIV negative controls from the South African National Blood Service satellite site. For group comparisons patients were divided into HAART (n=75) and HAART naïve (n=30) groups. Informed consent was obtained from all participants prior to obtaining all blood

samples. Plasma samples were processed on site and stored at -70^{o}C until analyses were performed. Refer to Chapter 2 for complete details.

The levels of tryptophan, kynurenine, quinolinic acid and nicotinamide were simultaneously determined by gas chromatography coupled to mass spectrometry (GC-MS) using the methodology as discussed in detail in Chapter 3. Briefly, elution of the chromatographic peaks was achieved with the retention time order of nicotinamide, followed by quinolinic acid, kynurenine and tryptophan on a DB-5-MS column. In terms of the mass spectrums for the pentafluoropropionic anhydride and pentafluoropropanol derivatives, the most abundant mass peaks for each of the relevant metabolites were used for quantification while the largest base peak was used for identification. Neopterin and cytokine levels were determined by ELISA and cytometric bead array flow cytometry respectively (Chapter 2). All other variables were determined by the National Health Laboratory Service at Kalafong.

Data are expressed as mean and standard deviation. Groups were compared by ANOVA and subgroups by non-parametric Kruskal-Wallis. Associations between variables were tested by non-parametric Spearman rank correlation coefficients. All testing was performed at a significance level of $p<0.05$ using SPSS (Version 22, IBM Inc.).

7.4 Results

Tryptophan levels were, as discussed in the previous chapter, significantly lower ($p<0.001$) in the total patient group (24.36 ± 4.14 µmol/l) than in the controls (43.57 ± 11.85 µmol/l). The HAART naïve group had lower tryptophan levels than the HAART group (22.04 ± 4.32 vs. 25.13 ± 3.80 µmol/l; $p=0.033$). Both kynurenine (3.21 ± 1.33 vs. 2.14 ± 0.45 µmol/l; $p<0.001$) and quinolinic acid (4.46 ± 2.32 vs. 0.25 ± 0.058 µmol/l; $p<0.001$) levels were significantly higher in the total patient group than in the controls. For kynurenine the levels were not significantly higher in the HAART naïve group as compared to the HAART group (3.58 ± 1.42 vs. 3.08 ± 1.28 µmol/l; $p=0.144$). The difference for quinolinic acid between the HAART naïve group and the HAART group bordered on significance (5.77 ± 2.65 vs. 4.03 ± 2.04 µmol/l; $p=0.072$). The nicotinamide levels were as follows: total patient group (14.25 ± 9.47 µmol/l), HAART group (13.31 ± 9.65 µmol/l), HAART-naïve group (16.93 ± 8.61 µmol/l)

and control group (12.92 ± 3.69 µmol/l). The nicotinamide levels of the HAART-naïve were significantly higher than that of the control group (p=0.046).

Patients with CD4 counts below 200 cells/µl presented with significantly higher kynurenine (3.92 ± 1.54 vs. 2.87 ± 1.01; p=0.002) and kynurenine/tryptophan (K/T) ratio (171.90 ± 78.11 vs. 116.41 ± 48.45; p=0.001) than patients with CD4 counts greater than 200 cells/µl. Figure 7.1 shows the comparisons for kynurenine and the K/T ratios for the different groups

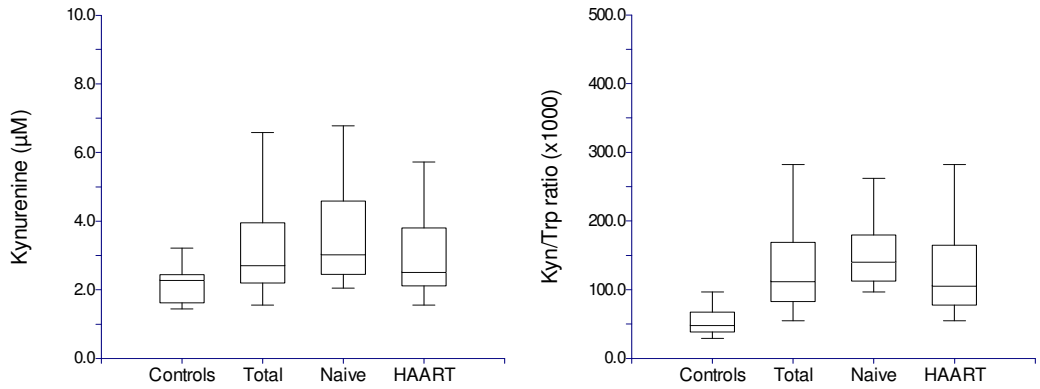

Figure 7.1 Box plots for kynurenine and K/T ratio for the control, total patient, HAART naïve and HAART groups

The results, as presented in Figure 7.1, show that kynurenine and the K/T ratio were significantly higher in the patient groups than in the controls. A search was conducted to gather a list of studies which have investigated metabolites of the kynurenine pathway in HIV/AIDS. Table 7.1 provides a list of these studies which have assessed one or more kynurenine pathway metabolites in HIV patients. From the table it can be seen that none of the other authors have assessed all of the said metabolites, as well as IFN-γ, simultaneously in a given study.

Table 7.1 Studies in which metabolites of the kynurenine pathway have been assessed in HIV patients.

		Tryptophan (µmol/l)	**Kynurenine** (µmol/l)	**K/T ratio**	**Quinolinic Acid** (µmol/l)	**Niacin / NAD precursor** (µmol/l)	**IFN-γ** (pg/ml)
Sub-Saharan Countries							
Present Study South Africa	**Patients**	24.36 ± 4.14	3.21 ± 1.33	136.03 ± 65.45	4.46 ± 2.32	14.25 ± 9.47	44.46 ± 22.46
	Controls	43.57 ± 11.85	2.14 ± 0.45	52.18 ± 16.95	0.25 ± 0.058	12.92 ± 3.69	24.85 ± 2.96
	p-value	<0.0001	0.0001	<0.001	<0.0001	0.198	<0.0001
Byakwaga *et al* 2014 [18] Uganda	**Patients**	18	2.157	131	Not done	Not done	Not done
	Controls	Not done	Not done	Not done	Not done	Not done	Not done
Martinez *et al* 2014 [19] Uganda	**Patients**	18.17	~2.22	122.2	Not done	Not done	Not done
	Controls	Not done	Not done	Not done	Not done	Not done	Not done
Developed Countries							
Fuchs *et al* 1991 [11] Austria	**Patients**	57.0 ± 2.8	3.45 ± 0.14	-	Not done	Not done	259 ± 7 (U/l)
	Controls	91.0 ± 6.63	2.31 ± 0.23	-	Not done	Not done	23.5 (U/l)
	p-value	<0.01	<0.01	-	Not done	Not done	<0.01
Huengsberg *et al* 1998 [12] Austria	**Patients**	50.1	2.55	50.5	Not done	Not done	Not done
	Controls	56.3	1.98	34.9	Not done	Not done	Not done
	p-value	<0.01	<0.001	<0.001	-	-	-
Look *et al*. 1998 [13] Germany	**Patients**	44.6	4.1	108.2	0.848	Not done	Not done
	Controls	52.6	2.7	51.4	0.303	Not done	Not done
	p-value	0.14	0.002	0.002	0.001	-	-
Zangerle *et al* 2002 [14] Austria	**Patients**	44.1 ± 13.3	3.01 ± 0.91	79.2 ± 60.3	Not done	Not done	Not done
	Controls	65.8 ± 12.8	2.02 ± 0.66	30.7 ± 8.7	Not done	Not done	Not done
	p-value	<0.001	<0.001	<0.001	-	-	-
Schroeksnadel *et al* 2008 [15] Austria	**Patients**	51.40	2.60	51.15	Not done	Not done	Not done
	Controls	Not done	Not done	Not done	Not done	Not done	Not done
Heyes *et al* 1998 [24] USA	**Patients**	Not done	Not done	Not done	16.85 ± 3.36	Not done	Not done
Skurnick *et al* 1996 [25] USA	**Patients**	Not done	Not done	Not done	Not done	43.9 ± 0.89	Not done
	Controls	Not done	Not done	Not done	Not done	37.4 ± 1.38	Not done
	p-value	-	-	-	-	0.0001	-
Heyes *et al* 2001 [26] USA	**Patients**	Not done	Not done	Not done	1.358 ± 0.939	Not done	Not done
	Controls	Not done	Not done	Not done	0.416 ± 0.122	Not done	Not done
Bogden *et al* 1990 [27] USA	**Patients**	Not done	Not done	Not done	Not done	43.86 ± 2.44	Not done
	Controls	Not done	Not done	Not done	Not done	Not done	Not done

Results expressed as mean ± SD. P-values represent comparisons between patients and control values, where available.
Not done: refers to studies which have not assessed the particular metabolite or inflammatory indicator.

Correlations were determined to delineate any positive or negative associations between the kynurenine pathway metabolites on the one hand and immune and nutritional indicators on the other. The correlations for kynurenine, K/T ratio, quinolinic acid and nicotinamide with markers of immune activity and indicators of nutrition are given in Table 7.2.

Table 7.2 Correlations for kynurenine, K/T ratio, quinolinic acid and nicotinamide for the total patient group

	Kynurenine		K/T		Quinolinic Acid		Nicotinamide	
Variable	**Rho**	**p-value**	**Rho**	**p-value**	**Rho**	**p-value**	**Rho**	**p-value**
CD4 count	-0.393	0.0008	-0.366	0.0027	-0.110	0.371	-0.082	0.516
Neopterin	0.514	<0.0001	0.538	<0.0001	0.309	0.0036	-0.014	0.904
IL-6	0.354	0.0007	0.362	0.0008	0.062	0.566	-0.112	0.317
IFN-γ	0.344	0.0010	0.366	0.0007	-0.030	0.781	-0.145	0.192
Albumin (g/l)	-0.342	0.0017	-0.399	0.0003	-0.323	0.003	-0.089	0.447
Globulin (g/l)	0.382	0.0002	0.490	<0.0001	0.203	0.054	0.287	0.073
A/G ratio	-0.365	0.0007	-0.448	<0.0001	-0.334	0.0023	-0.084	0.472
Hb (g/dl)	-0.329	0.0024	-0.362	0.0011	-0.311	0.0042	-0.189	0.101
RDW (%)	0.244	0.027	0.384	0.0005	0.163	0.144	0.062	0.596
BMI (kg/m^2)	-0.292	0.010	-0.309	0.0070	-0.237	0.037	-0.066	0.584

A/G = albumin/globulin ratio, Hb = Haemoglobin, RDW = red cell distribution width, BMI = Body mass index. Spearman Rho correlations; significance at $p<0.05$

Table 7.3 represents the comparison of neopterin levels between the present study and that of developed countries at corresponding levels of immune deficiency (CD4 counts). This has been shown in the previous chapter, but is reproduced here for the sake of convenience as it is referred to in the discussion.

Table 7.3 Neopterin levels in HIV patients compared to that of HIV patients from populations of developed countries

	Total patient groups		At lower CD4		At higher CD4	
Study	**NPT**	**CD4**	**NPT**	**CD4**	**NPT**	**CD4**
Present Study	45.57	257.97 cells/µl	70.48	<200 cells/µl	24.07	>200 cells/µl
Zangerle 2002 [14]	23.4	112 cells/µl	23.4	112 cells/µl	8.0	232 cells/µl
Schroeksnadel 2008 [15]	14.05	404 cells/mm^3	-	-	-	-
Mildvan 2005 [20]	16.03	75 cells/ml	20.4	50 cells/ml	9.9	200 cells/ml
Hanna 2009 [21]	-	-	24.4	<200 cells/µl	12.5	> 200 cells/µl
Kurz 2009 [22]	25.0	204 cells/mm^3	-	-	-	-
Bogner 1988 [23]	-	-	29.7	264 cells/µl	14.4	487 cells/µl

NPT: Neopterin (nmol/l). CD4 counts units given as cells per unit volume as indicated per publication.

Figure 7.2 illustrates the relationship between nicotinamide and quinolinic acid for the HAART and HAART-naïve group. Nicotinamide and quinolinic acid results were ordered from smallest to highest values for each group and thereafter plotted as shown on the graph.

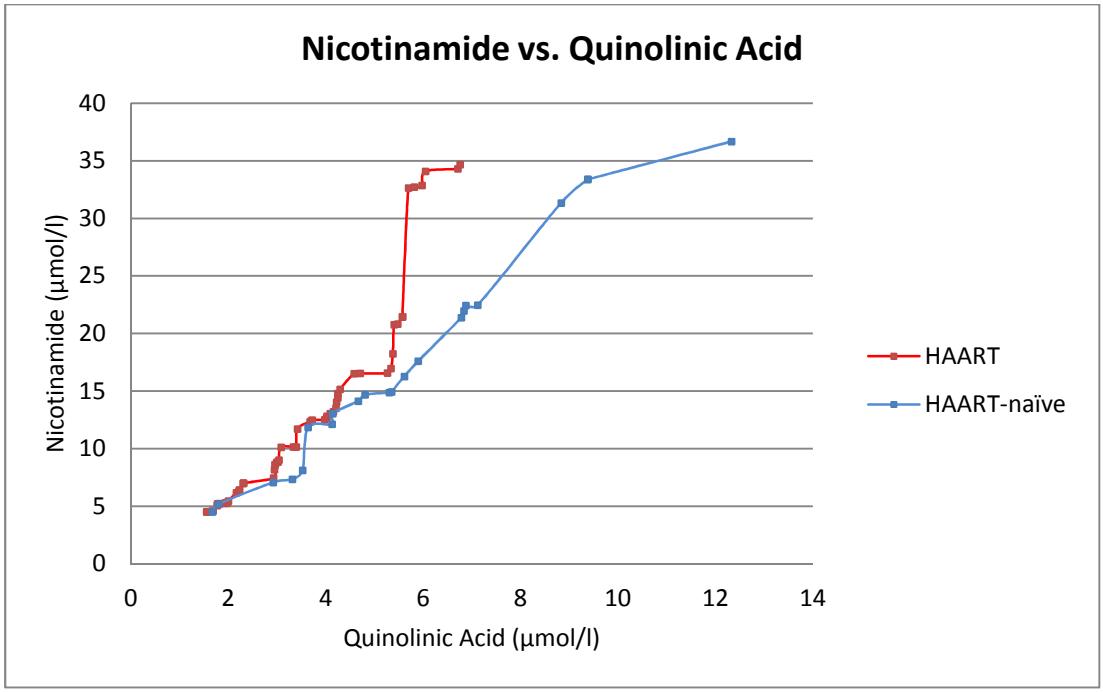

Figure 7.2 Relationship between nicotinamide and quinolinic acid. Information presented from ordered data sets (smallest to largest values).

7.5 Discussion

The previous chapter showed tryptophan depletion in a black low income HIV/AIDS sub-Saharan population to be markedly more pronounced than in HIV/AIDS patients from developed countries. In addition to malnutrition, it was postulated that higher levels of inflammation-induced oxidation of tryptophan to kynurenine to be the major contributor. This chapter investigated this possibility, i.e., that higher levels of tryptophan oxidation in the kynurenine pathway of HIV/AIDS patients from low income sub-Saharan populations contributes to tryptophan levels that are lower than that found in HIV populations from developed countries. The study further looked at two physiological important substances of the kynurenine pathway, i.e., the neurotoxin quinolinic acid and nicotinamide, the essential precursors for the *de novo* synthesis of nicotinamide adenine dinucleotide (NAD+) and speculated on the contribution of *de novo* synthesis of nicotinamide in this group of patients.

7.5.1 Oxidation of tryptophan to kynurenine

In the first segment of the kynurenine pathway, tryptophan is converted to kynurenine under influence of the rate limiting enzyme IDO [2]. The results of this study showed that, despite significantly lower tryptophan levels in the patients than in the controls (24.36 ± 4.14 µmol/l vs. 43.57 ± 11.85 µmol/l; p<0.001), kynurenine levels were significantly higher in the patients (3.21 ± 1.33 vs. 2.14 ± 0.45 µmol/l; p<0.001). This finding is in line with the known low Km of IDO [8,28], with previous reports [12-15] and with the assumed fact that the activity of the IDO enzyme is, in contrast to TDO, not substrate dependent [8]. Although higher levels were seen in the HAART naïve group than the HAART group, the difference was not statistically significant (kynurenine: 3.58 ± 1.42 vs. 3.08 ± 1.28 µmol/l; p=0.144). The association between inflammatory activity and IDO activity was subsequently investigated. The kynurenine/tryptophan (K/T) ratio is generally used as an indication of the activity of IDO [3,4,12,15]. The K/T ratios were significantly higher (p<0.001) in the patients than in the control group (Figure 7.1). When the K/T ratios were compared to the levels of neopterin, a marker of inflammatory activity (Chapter 4), and to that of the pro-inflammatory cytokine IL-6, significant positive correlations were found, both between the K/T ratio and neopterin (r=0.514; p<0.0001) and between the K/T ratio and IL-6 (r=0.354; p=0.00071). This is in agreement with the view of pro-inflammatory activity being the main stimulus for the conversion of tryptophan to kynurenine, in other words the major stimulus for IDO activation. Perhaps of more importance is the significant positive correlation found between the K/T ratios and the levels of IFN-γ (r=0.344; p=0.001), the pro-inflammatory cytokine considered to be the primary IDO inducer [3,4].

When the values of our patient group were compared to values obtained for HIV/AIDS patients from developed countries, the IDO activities, as indicated by the K/T ratio, were markedly higher in the patients from our population (Table 7.1). However, inflammatory activity was also much higher at comparable levels of immune deficiency (Table 7.3). In view of the high incidence of infections and malnutrition in low income sub-Saharan populations and the fact that malnutrition further stimulates inflammatory activity, the major contributors to the higher levels of inflammatory activity were most probably the presence of clinical and/or subclinical infections, as well as malnutrition-induced pro-inflammatory activity. This has been discussed in more detail in the previous chapter. These findings of

higher K/T ratios and higher pro-inflammatory activity in our population compared to populations from developed countries, as well as the highly significant positive associations found between the K/T ratio and inflammatory activity (neopterin: r=0.514; p<0.0001 and IL-6: r=0.354; p=0.00071), especially IFN-γ (r=0.344; p=0.001), and the negative associations between IFN-γ and tryptophan (r=-0.217; p=0.036), support our hypothesis that a higher degree of oxidation in the kynurenine pathway contributed to the lower tryptophan levels found in the black, low income, sub-Saharan population of this study.

Shortly after completion of this study, information emerged [18,19] on the first segment of the kynurenine pathway (tryptophan conversion to kynurenine) in a Ugandan population. The results, as shown in Table 7.1, were dispersed over two publications. This appears to be the first data published on the kynurenine pathway in an African population. The results of the Ugandan project and that of the present study are in agreement with regard to the findings of higher kynurenine levels and K/T ratios despite lower tryptophan levels in patients from resource-limited settings than in HIV populations from developed countries. While the present study showed higher pro-inflammatory activity (Table 7.3) as the most probable cause of the higher K/T ratio (Table 7.1), no such comparisons could be made between their and our results as neither relevant biomarkers of inflammatory activity, nor values for the markers of nutritional status, were reported in the Ugandan paper. As in the Ugandan study, associations were seen in the present study for CD4 counts with kynurenine levels and with the K/T ratios (Table 7.2). In the present study patients with CD4 counts below 200 cells/µl presented with significantly higher kynurenine levels (3.92 ± 1.54 vs. 2.87 ± 1.01; p=0.002) and K/T ratios (171.90 ± 78.11 vs. 116.41 ± 48.45; p=0.001) than patients with CD4 counts greater than 200 cells/µl. This could perhaps be seen as an association between kynurenine pathway activity and, as was suggested [18], immune deficiency and mortality. However, it is more likely a reflection of the higher inflammatory activity with a decline in immune deficiency (neopterin vs. CD4 count: r=-0.558; p<0.0001; Il-6 vs. CD4 count: r=-0.435; p=0.00012; IFN-γ vs. CD4: r=-0.271; p=0.02) coupled to the influence of inflammatory activity on IDO activity (Table 7.2). Such negative associations between inflammatory activity and CD4 counts and positive associations between inflammatory activity and disease progression have been demonstrated in Chapter 4 of this thesis and were published at completion of the work for that chapter [29]. It is, however, also known

that tryptophan deprivation of T lymphocytes may cause cell cycle arrest in the G1 phase and cell death [30], a phenomenon that implies the depletion of tryptophan through inflammation-induced oxidation in the kynurenine pathway as a contributing factor to a decline in CD4 count. In the present study an association was seen between the tryptophan levels and CD4 counts (r=0.341; p=0.0048), but this observation may be a mere epiphenomenon.

An interesting observation was the inverse correlations found between the K/T ratios and kynurenine levels with that of substances often considered indicators of nutritional status such as albumin, haemoglobin, the albumin/globulin ratio, the red cell distribution width and body mass index (Table 7.2). However, in view of the role of pro-inflammatory activity in the oxidation of tryptophan to kynurenine, and in the alterations in markers of nutritional status (discussed in Chapter 6), this is not unexpected.

7.5.2 Kynurenine to quinolinic acid

Kynurenine is further metabolized via 3-hydroxy-kynurenine, to 3-hydroxy-anthranilic acid (see Figure 1 page 4) which is metabolized to α-amino-ß-carboxymuconate-ε-semialdehyde (ACMS). Under normal conditions the majority of ACMS is converted to α-aminomuconate-ε-semialdehyde which is then further metabolised either to picolinic acid or, via the tricarboxylic acid cycle, to ATP, CO_2 and H_2O. Conversion of ACMS to α-aminomuconate-ε-semialdehyde is dependent on the rate-limiting enzyme ACMS decarboxylase (ACMSD), also known as picolinate carboxylase. Some ACMS, not converted to α-aminomuconate-ε-semialdehyde is non-enzymatically converted to quinolinic acid, the precursor of NAD+ and niacin [6,7,28]. ACMSD, due to its role as rate-limiting enzyme for the synthesis of α-aminomuconate-ε-semialdehyde, thus determines the fate of ACMS, and by implication, the amount of ACMS available for conversion to quinolinic acid. Quinolinic acid formation is said to be inversely correlated to ACMSD activity [7] and decreased ACMSD activity could therefore increase ACMS turnover towards quinolinic acid [31,32]. Although the conversion to quinolinic acid is considered to be spontaneously cyclised, indications are that ACMS metabolism, under conditions of NAD+ depletion or increased utilisation, may be shifted in the direction of quinolinic acid synthesis [7]. The activity of the rate-limiting enzyme ACMSD

has been shown to be down-regulated by diets low in protein and low in poly-unsaturated fatty acids [32,33]. ACMSD activity is also inhibited by dietary phthalic acid diesters, leading to an increase in quinolinic acid synthesis and in the conversion ratio of tryptophan to niacin [33,34]. Various enteric-coated medications are said to contain phthalates including the antiretroviral Didanosine [35].

Quinolinic acid is found in micromolar concentrations in the plasma and reported normal values are usually in the range of 0.2 to 0.5 µmol/l [13,24,26,36]. However, it is known to increase with several immune-associated disorders and quinolinic acid levels several times normal have been reported in CSF and plasma in neurological conditions such as Alzheimer's disease, Parkinson's disease and other neurocognitive psychiatric disorders [2,26,37-41].
In the present study plasma quinolinic acid levels for the control group corresponded to published values for normal. However, quinolinic acid levels were significantly higher for the total patient group than for the controls (4.46 ± 2.32 vs. 0.25 ± 0.058 µmol/l; $p<0.0001$). The difference in quinolinic acid levels between the HAART (4.03 ± 2.04 µmol/l) and the HAART naïve groups (5.77 ± 2.65 µmol/l) bordered on significance ($p=0.072$). This HAART-induced decline in quinolinic acid production is most probably related to the partial correction of the pro-inflammatory/anti-inflammatory balance brought about by anti-retroviral medication. The latter was shown and discussed in more detail in Chapter 6. When quinolinic acid levels were compared to markers of pro-inflammatory activity, significant positive correlations were seen with neopterin for the total patient group ($r=0.309$; $p=0.0036$) and the HAART group ($r=0.249$; $p=0.041$). Similar correlations were, however, not seen between quinolinic acid levels and levels of the main inducer of IDO activity, i.e. IFN-γ. As for kynurenine, patients with CD4 counts below 200 cells/µl presented with higher quinolinic acid levels as compared to patients with CD4 counts above 200 cells/µl (5.13 ± 2.67 µmol/l vs. 3.98 ± 2.02 µmol/l; $p=0.052$). Once again it is tempting to describe it as a reflection of the association between immune deficiency (CD4) and inflammatory activity (CD4 vs. neopterin: $r=-0.558$; $p<0.0001$).

Several factors probably contributed to the high levels of quinolinic acid synthesis seen in our HIV/AIDS population. Firstly, ACMS levels are dependent on the activities in the kynurenine pathway upstream from quinolinic acid, especially the levels of kynurenine

produced by the oxidative catabolism of tryptophan under the influence of IDO [31,32]. The high levels of kynurenine found in this study (Table 7.1) were discussed in a previous paragraph. As for kynurenine, inverse correlations were found between the levels of quinolinic acid and that of substances often considered indicators of nutritional status (Table 7.2) – indirectly supporting the notion of quinolinic acid levels being dependent on the pro-inflammatory status. Secondly, in addition to the increase in the upstream substrate levels for ACMS synthesis, there might have been be a shift in the metabolism of ACMS in favour of the non-enzymatic conversion to quinolinic acid. Such a shift, ascribed by the authors to an immune-induced suppression of ACMSD expression, has previously been reported in primary cultures of human macrophages stimulated by IFN-γ [41]. In addition, the fact that ACMSD activity can also be down-regulated by diets low in proteins and polyunsaturated fats [8,32,33] might have been a contributing factor in the population of the present study where, in the majority of patients, maize represented the staple food. Although the phthalate-containing enteric-coated antiretroviral medication Didanosine [35], which is on register at the hospital attended by the HIV/AIDS population of this study, could also have suppressed ACMSD activity, it is not as a rule prescribed and can thus in most patients be ruled out as a contributor to the high quinolinic acid levels.

Comparing the quinolinic acid plasma values of our study to values obtained for HIV/AIDS patients elsewhere proved to be difficult. It would appear that only a few groups in developed countries studied plasma quinolinic acid as part of the kynurenine pathway in HIV patients, whereas no such study could be found on patients from sub-Saharan Africa (Table 7.1). In the present study quinolinic acid levels were 21.1 fold higher in the HAART-naïve patients and 16.1 fold higher in the HAART patients than in the controls. These increments in quinolinic acid are much higher than the 3.3 fold indicated by Heyes *et al*, 2001 [26], and the 2.9 fold higher for HAART naïve and 1.6 fold higher than HAART patients in the study by Look *et al*, 2000 [13]. Despite the similarities in the control values the patient values of the present study were thus considerably higher than that of patients from developed countries.

The question is thus whether the higher inflammatory activity and the resultant higher kynurenine levels, coupled to the assumed protein malnutrition-induced shift away of ACMS

towards quinolinic acid production, could have caused the markedly higher levels of quinolinic acid seen in the population of this study, compared to populations from the developed world. Support for the occurrence of quinolinic acid at levels as high as those found in the present study were derived from studies on neurocognitive function in HIV/AIDS patients. Quinolinic acid has previously been implicated in AIDS dementia and a number of studies measured quinolinic acid in cerebrospinal fluid (CSF) of HIV/AIDS patients [42-46]. A few of these studies also measured it in plasma [47-49]. At least one such study [49] found plasma quinolinic acid levels (4041 ± 892 nM) comparable to that of the present study (4.46 ± 2.32 µmol/l). From papers where quinolinic acid were measured in both plasma and CSF it would appear that a correlation exists between plasma and CSF levels, but that plasma quinolinic acid levels are up to 10 times higher than in CSF [48]. As shown by the present study for plasma quinolinic acid (QA vs. neopterin: r=0.309; p=0.0036), quinolinic acid levels in CSF are reported to correlate with immune activity as indicated by neopterin levels [20,29,48]. Serious neurological/psychiatric effects such as in inflammatory brain disorders and AIDS dementia complex have been reported with quinolinic acid levels in the range of 0.5 to 1.2 µmol/l [37-40]. In view of the latter, the high quinolinic acid levels of this study, partially resulting from excessive inflammatory activity, do not augur well for the neuropsychiatric wellness of HIV/AIDS patients from the population of the present study.

7.5.3 Deliberations on nicotinamide in HIV/AIDS

Quinolinic acid formed in the kynurenine pathway is an essential precursor for niacin and for nicotinamide adenine dinucleotide (NAD+) and its phosphorylated form (NADP+) [6,7,28]. The term niacin is the generic name for the two compounds nicotinic acid and nicotinamide, the major precursors for NAD+ [50,51]. Nicotinamide is said to be the predominant and biologically active form of niacin in circulation, with nicotinic acid, after absorption, being converted to nicotinamide by hepatocytes [50,51].

Nicotinamide, as well as nicotinic acid can be metabolised to NAD+ – although in slightly different pathways [8,51]. Nicotinic acid reacts with 5-phosphoribosyl-1-pyrophosphate to form the nicotinic acid mononucleotide, which then condenses with ATP to form the nicotinic acid analogue of NAD+, which is subsequently converted to NAD+ by a reaction

with glutamine and ATP. In contrast, nicotinamide is converted to the pyridine nucleotide simply by reaction with phosphoribosyl-1-pyrophosphate [53]. Nicotinamide can, in turn, be recycled from NAD+. In conditions of excess nicotinamide, recycling of nicotinamide in the NAD cycle could lead to an increase in nicotinamide being metabolised to N-methylnicotinamide, as well as its metabolites, which is excreted in the urine and is sometimes used as an indirect measurement of niacin status [54,55]. Relevant to the present study is the *de novo* synthesis of NAD+ and its precursors, from quinolinic acid, along the kynurenine pathway. Several aspects of NAD+ synthesis from quinolinic acid are still debated. Quinolinic acid is metabolised to nicotinic acid mononucleotide, pyrophosphate and CO_2 under the influence of quinolinic acid phosphoribosyltransferase (QPRT) (in the presence of 5-phosphoribosyl-1-pyrophosphate). Nicotinic acid mononucleotide is converted to nicotinic acid adenine dinucleotide, which is converted to NAD+ and can thereafter be metabolised to nicotinamide [7,56]. The QPRT enzyme is the rate limiting enzyme for the first step in quinolinic acid catabolism [56]. QPRT activity has been shown to increase in response to increases in the levels of quinolinic acid [57]. However, indications are that QPRT activity is inhibited by pyrophosphate and, in the brain, by saturation of its receptor sites [56,58].

Although it is said that, under normal conditions, only about 2% of niacin is derived from *de novo* synthesis [28], there are indications that the contribution of the tryptophan-nicotinamide pathway of nicotinamide biosynthesis contribution is of much greater importance [7], even in normal healthy populations. Recent research from Japan reported nicotinamide synthesised from tryptophan in normal healthy Japanese to be almost equal to that from dietary intake [7].

Instead of assessment of plasma niacin or nicotinamide levels, deficiency is generally assumed by the appearance of symptoms of pellagra and occasionally by the determination of metabolites of niacin excreted in the urine [55,61]. Nevertheless, the normal plasma reference range of niacin is accepted to be 0.5 to 8.91 µg/ml (4.06 to 68.64 µmol/l) [62,63]. No reports of normal plasma levels of nicotinamide and nicotinic acid could be found.

Several papers refer to a niacin/NAD deficiency in HIV/AIDS patients [28,50,55,59,61,65]. However, no clinical study has proved niacin deficiency in HIV patients without pellagra. It

would appear that assumptions of a general niacin deficit in HIV are largely based on, firstly, the results from an *in vitro* study by Murray *et al* in 1995 [66], secondly, on reports of pellagra in HIV patients and thirdly, perhaps on therapeutic successes with niacin supplementation [59,61]. Although several papers refer to intracellular NAD+ depletion in HIV [61,65], this is usually done in reference to the *in vitro* cell culture study published by Murray *et al* (1995) [66]. No other studies could be found where intracellular NAD+ levels were assessed in HIV/AIDS patients. When studies on the excretion of niacin metabolites in urine were investigated for indications of niacin deficiency as a common occurrence in HIV/AIDS, none could be found, except in those patients with pellagra-like symptoms such as diarrhea. No indication of niacin deficiency could, for instance, be found In a study on nutritional status of HIV type-1 positive children, by Tremeschin *et al* 2007 [68], where niacin status was compared between HIV positive and control children by measuring urinary levels of N-methylnicotinamide. The occurrence of niacin depletion in HIV patients with pellagra, as in other conditions marked by chronic diarrhea, cannot be denied. Urinary excretion of N-methylnicotinamide has in fact been shown to be lower in HIV patients with diarrhea than in those without diarrhea [61]. However, the incidence of pellagra in HIV/AIDS is probably overestimated. Pitche P *et al*, 1999, for instance, found the incidence of pellagra and pellagra-like erythema in HIV patients in Togo not to be higher than that in the general population [67]. Reports on successes in trials on niacin supplementation in order to counteract the tryptophan drain and the HAART-induced dyslipidemia, to protect endothelial cell functions and as antimicrobial, abound [2,50,59,69-72]. However, therapeutic successes, without assessment of niacin/NAD+ status in patients, do not confirm niacin deficiency as a general nutritional deficiency in HIV/AIDS.

Two groups seem to have actually measured the plasma or serum levels of niacin in HIV patients, one in 1990 and the other in 1996. In both studies a number of patients were taking vitamin supplements, but no distinction is made between the values of those on supplements and those not on supplements. Skurnick *et al*, 1996, reported niacin serum levels for HIV patients to be significantly ($p=0.0001$) higher than that of their HIV negative control group [25]. Bogden *et al*, 1990, reported niacin levels of 6.08 ± 1.1 ng/ml in asymptomatic HIV patients and 5.79 ± 0.68 ng/ml in HIV/AIDS patients while control levels

were given as 3.5-7 ng/ml [27]. No *in vivo* study could be found where nicotinamide or NAD+ levels were assessed in HIV/AIDS patients.

The population studied in the present study involved HIV/AIDS patients with a mean viral load of 2.75 ± 1.36 $\log_{10}$ copies/ml and a CD4 count of 257.97 ± 193.06 cells/µl. It would therefore be feasible to expect them to have, as previously reported for HIV/AIDS patients [59], higher needs and utilisation rates for niacin/NAD. In addition, the population studied were generally of low income or unemployed with some families surviving on a single grant or pension. Maize meal, known to have a low niacin content [55,60] was the staple diet of the population. In view of an assumed increase in utilisation, coupled to a deficient dietary supply, it would not be unreasonable to expect niacin levels to have been subnormal. Patients were, however, provided with vitamin B complex supplements (B.CO, containing 10mg nicotinamide per tablet, prescribed as two tablets per day). Patients on HAART were more likely to comply with supplement intake than patients not receiving HAART, but actual intake and compliance could not be accounted for (personal communication with clinical and pharmacy staff).

Despite strong reservations about patient compliance, and about the adequacy of the prescribed dosage, it must be assumed that supplementation contributed to the nicotinamide levels in at least some of the patients studied. Nevertheless, plasma nicotinamide levels were assessed and correlated to that of quinolinic acid. In the present study nicotinamide levels were numerical higher for the total patient (HAART plus HAART-naïve) group than for the controls, but this difference was not statistically significant (14.25 ± 9.47 vs. 12.92 ± 3.69 µmol/l; p=0.198). However, although the difference between the HAART and HAART-naïve patients were not of statistical significance (13.31 ± 9.65 vs. 16.93 ± 8.61 µmol/l; p=0.108), nicotinamide levels were significantly higher in the HAART-naïve patients compared to the controls (16.93 ± 8.61 vs. 12.92 ± 3.69 µmol/l; p=0.046). As increases in niacin have previously been reported in HIV negative patients with *M tuberculosis* infection [64], a comparison was made between the HIV patients with and without TB co-infection. In the present study twenty four patients were co-infected with TB of which 14 were on HAART. No difference in the nicotinamide levels was observed between the TB negative patients on HAART and the TB positive patients on HAART (13.17 ±

9.60 vs 13.97 ± 10.34; p = 0.817). Although the nicotinamide levels were almost 20% higher in the HAART-naïve/TB positive patients than in the HAART-naïve/TB negative patients, the difference was also statistically not significant (18.56 ± 8.83 vs. 14.99 ± 8.37; p = 0.343). However, this statistic non-significance does not rule out the possibility of higher nicotinamide levels in TB co-infected HIV positive patients as all of the TB positive patients in this study were on anti-tuberculosis treatment.

In short, although niacin deficiency in HIV/AIDS is assumed by many [28,50,55,59,61,65,66], no convincing supportive evidence could be found from published papers. In addition, the present study could also not find any indication of low nicotinamide plasma levels in the HIV patients of the population studied, despite indications of low compliance with regard to supplement intake, their resource-limited setting and indications of malnutrition (Chapter6). Furthermore, none of the patients showed overt signs of pellagra on clinical examination. These findings are in no way meant to dispute the value of niacin supplementation in HIV/AIDS. The question therefore arises whether *de novo* synthesis could, at the cost of tryptophan levels, have made a significant compensatory contribution for the dietary deficiencies, the absorption problems associated with HIV/AIDS, and the increase in NAD+ utilisation reported to occur in HIV/AIDS patients.

As the activity of the rate limiting enzyme, QPRT, is known to increase in response to increases in the levels of quinolinic acid [57], it was of interest to investigate the relationship between the plasma levels of quinolinic acid and that of nicotinamide. It is known that human cerebral neurons can take up exogenous quinolinic acid, but can only catabolise a certain amount due to saturation of QPRT [73]. Neuronal QPRT activity is saturated when quinolinic acid concentration exceeds 500 nM (0.5 µmol/l), and it is suggested that this may play a role in the toxic accumulation of quinolinic acid [73]. It was of interest to know whether saturation also limits the conversion outside the central nervous system. In Figure 7.2 the relationship between nicotinamide and quinolinic acid in plasma was shown. Nicotinamide levels increased with increases in quinolinic acid concentration up to a plasma level of 5 µmol/l in HAART patients and up to about 8 µmol/l in HAART-naïve patients after which hardly any further increases occurred. The correlation between nicotinamide and quinolinic acid concentrations for the HAART patients, up to a plasma level of 5 µmol/l, was (r = 0.545; p=0.00006) and for the HAART-naive patients, up to 8 µmol/l (r = 0.882; p

<0.0001). These significant positive correlations suggest a significant portion of the circulating nicotinamide to be derived from *de novo* synthesis in the kynurenine pathway. From the levelling-off in niacin levels seen in Figure 7.2 one could speculate that QPRT becomes saturated at plasma levels around 5 µmol/l in patients on HAART and just above 8 µmol/l in HAART-naïve patients. Of interest is the fact that it would appear from literature that quinolinic acid are on average up to 10 times higher in plasma than in CSF [48], which would be in line with the observation that nicotinamide levels started to level off in plasma at quinolinic acid levels 10 times higher than that reported for CSF.

7.6 Summary

This is the first study to assess plasma tryptophan levels, kynurenine levels, IDO activity, quinolinic acid levels and nicotinamide levels, as well as pro-inflammatory status and IFN-γ levels, simultaneously in one population. Patients of the present study were all from a black, low income sub-Saharan population where malnutrition and higher rates of clinical and subclinical infections are bound to have an influence.

In the HIV/AIDS patients of this study tryptophan levels were significantly lower, while IDO activity, as well as kynurenine and quinolinic acid levels were significantly higher than in the control group. Nicotinamide levels in the HAART-naïve patients were also significantly higher than in the control group. Although some of the patients were taking vitamin B complex supplements, the influence of quinolinic acid levels and, by implication *de novo* synthesis of nicotinamide in the kynurenine pathway, was evident from the correlation between quinolinic acid and nicotinamide levels up to quinolinic acid levels of 5 µmol/l and 8.8 µmol/l in HAART and HAART-naïve patients, respectively. Saturation of QPRT appears to have occurred at levels of quinolinic acid above that.

This was a cross-sectional study. However, keeping in mind that patients only enter the HAART program at a CD4 count at or below 200 cells/µl, the influence of effective antiretroviral treatment could be seen in the significantly higher CD4 counts in patients on HAART than in HAART-naïve patients. Patients on HAART showed, not only significantly higher CD4 counts, but also lower inflammatory activity, lower IFN-γ, higher tryptophan

levels, lower kynurenine levels and lower quinolinic acid levels than patients not on HAART. Although nicotinamide levels were significantly higher in HAART-naïve patients than in controls, the levels in patients on HAART were not statistically different from that of the controls. This despite the fact that patients on HAART were more likely to have taken vitamin B Complex supplementation.

An attempt to compare the values obtained in this study to values obtained elsewhere was confounded by a lack of published information on the kynurenine pathway, downstream from kynurenine, in HIV/AIDS .The only information on the kynurenine pathway metabolites on a sub-Saharan population is that on an Ugandan population in whom the kynurenine levels were published at the time when the present study was completed. The kynurenine levels reported in the Ugandan study were in line with that found in the present study, but no information was available on metabolites downstream from kynurenine or on the inflammatory status of the patients. By necessity, comparisons of levels of kynurenine pathway metabolites between the present study and that of populations from developed countries were made to levels of metabolites from studies where only one or two metabolites were measured in a study. Tryptophan depletion and the increase in IDO activity, as well as kynurenine and quinolinic acid levels were generally greater than in populations from developed countries. However, inflammatory activity, the main driver of the kynurenine pathway was also markedly higher at comparable CD4 counts than that in HIV patients from developed countries. The results of this study support our hypothesis that higher levels of inflammatory activity, at comparable levels of immune deficiency, contributed to a higher degree of tryptophan depletion in this low income sub-Saharan population than in populations from developed countries. This, as shown in the results, contributed to higher levels of kynurenine pathway metabolites. Largely due to the high inflammatory activity, but ostensibly also due to the effects of dietary insufficiencies on ACMSD activity, quinolinic acid levels were above the saturation level for QPRT activity and for several patients within the range associated with the development of HIV/AIDS-associated neurocognitive dysfunction. Associations between quinolinic acid and nicotinamide levels suggested a sizeable contribution of the kynurenine pathway to the maintenance of NAD+ and the precursors of NAD+ in patients with HIV/AIDS.

7.7 References

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Chapter 8

Final summation

The study investigated the dysregulation of tryptophan metabolism along the tryptophan-kynurenine pathway in a HIV/AIDS population from the Gauteng Province of South Africa. The patients were all from a black, low income sub-Saharan population where malnutrition and higher rates of clinical and subclinical infections are bound to have had an influence. All individuals who gave informed consent to participate had a thorough medical examination before enrolment in the study.

A novel gas chromatography-mass spectrometry (GC-MS) method to enable simultaneous reliable quantification of the plasma levels of tryptophan, kynurenine, quinolinic acid and nicotinamide was developed and evaluated. Subsequently, a search was launched for the best biomarker against which to express the tryptophan-kynurenine pathway results. Although viral load and CD4 counts are considered the diagnostic gold standards for HIV, soluble markers may add valuable information about immune activation and other aspects of general wellness. In view of the influence of inflammation on the kynurenine pathway it was of primary interest that the marker be a good indicator of inflammatory status. In addition, cost-effective, reliable biomarkers could be of benefit in resource-limited settings where restrictions are placed on the frequency of laboratory investigations such as viral loads and CD4 counts.

This initial study showed neopterin to be superior to procalcitonin and C-reactive protein as biomarkers for the degree of immunodeficiency, the efficacy of antiretroviral therapy, co-infection with tuberculosis, and as predictor of disease progression. The potential of neopterin as non-specific biomarker, and especially as indicator of pro-inflammatory activity in patients with advanced HIV/AIDS was thus investigated further. Neopterin was found to be superior to C-reactive protein and to individual cytokines as indicator of immune deficiency and of active inflammatory activity. It was further shown to be a good non-specific biomarker in HIV/AIDS patients with regard to the predictive value for underlying active disease, the perpetuation of inflammation-associated co-morbidities, and for monitoring the HAART response. Increased neopterin levels were associated with a decline

in albumin, haemoglobin and the albumin/globulin ratio, and with an increase in red cell distribution width. Neopterin therefore mirrored the inflammation-induced deterioration in nutritional status.

The first substance of the tryptophan-kynurenine pathway to be investigated was tryptophan. Tryptophan depletion was examined, among other, in context of the general nutritional and inflammatory status. Patient tryptophan levels were lower than those generally reported for developed countries. Tryptophan levels correlated positively with CD4 counts and negatively with pro-inflammatory activity. As for tryptophan, nutritional indicators such as albumin and haemoglobin also correlated positively with tryptophan and negatively with the pro-inflammatory markers neopterin, IL-6 and CRP. As oxidation of tryptophan, due to inflammatory activity, is considered the rate-limiting step in kynurenine pathway activation, the results thus indicated tryptophan depletion to form part of the much wider effect of pro-inflammatory activity on the nutritional profile of HIV/AIDS.

We hypothesized that the lower tryptophan levels of patients in the present study could be ascribed, in addition to malnutrition, to higher levels of inflammation in resource-limited populations of sub-Saharan countries than in developed countries. The validity of this hypothesis was supported by findings of higher K/T ratios and higher pro-inflammatory activity in our study population, compared to populations from developed countries, highly significant positive associations between the K/T ratios and inflammatory activity, and negative associations between IFN-γ and tryptophan levels.

The study further showed above normal kynurenine levels, quinolinic acid levels and IDO activity in HAART-naïve patients, and these increases correlated with increases in inflammatory activity. Tryptophan depletion, IDO activity and the levels of the kynurenine pathway metabolites, kynurenine and quinolinic acid, were lower in HAART than in HAART-naïve patients – indicating a partial correction by HAART. The lower inflammatory activity in patients on HAART suggests that these changes have occurred secondary to a partial correction of the inflammatory status by HAART. This, in turn, correlated with the HAART-induced improvement in the state of immune deficiency as reflected by CD4 counts. In line with inflammatory activity being the main drive for *de novo* niacin and NAD+ synthesis in the kynurenine pathway, higher levels of nicotinamide were seen in the HAART-naïve than

in the HAART patients. It was shown that nicotinamide levels increase with increases in quinolinic acid up to a quinolinic acid level where saturation of quinolinate phosphoribosyl transferase occurred. The study showed *de novo* synthesis of nicotinamide in the kynurenine pathway to make a marked contribution to the levels of nicotinamide. It is therefore reasonable to suggest that *de novo* synthesis of nicotinamide offers some protection against nicotinamide deficiency and NAD+ depletion in HIV/AIDS patients with inadequate dietary intake.

8.1 Contribution of the study

This is the first study in HIV/AIDS patients to simultaneously investigate plasma tryptophan, kynurenine, quinolinic acid and nicotinamide levels, as well as the activity of IDO, and that of the relevant immunological factors in the same population. It is also the first to investigate the kynurenine pathway in a sub-Saharan HIV/AIDS population beyond the initial oxidation step. The study confirmed the value of neopterin as indicator of inflammatory status. In addition, it showed plasma neopterin to be superior as inflammatory and non-specific biomarker when compared to procalcitonin, C-reactive protein, the cytokines IL-2, IL-4, IL-6, IL-10, TNF, and IFN-gamma, as well as to factors routinely measured and elsewhere described as biomarkers in HIV, i.e., albumin, the albumin/globulin ratio, haemoglobin and red cell distribution width.

The population studied is typical of resource-limited populations from developing countries where clinical and subclinical infections, as well as malnutrition, are common. A multitude of studies exist on the nutritional profiles of such populations. However, the effects of pro-inflammatory activity are usually ignored and the nutritional profiles simply ascribed to poverty-associated malnutrition. The present study showed the overt tryptophan depletion, with accumulation of kynurenine pathway metabolites, to be part of the much wider effect of pro-inflammatory activity on the nutritional profile. However, while levels of substances like albumin and haemoglobin are adversely affected by pro-inflammatory activity, *de novo* synthesis of nicotinamide increases with increased inflammatory activity and probably offers some protection against NAD+ depletion.

8.2 Shortcomings of the study

Highly significant correlations between nicotinamide and quinolinic acid levels confirmed a major contribution of *de novo* synthesis of nicotinamide to the nicotinamide levels. However, despite reservations about patient compliance and prescribed dosages - especially those patients not yet on the HAART program (HAART-naïve patients) - the fact remains that some patients were probably taking the B. Co tablets (containing 10 mg nicotinamide per tablet) that were made available.

8.3 Suggestion for further study

In view of the importance of niacin and NAD+ in physical, as well as neuropsychiatric health, further studies are necessary, starting with baseline values for these substances, i.e., before niacin supplementation. Withholding any potentially beneficial treatment from patients would be unethical. However, the opportunity exists with first time diagnosed patients, not previously taking niacin supplements, who have a significant deficit in CD4 counts, but who do not as yet meet the stage of immunodeficiency where they qualify for anti-retroviral treatment. In addition to comparing the tryptophan-to-niacin (and perhaps NAD+) profile before and after supplementation, other claimed benefits for niacin supplementation should be further investigated. The benefit of niacin supplementation to counteract the HAART-associated dyslipidaemia [1-4] is well-researched, but other claims need further investigation, including:

- Nicotinamide may increase intracellular NAD levels in HIV-associated intracellular pellagra [5]
- Nicotinamide may inhibit HIV-1 infection and is suggested as an AIDS preventive factor and to be beneficial as adjunct to HAART in controlling the disease progression [6,7]
- Niacin may improve endothelial function in HIV-infected patients [8]
- Nicotinamide treatment may increase plasma tryptophan levels in HIV infected patients and may prevent the tryptophan drain/depletion associated with HIV [9,10]
- Niacin therapy increases adiponectin, is beneficial for migraine and loss of beta-cell function [11,12]
- Niacin may reduce inflammation, particularly vascular inflammation [13,14]

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