Dietary habits and the association of neopterin with niacin and tryptophan in university students.

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ABSTRACT

Background: University students are potentially nutrient compromised as a result of social (demographics, socio-economic status) and other factors (such as transitioning from school to university - independent living). Poor eating habits compounded by lack of variety (including fruits, vegetables and dairy) may lead to students being at risk for malnutrition and non-communicable diseases of lifestyle. Adding to their stressful academic studies, the possible presence of chronic low-grade inflammation may negatively influence nutrition status, more so considering the effect of inflammation on malnutrition. Niacin is an essential nutrient and mild deficiencies are usually associated with irritability, memory problems, poor concentration and fatigue. The importance of this study was to investigate niacin micronutrient levels together with other biomarkers of nutrition, namely tryptophan and anthropometric indicators, in order to identify possible niacin deficiencies in apparently healthy individuals. Furthermore, it was important to investigate any presence of low-grade inflammation as this may have consequences for altered niacin and tryptophan micronutrient levels and nutrient metabolism among university students.

Methods: A total of n=40 participants (70% female), with a mean age 23,6 ± 1,41 years, were recruited from the Faculty of Health Sciences at the University of Pretoria Prinshof Campus. Participants were divided into three malnutrition risk groups after completing questionnaires relating to university demographics, dietary habits and nutrition. Urine samples were collected for the assessment of niacin, tryptophan and the inflammatory biomarker neopterin via ELISA kits.

Results: The mean BMI was 25.18 ± 5.14 kg/m^2. The mean niacin and tryptophan levels were normal according to normative reference ranges. A total of n=19 (47.5%) participants had marginally above normal neopterin levels. There were no significant differences noted for niacin, tryptophan and neopterin between the malnutrition risk groups. However, neopterin correlated negatively with niacin, tryptophan and BMI, but positively with malnutrition risk scores. 1 in 4 participants consumed high-fat meat and processed foods daily and were at moderate risk for malnutrition. Less than 50% consumed dairy products or adequate servings of fruits and vegetables weekly with 85% of participants skipping at least one meal per day.

Conclusion: Neopterin, as a relatively good marker of underlying inflammation, was negatively associated with nutrition parameters such as niacin and tryptophan. Although there were no deficiencies indicated by the niacin and tryptophan urine levels, university students

may still be at risk for malnutrition as a result of underlying inflammation and the negative association of inflammation with dietary variables tested. Further research is required to support these findings.

Keywords

Biomarker

Dietary habits

ELISA

Essential nutrients

Inflammation

Malnutrition

Neopterin

Niacin

Tryptophan

University students

DECLARATION

By submitting this dissertation, I declare that the work contained therein is my own, that all the sources that I have used, have been indicated and acknowledged as complete references, that reproduction and publication thereof by University of Pretoria will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Love, Tanya

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AGP	α1-acid glycoprotein
Alb	Albumin
APP	Acute Phase Protein
APR	Acute phase response
ATP	Adenosine Triphosphate
BH4	Tetrahydrobipterin
BMI	Body Mass Index
CRP	C-reactive proteins
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
GTP	Guanosine triphosphate
GTP-CH	Guanosine triphosphate cyclohydrolase
HPLC	High performance liquid chromatography
IL	Interleukins
MR	Malnutrition Risk

NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NCD	Non-Communicable Disease
NE	Niacin Equivalence
NH2TP	Dihydroneopterintriphosphate
NPT	Neopterin
PCT	Procalcitonin
RBP	Retinol binding protein
RDA	Recommended dietary allowance
Th1	T-lymphocytes type I
TNF	Tumour Necrosis Factor
TRP	Tryptophan
WBC	White Blood Cell

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

The World Health Organization defines malnutrition as a deficiency, excess or imbalance in the intake of energy and/or nutrients of an individual. Malnutrition covers two groups, namely overnutrition and undernutrition. Undernutrition includes stunting, wasting, underweight, micronutrient deficiencies or insufficiencies (1). Increased consumption of nutrient dense meals is also a contributing factor for malnutrition and is cause for concern.

University students are potentially nutrient compromised. Factors that could play a role in poor dietary choices include social factors, such as socio-economic status and transitioning from school to university/independent living, as well as eating habits, physical activity and sedentary behaviour, which leads to predisposed vulnerability to malnutrition and non-communicable diseases of lifestyle (2). It would also appear that time plays an essential role in the food selections that students make. Research has also indicated an increase in fatty food and alcohol consumption during the first year at university (as compared to high school years) (2). This is worsened by a lack of intake of fruits, vegetables and dairy. Evidence has shown that academic performance is affected by nutritional status (3,4) and that the water-soluble vitamins are most relevant when it comes to cognitive function (4). In this study two important nutrients, namely niacin and tryptophan, were investigated.

Niacin, also referred to as nicotinic acid or nicotinamide, is the water-soluble form of Vitamin B_3 (5). Niacin was first synthesized in 1867 by means of nicotine oxidation, for purposes unrelated to food or health. Niacin was isolated for the first time in 1912 by Casimir Frunk, a Polish-American biochemist (6). Although Casimir showed that niacin had some nutritional value, it was only in 1915 that the connection between niacin and nutritional deficiencies was made by Austrian-American physician, Joseph Goldberger. American biochemist Conrad Arnold Elvehjem subsequently discovered the chemical structure of niacin in 1937 (6).

As an essential nutrient, niacin plays a vital role in many metabolic functions. Mild deficiency in niacin leads to numerous side effects such as irritability, memory problems, poor concentration and fatigue. Severe deficiency is associated with pellagra, which in turn, is commonly associated with diets based on non-alkali-treated maize (7). Both forms of niacin (nicotinamide and nicotinic acid) can be metabolized to Nicotinamide adenine dinucleotide (NAD), but the pathways of metabolism differ somewhat. Nicotinamide is metabolised to the pyridine nucleotide while nicotinic acid reacts with 5-phosphoribosyl-1-pyrophosphate to form

the nicotinic acid mononucleotide which is subsequently converted to NAD by reaction with glutamine and adenosine triphosphate (ATP) (8,9).

Niacin can also be synthesised *in vivo* from tryptophan along the kynurenine pathway (9). Tryptophan is an essential amino acid that serves as a substrate for important metabolites. It participates in the synthesis of proteins, kynurenine, serotonin and niacin, to name a few (10). The metabolism of tryptophan along the kynurenine pathway gives rise to the *de novo* synthesis of niacin (11).

Inflammation is usually defined as a localized reaction to injury or trauma, characterized by redness, swelling, pain and heat (12). Inflammation is designed to be protective and to neutralize and remove invaders in order to repair the damage that has been caused by the invader. However, the changes involved in the inflammatory response are metabolically demanding and can potentially be destructive if it continues for prolonged periods of time (13). Recent studies have also given rise to the term "meta-inflammation", which describes the chronic low-grade inflammation present in individuals with obesity (14). Furthermore, underlying or low-grade inflammation may also be present in supposedly healthy people.

Neopterin is a pteridine compound of low molecular weight (15) and is a by-product of the guanosine triphosphate-biopterin pathway (16). Elevated neopterin levels have been measured in the urine of individuals with numerous disease states where cell-mediated immunity is active (15). Neopterin has shown to be a good indicator of inflammation and underlying inflammation, including low-grade inflammation in healthy individuals (17,11).

Recently the effect of inflammation on malnutrition has become evident. Research has shown that the presence of inflammation is associated with low plasma levels of micronutrients, with numerous essential nutritional biomarkers being influenced by the presence of inflammation (18,19). Underlying or low-grade inflammation may therefore underscore a vulnerability for malnutrition or essential nutrient deficiency in university students due to consumption of nutrient poor diets (20).

1.2 MOTIVATION FOR RESEARCH STUDY

University students are potentially nutrient compromised as a result of social (demographics, socio-economic status) and other factors (transition from school to university - independent living) (21). Poor eating habits compounded by lack of variety (including fruits, vegetables and dairy) could make students vulnerable to malnutrition (2). The possible presence of chronic low-grade inflammation could negatively influence nutritional status. Malnutrition in this context may not only refer to under nutrition but also to consumption of nutrient poor diets, such as unhealthy fast-foods. Adding to this, their stressful academic studies may negatively influence nutritional status. Taking the important physiological functions of niacin into consideration, there could be an imbalance or underlying niacin and/or tryptophan deficiency in this cohort. The importance of this study was to investigate niacin micronutrient levels together with other biomarkers of nutrition, namely tryptophan and anthropometric indicators, as niacin deficiency could lead to side effects such as memory problems, apathy, poor concentration and sleep disturbances (7). Furthermore, reduced tryptophan may imply a reduced availability for serotonin and melatonin biosynthesis. All of the aforementioned could negatively impact students' academic performance. It was important to also investigate the possible presence of low-grade inflammation as this may have consequences for altered niacin and tryptophan micronutrient levels and nutrient metabolism among university students.

1.3 AIM

The aim of this MSc study was to investigate the dietary habits as well as the association of urinary neopterin with urinary niacin and tryptophan levels in a cohort of university students.

1.4 OBJECTIVES

The objectives of this research study were:

- To assess urinary niacin and tryptophan levels using ELISA kits.
- To evaluate nutritional status and malnutrition scores by self-reported questionnaires and demographic indices.
- To assess urinary neopterin/creatinine ratios as biomarker of low-grade inflammation in apparently healthy individuals.
- To compare niacin, tryptophan, neopterin, anthropometric and nutrition surveys between participants divided into low, moderate and high-risk malnutrition groups.
- To correlate the data and investigate any associations between the measured parameters.

The following chapter delves deeper into the existing literature on niacin, tryptophan and the inflammatory response including food sources and physiological interactions.

CHAPTER TWO

LITERATURE REVIEW

2 LITERATURE REVIEW

2.1 Niacin and Tryptophan

As previously mentioned, niacin is the water-soluble form of Vitamin B_3. Nicotinamide (the amide derivative of niacin) has higher water solubility than nicotinic acid and both compounds are stable in dry and solution form (5) and plays a vital role in many metabolic functions. Niacin deficiency is associated with the development of pellagra, which. is commonly associated with diets based on non-alkali-treated maize, such as is found in developing countries (7). Niacin within cereal grains – in particular, maize – is mainly bound and is poorly available, thus treating maize with alkali increases the percentage of niacin absorbed.

Pellagra is divided into primary and secondary forms with primary pellagra being the result of inadequate niacin and/or tryptophan consumption and secondary pellagra being the result of disease that affect the niacin levels in the body (22), therefore not all niacin deficiency cases are the result of malnutrition. Nicotinic acid and nicotinamide deficiency may also affect physical and neurocognitive function as they are major precursors for NAD and nicotinamide adenine dinucleotide phosphate (NADP). These two compounds participate in cellular oxidation-reduction reactions as well as in ADP-ribosylation reactions, including DNA repair, deacetylation and calcium mobilization (22). NAD also plays a role in mediating energy metabolism and mitochondrial functions and NADP helps modulate key factors in cell death (23).

Niacin can be used to treat atherosclerosis and dyslipidaemias[1] and evidence suggests that niacin may improve acute and chronic headaches (22). Recent studies by James Brugarolas *et al* indicated that niacin may also play a role in regulating the mammalian rapamycin (mTOR) signalling pathway, which is involved in cell proliferation, protein synthesis and transcription (24). Studies suggest that optimising the homeostasis of niacin in the body can protect against age-related cognitive decline as well as Alzheimer's disease (25). The recommended dietary allowance (RDA) for adult males is 16mg/day and for adult females is 14mg/day (26). Although niacin plays a vital nutritional role, it is hardly ever measured in nutritional surveys.

Niacin can be obtained from endo- and exogenous sources. It is abundant in a variety of food groups – as can be seen in **Tables 1 and 2** – including dairy products, cereals, nuts and seeds,

[1] A disorder of lipoprotein metabolism, including lipoprotein overproduction or deficiency.

fish, certain vegetables and meat products as well as coffee and tea (27). One exogenous source of niacin that has been ignored for many years, as it was thought to have been of no nutritional value and lost in the faeces, is the niacin that is produced by microflora in the large intestine. This niacin must be absorbed in order to be bioavailable, and until recently, very little was known about the ability of the large intestine to absorb this niacin. Recent studies by Kumar *et al* have shown clear evidence for the existence of specialized, high-affinity, carrier-mediated systems for the uptake and absorption of luminal nicotinic acid by colonocytes. Kumar *et al* also found evidence that the process of nicotinic acid uptake is influenced by both intra- and extracellular factors (28). However, further studies are needed.

Table 1 Raw food sources of niacin (*Sorted from high to low per Food Group)*

Food group	Sources*	Content per 100g serving	% Daily value
Dairy products	Non-fat cheese	5.6 mg	28%
	Egg white dried	0.9 mg	4%
	Dried buttermilk	0.9 mg	4%
Fish	Canned anchovy in oil	19.9 mg	100%
	Canned Tuna	13.3 mg	66%
	Raw macherel	9.1 mg	45%
Fruit & Vegetables	Sun-dried tomatoes	9.1 mg	45%
	Mushrooms	3.6 mg	18%
	Avocado (medium size)	3.5 mg	17.5%
	Green peas	2.1 mg	10%

Food group	Sources*	Content per 100g serving	% Daily value
Meat	Raw liver – beef	13.2 mg	69%
	Raw liver – lamb	13.7 mg	56%
	Raw liver – turkey	11.2 mg	59%
Nuts and seeds	Dried ginkgo nuts	11.7 mg	59%
	Dried sunflower seed kernels	8.3 mg	42%
	Peanuts roasted with nut oil	7.7 mg	39%
Beverages	Instant coffee powder	28.2 mg	141%
	Chocolate powder	25.6 mg	128%
	Instant tea powder	10.8 mg	54%

*List of high nutrient ranking sources from each group [USDA National Nutrient Database for Standard Reference, Release 27] (29,30)

Table 2 Cooked food sources of niacin

Food group	Sources (amount in grams) *	Content per serving	% Daily value
Meat	Cooked chicken breast (1 cup, 140g)	19.2 mg	96%
	Cooked liver – beef (90g)	14.7 mg	73.5%
	Cooked salmon (85g)	8.6 mg	43%
	Cooked turkey (87g)	6.5 mg	33%
	Cooked lean pork cuts (85g)	6.3 mg	31%
	Cooked lean beef mince (85g)	6.2 mg	31%
Vegetables	Potatoes (1 medium, 173g)	2.4 mg	12%
Starch	Brown rice (1 cup, 195g)	3.0 mg	15%

*List of high nutrient ranking sources from different food groups (29,30)

Both forms of niacin, namely nicotinamide and nicotinic acid, can be metabolised to NAD. The pathways of metabolism differ somewhat. Nicotinamide is metabolised to the pyridine nucleotide while nicotinic acid, on the other hand reacts with 5-phosphoribosyl-1-pyrophosphate to form the nicotinic acid mononucleotide which is subsequently converted to NAD by a reaction with glutamine and adenosine triphosphate (ATP) (8,9).

Niacin can also be synthesised in the body from tryptophan. About 60mg of tryptophan contributes to about 1mg of metabolically derived niacin (9,27). This metabolism occurs along the kynurenine pathway (9) shown in **Figure 1** (31). Methylation of excess niacin into N^1-methyl-nicotinamide occurs in the liver and is then excreted in urine along with its oxidation products. Niacin equivalence (NE) is often referred to as tryptophan-derived-niacin in conjunction with dietary niacin intake (27). The absorption of niacin by the small intestinal epithelial cells is a high-affinity and specific carrier-mediated mechanism that is acidic pH-dependent (32).

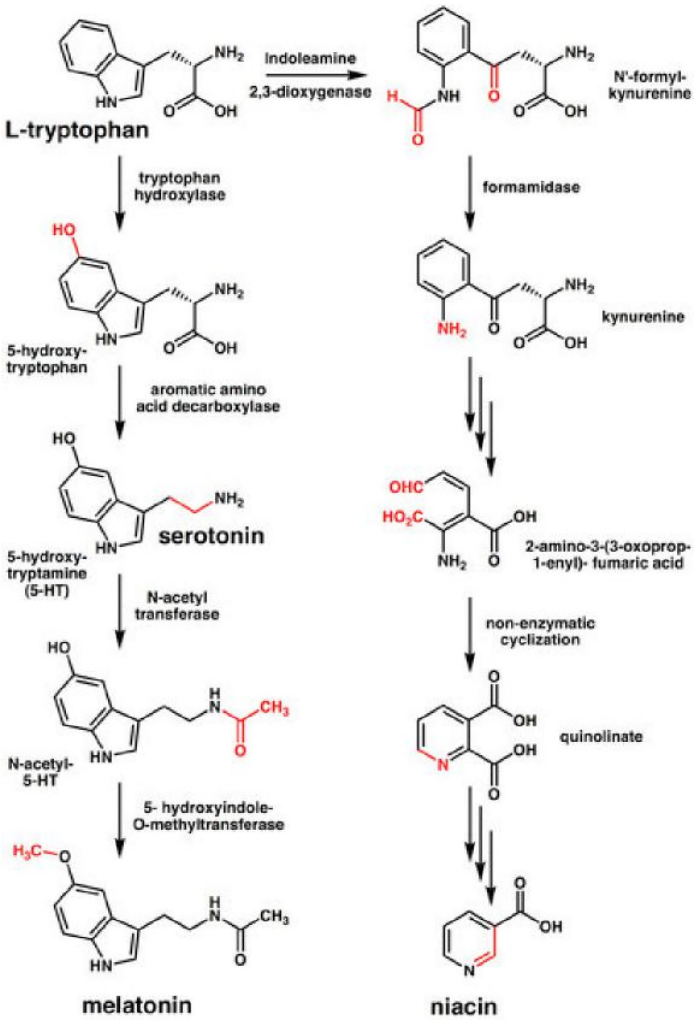


Figure 1: Kynurenine Pathway Synthesis of serotonin and melatonin via tryptophan (left) and shifting of the metabolism of tryptophan to the kynurenine-pathway via the enzyme indoleamine 2,3-dioxygenase (right) resulting in the *de novo* synthesis of niacin (31).

Tryptophan is an essential amino acid which serves as the substrate for important metabolites. Tissue storage levels of tryptophan in humans is relatively low, however it participates in the synthesis of proteins, kynurenine, serotonin, melatonin, tryptamine, NAD/NADP and niacin (10). Sources of tryptophan include proteins, milk, eggs (27), oats, bananas, dried prunes, cheese, bread, turkey, chicken, chocolate, peanuts and tuna (10).

The metabolism of tryptophan along the kynurenine pathway (see Figure 1) gives rise to the *de novo* synthesis of niacin (11). The key enzyme, indoleamine 2,3 dioxygenase, has also been shown to be affected by inflammation. Thus, reduced intake of tryptophan and/or niacin coupled with inflammation-altered metabolism, may lead to reduced niacin availability.

2.2 Inflammation and nutritional status

Inflammation includes physical and biochemical changes that occur in response to tissue damage or the presence of foreign objects or organisms. The changes involved in the inflammatory response are metabolically demanding and can be destructive if it continues for longer than 9 – 10 days (19), as these responses take precedent over normal body metabolism (13). Over the years, research has proven that the presence of inflammation is associated with low plasma levels of micronutrients. Inflammation was shown to be a primary cause of changes in micronutrient level in plasma and urine (18). Infection is said to alter concentrations of numerous micronutrients and nutrition biomarkers by causing endogenous consumption of macronutrient stores (13) and leading to impaired absorption as well as increased utilization and excretion of nutrients (33).

Evidence now shows that low grade-inflammation is associated with diet composition and health (34). The concentrations of numerous essential nutritional biomarkers are influenced by inflammation. These nutrients include retinol, vitamins C and D, iron, carotenoids, zinc, selenium, ferritin (19), vitamin E, copper, thiamine and niacin (18).

Dietary fats and carbohydrates affect inflammatory markers. Studies by Kasim-Karakas *et al* suggest that a high-fat and very-low-carbohydrate or low-fat diets reduce inflammatory markers (35), however studies by Giugliano *et al* (36) somewhat contradicts this by focussing on specific fatty acids in their study. Their study shows that Omega-3 fatty acids appear to have anti-inflammatory activity, but that trans-fats and saturated fats are associated with an increase in

inflammatory biomarkers (36). Previous studies have also found a positive correlation between the consumption of saturated fats and the number of inflammatory biomarkers present in the plasma (36, 37, 38). Thus, junk food (such as burgers containing processed meats, most pizza's, potato chips fried in oil, sugary drinks, ice cream, condiment sachets high in sodium) and other nutrient poor intake may give rise to low-grade inflammation (36, 37, 38).

2.3 Biomarkers of inflammation

Biomarkers were defined by the National Institutes of Health Biomarkers Definitions Working Group as a characteristic that is objectively measured and evaluated as an indicator of normal, biological, pathogenic, or pharmacological responses to a therapeutic intervention (39). Common inflammatory biomarkers include positive acute phase proteins (APPs), including C-reactive proteins (CRP), procalcitonin (PCT), α-1 antitrypsin, neopterin and α1-acid glycoprotein (AGP) as well as negative APPs, including albumin, retinol binding protein (RBP) and high-density lipoprotein A1 (18). APPs are synthesized by the liver as part of the acute phase response[2] (APR) and function to restore the balance of homeostasis that gets disturbed by injury, infection or tissue necrosis. Cytokines are another biomarker of inflammation. They are released by macrophages and neutrophils and the most common include the Interleukins (IL), specifically IL1 and IL6, and tumour necrosis factor (TNF). Cytokines function to induce and regulate the production of APPs (19). However, cytokines have a short half-life (33) and are cleared from the circulation within hours. APPs however, remain in the blood for longer. Thus, to monitor health, cytokines are not as useful as APPs (40), which can ideally be used to match its levels with changes in nutrient biomarker concentration (33).

The APR has a metabolic and nutritional impact on the body. The accompanying changes in APP as a result of the APR causes the following to occur: a decrease in nutrient intake and impaired nutrient metabolism, which includes a decrease in nutrient digestion, increased rates of endogenous losses of nutrients as well as changes in the transport and regulation of nutrients (33).

[2] A group of physiological and biochemical processes that occur due to infection, trauma or inflammation and function to aid in repair of damaged tissues.

Neopterin is a pteridine compound of low molecular weight (15) and is a by-product of the guanosine triphosphate-biopterin pathway. It is produced from guanosine triphosphate (GTP) by active macrophages and monocytes, using the enzyme GTP cyclohydrolase (GTP-CH). The activity of GTP-CH is enhanced by interferon-γ which, in turn, is released by helper T-lymphocytes type I (Th1) (16). Neopterin is said to activate cell-mediated immunity (15) and can reflect the level of oxidative stress due to the activation of the immune system as well as provide information on the current state of a cellular immune response. Neopterin is excreted in an unchanged form in urine and is thus a very useful inflammatory biomarker (16). Elevated neopterin levels have been measured in the urine of individuals with numerous disease states where cell-mediated immunity is active (15).

Non-communicable diseases (NCD), such as type 2 diabetes mellitus, cancer and cardiovascular disease (CVD) are characterized by a 2- to 3-fold increase in plasma concentrations of several cytokines, including TNF, IL6 and CRP (33). Each of the major NCDs has major inflammatory components (23) and most chronic diseases are caused by chronic inflammation (39). To date, how chronic, subclinical inflammation affects nutritional status has not been well studied (33, 39).

Subclinical inflammation has two phases – initial and second phase. The initial phase is the short period where pathogens invade and multiply in tissues. This phase may or may not be followed by clinical symptoms. The second phase occurs during convalescence after an acute illness. In both the initial and the second phases, subclinical inflammation may only be detected via biochemical methods (33). During subclinical inflammation, biochemical changes in inflammatory biomarkers occur. There are numerous inflammatory biomarkers, such as C-reactive proteins, procalcitonin, albumin, retinol binding protein and neopterin which may indicate the presence of underlying inflammation. Neopterin is unique biomarker of innate inflammation since it is synthesized and released solely by activated macrophages and monocytes (41). In the absence of infection, low grade inflammation may still be prevalent in normal, healthy individuals. There is evidence to suggest an indication of altered cell mediated immunity in healthy subjects undergoing normal physiological and psychological stress, such as in students undertaking examinations (15). In the study by Dunbar *et al* urinary neopterin, as a biomarker of cell-mediated immunity, was used as an indicator associated with the normal inflammatory response to stress (15).

2.4 Niacin and Neopterin analyses

Assessing biomarkers of inflammation and correlating that data to the levels of niacin and other biomarkers of nutritional status, can give an indication of the association of inflammation with micronutrients – in particular niacin and tryptophan. The structure of neopterin can be seen in **Figure 2** (42). As a stable molecule, neopterin is excreted chemically unchanged in urine.

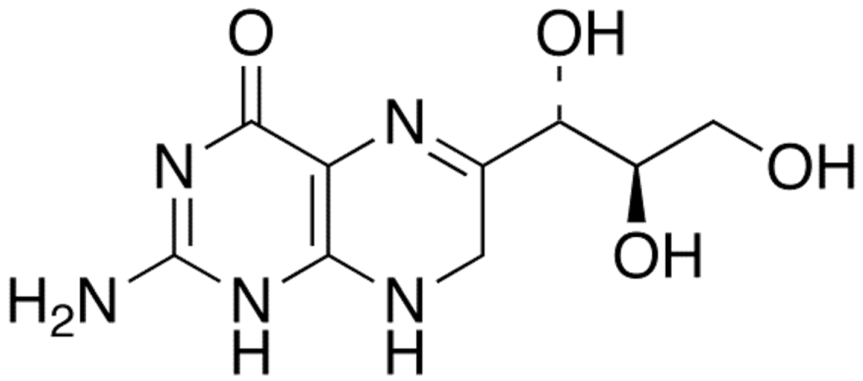


Figure 2: Chemical structure of neopterin (42).

Measuring biomarkers of inflammation has become standard practice, and methods such as saliva and urine are becoming more popular. These methods hold several advantages, such as (39):

- The biofluids do not clot, thus anticoagulation treatment after sample collection is not necessary.
- They are less likely to transmit diseases.
- They are quiescent[3].
- They contain very few interfering proteins and self-collection of specimens by patients is possible.

[3] Their molecular activity does not change much after sampling.

Neopterin has been shown to be a better indicator of inflammation as compared to CRP, procalcitonin and individual cytokines (17, 11). In addition, neopterin may be a good indicator of underlying inflammation.

Neopterin is synthesized from GTP into Dihydroneopterintriphosphate (NH2TP) via the enzyme GTP-CH. NH2TP, under normal conditions, will be synthesized into tetrahydrobipterin (BH4). BH4 participates in the hydroxylation of phenylalanine into tyrosine within the liver and then of tyrosine into L-dopa (43). Within the neuroendocrine tissues, BH4 also participates in the conversion of tryptophan into 5-hydroxy-tryptophan to synthesize cathecholamines or serotonin (44). In human macrophages and monocytes however, NH2TP does not get converted into BH4 due to a lack of 6-pyruvoyl- tetrahydropterin synthetase, which leads to a build-up of NH2TP. NH2TP then gets hydrolysed into dihydroneopterin or neopterin, as indicated in pathway depicted in **Figure 3** (45).

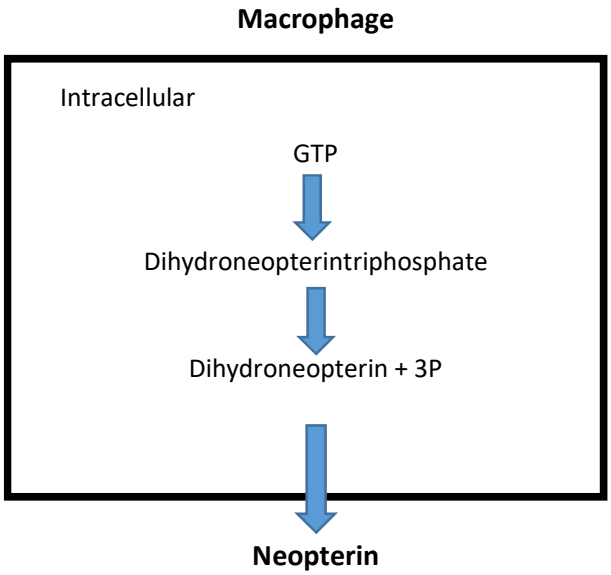


Figure 3: Conversion of GTP to neopterin in macrophages (45).

Since the activity of GTP-CH is enhanced by interferon- γ, it can be concluded that an increase in neopterin synthesis during inflammatory diseases is primarily due to interferon- γ activated macrophages and monocytes (45).

A few factors are important to keep in mind when assessing the influence of inflammation on nutritional biomarkers. Firstly, the type of inflammation present is important (acute, chronic, subclinical). Secondly, individual factors such as diet/food intake, age, breastfeeding and socioeconomic factors need to be considered as this may affect the APR. Lastly, environmental conditions such as poor sanitation and water quality may also predict the presence of inflammation (33).

Recent studies on obesity as a form of malnutrition found that immunology and nutrient physiology are inextricably linked (14). Varying forms of malnutrition are known to impair immune function (46), whilst on the other hand, the presence of inflammation also alters nutrient absorption and homeostasis (26). This close interaction between micronutrient status and inflammation, often makes it relatively difficult to identify which is the prime driving factor (46). It appears to be a snowball effect, with the presence of inflammation altering the nutrient status, putting one at possible risk for malnutrition; whilst malnutrition leads to the presence of inflammation.

The following chapter discusses the materials and methods used to conduct this study.

CHAPTER THREE

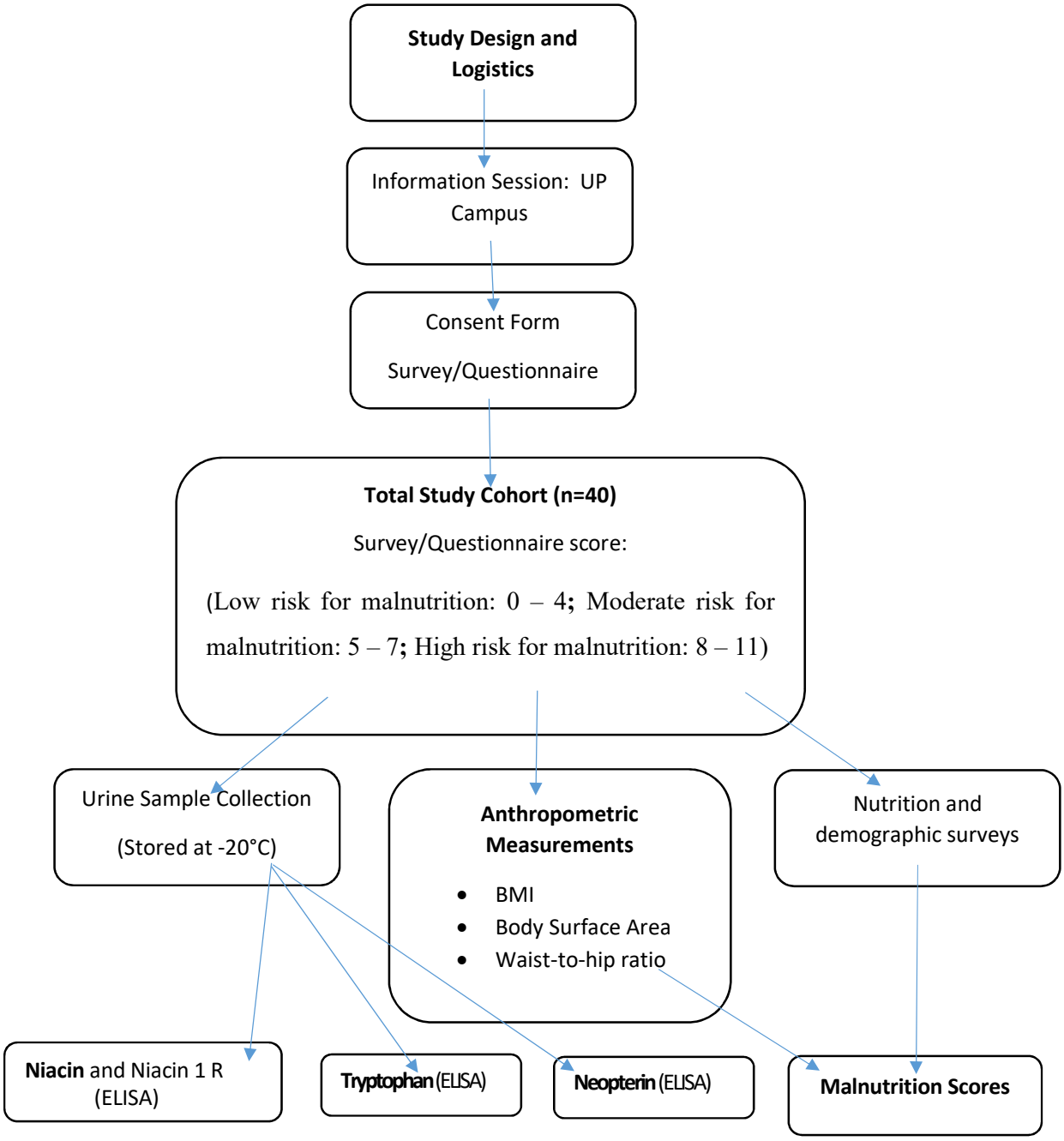
MATERIALS AND METHODS

3.1 INTRODUCTION

This was a non-intervention, non-invasive study that involved male and female students from the University of Pretoria. All participants completed a questionnaire consisting of three parts; Dietary-, Nutrition- and Demographic (Annexure A), after which urine samples of the participants were collected and analysed in the laboratory using ELISA- and biomarker kits, all of which are discussed in detail in this chapter.

3.2 RECRUITMENT AND ETHICS

Students from the University of Pretoria were recruited to participate in this study. There were no exclusions for race, gender, nationality, year of study or degree programme. A sample size of 30 participants was deemed statistically sufficient, however it was decided to proceed until a sample size of 40 participants were successfully recruited. This was also in accordance with each of the ELISA kits able to assay a maximum sample size of n=40 using duplicate analyses. An overview of the study is illustrated in the following flow-chart:



This research study was approved, in accordance with the Declaration of Helsinki, by the Faculty of Health Sciences Research and Ethics Committee of the University of Pretoria (259/2017) prior to recruitment commencing.

3.3 MATERIALS AND METHODS

Analysis of niacin can be accomplished by means of proper quantification from body fluids (plasma and urine). High performance liquid chromatography (HPLC) is often used for multi-analyte measurements (5). However, when using HPLC, sensitivity and selectivity can be a problem. Mass spectrometry coupled to HPLC may increase sensitivity and selectivity; however, this requires use of specialised equipment. In addition, the development and validation of a suitable HPLC, or other chromatography method is required thereby limiting readily availability. Various commercial kits are now also available for the determination of niacin levels in blood (serum and plasma) as well as in urine. In this study we made use of the less invasive method in the form of Niacin ELISA (enzyme-linked immunosorbent assay) kits to analyse niacin, tryptophan and neopterin levels in urine samples. Commonly urinary creatinine is measured and neopterin levels are expressed as neopterin/creatinine ratios.

3.3.1 Materials

The following laboratory materials were used for purposes of the assays:

- Gilson multi-channel pipette (8 channel at 200µl)
- 20µl, 100µl and 200µl calibrated pipettes with tips
- Sterile 50ml urine collection containers (Italy) from Lasec South Africa
- 1.5 ml tubes from Lasec South Africa
- Ultra-pure water (free of particles >0.2µm; electrical conductivity ≥18.2MΩ cm)
- IDK (ImmunDiagnostik – Germany) Niacin ELISA from Biocom Africa
- IDK (ImmunDiagnostik – Germany) Tryptophan ELISA from Biocom Africa
- Demeditec (Germany) Neopterin ELISA from Biocom Africa

The surveys used in this dissertation were adapted from NUTRITIONAL STATUS & LIFESTYLE QUESTIONNAIRE: *A Guide for Personal Assessment RenaiSante Institute of Integrative Medicine.* The surveys were chosen for a general population and could be easily self-administered. Due to the University of Pretoria having international/foreign students, the survey/questionnaire was not standardized for a South African population.

3.3.2 Neopterin Kit

The methods for the assay procedure were drafted from the User Manual of the Neopterin ELISA (DE59321).

Specific amounts and measurements for the procedures below can be found in the user manual.

Manual Procedure

Neopterin standards, controls and participant urine samples were added to the appropriate pre-coated wells. Enzyme conjugate was added to each well and then neopterin antiserum was added to each well. The plate was covered with black adhesive foil and incubated at room temperature for 90 minutes, after which it was washed 4 times with wash buffer. TMB substrate solution was then added to all the wells and incubated for 10 minutes at room temperature. TMB stop solution was added to all wells and absorbance was read at 450nm and 620nm within 15 minutes. Calculation of results from the calibration standards used a 4 Parameter Logistics curve with r^2 indicating a good fit of the curve. Validation was ensured from reference targets of the quality controls.

3.3.3 Niacin Kit

The methods for sample preparation, assay procedure, test initiation as well as the methods for measurement were drafted from the User Manual of ID-Vit® Niacin (KIF003).

Specific amounts and measurements for the procedures below can be found in the user manual.

Preparation of Controls:

The controls were resuspended with water from the test kit, then homogenised using a vortex.

Preparation of Standard Curve:

Standard concentrate was prepared by resuspending the standard with water from the test kit, then homogenised using a vortex. A standard curve was prepared using the scheme indicated in **Table 3**.

Table 3: Niacin dilution scheme

Niacin [µg/l]		Water [DIL] [µl]	+	Standard concentrate [µl]	=	Total volume [µl]
Blank:	0	500	+	0	=	500
Standard 1:	2	475	+	25	=	500
Standard 2:	8	400	+	100	=	500
Standard 3:	16	300	+	200	=	500
Standard 4:	24	200	+	300	=	500
Standard 5:	40	0	+	500	=	500

Preparation of Sterile Assay Medium:

This medium was prepared freshly prior to the test. The desiccant bag was removed from the assay medium bottle after which water was added to the bottle. The bottle was warmed in a water bath and then quickly cooled. The medium was then filtered using a disposable syringe and a 0.2 µm PES filter into a sterile centrifuge tube.

Sample Dilution:

The samples and controls were diluted to 1:4 (dilution factor) with sample preparation buffer from the kit prior to analysis, as shown below.

100 µl sample/control + 300 µl sample preparation buffer

Assay Procedure:

Sterile assay medium was added to the cavities of the microtiter strips, after which standards and controls were added to the respective cavities. The cavities were sealed with adhesive foil and incubated at 37°C for 48 hours.

Measurement:

The turbidity was read with the ELISA-Reader at an absorbance wavelength of 610 – 630 nm. Results were calculated using a 4-parameter algorithm.

3.3.4 Tryptophan Kit

The methods for reagent preparation and storage, as well as the method for the assay procedure were drafter from the User Manual of the IDK® Tryptophan ELISA Kit (K7730).

Specific amounts and measurements for the procedures below can be found in the user manual.

Reagent and Sample Preparation:

The wash buffer concentrate was diluted with distilled water to a final volume for 1000 ml. The standards and controls were reconstituted with reaction buffer and placed on a horizontal shaker for 15 minutes. Derivatisation agent was dissolved in DMSO immediately before use. Vials of Tryptophan antibodies were dissolved in wash buffer and peroxidase conjugate was diluted with conjugate stabilising buffer

Assay Procedure:

Standards, controls and samples were added to corresponding vials. Reaction buffer was then added to all vials followed by freshly prepared derivatisation agent. The vials were then incubated for 45 minutes at room temperature on a horizontal shaker.

Test Procedure:

Prepared standards, controls and samples were pipetted into the appropriate wells of the tryptophan microtiter strips after which Tryptophan antibodies were pipetted into all wells and mixed. The plate was then covered and incubated for 2 hours at room temperature on a horizontal shaker. Each well was washed with wash buffer 5 times and blotted dry. Peroxidase conjugate was added to all wells and the plate was then incubated for 1 hour at room temperature on a horizontal shaker. The wells were again washed 5 times with wash buffer after which substrate was added to each well. The plate was incubated for 15 minutes at room temperature in the dark. Stop solution was then added to each well and the absorbance of the solution was immediately read at 450 nm and 620 nm. Results were calculated using a 4-parameter algorithm.

This concludes Chapter 3. The results of the assays and sample analyses as well as the dietary and nutrition data are presented in Chapter 4.

CHAPTER FOUR

RESULTS

This chapter presents the results obtained from the analyses. Demographic and nutritional results are indicated first followed by the urine analytes and statistical results.

4.1 DEMOGRAPHICS AND NUTRITIONAL DATA

A total of n=40 participants were recruited from the Faculty of Health Sciences at the University of Pretoria Prinshof Campus. The mean age was 23,6 ± 1,47 years and 70% (n=26) of the participants were female. The mean BMI for the total group was 25.18 ± 5.14 kg/m^2 (see **Table 4**).

Table 4: Demographic data

Participant no.	Gender	Age	Smoking	Alcohol	Chronic meds	Height (m)	Weight (kg)	BMI
C101	M	24	No	15/week	No	1.8	70	21.60
C102	M	23	4/day	15/week	Anti-anxiety	1.78	78.5	24.78
C103	F	22	No	0,5/week	No	1.6	60	23.44
C104	M	24	7/day	5/week	No	1.77	79	25.22
C105	M	23	No	12/week	No	1.78	87	27.46
C106	F	26	No	4/week	Contraception	1.71	60	20.52
C107	F	27	No	No	No	1.76	82	26.47
C108	M	24	No	1/week	No	1.69	60.1	21.04
C109	F	25	No	No	No	1.5	65	28.89
C110	F	24	No	No	No	1.6	80	31.25
C111	M	23	5/day	12/week	No	2	120	30.00
C112	M	23	No	10/week	No	1.83	83	24.78
C113	F	26	No	No	No	1.58	65	26.04
A102	M	26	20/day	2/week	No	1.89	131	36.67
A103	F	23	No	1/week	Contraception	1.55	56	23.31
A104	F	23	No	1/week	No	1.61	59	22.76
A105	F	23	No	No	No	1.57	65	26.37
A106	F	24	No	3/week	Asthma, allergies	1.61	112	43.21
A107	F	22	No	3/week	No	1.61	55	21.22
45	F	22	No	5/week	No	1.69	67	23.46
46	F	22	No	1/week	No	1.6	50	19.53
48	F	24	No	1/week	No	1.72	60	20.28
51	F	23	No	1/week	No	1.68	59	20.90
52	M	22	12/day	7/week	No	1.88	80	22.63
53	M	24	No	15/week	No	1.8	70	21.60

Participant no.	Gender	Age	Smoking	Alcohol	Chronic meds	Height (m)	Weight (kg)	BMI
78	M	24	No	No	No	1.8	85	26.23
81	F	22	No	No	No	1.72	68	22.99
84	F	22	No	No	No	1.79	76	23.72
85	M	21	No	No	No	1.7	75	25.95
90	F	23	No	3/week	No	1.65	53	19.47
92	F	24	No	No	No	1.67	70	25.10
95	F	22	No	No	Contraception	1.82	71	21.43
104	F	24	No	2/week	No	1.73	55	18.38
108	F	22	No	1/week	No	1.56	57	23.42
MS10	F	25	No	No	No	1.54	48	20.24
MS11	F	23	No	No	No	1.2	52	36.11
MS12	M	24	No	No	No	1.8	85	26.23
MS14	F	25	No	No	No	1.6	84	32.81
MS15	F	27	No	No	No	1.5	62	27.56
B101	M	22	No	2/week	No	1.8	78	24.07
Mean		**23.55**				**1.69**	**71.82**	**25.18**
SD		**1.414**				**0,14**	**18.03**	**5.14**
Min		**21**				**1.2**	**48**	**18.38**
Max		**27**				**2**	**131**	**43.21**

According to the demographic survey results (**Table 5**), 32,5% of participants reported staying on university campus, while the majority of participants (60%) reported independent accommodation such as private commune or flat, of which 47,5% live more than 5km away from campus. In addition, 52,5% of participants had private transport, whilst 42,5% made use of university transport or walked to campus. In terms of student financial well-being, 65% of participants received an allowance from a parent/guardian or other family member and 27,5% made use of university scholarships, grants and/or bursaries. A few participants (2,5%) were employed and paid for their own expenses.

Table 5: Demographic survey results

General Demographic Survey	Number of Participants	Percentage	Variance	Standard Deviation
1) Please indicate your type of accommodation as a university student.				
§ - University residence	13	32.50%	0.22	0.47
§ - Private or rental accommodation, such a flat or commune	24	60.00%	0.24	0.49
§ - Home with parent/s or guardian	3	7.50%	0.07	0.26
2) How far is your accommodation from campus?				
§ < 1 km	15	37.50%	0.23	0.48
§ < 5 km	6	15.00%	0.13	0.36
§ > 5 km	19	47.50%	0.25	0.50
3) How do you commute between your accommodation and campus?				
§ - Walk or university transport	17	42.50%	0.24	0.49
§ - Private transport	21	52.50%	0.25	0.50
§ - Public transport such as bus, taxi, train	2	5.00%	0.05	0.22
4) As a student, how many meals do you have on average per day?				
§ - 1 meal per day	1	2.50%	0.02	0.16
§ - 2 meals per day	23	57.50%	0.24	0.49
§ - 3 or more meals per day	15	37.50%	0.23	0.48
§ - Did not answer	1	2.50%		
5) Do you generally skip a meal due to lack of time owing to your studies or other university activities or busy schedule?				
§ - No	5	12.50%	0.11	0.33
§ - Yes, some of the time	17	42.50%	0.24	0.49
§ - Yes, most of the time	17	42.50%	0.24	0.49
§ - Did not answer	1	2.50%		
6) While on campus, do you generally skip a meal due to financial constraints?				
§ - No	17	42.50%	0.24	0.49
§ - Yes, some of the time	19	47.50%	0.25	0.50
§ - Yes, most of the time	3	7.50%	0.07	0.03
§ - Did not answer	1	2.50%		

General Demographic Survey	Number of Participants	Percentage	Variance	Standard Deviation
7) At your accommodation, do you generally skip a meal due to financial constraints or lack of food security?				
§ - No	29	72.50%	0.17	0.41
§ - Yes, some of the time	11	27.50%	0.20	0.45
§ - Yes, most of the time	0	0.00%	0.00	0.00
8) As a student, which of the following applies to you regarding pocket expenses for food and daily subsistence?				
§ - I receive an allowance from my parents / guardian / other family member	26	65.00%	0.23	0.48
§ - I am employed and pay for my own expenses	1	2.50%	0.02	0.16
§ - I have a university related grant / scholarship / bursary	11	27.50%	0.20	0.45
§ - I do not receive an allowance and find it financially constraining	2	5.00%	0.02	0.22

4.1.1 Dietary Habits

The dietary intake of participants was determined via self-reported semi-quantified food frequency questionnaires that were adapted from NUTRITIONAL STATUS & LIFESTYLE QUESTIONNAIRE: *A Guide for Personal Assessment RenaiSante Institute of Integrative Medicine.* The results were captured on an excel sheet, see **Table 6** and **Table 7,** and is summarized below.

Dietary habits varied and 1 in 4 participants consumed high-fat meat and other related processed foods daily. Less than 50% consumed dairy products and adequate servings of fruit and vegetables weekly and 45% of participants drank more than 3 cups of coffee a day.

The majority of participants (85%) admitted to skipping at least one meal per day while on campus due to their busy schedules, whilst 55% of participants skipped at least one meal per day while on campus due to financial constraints. Furthermore, 57.5% of the participants surveyed, consumed less than 3 meals per day. In addition, 10% of participants also indicated that they were on a calorie restricting diet whilst participating in this study.

Table 6: Dietary survey results

B. Vitamin and Mineral Supplementation Assessment	Number of Participants	Percentage	Variance	Standard Deviation
Dietary Habits				
1. Do you have fewer than 5 servings of fruits and vegetables per day on average?				
Yes	35	87.50%	0.11	0.33
No	5	12.50%	0.11	0.33
2. Do you consume citrus fruits fewer than 4 times per week on average?				
Yes	27	67.50%	0.22	0.47
No	13	32.50%	0.22	0.47
3. Do you consume 1 serving of orange-yellow fruits and vegetables fewer than 5 times per week on average?				
For example:				
- ▪ 1 whole carrot				
- ▪ 8 large apricots halves				
- ▪ ¼ of a cantaloupe				
- ▪ ½ cup melon squash				
- ▪ 1 baked sweet potato				
- ▪ 1 whole peach/nectarine				
Yes	24	60.00%	0.24	0.49
No	16	40.00%	0.24	0.49
4. Do you consume cruciferous vegetables (cabbage, cauliflower, broccoli, Brussel sprouts) less than 5 times per week on average?				
Yes	28	70.00%	0.21	0.46
No	12	30.00%	0.21	0.46
5. Do you eat smoked meat or fish more than once per week on average?				
Yes	6	15.00%	0.13	0.36
No	34	85.00%	0.13	0.36
6. Do you eat luncheon meats, processed meats, sausages, bacon, bologna or any other nitrate salt containing meat once per week or more on average?				
Yes	20	50.00%	0.25	0.5
No	20	50.00%	0.25	0.5

B. Vitamin and Mineral Supplementation Assessment	Number of Participants	Percentage	Variance	Standard Deviation
7. Do you eat barbecued foods that are charred (braai), once per week or more on average?				
Yes	13	32.50%	0.22	0.47
No	27	67.50%	0.22	0.47
8. Do you drink 3 or more cups of coffee per day on average?				
Yes	18	45.00%	0.25	0.5
No	22	55.00%	0.25	0.5
9. Do you consume less than two dairy servings per day on average? 1 serving = glass of milk or 100ml of yoghurt or 100g of cheese.				
Yes	21	52.50%	0.25	0.5
No	17	42.50%	0.24	0.49
Did not answer	2	5.00%		
10. Are you currently on a diet to lose weight or on a calorie-restricted programme?				
Yes	4	10.00%	0.09	0.3
No	36	90.00%	0.09	0.3
11. Do you consume poultry or fish less than four times per week?				
Yes	20	50.00%	0.25	0.5
No	20	50.00%	0.25	0.5

Table 7: Nutrition survey results

Nutrition Survey	Number of Participants	Percentage	Variance	Standard Deviation
A. Dietary Information				
1) How often, on average, do you eat any of the following foods?				
- ▪ ground beef				
- ▪ bacon				
- ▪ pork products				
- ▪ burgers				
- ▪ spare ribs				
- ▪ chicken wings				

Nutrition Survey	Number of Participants	Percentage	Variance	Standard Deviation
- ▪ processed luncheon meats (ex. salami)				
a) daily	2	5.00%	0.05	0.22
b) once per week	8	20.00%	0.16	0.4
c) 1-2 times per week	15	37.50%	0.23	0.48
d) 2-3 times per month	9	22.50%	0.17	0.42
e) less than 2 times per month	6	15.00%	0.13	0.36
2) How often, on average, do you consume any of the following foods:				
- ▪ cheeses that are more than 20% milk fat (eg. cheddar cheese, mozzarella, brick, cream cheese, parmesan)				
- ▪ homogenized milk				
- ▪ yogurt that is more than 1% milk fat				
- ▪ ice cream				
a) daily	13	32.50%	0.22	0.47
b) once per week	9	22.50%	0.17	0.42
c) 1-2 times per week	10	25.00%	0.19	0.43
d) 2-3 times per month	4	10.00%	0.09	0.3
e) less than 2 times per month	4	10.00%	0.09	0.3
3) Do you use cream/milk in your coffee or tea?				
Yes	7	17.50%	0.14	0.38
No	33	82.50%	0.14	0.38
4) Do you routinely use butter on bread products such as bagels, toast, crackers, etc.?				
Yes	28	70.00%	0.21	0.46
No	6	15.00%	0.13	0.36
Infrequently	6	15.00%	0.13	0.36
5) Do you routinely use butter for cooking or on baked potatoes or other vegetables?				
Yes	16	40.00%	0.24	0.49
No	13	32.50%	0.22	0.47
Infrequently	10	25.00%	0.19	0.43
Did not answer	1	2.50%		
6) Do you use regular sour cream or high fat salad dressings (ex. French, Thousand Islands, Blue Cheese) more than once per week?				
Yes	9	22.50%	0.17	0.42

Nutrition Survey	Number of Participants	Percentage	Variance	Standard Deviation
No	31	77.50%	0.17	0.42
7) What is your weekly whole egg consumption on average?				
a) 12 or more eggs per week	2	5.00%	0.05	0.22
b) 8-11 eggs per week	0	0.00%	0	0
c) 5-7 eggs per week	11	27.50%	0.2	45
d) 2-4 eggs per week	9	22.50%	0.17	0.42
e) less than 2 eggs per week	17	42.50%	0.24	0.49
Did not answer	1	2.50%		
8) How often do you eat fried foods?				
a) 7 or more times per week	3	7.50%	0.07	0.26
b) 5-6 times per week	1	2.50%	0.02	0.16
c) 2-4 times per week	19	47.50%	0.25	0.5
d) 0-1 times per week	17	42.50%	0.24	0.49
9) Do you choose poultry or fish in place of red meat, pork or fried foods in most situations?				
Yes	26	65.00%	0.23	0.48
No	14	35.00%	0.23	0.48
10) Are you a vegetarian or near vegetarian? If yes, please describe (that is, are you vegan)				
Yes	7	17.50%	0.14	0.38
No	33	82.50%	0.14	0.38
11) How often, on average, do you consume any of the following:				
- ▪ 2% milk				
- ▪ low fat sour cream				
- ▪ yogurt that is 2% milk fat				
a) 7 or more times per week	13	32.50%	0.22	0.47
b) 5-6 times per week	8	20.00%	0.16	0.4
c) 2-4 times per week	9	22.50%	0.17	0.42
d) 0-1 times per week	10	25.00%	0.19	0.43
12) How often, on average, do you consume any of the following foods?				
▪ pastries such as cakes, croissants, wraps				
▪ ice cream				
- ▪ donuts				
- ▪ cookies (3 or more)				
- ▪ high fat muffins				

Nutrition Survey	Number of Participants	Percentage	Variance	Standard Deviation
- ▪ rich desserts (ex. cheesecake, brownies)				
a) 7 or more times per week	0	0.00%	0	0
b) 5-6 times per week	9	22.50%	0.17	0.42
c) 2-4 times per week	11	27.50%	0.2	0.45
d) 0-1 times per week	20	50.00%	0.25	0.5
13) How often, on average, do you consume any of the following snack foods?				
- ▪ potato chips				
- ▪ nachos				
- ▪ any type of fried snack				
- ▪ chocolate bars				
a) 7 or more times per week	2	5.00%	0.05	0.22
b) 5-6 times per week	10	25.00%	0.19	0.43
c) 2-4 times per week	12	30.00%	0.21	0.46
d) 0-1 times per week	16	40.00%	0.24	0.49
14) How often, on average, do you consume any of the following snacks or drinks?				
- ▪ regular soft drinks				
- ▪ hard candy				
- ▪ jujubes				
- ▪ gummi bears or anything similar				
- ▪ liquorice				
a) 7 or more times per week	3	7.50%	0.07	0.26
b) 5-6 times per week	4	10.00%	0.09	0.3
c) 2-4 times per week	12	30.00%	0.21	0.46
d) 0-1 times per week	21	52.50%	0.25	0.5
15) On average, do you consume garden type vegetables (eg. carrots, broccoli, cauliflower, peppers, romaine lettuce, spinach, collard greens, kale)?				
a) 7 or more times per week	10	25.00%	0.19	0.43
b) 5-6 times per week	10	25.00%	0.19	0.43
c) 2-4 times per week	13	32.50%	0.22	0.47
d) 0-1 times per week	7	17.50%	0.14	0.38
16) On average, how many servings per day do you consume of any of the following: pasta, rice, beans, peas, corn, barley, oatmeal?				

Nutrition Survey	Number of Participants	Percentage	Variance	Standard Deviation
NOTE: each of the following is equal to one serving:				
- ▪ ½ cup of pasta, rice, beans, peas, corn, oatmeal, etc. (before cooking)				
- ▪ 1 slice of bread				
- ▪ ½ bagel				
- ▪ ½ English muffin				
- ▪ ¼ cup of most fibre cereals				
- ▪ low-fat, high-fibre muffin				
a) 5 or more servings per day	5	12.50%	0.11	0.33
b) 3-4 servings per day	17	42.50%	0.24	0.49
c) 1-2 servings per day	15	37.50%	0.23	0.48
d) 0 servings per day	3	7.50%	0.07	0.26
17) On average, how many servings of fruit do you have per day? Note: 1 serving = 1 whole fruit				
(e.g., apple, orange, peach) = Vi cup chopped fruit (i.e., fruit salad).				
NOTE: each of the following is equal to one serving:				
- ▪ 1 whole fruit (ex. apple, orange, peach)				
- ▪ ½ cup of chopped fruit (i.e. fruit salad)				
a) 5 or more servings per day	0	0.00%	0	0
b) 3-4 servings per day	9	22.50%	0.17	0.42
c) 1-2 servings per day	20	50.00%	0.25	0.5
d) 0 servings per day	11	27.50%	0.2	0.45
18) What is your average alcohol consumption? (Note: 1 drink = 1 beer or 1 cocktail)				
a) 3 or more drinks per day	0	0.00%	0	0
b) 1-2 drinks per day	2	5.00%	0.05	0.22
c) 2-3 drinks per week	8	20.00%	0.16	0.4
d) 2-3 drinks per month	17	42.50%	0.24	0.49
e) none	13	32.50%	0.22	0.47
19) How often, on average, do you consume any food or drinks that are highly processed and contain preservative				
NOTE: these foods would primarily include:				
- ▪ diet and regular soft drinks, sugary fruit drinks				

Nutrition Survey	Number of Participants	Percentage	Variance	Standard Deviation
- ▪ potato chips, nachos, cheesies, corn chips etc.				
- ▪ licorice, jujubes, gummy bears, gelatins etc.				
- ▪ ice cream, fruit ices, sherbet etc.				
a) 3 or more per day	3	7.50%	0.07	0.26
b) 1-2 per day	6	15.00%	0.13	0.36
c) 2-3 per week	15	37.50%	0.23	0.48
d) once per week or less	16	40.00%	0.24	0.49
20) Do you take a multivitamin and mineral supplement daily?				
Yes	10	25.00%	0.19	0.43
No	30	75.00%	0.19	0.43

Consumption of diet patterns varied amongst the participants. A breakdown of the primary dietary habits can be summarised as indicated in the pie charts below.

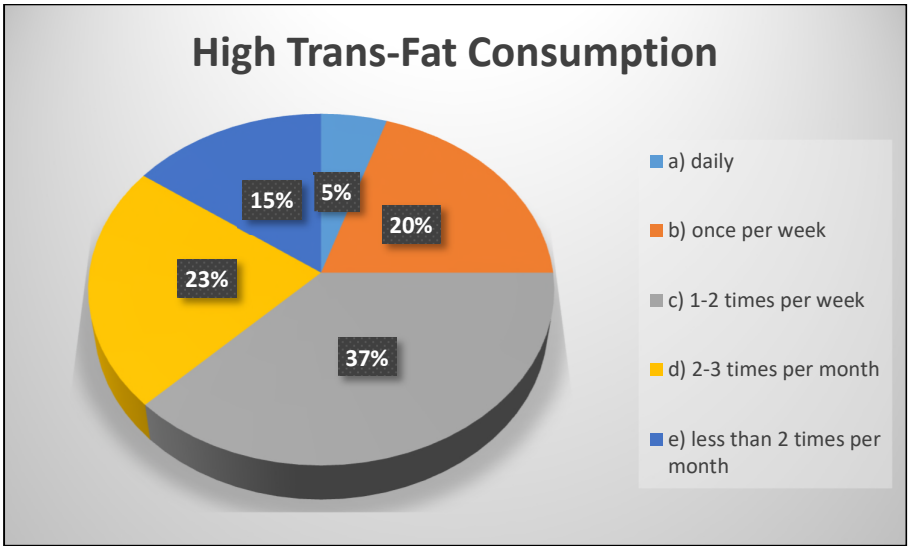


Figure 4: High Trans-Fat consumption amongst the participants

Up to 62% of participants consume trans-fats at least 1-2 times per week, with 5% of these consuming trans-fats even more frequently. A small percentage of participants (15%) consumed trans-fats less than twice a month (Figure 4). 50% of participants consumed vegetables at least 5 times per week, however only 7 participants were vegetarians (Figure 5). Only 25% of the participants take multivitamins and/or mineral supplements on a daily basis (Table 7).

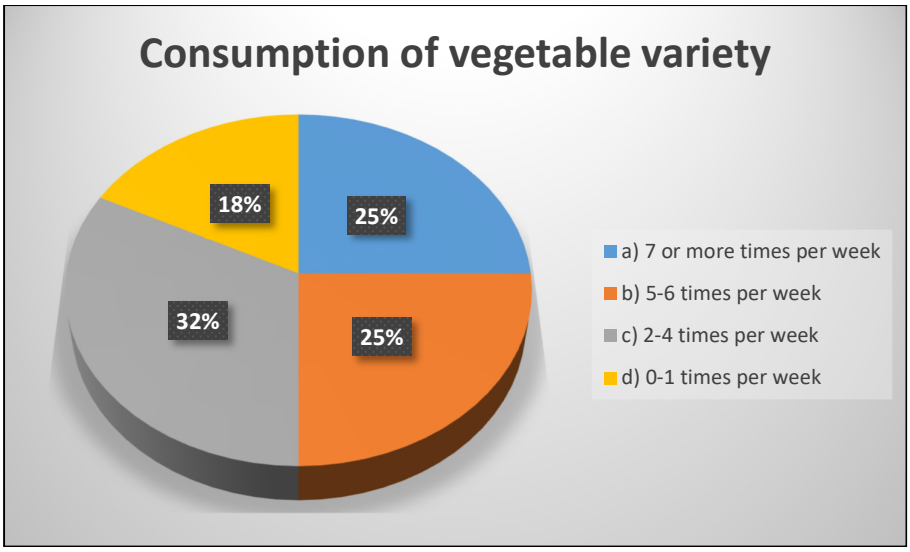


Figure 5: Vegetable variety consumption amongst the participants

4.1.2 Malnutrition Risk Scores

For the calculation of the risk of malnutrition, a score was given according to the Nutrition Survey in Annexure A (**Yes =1 and No = 0**), where the scores determined into which malnutrition group the participants fell:

Low risk for malnutrition: 0 – 4
Moderate risk for malnutrition: 5 – 7
High risk for malnutrition: 8 – 11

4.2 NEOPTERIN RESULTS

Neopterin results were calculated using 4-parameter logistics from the calibration standards as indicated in **Figure 6**. The R^2 value obtained for the calibration curve indicated a good fit of the standards. In addition, the kit quality controls calculated from the calibration curve were within range ensuring validation of the analyses.

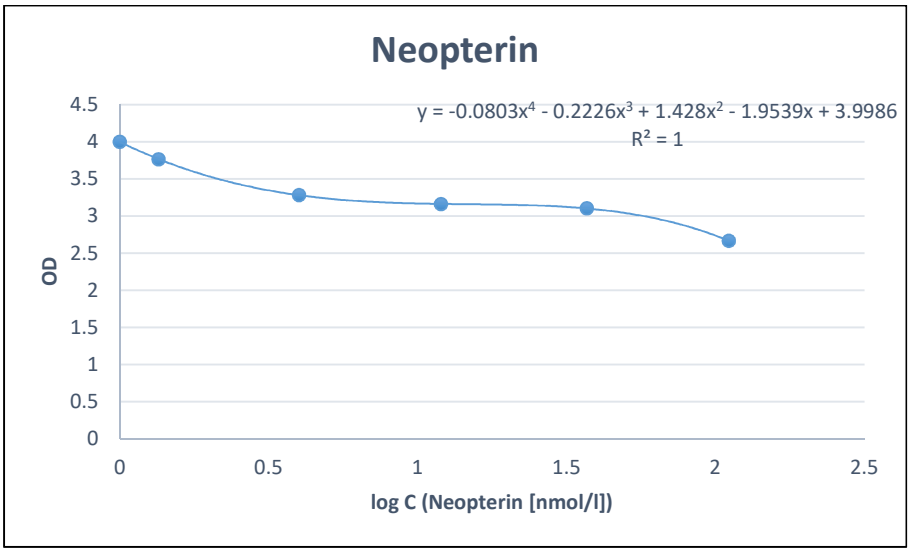


Figure 6: Neopterin calibration curve using 4-parameter algorithm. Optical density (OD) at 450nm**.**

The urinary neopterin results as calculated were expressed as ratio per unit creatinine, i.e. (µmol neopterin/mol creatinine) and a representation of the results is indicated by the box plot in **Figure 7**.

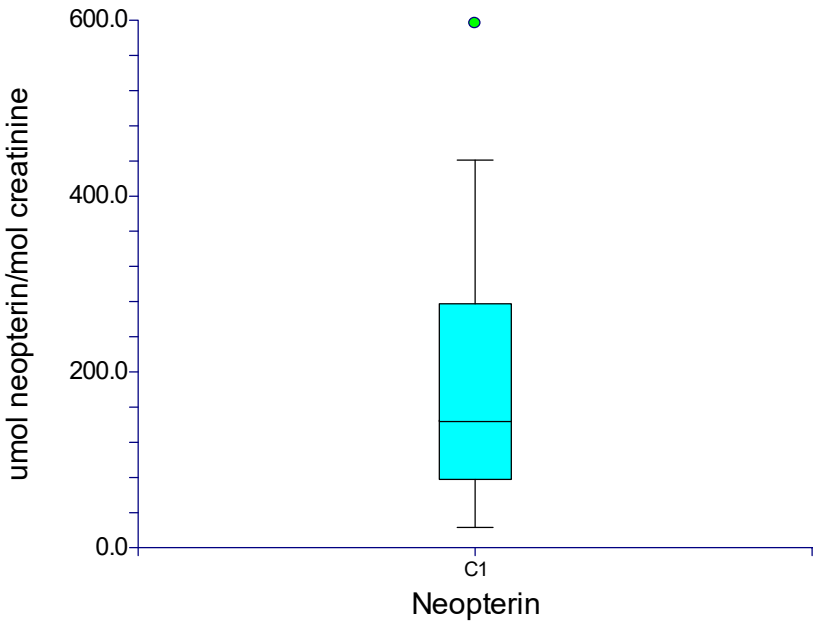


Figure 7: Neopterin box plot

The majority of participants displayed neopterin levels within the normal range. The mean value was 338.367µmol neopterin/mol creatinine and the standard deviation was 385.914µmol neopterin/mol creatinine. The inter-quartile range was 80-280µmol neopterin/mol creatinine, with the median being 130µmol neopterin/mol creatinine. A total of n=19 (47.5%) participants had marginally above normal neopterin levels when applying a normal neopterin reference of <210µmol/mol creatinine (according to kit biological reference). One participant displayed very high levels of neopterin (the outlier in the box plot above) with a measurement of 600µmol neopterin/mol creatinine.

4.3 NIACIN RESULTS

Niacin results were calculated using the 4-parameter algorithm. The calibration curve is indicted by **Figure 8**. The R^2 obtained indicated a good fit of the calibration standards. Results were checked using 4-parameter logistics and algorithm software (© Andreas Swart 2010-2018). The box plot is depicted in **Figure 9**.

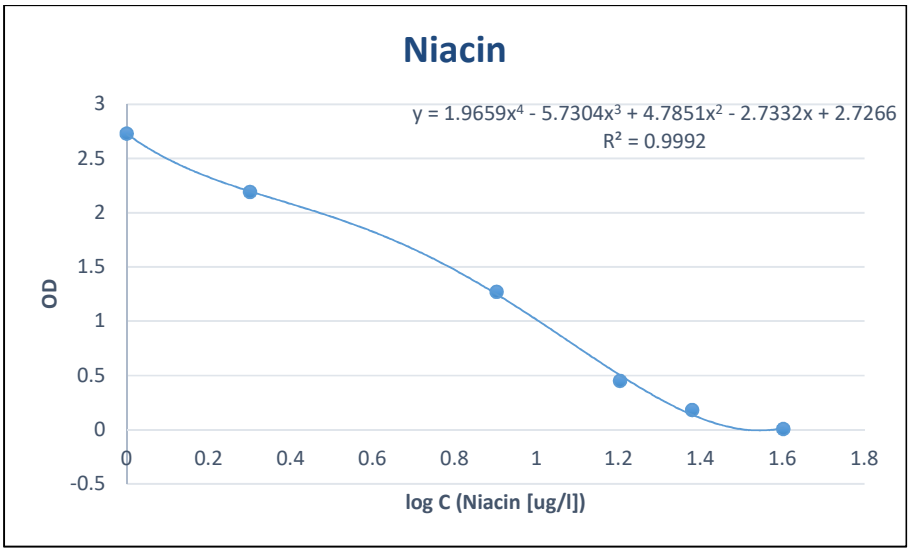


Figure 8: Niacin calibration curve with turbidity read at E 610-630nm

Urinary niacin content in adults should be above 2.84µg/l (4). The lowest niacin level measured was 2.24µg/l. The inter-quartile range was 3.88-5.29µg/l. There were 3 outliers with high levels of niacin, with one measuring a niacin level of 14.12µg/l.

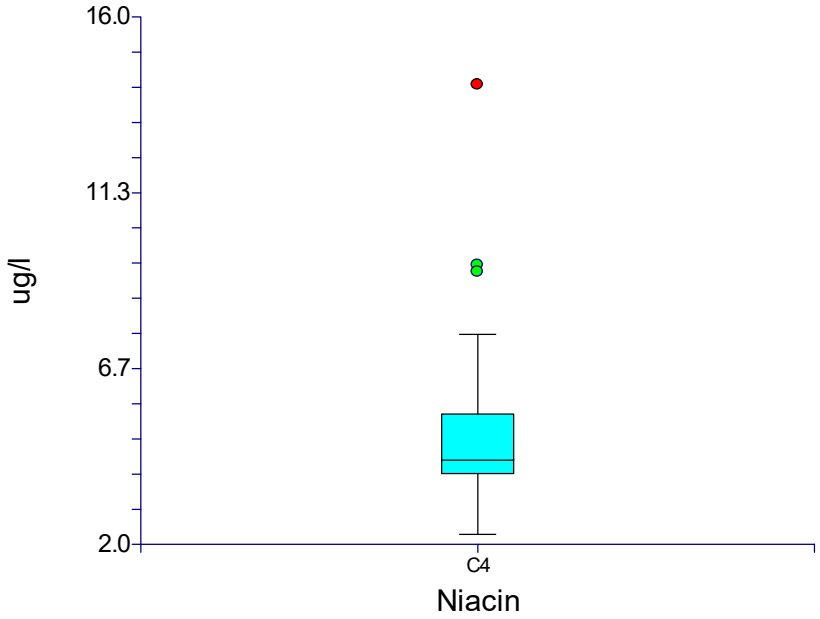


Figure 9: Niacin box plot

4.4 TRYPTOPHAN RESULTS

The 4-parameter algorithm was used for calculation of the urinary tryptophan results. The calibration curve obtained is indicated by **Figure 10**. The R^2 obtained indicated a good fit of the calibration standards. The box plot is depicted in **Figure 11**.

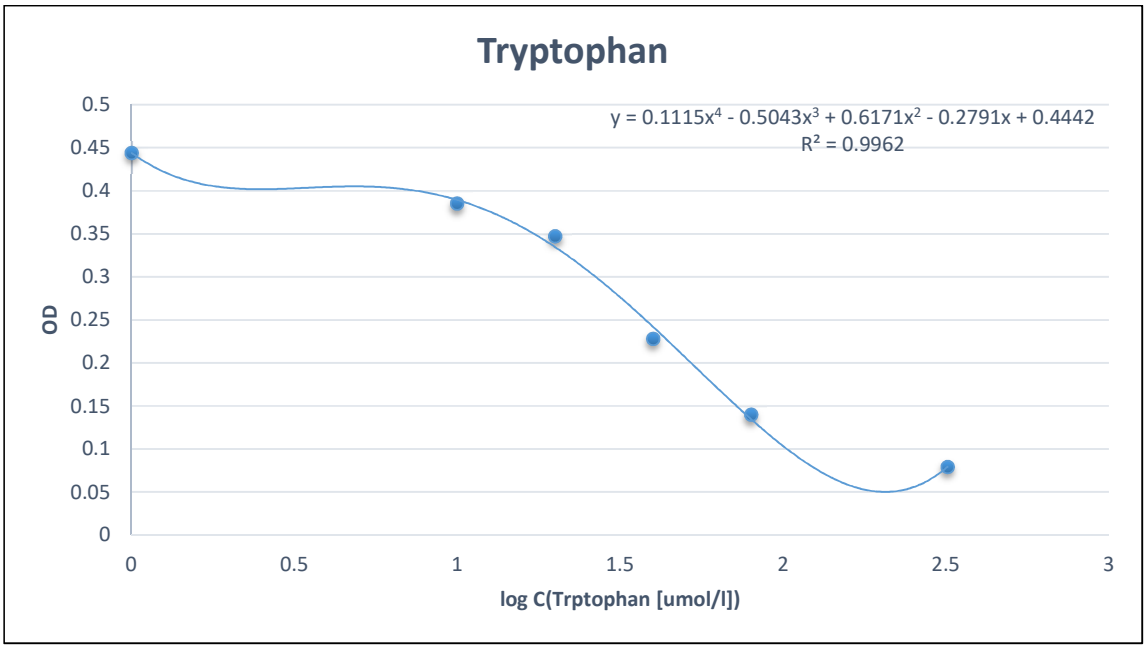


Figure 10: Tryptophan calibration curve. OD at 450nm**.**

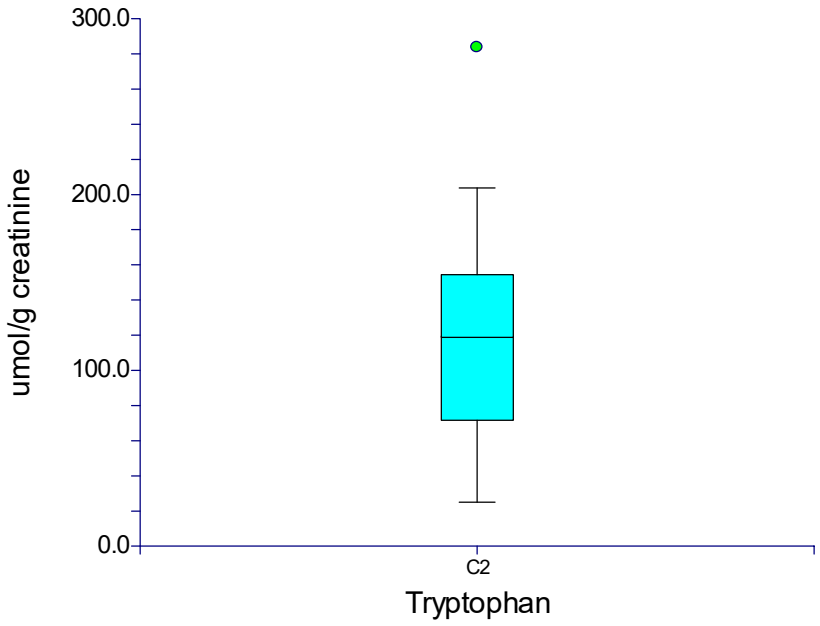


Figure 11: Tryptophan box plot

The box plot displays a minimum of 20µmol/g creatinine and a maximum of 200µmol/g creatinine. The inter-quartile range was 70-153,3µmol/g creatinine. There was one outlier with a tryptophan level of 280µmol/g creatinine. The median was measured at 120µmol/g creatinine, which is somewhat higher than the reference range median of 54.6µmol/g creatinine (according to kit reference).

4.5 STATISTICS

Statistical results were computed with 95% confidence interval and a significance level of $p<0.05$ unless otherwise indicated. The statistical software packages utilized were STATA 15 and IBM SPSS Statistics version 25.

Associations were tested for neopterin with niacin and tryptophan. Other pairwise correlation distributions were also computed to assess associations between variables. Outliers were removed from main computations where valid listwise numbers were indicated. The main results are indicated in the accompanying tables and graphs.

4.5.1 Descriptive Statistics

The mean values for neopterin, tryptophan and niacin were within normal range according to the kit validation parameters. However, variance and outliers were noted. A summary of the descriptive statistics is indicated in **Table 8**.

Table 8: Descriptive statistics

	N	Minimum	Maximum	Mean	Std. Deviation
NPT	33	23.11	596.10	182.2616	129.14236
TRP	39	24.93	283.46	119.6995	56.10589
Niacin	40	2.27	14.20	5.1351	2.13277
Valid N (listwise)	32				

4.5.2 Statistical Correlations

Pairwise distributions and Pearson correlation were calculated with 95% confidence and significance level at 0.01. The results are given in Tables 9 and 10. Spearman's correlation is depicted in Table 11. Figures 12 – 14 indicate the distribution between the variables.

The mean, mode and variance for neopterin, tryptophan and niacin are listed in **Table 9**, along with the credible intervals for each analyte.

Table 9: Pairwise correlations

Posterior Distribution Characterization for Pairwise Correlations[a]

			NPT	TRP	Niacin
	N		33	32	33
NPT	Posterior	Mode		-.114	-.334
		Mean		-.104	-.308
		Variance		.028	.023
	95% Credible Interval	Lower Bound		-.428	-.593
		Upper Bound		.222	-.005
	N		32	39	39
TRP	Posterior	Mode	-.114		.441
		Mean	-.104		.414
		Variance	.028		.017
	95% Credible Interval	Lower Bound	-.428		.158
		Upper Bound	.222		.659
	N		33	39	40
Niacin	Posterior	Mode	-.334	.441	
		Mean	-.308	.414	
		Variance	.023	.017	
	95% Credible Interval	Lower Bound	-.593	.158	
		Upper Bound	-.005	.659	

a. The analyses assume reference priors (c = 0).

Figures 12 to 14 indicate the distributions of neopterin with tryptophan and niacin as well as niacin with tryptophan. The plots indicate uneven distributions for the association of the analytes with each other.

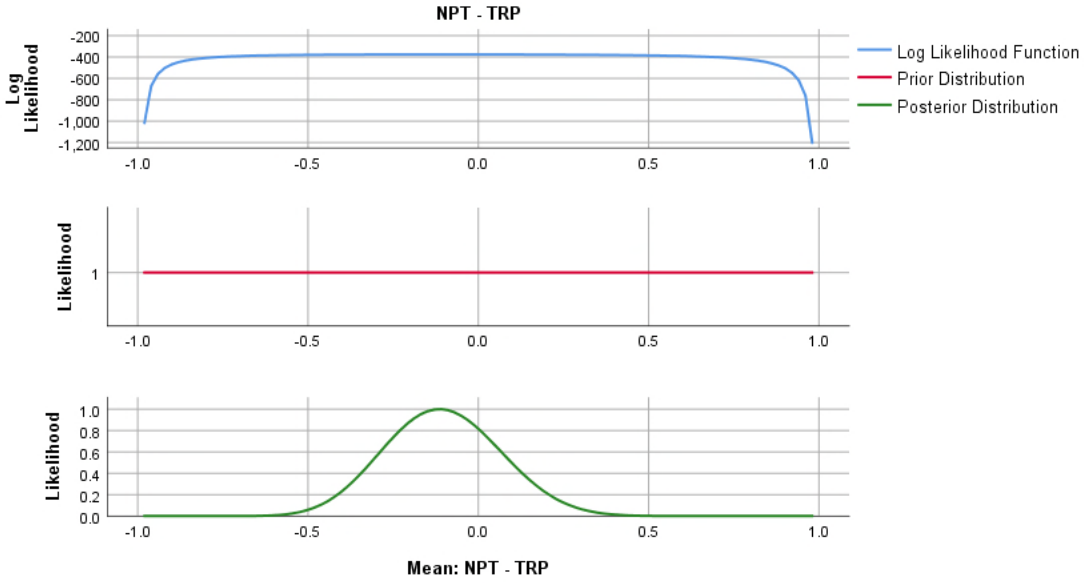


Figure 12: Neopterin-Tryptophan distribution

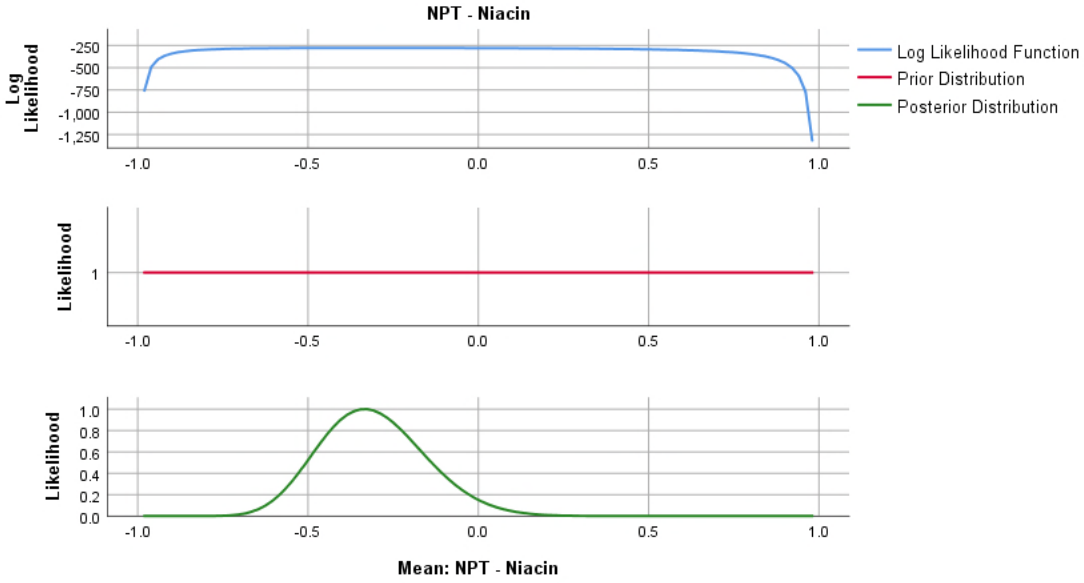


Figure 13: Neopterin-Niacin distribution

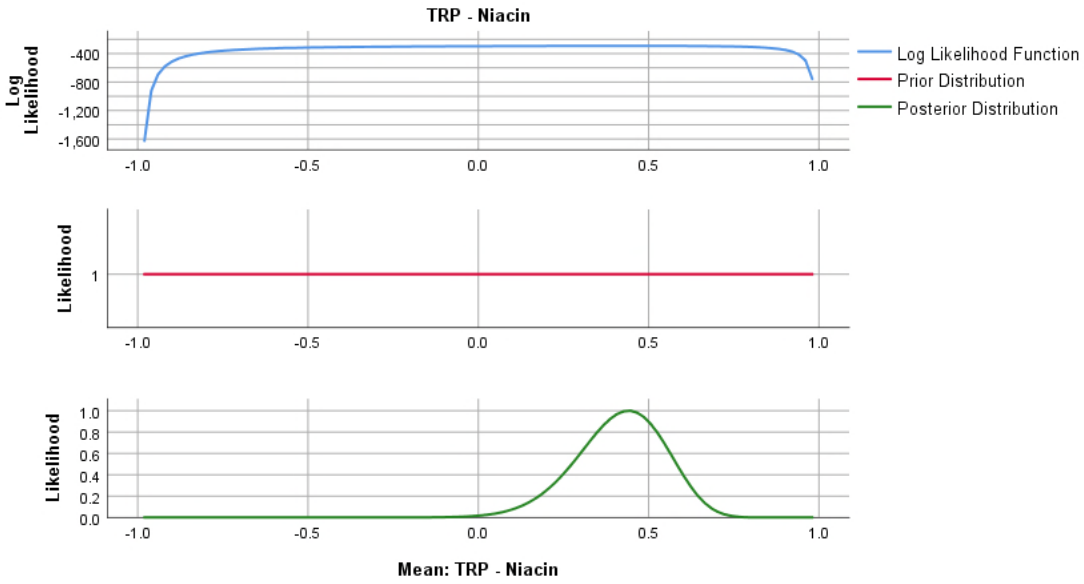


Figure 14: Tryptophan-Niacin distribution

A significant positive correlation was identified between tryptophan and niacin (**Table 10**). This could be expected as tryptophan is converted to niacin via the kynurenine pathway, as discussed in previous chapters.

Table 10: Pearson correlations

Correlations

		NPT	TRP	Niacin
	N	33	32	33
NPT	Pearson Correlation	1	-.116	-.339
	Sig. (2-tailed)		.527	.054
	N	32	39	39
TRP	Pearson Correlation	-.116	1	.446**
	Sig. (2-tailed)	.527		.004
	N	33	39	40
Niacin	Pearson Correlation	-.339	.446**	1
	Sig. (2-tailed)	.054	.004	

**. Correlation is significant at the 0.01 level (2-tailed).

Significant correlations were seen between tryptophan and niacin, neopterin and BMI as well as neopterin and malnutrition risk score. Neopterin correlated negatively with BMI, whilst the correlations between tryptophan, niacin, neopterin and malnutrition scores were positive (**Table 11**).

Table 11: Spearman's correlations

Correlations

			NPT	TRP	Niacin	BMI	MR_Score
		N	33	32	33	22	21
Spearman's rho	NPT	Correlation Coefficient	1.000	-.134	-.262	-.548**	.447*
		Sig. (2-tailed)	.	.463	.140	.008	.042
		N	32	39	39	25	24
	TRP	Correlation Coefficient	-.134	1.000	.476**	.196	-.136
		Sig. (2-tailed)	.463	.	.002	.347	.528
		N	33	39	40	26	25
	Niacin	Correlation Coefficient	-.262	.476**	1.000	.264	-.078
		Sig. (2-tailed)	.140	.002	.	.193	.711
		N	22	25	26	26	25
	BMI	Correlation Coefficient	-.548**	.196	.264	1.000	-.382
		Sig. (2-tailed)	.008	.347	.193	.	.059
		N	21	24	25	25	25
	MR_Score	Correlation Coefficient	.447*	-.136	-.078	-.382	1.000
		Sig. (2-tailed)	.042	.528	.711	.059	.

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 12 indicates the mean and standard deviation of the measured variables within the three different malnutrition risk groups. Analysis of variance (ANOVA) indicated no significant differences across the three groups for all the variables measured.

Table 12: Variables divided according to malnutrition risk scores

MR risk group		Neopterin	Tryptophan	Niacin	BMI
Low (0-4)		152.98 ± 111.44	118.97 ± 57.94	5.89 ± 3.07	25.38 ± 9.82
Moderate (5-7)		210.25 ± 156.08	113.78 ± 47.98	4.59 ± 1.18	23.70 ± 3.32
High (8-11)		179.01 ± 49.75	139.58 ± 78.17	4.95 ± 1.12	25.87 ± 3.23
ANOVA*	F	.700	.452	1.617	.393
	Sig.	.505	.640	.212	.678
Kruskal-Wallis		.495	.886	.691	.380

*Significance at 0.05 level and non-parametric Kruskal-Wallis at 95% confidence interval

In summary, the results indicate that many participants do not consume a healthy diet, consisting of a variety of fruit, vegetables and dairy products, with 15% of participants having a high risk of malnutrition. Moreover, 47,5% of participants had an above normal neopterin level, which could be indicative of the presence of low-grade inflammation. Within our participant group, 10 of the 24 female participants (38,5%) were considered to be overweight, whilst 7 of the 14 males (50%) were considered to be overweight. There was only one participant that was considered to be underweight according to BMI scores – using the European World Health Organization Body Mass Index Scale (47). Some significant correlations and associations between the measured parameters were identified. Neopterin correlated negatively with BMI and positively with malnutrition risk scores, whilst tryptophan correlated positively with niacin. These correlations and what can be deduced from them are discussed in detail in Chapter 5.

CHAPTER FIVE

DISCUSSION

5.1 DISCUSSION

In this study we investigated the dietary habits and the association of urinary neopterin with urinary niacin and tryptophan levels in a cohort of university students. This chapter evaluates the results we obtained and the associations and links that could be made from it.

The dietary habits of each participant were evaluated via dietary- and nutritional surveys. From the results of the surveys, it is apparent that the participants did not consume a variety of foods containing niacin and/or tryptophan. Of the 40 participants, 19 were at moderate risk for malnutrition and 6 (15%) were at high risk for malnutrition. The reason for this could vary amongst the participants. It could be due to financial constraints affecting the frequency and variety of food consumed, with 7,5% of participants skipping meals due to financial constraints and 47,5% of participants skipping a meal some of the time due to financial constraints. Another possible and perhaps more likely reason is time constraints. 85% of our study participants admitted to skipping at least one meal per day due to their busy schedules on campus. There were 10 participants (25%) that consumed a multi-vitamin on a daily basis. Previous studies have proven that the daily consumption of a '100% Daily Value' multivitamin can improve micronutrient status (48) and decrease nutrient deficiencies. The majority of our participant population however, at 75%, did not consume any form of multivitamin.

Urinary neopterin levels were measured to identify possible sub-clinical inflammation in apparently healthy individuals in order to ascertain whether underlying, low-grade inflammation in supposedly healthy individuals will alter their nutritional status, namely niacin, tryptophan and risk of malnutrition and possibly lead to deficiencies. Previous studies have identified elevated neopterin levels in the urine of healthy individuals where cell-mediated immunity is active (15) and clinical studies conducted by Scrimshaw *et al* also identified that subclinical infections play an important role in influencing micronutrient status (49). There were no significant differences between the variables divided according to malnutrition risk scores. However, from the results it could be seen that neopterin correlated negatively with almost all other variables, including – niacin, tryptophan and BMI, but an inverse relationship was seen with the malnutrition risk scores. The lack in variety of food intake could have predisposed the participants to malnutrition, resulting in the significant positive correlation we identified between neopterin and malnutrition risk scores. In other words, participants with a higher risk for malnutrition may have had a higher degree of

underlying inflammation, as reflected by neopterin. It can be deduced that underlying inflammation in supposedly healthy individuals may therefore pose a risk factor for malnutrition. This is in agreement with other studies which found that micronutrient status is altered by the presence of inflammation (33,50,51).

Urinary niacin levels were measured by means of an ELISA kit, in order to identify niacin levels within the different malnutrition risk groups and correlate this data to neopterin levels to identify whether neopterin (i.e. inflammation), has affected the niacin levels within our study participants. Another objective was to establish the association between tryptophan and niacin as well as that between tryptophan and neopterin. Tryptophan can be metabolised to niacin (9, 27) via the kynurenine pathway. The enzyme indoleamine-2,3-dioxygenase is the main enzyme allowing tryptophan to be metabolised to niacin and it is also the enzyme that is upregulated by the presence of inflammation. Recent research has shown that indoleamine-2,3-dioxygenase is susceptible to modulation by factors such as dietary components and physical exercise (52,53). There is a strong relationship between tryptophan breakdown via the kynurenine pathway (induced by the activation of the immune system), and increased levels of biomarkers such as neopterin (52). From the correlations tested, a significant positive correlation between niacin and tryptophan was found. This could be expected since tryptophan is converted to niacin via the kynurenine pathway (11,53,54), and an increase in tryptophan leads to an increase in *de novo* synthesis of niacin (55). This tryptophan to niacin turnover could possibly be a reason for not seeing any notably lower urinary niacin levels in our study. In fact, elevated niacin outliers were seen for some of the participants. A negative association was also seen between niacin and neopterin as well as tryptophan and neopterin.

Dietary habits play a larger role in underlying inflammation than we may realize. Studies have found that dietary patterns with high monounsaturated to saturated fat ratio's and abundance of fruits and vegetables show anti-inflammatory effects when compared to dietary patterns containing many processed foods and meats without many fruits and vegetables (56). From our study surveys, we identified that 1 in 4 participants consumed high-fat meat and other processed foods on a daily basis, with less than half of the participants consuming adequate fruit and vegetable portions on a weekly basis. These dietary habits, along with their stressful study environment, could contribute to the presence of underlying inflammation.

Something interesting to note is that 67.5% of participants drink alcohol at a minimum of 2-3 times per month, with 25% drinking alcohol more frequently (at least 2-3 times per week). 13% of participants were smokers. All the smokers also consumed alcohol. Although the alcohol consumption could be considered as normal within an adult population, previous literature has proven that drinking, as well as smoking, influences the intake of nutrients. Nutrient intake of smokers and individuals who consume alcohol on a weekly basis vary markedly compared to non-smokers and -drinkers (18,57,58).

Our results indicated that there was a significant negative correlation between neopterin and BMI. This would suggest that a decrease in BMI levels would lead to an increase in neopterin levels and vice versa. This is in contrast to previous literature by Kahraman *et al.* and Zampieri *et al.* which indicates a non-linear relationship between BMI and inflammation. Individuals that have a high or low BMI score were more likely to have inflammation than those with a normal BMI score (59,60). Further research is necessary to corroborate the association.

Of importance to note is that urinary niacin and tryptophan levels may not be an entirely true reflection of the bioavailability from dietary consumption. A dysregulation of metabolism along the kynurenine pathway may alter the levels which ultimately spill over to the urine (27). In this regard the assumptions made require further investigation.

Nonetheless, the associations between the variables provide an important and relevant scientific basis for understanding the physiological relationship of inflammation with malnutrition. Dietary habits could have an influence on sub-clinical inflammation in university students, thereby putting them at an increased risk for malnutrition. Previous literature by Meyer *et al.* and Bonaccio *et al* substantiates these findings (61, 62). Our understanding and knowledge of the intricate relationship between nutrition, immune function and the inflammatory response continues to evolve (33).

To summarise; dietary habits could have an influence on sub-clinical inflammation in university students and put them at an increased risk for malnutrition (52). The associations observed in the results indicated that participants displaying a higher risk for malnutrition had higher neopterin levels. This could be due to the presence of underlying inflammation (50,51). Niacin levels appeared normal in the majority of participants and no significant association was identified between niacin and neopterin. The associations identified, and assumptions made require further investigation.

CHAPTER SIX

CONCLUSION

6.1 CONCLUSION

Neopterin, as a relatively good indicator of underlying inflammation, was negatively associated with nutrition parameters such as niacin, tryptophan and BMI whilst being positively associated with malnutrition risk scores.

Niacin, tryptophan, neopterin and nutrition surveys were compared between participants to subsequently divide them into low, moderate and high malnutrition risk groups. 15% of participants were at high risk for malnutrition and 25% of participants were at moderate risk for malnutrition. There is a close interaction between nutritional status and inflammation and university students may be at risk for malnutrition as a result of underlying inflammation and associated variables such as poor dietary habits. The associations between the measured variables provide an important and relevant scientific basis for understanding the physiological relationship of inflammation with malnutrition.

Further research is required to support these findings.

6.2 STUDY LIMITATIONS AND FUTURE PERSPECTIVE

The participant population used for this study was not diverse. We only made use of students on the Health Science Campus (Prinshof Campus) at the University of Pretoria. Future studies could include a more diverse student population across campuses and across universities.

Our sample size was sufficient for the purposes of this study; however, future studies could include larger sample sizes to corroborate the findings of this study.

We used self-controls. What could be a true control for future studies? Comparable controls could include graduates who are permanently employed. However, stress and other factors need to be controlled.

Outliers were removed from comparisons where statistically fitting.

For dietary habit and malnutrition risk scores, we are relying on the honesty and/or accuracy of participants' answers in their surveys. A more relevant survey standardized for certain demographics should ideally be used.

Future studies should aim to measure additional niacin and tryptophan metabolites in both urine and blood samples.

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ANNEXURES

Annexure A –Dietary-, Nutrition and Demographic Surveys

Dietary and Nutrition Survey

Participant number: _____ (As assigned to you)

Please answer the following questions to help us assess your nutrition and wellness status.

A. Dietary Information

1) How often, on average, do you eat any of the following foods?

- ground beef
- bacon
- pork products
- burgers
- spare ribs
- chicken wings
- processed luncheon meats (ex. salami)

a) daily	
b) once per week	
c) 1-2 times per week	
d) 2-3 times per month	
e) less than 2 times per month	

2) How often, on average, do you consume any of the following foods:

- cheeses that are more than 20% milk fat (eg. cheddar cheese, mozzarella, brick, cream cheese, parmesan)
- homogenized milk
- yogurt that is more than 1% milk fat
- ice cream

a) daily	
b) once per week	
c) 1-2 times per week	
d) 2-3 times per month	
e) less than 2 times per month	

3) Do you use cream/milk in your coffee or tea? If yes, how many cups of coffee/tea per day do you average? _____

Yes		**No**	

4) Do you routinely use butter on bread products such as bagels, toast, crackers, etc.?

Yes		**No**		**Infrequently**	

5) Do you routinely use butter for cooking or on baked potatoes or other vegetables?

Yes		**No**		**Infrequently**	

6) Do you use regular sour cream or high fat salad dressings (ex. French, Thousand Islands, Blue Cheese) more than once per week?

Yes		**No**	

7) What is your weekly whole egg consumption on average?

a) 12 or more eggs per week	
b) 8-11 eggs per week	
c) 5-7 eggs per week	
d) 2-4 eggs per week	
e) less than 2 eggs per week	

8) How often do you eat fried foods?

a) 7 or more times per week	
b) 5-6 times per week	
c) 2-4 times per week	
d) 0-1 times per week	

9) Do you choose poultry or fish in place of red meat, pork or fried foods in most situations?

Yes		**No**	

10) Are you a vegetarian or near vegetarian? If yes, please describe (that is, are you vegan, lacto-ovo etc.?): _____

Yes		**No**	

11) How often, on average, do you consume any of the following:

- 2% milk
- low fat sour cream
- yogurt that is 2% milk fat

a) 7 or more times per week	
b) 5-6 times per week	
c) 2-4 times per week	
d) 0-1 times per week	

12) How often, on average, do you consume any of the following foods?

- pastries such as cakes, croissants, wraps
- ice cream
- donuts

- cookies (3 or more)
- high fat muffins
- rich desserts (ex. cheesecake, brownies)

a) 7 or more times per week	
b) 5-6 times per week	
c) 2-4 times per week	
d) 0-1 times per week	

13) How often, on average, do you consume any of the following snack foods?

- potato chips
- nachos
- any type of fried snack
- chocolate bars

a) 7 or more times per week	
b) 5-6 times per week	
c) 2-4 times per week	
d) 0-1 times per week	

14) How often, on average, do you consume any of the following snacks or drinks?

- regular soft drinks
- hard candy
- jujubes
- gummi bears or anything similar
- liquorice

a) 7 or more times per week	
b) 5-6 times per week	
c) 2-4 times per week	
d) 0-1 times per week	

15) On average, do you consume garden type vegetables (eg. carrots, broccoli, cauliflower, peppers, romaine lettuce, spinach, collard greens, kale)?

a) 7 or more times per week	
b) 5-6 times per week	
c) 2-4 times per week	
d) 0-1 times per week	

16) On average, how many servings per day do you consume of any of the following: pasta, rice, beans, peas, corn, barley, oatmeal?
NOTE: each of the following is equal to one serving:

- ½ cup of pasta, rice, beans, peas, corn, oatmeal, etc. (before cooking)
- 1 slice of bread
- ½ bagel
- ½ English muffin
- ¼ cup of most fibre cereals
- low-fat, high-fibre muffin

a) 5 or more servings per day	
b) 3-4 servings per day	
c) 1-2 servings per day	
d) 0 servings per day	

17) On average, how many servings of fruit do you have per day? Note: 1 serving = 1 whole fruit (e.g., apple, orange, peach).

NOTE: each of the following is equal to one serving:

- 1 whole fruit (ex. apple, orange, peach)
- ½ cup of chopped fruit (i.e. fruit salad)

a) 5 or more servings per day	
b) 3-4 servings per day	
c) 1-2 servings per day	
d) 0 servings per day	

18) What is your average alcohol consumption? (Note: 1 drink = 1 beer or 1 cocktail)

a) 3 or more drinks per day	
b) 1-2 drinks per day	
c) 2-3 drinks per week	
d) 2-3 drinks per month	

19) How often, on average, do you consume any food or drinks that are highly processed and contain preservative, artificial flavours, colours, and related chemicals?
NOTE: these foods would primarily include:

- diet and regular soft drinks, sugary fruit drinks
- potato chips, nachos, cheesies, corn chips etc.
- licorice, jujubes, gummy bears, gelatins etc.
- ice cream, fruit ices, sherbet etc.

a) 3 or more per day	
b) 1-2 per day	
c) 2-3 per week	
d) once per week or less	

20) Do you take a multivitamin and mineral supplement daily?

Yes		**No**	

B. Vitamin and Mineral Supplementation Assessment

Dietary Habits

1. Do you have fewer than 5 servings of fruits and vegetables per day on average?

Yes		**No**	

2. Do you consume citrus fruits fewer than 4 times per week on average?

Yes		**No**	

3. Do you consume 1 serving of orange-yellow fruits and vegetables fewer than 5 times per week on average? For example:

- 1 whole carrot
- 8 large apricots halves
- ¼ of a cantaloupe
- ½ cup melon squash
- 1 baked sweet potato
- 1 whole peach/nectarine

Yes		**No**	

4. Do you consume cruciferous vegetables (cabbage, cauliflower, broccoli, Brussel sprouts) fewer than 5 times per week on average?

Yes		**No**	

5. Do you eat smoked meat or fish more than once per week on average?

Yes		**No**	

6. Do you eat luncheon meats, processed meats, sausages, bacon, bologna or any other nitrate salt containing meat once per week or more on average?

Yes		**No**	

7. Do you eat barbecued foods that are charred (braai), once per week or more on average?

Yes		**No**	

8. Do you drink 3 or more cups of coffee per day on average?

Yes		**No**	

9. Do you consume less than two dairy servings per day on average? 1 serving = glass of milk or 100ml of yogurt (preferably low-fat varieties) or 100g of cheese (preferably low-fat varieties)

Yes		**No**	

10. Are you currently on a diet to lose weight or on a calorie-restricted programme?

Yes		**No**	

11. Do you consume poultry or fish less than four times per week?

Yes		**No**	

C. Clinical Status - Inflammation

1. Do you suffer from chronic pain or injury?

Yes		**No**	

If yes, please describe the nature and location of the injury(s)?
Additionally, please indicate if any of the following chronic pain disorders apply to you.

__ Fibromyalgia __ Ulcerative Colitis __ Complex Regional Pain Syndrome
__ Chronic Pain Syndrome __ Crohn's Disease __ Carpal Tunnel Syndrome
__ Tendinitis __ Rheumatoid Arthritis __ Sciatica
__ Muscle Strain __ Osteoarthritis __ Thoracic Outlet Syndrome

__ Chronic Fatigue Syndrome __ Autoimmune __ Candidiasis infection
__ Spasms __ Prostate/Prostatitis __ Dysmenorrhea
__ Numbness/Tingling __ Other:_____

2. How often do you suffer from the pain and/or injury?

a) Less than once per year	
b) Approximately once per year	
c) A few times per year	
d) Once per month	
e) A few times per month	
f) Once per week	
g) More often than once per week	
h) Constant Pain/Injury	

3. Please indicate if the pain and/or injury require over-the-counter medication?

a) Yes	
b) Sometimes	
c) No	

Please indicate type/frequency:_____

4. Are you on any chronic antibiotics / antifungals for long term infection?

Yes		**No**	

5. Have you ever been diagnosed with any condition related to nutrient malabsorption such as Chrohn's disease, Hartnup's disease or irritable bowel syndrome?

Yes		**No**	

Demographic Survey [Adapted for purposes of study to account for student socioeconomic status and food security]

Participant number: _____ (As assigned to you)

Please answer the following questions to help us assess student wellness.

1) Please indicate your type of accommodation as a university student.

- University residence
- Private or rental accommodation, such a flat or commune
- Home with parent/s or guardian

2) How far is your accommodation from campus?

< 1 km

< 5 km

> 5 km

3) How do you commute between your accommodation and campus?

- Walk or university transport

- Private transport

- Public transport such as bus, taxi, train

4) As a student, how many meals do you have on average per day?

- 1 meal per day

- 2 meals per day

- 3 or more meals per day

5) Do you generally skip a meal due to lack of time owing to your studies (lectures, tests, exams), other university activities or busy schedule?

- No

- Yes, some of the time
- Yes, most of the time

6) While on campus, do you generally skip a meal due to financial constraints?

- No

- Yes, some of the time

- Yes, most of the time

7) At your accommodation, do you generally skip a meal due to financial constraints or lack of food security?

- No

- Yes, some of the time

- Yes, most of the time

8) As a student, which of the following applies to you regarding pocket expenses for food and daily subsistence?

- I receive an allowance from my parents / guardian / other family member
- I am employed and pay for my own expenses
- I have a university related grant / scholarship / bursary
- I do not receive an allowance and find it financially constraining

BIOSTATISTICS UNIT

8 May 2017

To whom it may concern,

This letter confirms that **T. Fouché** from the **Department of Physiology, Faculty of Health Sciences of the University of Pretoria** discussed her project: **"Dietary habits and the association of neopterin with niacin and tryptophan in university students"** with me. I confirm that I will assist with the statistical analysis of the study data.

Data analysis

The inflammatory and other physiological parameters will be describes using mean, median, standard deviation and range, with 95% confidence intervals. Pearson's correlation coefficient will be calculated to test the correlations with the parameters and niacin levels. The t-test, or non-parametric alternative will be used to determine if niacin differs across high and low risk neopterin values. Tests will be evaluated at 5% level of significance. All analysis will be done using STATA 14.

Sample size

A sample size of 30 is deemed sufficient for the study, with 15 per group for the high and low risk neopterin.

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Annexure C – Ethical Clearance

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

29/06/2017

Approval Certificate
New Application

Ethics Reference No,: 259/2017

Title: Dietary habits and the association of neopterin with niacin and tryptophan in university students

Dear Tanya Fouche

The **New Application** as supported by documents specified in your cover letter dated 20/06/2017 for your research received on the 23/06/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 28/06/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**259/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your researdE,

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports,** and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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SUMMARY

The aim of this study was to investigate the dietary habits and the association of urinary neopterin with urinary niacin and tryptophan levels in a cohort of university students.

It was a non-intervention, non-invasive study involving male and female students from the University of Pretoria. All participants completed a questionnaire consisting of three parts; Dietary-, Nutrition- and Demographic, after which urine samples were collected and analysed in the laboratory using ELISA- and biomarker kits. There were no exclusions for race, gender, nationality, year of study or degree programme. A sample size of 40 participants were successfully recruited. The mean age was 23,6 ± 1,47 years and 70% of the participants were female. The mean BMI for the total group was 25.18 ± 5.14 kg/m^2.

Dietary habits varied with less than 50% consuming dairy products and adequate servings of fruit and vegetables weekly. 85% of participants skipped at least one meal per day while on campus due to their busy schedules, whilst 55% of participants skipped at least one meal per day while on campus due to financial constraints. 57.5% of the participants consumed less than 3 meals per day. Up to 62% of participants consumed trans-fats at least 1-2 times per week, with 5% of these consuming trans-fats even more frequently. The results indicated that many participants do not consume a healthy diet, consisting of a variety of fruit, vegetables and dairy products, with 15% of participants having a high risk of malnutrition. 47,5% of participants had an above normal neopterin level, which could be indicate the presence of low-grade inflammation. 10 of the 24 female participants (38,5%) and 7 of the 14 males (50%) were considered to be overweight according to BMI scores. From the results of the surveys, it is also apparent that the participants did not consume a variety of foods containing niacin and/or tryptophan. There were 10 participants that consumed a multi-vitamin on a daily basis, which can improve micronutrient status and decrease nutrient deficiencies. The majority of our participant population however (75%) did not consume any form of multivitamin.

Some significant correlations and associations between the measured parameters were identified. Neopterin correlated negatively with BMI, and positively with tryptophan, niacin, neopterin and malnutrition scores.

The majority of participants displayed neopterin levels within the normal range. 19 participants had marginally above normal neopterin levels and 1 participant displayed very high levels of neopterin. There were no significant differences between the variables divided according to malnutrition scores. The lack in variety of food intake could have predisposed the participants to malnutrition, resulting in the significant positive correlation identified between neopterin and malnutrition scores. Niacin levels appeared normal in most participants and no significant association was identified between niacin and neopterin.

Dietary habits could have an influence on sub-clinical inflammation in university students and increase their risk for malnutrition. The associations observed indicated that participants displaying a higher risk for malnutrition had higher neopterin levels, which could be due to the presence of underlying inflammation.