MSc Dissertation

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NEOPTERIN AND NEUROPHYSIOLOGICAL MEASUREMENTS AS MARKERS OF ANXIETY AND STRESS

by

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Executive Summary

Mental health is an increasing global crisis. It has large social, economic, and health costs, with stress and anxiety disorders accounting for a large portion of the impact. Subjective measures, misdiagnosis, and social stigma may interfere with the detection, prevention, and treatment of mental health problems. As a result, more objective measures such as biomarkers are necessitated to aid in the identification and treatment of mental health disorders. As aberrations in the autonomic nervous system (ANS) are implicated in stress and anxiety, biomarkers measuring ANS activity were investigated in this study, namely heart rate variability (HRV), blood pressure (BP), blood-volume pulse (BVP), electrodermal activity (EDA), and quantitative encephalography (qEEG). Additionally, inflammation appears to have a bidirectional relationship with stress and anxiety. Therefore, the inflammatory marker neopterin was also investigated as a possible biomarker of stress and anxiety symptoms. The study aimed to determine whether the aforementioned biomarkers correlated with scores of stress and anxiety. Thus, the Depression, Anxiety, and Stress Scale (DASS-21) scoring system was used to stratify participants between Group A (n = 20), who had high levels of symptoms, and Group B (n = 20) who had normal levels of stress and anxiety. The participants were students from the Faculty of Health Sciences who were recruited using an online questionnaire (n = 158) with biographical questions and the DASS-21 questionnaire. Respondents (n = 74) who met the inclusion criteria were invited to partake in the neurophysiological measurements and provide a urine sample, which was tested for neopterin using an enzyme-linked immunosorbent assay (ELISA).

Group A had higher mean neopterin and delta power compared to Group B ($p < 0.05$). Group A also had lower HRV and alpha waves. Significant correlations ($p < 0.05$) were as follows: anxiety, depression, neopterin, delta waves, and hibeta waves, all positively correlated with the stress score. Mean HRV and alpha waves were negatively correlated with the stress score. Stress, depression, neopterin, delta waves, and hibeta waves, positively correlated with the anxiety score. Mean HRV, minimum BVP amplitude, mean BVP amplitude, and alpha waves, negatively correlated with the anxiety score. In summary, neopterin positively correlated with the stress and anxiety scores, while HRV and BVP were negatively correlated with these scores. In terms of qEEG, delta and hibeta wave activity increased in the left and frontal brain regions of participants with mental health struggles, whereas alpha wave activity decreased in these regions.

The prevalence of stress and anxiety is high in this Health Sciences student population and is associated with physiological changes. According to the results, BP and EDA did not correlate

with stress and anxiety scores. Whereas, neopterin and parameters of HRV, BVP, and qEEG, did correlate with these scores and could therefore be used as potential biomarkers in conjunction with existing measures.

The associations between inflammation, neurophysiology, and mental health need to be addressed and further investigated to mitigate further health and economic burden. Especially given that anxiety is the leading mental health disorder, and it is associated with inflammation, another large contributor to disease. As such, universities have a responsibility to help reduce and prevent mental health conditions and mitigate their effects.

Keywords: anxiety, autonomic nervous system, biomarkers, brainwaves, depression, health science, heart rate variability, inflammation, mental health, neopterin, stress

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Declaration by Student

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.

Sign: Rouxzan Cronjé

Date: 15 February 2023

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ANS	Autonomic nervous system
APs	Action potentials
BBB	Blood-brain barrier
BCIA	Biofeedback Certification International Alliance
BH_4	Tetrahydrobiopterin
BP	Blood pressure
BVP	Blood-volume pulse
CI	Confidence interval
CNS	Central nervous system
CVD	Cardiovascular disease
CRH	Corticotrophin-releasing hormone
DASS	Depression, Anxiety and Stress Scale
DHNTP	7,8-Dihydroneopterin triphosphate
DSM	Diagnostics and Statistical Manual of Mental Disorders
EDA	Electrodermal activity
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay
FFT	Fast Fourier transform
GABA	Gamma-aminobutyric acid
GAD	Generalized Anxiety Disorder
GTP	Guanosine-5′-triphosphate
HF	High-frequency
HIV	Human immunodeficiency virus

HPA	Hypothalamic-pituitary-adrenal
HPSCA	Health Professions Council of South Africa
HRV	Heart rate variability
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon-gamma
IFNα	Interferon-alpha
IL	Interleukin
IQR	Interquartile range
LF	Low-frequency
MDD	Major depressive disorder
mRNA	Messenger ribonucleic acid
NN	Normal-to-normal
NSAIDS	Non-steroidal anti-inflammatory drugs
PIC	Pro-inflammatory cytokine
PNS	Parasympathetic nervous system
PSPs	Post-synaptic potentials
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
qEEG	Quantitative electroencephalography
RMSSD	Root mean square of successive differences between normal heartbeats
ROS	Reactive oxygen species
SAM	Sympathetic-adreno-medullar pathway
SD	Standard deviation
SDNN	Standard Deviation of Normal-to-Normal
SNS	Sympathetic nervous system
TMB	3,3',5,5'-Tetramethylbenzidine
TNFα	Tumour necrosis factor-alpha
WHO	World Health Organization

1. Introduction

1.1 Mental Health

Good mental health is defined by the World Health Organisation (WHO) as a state of well-being in which the individual can cope with the normal stresses of life, can work productively, can contribute to their community, and realise their abilities.[1] Conversely, when people have mental health struggles they may suffer from mental health disorders such as anxiety, depression, anorexia, bulimia, or bipolar disorder.[2]

The number of people who suffer from mental health conditions is increasing. Between 1990 and 2019, cases of mental health disorders increased by 48%.[2] Of these, the cases of anxiety disorders increased by nearly 50% and depressive disorders increased by 64%.[2-3] In South Africa, almost one in every three people experience a mental health disorder in their lifetime, with anxiety disorders being the most common.[4] The effects of living with a mental health condition permeates into all areas of life, affecting both personal and professional aspects, such as impacting performance, productivity, and relationships.[5-8] Mental health conditions can cause disability to a similar degree as some physical conditions, like heart disease and arthritis.[9] Some ways in which mental health conditions can be debilitating is by having a negative impact on concentration, self-care (e.g. washing and eating), the ability to perform daily activities, social interactions, and leaving the house.[9] It is then not surprising that mental health disorders are among the leading contributors to the burden of disease worldwide,[2] with financial, social, and medical consequences. Even in sub-Saharan Africa, a region with the highest burden of contagious diseases, mental disorders account for nearly 10% of the total burden of disease.[10] Mental health conditions are among the most costly disorders in terms of projected healthcare expenditures needed to treat them,[10] accordingly, the global economic impact of these conditions is estimated to be US$3-7 trillion each year (due to medical costs, disability, and lost productivity).[11] Despite the pervasive and increasing effects of mental health, the medical resources, interventions, and funding allocated to treating them are not proportional to the actual burden. In many countries, less than 1% of government health expenditure goes towards mental health services, with the average expenditure being only 2.8%.[12-14]

Not only is mental health a major concern globally, but it is also of particular importance for medical students who have a high incidence of anxiety, depression, burnout, and mental health struggles.[15-16] Studies suggest that medical students often have higher levels of psychological distress than the general population and their age-matched peers.[17-19] This may

have an adverse effect on academic performance, empathy, and the care of their patients, as well as contributing to other negative professional and personal aspects.[20-22]

As mental health conditions are highly prevalent with extensive negative ramifications,[2,5-9] it raises the question of why so many people are suffering and what can be done about it? How can the immense impact on aspects such as well-being, daily activities, social relationships, and cost be reduced?[9,11] This is particularly prudent for Health Science students who are not only at greater risk for mental health struggles, but also represent the future of the medical profession and the prospective quality of patient care.

In this chapter, stress, anxiety, and depression will be discussed. Due to the emerging role of inflammation in mental health, aspects of inflammation and its relation to mental health are also considered. Biomarkers may be of use in improving the management of mental health conditions. Thus, potential biomarkers of inflammation and neurophysiological activity are discussed and any associations they may have with mental health aspects.

1.1.1 Stress

Stress is commonly defined as the response to a real or perceived threat to homeostasis.[23-25] This response involves physiological, endocrine, and cognitive reactions, which aid in survival.[25] Behavioural effects of the stress response include enhanced cognition, awareness, analgesia, and euphoria. Physiological effects include vasoconstriction, and inhibition of growth, metabolism, digestion, reproduction, and immune function.[24,26]

The stress response involves two main pathways: the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. The SNS is part of the autonomic nervous system (ANS), thus providing a rapid response to stress with short-term effects. The secondary phase of the stress response involves the slower endocrine response of the HPA axis, which has longer-lasting effects.[25,27]

When the SNS is stimulated by a stressor it activates the ‘fight-or-flight’ response. This results in the release of catecholamines, namely adrenaline and noradrenaline, from sympathetic neurons and the adrenal medulla into circulation. Catecholamines interact with adrenergic receptors in the central nervous system (CNS) and on the cell membranes of smooth muscles and other organs. The activation of these receptors have many effects such as increasing blood glucose levels, metabolic rate, heart rate, cardiac output, vasoconstriction, oxygen consumption, thermogenesis, muscle contraction, and blood flow to skeletal muscles.[28-29] Additionally, SNS activation enhances arousal, alertness, vigilance, cognition, attention, and

analgesia.[28] Responses of the SNS are very fast and occur almost simultaneously with the detection of a threatening stimulus.[25]

In terms of the HPA axis, the secondary phase of the stress response, the main effectors are the paraventricular nucleus (PVN) of the hypothalamus, the anterior pituitary gland, and the adrenal gland. The PVN is innervated by afferent projections from many brain regions, which provide psychological, visceral, humoral, and endocrine information.[23] Activation of the HPA axis causes the sequential release of three main molecules: corticotrophin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and glucocorticoids.[23]

CRH is produced in the PVN and is released into the hypophyseal portal vessels in response to stress. This carries CRH to the anterior pituitary gland, where it binds to its receptor and causes the release of ACTH.[23] The main target of ACTH is the zona fasciculata of the adrenal cortex, where it stimulates the synthesis and secretion of glucocorticoids, namely cortisol, which affects metabolic, cardiovascular, immune, and behavioural processes.[24,26]

Activation of the HPA axis leads to an increase of glucocorticoids in circulation – peak levels are reached within 30 minutes after the commencement of a stressor.[30] Thereafter, glucocorticoids provide negative feedback by inhibiting further CRH and ACTH release from the hypothalamus and pituitary gland, thereby regulating the duration and magnitude of HPA activation.[23]

In summary, danger signals in the form of neuroendocrine mediators are released in response to stressors. The primary mediators are glucocorticoids (released by the HPA axis) and catecholamines (released by the SNS). The effects of these mediators assist individuals with responding to and surviving a threat.[25]

Although the stress response is a normal and healthy physiological process to help restore homeostasis, prolonged stress can become maladaptive and detrimental. Chronic stress is an important risk factor for the development of many disorders, including anxiety,[31] depression,[32] and cardiovascular disease (CVD).[33] Anxiety and depression are discussed in the next sections.

1.1.2 Anxiety

Anxiety is a physiological, psychological, and behavioural state induced by an actual, perceived, or potential threat.[34] As such, fear and anxiety function as danger signals and

trigger appropriate adaptive responses to promote survival and reduce harm.[34] However, pathological levels of anxiety interfere with quality of life, impacting health, emotion regulation, social and occupational functioning, and the ability to cope successfully with challenges.[34-35] Anxiety disorders are among the most prevalent and disabling mental illnesses worldwide.[9] They are characterised by excessive worry, fear, and other related behavioural alterations, such as avoidance and panic.[36]

Fear and anxiety are largely similar and overlap in terms of neurocircuitry and behavioural responses.[37] However, fear is usually associated with SNS activation for the fight-or-flight response, escape behaviours, and thoughts of immediate danger. Whereas, anxiety more often involves hypervigilance, avoidant behaviours, muscle tension, and anticipation of future threats.[36] As such, anxiety disorders are distinguished from normal levels of fear or anxiety by their excessive and persistent nature. This can in part be caused by dysregulation of the fear circuitry leading to fear in response to benign or non-threatening stimuli, and then failing to extinguish fear responses. This contributes to exaggerated fear, recurrence of traumatic memories,[38] and the promotion of a chronic state of hypervigilance.[39]

In terms of neurocircuitry in anxiety, the amygdala plays a key role and is often found to be hyperresponsive.[40] Pathways between the amygdala, the medial prefrontal cortex, and the hippocampus are important in anxiety and signalling safety.[37,41] The amygdala plays an important role in threat detection, as well as being involved in fear conditioning (creating an association between a stimulus and fear) and the emotional enhancement of memories.[42-44] During fear conditioning, the amygdala can be activated even when the conditioned stimulus is below perceptual thresholds.[40] This means that the amygdala can orchestrate a response without an individual being consciously aware of the threat-causing stimulus and can coordinate a response to the threatening signals.[38-39] Brain regions involved in anxiety are also implicated in the stress response, suggesting a relationship between stress and anxiety.[40] For example, the amygdala can modulate autonomic activity and activate the fight-or-flight response.[45] It is then not surprising that the ANS may be involved in anxiety.[46-47] This is supported by a study which found autonomic hypersensitivity in patients with anxiety when they were administered an adrenergic receptor agonist.[48]

Factors affecting the development of anxiety disorders include genetic predisposition, psychological vulnerability (e.g. adverse early life experiences), and other environmental factors.[34] Anxiety is a complex phenomenon that involves many neurotransmitters, peptides, and hormones.[34] Neurotransmitters that are implicated in anxiety-related behaviours include changes in noradrenaline,[49] serotonin,[50] γ-aminobutyric acid (GABA)[51-52] and their related

receptors, which are the targets of several anxiolytic drugs. It should be noted that the brain region and type of receptor are essential in determining the anxiogenic, anxiolytic or neutral effects of these neurotransmitters.[34] For example, serotonin has both anxiolytic and anxiogenic effects. The nature of the effect is dependent on the receptor subtype and the brain region. Activation of the 5-HT_{2A} receptor promotes or increases anxiety-like behaviour, whereas activation of 5-HT_{1A} receptors inhibit anxiety-like behaviour and have anxiolytic effects.[53-54] The paradoxical effect of serotonin is also supported by clinical evidence – benzodiazepines have an anxiolytic effect by decreasing serotonin,[55] whereas some antidepressants increase serotonin and are beneficial in several anxiety disorders.[56] In terms of having effects in different brain regions, the dual serotonin fear hypothesis suggests that serotonin may enhance conditioned fear in the amygdala while inhibiting innate fear in the posterior periaqueductal gray matter.[57] Considering the aforementioned, anxiety is a complex condition, which complicates its management and contributes to the potential heterogeneous treatment thereof.

In addition to neurotransmitters, HPA axis hormones, such as CRH and glucocorticoids, are also involved in anxiety-like behaviour.[34,58] For example, a study found that rats who were administered glucocorticoids showed increased anxiety and altered dendritic plasticity in the amygdala.[59] Furthermore, a murine study found that mutations in glucocorticoid receptors resulted in altered anxiety-related behaviours.[60] Another study involving rats found that exposure to gestational stress had long-lasting consequences for the offspring, such as impaired regulation of the HPA axis to stress, increased CRH, increased anxiety, and altered brain morphology in adulthood.[61] Therefore, stress is thought to precipitate anxiety.

In summary, anxiety disorders are a considerable problem worldwide,[9] and are characterised by excessive fear and other psychological and physiological alterations.[34-36] Aberrations in neurotransmitters,[49-52] hormones,[34,59] and the ANS[46-48] are thought to be involved in its pathophysiology. In addition to stress, genetic, environmental, or experiential factors also contribute to the risk of developing anxiety.

1.1.3 Depression

According to the Diagnostics and Statistical Manual of Mental Disorders (DSM), depressive disorders are characterised by feelings of sadness, emptiness, and/or irritability, which are accompanied by somatic and cognitive changes that significantly impact a person's capacity to function.[36] It should be noted that these symptoms are present every day and are distinguished from normal feelings of sadness or grief, which reduce in intensity over time.[36] The incidence of depression is increasing and it is already a leading cause of disability

worldwide, ranking the highest of all mental disorders.[2] Additionally, depression contributes to significant morbidity, mortality, and economic burden globally.[11] Part of the burden of depression is due to its association with several comorbidities, including diabetes,[62] CVD,[63] and cognitive impairment.[64]

Many factors contribute to the risk of developing depression in university students, these include confidence, personality, academic pressure, pre-existing conditions, lifestyle choices, social support, and financial struggles.[65]. In addition to these, stress is also a risk factor for depression.[32,66] There are many ways in which stress can contribute to depression, one such mechanism is through the aberration of CRH levels.[67-68]

In terms of aetiology, the monoamine hypothesis is often used to explain the pathophysiology of depression. In brief, this hypothesis suggests that a reduction in brain monoamines (serotonin, noradrenaline, and dopamine) can result in depressive symptoms.[69-70] As such, the main pharmacological focus of antidepressants is to increase the levels of these neurotransmitters in the brain.[71] Reduced serotonin in depression has been largely studied. Factors which contribute to decreases in serotonin levels include stress, cytokines, and tryptophan depletion.[69,72-73] However, the monoamine (or serotonin) hypothesis alone cannot explain all the facets of depression e.g. onset, course, or remission.[74] For example, not all depressed patients respond to drugs that enhance serotonergic neurotransmission,[75] and other drugs which do not affect the serotonergic system also show antidepressant effects.[71] Therefore, serotonin and other monoamines are not solely involved. There are other hypotheses such as the glutamate hypothesis which postulates that glutamate is increased in depressed patients, leading to neuronal damage through excessive glutamate excitation. The results of this excitation include dendritic atrophy, neuronal death, and changes in brain function.[70,72] Additionally, a glutamate receptor antagonist, ketamine, has anti-depressive effects, which support the involvement of glutamate in depression.[70] There is also the neurotrophic hypothesis which suggests that decreased growth factors, such as brain-derived neurotrophic factor, contribute to depression via decreased neurogenesis and increased neuronal atrophy.[76] Of particular interest to the present study is the cytokine (or macrophage) hypothesis in which inflammation and cytokines contribute to depression through various mechanisms.[77] For example, cytokines can alter neurotransmitter metabolism, thereby decreasing the availability of serotonin.[69,72] Lastly, the microbiota-inflammasome hypothesis suggests that stress activates the NLRP3 inflammasome and can cause gut dysbiosis, resulting in bacteria and concomitate molecules leaking from the gut, which induces the release of inflammatory cytokines from the immune system. Together, inflammasomes and

cytokines lead to neuroinflammation which increases depression and anxiety.[78-79] The relation between inflammation and mental health is discussed further in later parts of this chapter.

Considering the aforementioned, depression is a complex condition with the pathophysiology being affected by many factors including stress, neurotrophins, inflammation, cytokines, neurodegeneration, intestinal microbiota, and the dysregulation of neurotransmitters (e.g. serotonin and glutamate).[69-70,72,77,80-82]

In summary, stress, anxiety, and depression are highly prevalent and largely contribute to the burden of disease worldwide.[2,9,11] In order to mitigate their effects, quantitative evaluation is necessitated to facilitate diagnosis, treatment, and the understanding of mental health conditions, thus improving outcomes and reducing associated stigma.[83] Questionnaires and biomarkers could be useful in assessing these conditions – to paraphrase physicist Lord Kelvin "if you cannot measure it, you cannot improve it".[84] Therefore, reliable ways to measure and assess mental health parameters are of importance.

1.2 Questionnaires

One method that can be used to measure mental health symptoms are questionnaires. Numerous studies have been conducted to verify the validity and reliability of questionnaires in assessing mental health conditions, such as anxiety and depression.[85-89] If administered properly, they can be very useful research tools. Questionnaires allow researchers to gather a significant amount of data quickly and at relatively little cost. They are also considered to be a very good method of obtaining quantitative data.[90]

1.2.1 DASS-21

One questionnaire that has been used to assess mental health parameters is the Depression, Anxiety, and Stress Scale (DASS). This questionnaire was designed to measure mental health aspects on three scales – Depression, Anxiety, and Stress. The Depression scale reflects self-esteem and motivation, while the Anxiety scale reflects feelings of fear, panic, and arousal. The third scale, Stress, measures tension, irritability, and difficulty relaxing.[91] The questions are in the format of a Likert scale and interpretation is based on the use of cut-off scores for a severity rating of 'normal' to 'extremely severe' (Appendix D). The short-form version of the DASS, which consists of 21 questions (DASS-21), was used in this study.

One way to think about and visualise the symptoms of depression and anxiety is as a Venn diagram, whereby some symptoms appear in both conditions while some are unique to either

depression or anxiety.[92] For example, low mood and negative emotions are unique to depression, whereas hyperarousal is unique to anxiety.[92] Each scale of the DASS assesses unique features of each of the three conditions, which reduces the overlapping or intercorrelation of the measurements, thus increasing the ability to distinguish between depression, anxiety, and stress. Due to this setup, the DASS scales were found to have less overlap than other questionnaires such as the commonly used Beck Anxiety Inventory and Beck Depression Inventory,[93] which means that the DASS may be better at discriminating between conditions than these other questionnaires.

The DASS questionnaire was first developed in 1995 by Lovibond and Lovibond[91] using a sample of almost 3000 participants. It has since been found to be a reliable and validated measure in a number of different populations including Canadian outpatients,[94] Chinese hospital workers,[95] rural Vietnamese women,[96] Nepalese adults,[97] British adults,[86,98] Dutch substance-use disorder inpatients,[99] Turkish university students,[100] and Dutch first-year university students.[101] A study conducted in a South African sample also found this to be true of the DASS-21 questionnaire.[102] Thus, the DASS-21 can be considered reliable and valid in measuring depressive, anxious, and stressed symptoms in both clinical and non-clinical samples.

In addition to the body of evidence suggesting the reliability and validity of the DASS-21 questionnaire, its use is also favourable for other reasons. Unlike some questionnaires, the DASS is in the public domain, which means it can be used without permission or cost. Furthermore, the DASS is easy to use and administer and the scores are not significantly affected by gender,[95] age or years of education,[98] which simplifies interpretation. The short-form version is preferable to the longer version with 42 questions because it is quicker to fill in, reducing the burden on respondents and potentially increasing response rates. The DASS-21 also produces similar reliability and validity compared to the longer version.[94,103]

The DASS questionnaire is a dimensional rather than a categorical measure. A categorical approach uses diagnostic criteria, such as in the DSM, to determine the presence or absence of abnormal behaviours, which is used to diagnose a patient with a disorder. Whereas, a dimensional assessment considers these disruptive behaviours on a continuum of frequency and/or severity.[104] In other words, a dimensional approach comments on the severity of symptoms rather than the presence or absence of a diagnosed disorder. As such, The DASS should not be used to diagnose participants into discrete categories proposed in classification systems such as the DSM, but rather should be used as a screening tool to assess symptom severity.

Although mental health aspects can be determined through the administration of self-assessment questionnaires, suitable physiological measurements may be necessary to complement and substantiate the questionnaire facets surveyed. Adjunct physiological biomarkers may contribute to the scientific understanding of mental well-being and may be advantageous in improving the management thereof. For example, some but not all depressed patients present with elevated inflammation,[105] as such an inflammatory biomarker could be useful in identifying this subset of patients and thus prescribing an appropriate course of treatment. The contribution of biomarkers to mental health aspects is further discussed in the next section.

1.3 Biomarkers

Biomarkers have been gaining interest as tools to assist with the management of mental health disorders. Biomarkers are features that can be measured as an indicator of normal biological processes, pathogenic processes, or responses to an intervention.[106] Suitable biomarkers are non-invasive, affordable, and easy to measure. These markers can be measured in many different ways, including the use of sensors, electrodes, imaging technology, or from samples of body fluids or tissues. For example, a biomarker that is regularly used in clinical practice is low-density lipoprotein found in the blood, which is used to assess cholesterol levels and the risk of atherosclerosis.[107]

In relation to mental health, biomarkers could be used to facilitate diagnosis, elucidate underlying pathophysiology, predict and monitor clinical outcomes, and improve the understanding of these disorders.[108-109] Additionally, biomarkers may help to improve the personalisation of mental health management and therapeutic outcomes,[108] which is essential given the heterogeneous nature of mental health conditions.[110] They could also be useful in the development of new drugs to treat mental health disorders[107] and to predict the outcome of treatment, such as anticipating the efficacy and the likelihood of developing side effects.[110] For example, quantitative electroencephalography (discussed later in this chapter) has been used to predict antidepressant effectiveness.[111-112]

Furthermore, the use of diagnostic criteria, such as those found in DSM, can lead to subjectivity and underdiagnosis or misdiagnosis,[113] which largely contributes to the clinical and financial burden of mental health conditions.[114] The use of biomarkers in conjunction with questionnaires may be necessitated to minimise this, by providing a potential solution to reduce diagnostic and treatment errors. For example, a patient may meet the criteria for

anxiety or depression, but the cause could be due to a different physical condition, such as autoimmune encephalitis,[115] tumours,[116-117] vitamin B12 deficiency,[118] hypothyroidism, or Parkinson's disease,[119] to name a few.[109] Therefore, biomarkers may be useful in confirming the mental condition diagnosis and prompting the physician to do further investigation and testing if necessary. Besides the use of biomarkers in verification to reduce misdiagnosis and to prescribe appropriate treatment, biomarkers may help to reduce the stigma associated with mental health. Biomarkers may also help patients to have more confidence and acceptance in that they were accurately diagnosed, which is something some patients struggle with.[120] Furthermore, the use of biomarkers in conjunction with a questionnaire may be of use given that people suffering from anxiety may have altered perceptions and introspection, thus physiological markers could be used to verify self-rated aspects of patient evaluation.[121-123]

In addition to possible misdiagnosis of a physical disorder as a mental disorder, there is also a high rate of misdiagnosis between mental disorders, such as misdiagnosing bipolar disorder as major depressive disorder (MDD),[124] which increases burden and cost.[125-127] In these situations, biomarkers can also be of use; a recent study combined a questionnaire and blood biomarkers to create an algorithm to distinguish between bipolar disorder and MDD. The algorithm was found to improve the accuracy of diagnosis, and to reduce misdiagnosis of bipolar disorder as MDD.[114] Considering this, biomarkers could aid in the identification and treatment of mental health disorders, although further research is required.[114]

In summary, biomarkers may be of great assistance in reducing the increasing problem of mental health disorders.[2] They could be used to confirm a diagnosis, identify subtypes of a disorder, identify the stage and severity of a disorder, help to redefine diagnostic categories, identify at-risk individuals, personalise treatment, predict treatment outcomes, and help to identify pathophysiology in mental health disorders.[110,128] Additionally, current high rates of misdiagnosis and low response rates to treatment fuel the need for further research to improve the management and treatment of mental health conditions.[110] However, sufficient evidence has not been established to verify the reliability, validity, and reproducibility of biomarkers in clinical use for mental health, therefore further research is required.

Potential biomarkers of inflammation, autonomic activation, and brain activity in relation to mental health are discussed further in the upcoming sections. These biomarkers involve taking measurements from the fingers, heart, scalp, and urine.

1.4 Inflammation

1.4.1 What is Inflammation?

The inflammatory response is produced by the immune system. It is a defence mechanism against infection, general tissue injury, stress, and malfunction.[129] It can be triggered by both infectious and non-infectious agents.[130] These agents include exogenous inducers (i.e. microbes, irritants, allergens, and toxic compounds) and endogenous inducers (i.e. signals produced by stressed, damaged or otherwise malfunctioning tissues).[129] Irrespective of the cause, inflammation is thought to be an adaptive response tasked with restoring homeostasis and removing detrimental stimuli.[129] Acute inflammation is considered beneficial, however, when it becomes chronic, it can contribute to a variety of chronic inflammatory diseases, such as CVD, stroke, cancer, obesity, and diabetes.[131-132] This is a concern given that inflammatory diseases are the largest cause of death worldwide.[131]

Inflammatory responses are complex, involving many signalling molecules, cells, and elaborate regulatory networks. The process also depends on the location in the body and the nature of the initial stimulus.[130] A generic inflammatory pathway consists of inducers, sensors, mediators, and effectors. Inducers are molecules that can cause an inflammatory response and they are detected by sensors. Sensors activate mediators which then alter the functions of effectors (cells and tissues).[129] For example, cell-surface pattern receptors (sensors) can recognise detrimental stimuli (inducers) and cause the release of inflammatory mediators which recruit immune cells (effectors) to the affected site.[130]

A site of inflammation is marked by increased blood flow, increased capillary permeability, leukocyte infiltration/recruitment, and localised mediator production – these aforementioned are the four main events of an inflammatory response.[133] Inflammatory mediators include peptide mediators (e.g. cytokines, chemokines), lipid mediators (e.g. prostaglandins, eicosanoids, leukotrienes), reactive oxygen species (ROS), vasoactive amines (e.g. histamine), enzymes (e.g. matrix proteases), and products of proteolytic cascades.[129,133] The type of mediators released depend on the trigger of inflammation, the inflammatory response stage, and the tissue involved.[133] The production of mediators by leukocytes, namely tissue-resident macrophages, after the initial recognition of infection or injury, have many effects. These effects include changes in vasculature permeability, which allows an exudate to form locally. This allows leukocytes and plasma proteins, which are normally restricted to the blood vessels, to gain access to the site.[129] Particular mediators can act as chemoattractants to amplify the inflammatory process, and other mediators can leave the site of inflammation, entering circulation and exerting systemic effects. Consequently, inflammation at one site can

cause inflammation-driven changes in other sites.[133] The release of chemoattractants from the inflammatory site promotes attraction and migration of leukocytes from the capillaries to the site,[133] where they fight the infection or injury via phagocytosis, antibodies, antigen presentation, and/or the release of cytotoxic molecules.[134] For example, neutrophils release toxic compounds, such as ROS, which are highly potent and nonspecific, thus damaging both microbial and host cells.[135]

In a successful acute inflammatory response, the offending agent is eliminated and there is a resolution and repair phase. This is mediated mainly by tissue-resident and recruited macrophages.[136-137] As such, macrophages are critical in the initiation, maintenance, and resolution of inflammation.[138] When the acute response is not successfully resolved, chronic inflammation may occur. Reasons for unsuccessful resolution include genetic disorders, systemic metabolic dysregulation, macrophage dysfunction, and continuous insult.[137]

Due to the cascade of molecules released during the inflammatory response, there are a number of potential biomarkers of inflammation. One such biomarker is discussed next.

1.4.2 Neopterin: a biomarker of inflammation

Neopterin is a molecule that forms part of the pteridine family. It is also known as 2-amino-4-hydroxy-(erythro-1,2,3-trihydroxypropyl)-pteridine.[139] Neopterin is synthesized from guanosine-5′-triphosphate (GTP) and forms part of the tetrahydrobiopterin (BH_4) synthetic pathway.[140]

The main source of neopterin in humans is monocytes and macrophages, which produce neopterin when stimulated by interferon-γ (IFNγ); a cytokine produced by immune cells (Figure 1).[141] Activated T-lymphocytes, particularly Th1 cells, produce IFNγ which stimulates macrophages, resulting in the production of neopterin.[141-142] Therefore, neopterin is reflective of immune activation and is considered to be a non-specific biomarker of cell-mediated immunity,[143-144] because it reflects the production and effects of IFNγ, in addition to Th1 cell and macrophage activity. As such, neopterin has been used as a marker of immune activation during inflammation in a broad range of conditions, including cancer,[143] CVD,[145-146] and infectious diseases.[147]

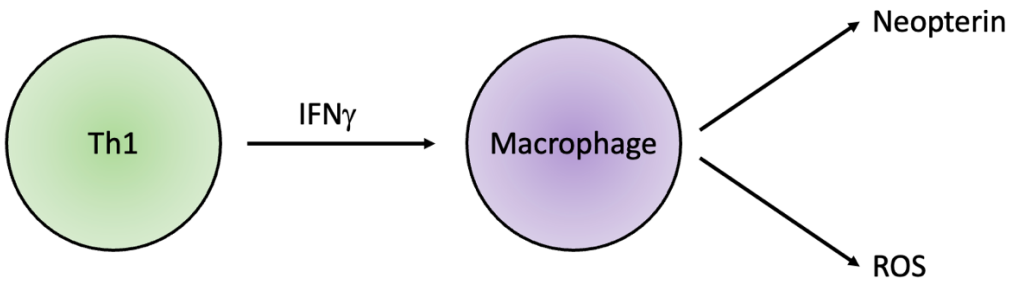

Figure 1: Neopterin production. *When macrophages are stimulated by interferon-γ (IFNγ), which is produced by Th1 cells (a type of T-lymphocyte), they produce neopterin and reactive oxygen species (ROS). Adapted from Murr et al. 2002.*[148]

The synthesis of neopterin starts with the conversion of GTP to 7,8-dihydroneopterin triphosphate (DHNTP) by the enzyme GTP-cyclohydrolase I. DHNTP is then converted to 7,8-dihydroneopterin, which is converted to neopterin (Figure 2).[140] Interferon-activated macrophages largely produce neopterin because IFNγ stimulates the activity of GTP-cyclohydrolase I, which leads to a build-up of DHNTP.[149] In macrophages, DHNTP can be converted to 7,8-dihydroneopterin faster than it can be converted to BH_4, because the BH_4-synthesising enzyme is slower and rate-limiting. As a result, IFNγ increases neopterin production.[149]

Neopterin and BH_4 share a pathway, whereby the precursor DHNTP can be converted to either 7,8-dihydroneopterin or BH_4 (Figure 2).[140] BH_4 is an essential cofactor for the biosynthesis of dopamine, serotonin, and tyrosine.[140,150] Inflammation and oxidative stress can result in decreased levels of BH_4, which consequently decreases the availability of neurotransmitters such as dopamine.[81,150-152] As such, neopterin may provide a link between mental health and inflammation, as it could reflect one mechanism by which immune system activation can affect neurotransmitters.[152] In addition, neopterin levels have been found to change significantly during periods of psychological stress, suggesting a correlation between mental state and alterations in cell-mediated immunity.[153] Furthermore, inflammation-induced stimulation of indoleamine 2,3-dioxygenase (IDO) and the kynurenine pathway can contribute to tryptophan depletion and decreased serotonin, which has been associated with depression[69,72-73,80,154-155] and anxiety.[50,156] This relates to neopterin as tryptophan depletion and increased kynurenine have been found to be correlated with neopterin.[154,157] Despite the potential mental health effects, tryptophan depletion by the immune system is purposeful, as it can reduce microbial proliferation.[158] Considering these findings, neopterin has the potential to be an immunological marker for mental health conditions.

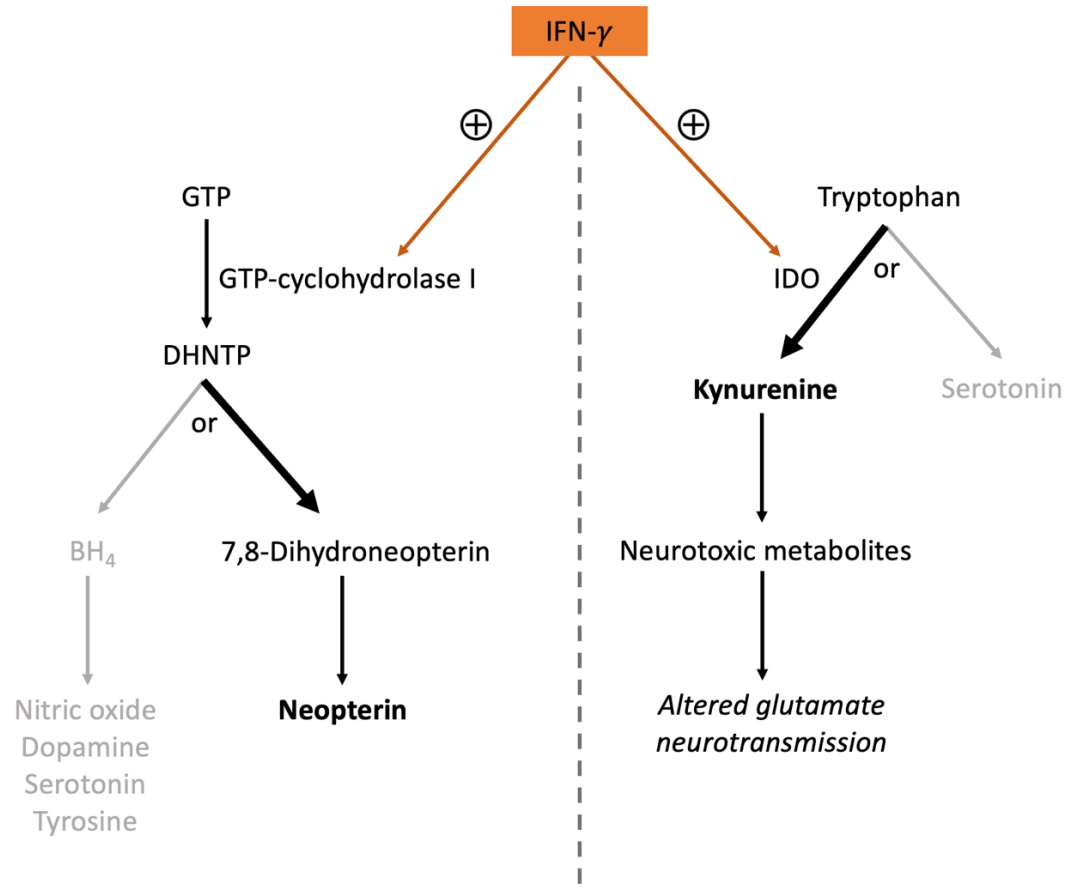

Figure 2: Inflammation, neurotransmitters, and neopterin synthesis. *The left arm of the diagram shows neopterin synthesis. The enzyme GTP-cyclohydrolase I converts guanosine-5′-triphosphate (GTP) to 7,8-dihydroneopterin triphosphate (DHNTP). DHNTP can then be converted to 7,8-dihydroneopterin or tetrahydrobiopterin (BH_4). BH_4 is a cofactor of several enzymes and is involved in the production of nitric oxide, dopamine, serotonin, and tyrosine. Tyrosine is used to make dopamine, noradrenaline, and adrenaline. When interferon-γ (IFNγ) stimulates the activity of the enzyme GTP-cyclohydrolase I, it leads to a build-up of DHNTP which exceeds the capacity of the rate-limiting enzyme that converts DHNTP to BH_4. This means more DHNTP is converted to 7,8-dihydroneopterin. Therefore, IFNγ stimulation increases neopterin production. IFNγ also stimulates the activity of indoleamine 2,3-dioxygenase (IDO) which converts tryptophan to kynurenine. This reduces the availability of tryptophan for the synthesis of serotonin. Kynurenine has some neurotoxic metabolites which affect glutamate neurotransmission and cause excitotoxic neuronal damage.*[72,81,159-160] *Kynurenine metabolites also contribute to oxidative stress, neurodegeneration, depression, anxiety, and changes in cognition.*[69,81,159,161-163] *Therefore, IFNγ stimulation leads to greater production of neopterin and kynurenine, and it provides a mechanism by which inflammation can alter levels of neurotransmitters, namely, dopamine, serotonin, and glutamate. This diagram was designed with information from multiple sources.*[72,140,159,164]

Neopterin is not just a by-product of the BH_4 pathway, it also has physiological roles. The precursor of neopterin, 7,8-dihydroneopterin, is an antioxidant which can react with and neutralize free radicals, such as ROS, to protect macrophages from oxidants through a scavenging reaction.[165] Neopterin is a result of this reaction as oxidation of 7,8-dihydroneopterin creates neopterin.[140,165] Neopterin is also involved in modulating the cytotoxic effects of ROS released by activated macrophages.[166] Therefore, neopterin levels can be considered an indirect marker of the amount of ROS and oxidative stress induced by the immune system.[145,165]

In terms of assessing immune activity, cytokines, such as IFNγ, can be measured. However, monitoring neopterin instead may be superior as it is biochemically inert and has a longer half-life. These properties allow neopterin to reach and stay in circulation, unlike other cytokines, which have a short half-life and may not reach circulation.[145] Once in circulation, neopterin levels can be measured with ease due to its unchanged excretion by the kidneys,[143] allowing it to be quantified in the urine using validated assays such as an enzyme-linked immunosorbent assay (ELISA).[145,165] Thus, neopterin is a good potential biomarker as it is relatively easy to measure and can be obtained non-invasively.

As neopterin is an inflammatory marker and inflammation is related to mental health (discussed in the next section), neopterin may be a viable biomarker for mental health symptoms and was therefore investigated in the present study.

1.4.3 Inflammation and Mental Health

Inflammation is thought to have many effects on the brain, resulting in cognitive decline, abnormalities in brain structure, changes in brain metabolism, and in the pathogenesis of neurodegeneration.[82,167-168] As such, it is not surprising that there is increasing evidence suggesting a causal link between inflammation and the pathogenesis of neuropsychiatric disorders.[82,169] One way in which inflammation may be involved in the pathophysiology of mental health conditions is the release of pro-inflammatory cytokines (PICs) which can influence feelings of anxiety and depression.[170-174] This is due to PICs, such as IFNγ, affecting the brain by altering neurotransmitter metabolism, neuroendocrine activity (HPA axis hormones), brain function, and synaptic plasticity. These changes have emotional, cognitive, and behavioural effects (Figure 3).[77,81,173,175] Additionally, Bauer and Teixeira[169] stated that "depression facilitates inflammatory reactions and inflammation promotes depression and other neuropsychiatric disorders", which suggests a bidirectional effect between inflammation and mental health.

Negative emotional states can be influenced by inflammation; daily social interactions that are negative and competitive are associated with heightened PIC activity[176] and higher cytokine levels are associated with increased negative mood.[177] Supporting this, interferon-α (IFNα) treatment produces negative biases in emotional processing, whereby patients can more accurately detect negative rather than positive facial expressions.[178] Stimulated increases in PICs are also associated with negative mood, cognitive impairment, fatigue, changes in appetite, anhedonia, and social withdrawal.[170] One mechanism by which inflammation can cause these changes is by affecting serotonergic neurotransmission. PICs, like IFNγ, increase

the activity of the enzyme IDO, which leads to increased conversion of tryptophan to kynurenine instead of serotonin (Figure 2).[170,179-180] Thus, PICs can increase tryptophan catabolism, leading to serotonin deficiency. In addition, increased kynurenine and its neurotoxic metabolites can affect glutamate neurotransmission and cause excitotoxic neuronal damage.[72,81,159-160,164] These kynurenine metabolites also contribute to neurodegeneration, depression, anxiety, and changes in cognition.[69,81,159,161-163] Figure 2 demonstrates one way in which inflammation can affect the neurotransmitters, namely serotonin, dopamine, and glutamate, which as discussed previously, contribute to the pathophysiology of anxiety and depression.[50,69-70,72-73] Therefore, inflammation is important when discussing mental health. In addition, inflammation may increase the interplay between emotional negativity and mental health vulnerability, and vice versa.

The body of research investigating the links between mental health and inflammation is growing. Studies involving the effects of PICs on the brain suggest that inflammation may have a pivotal role in the pathophysiology and symptom severity of stress, anxiety, and depression, which is discussed next.[105,169,181-187]

1.4.3.1 Inflammation and Stress

Stress can influence inflammation as immune cells can respond to SNS and HPA axis signals, due to their expression of adrenergic and glucocorticoid receptors.[188] This means that immune cells can be 'informed' about a stressor via catecholamines and glucocorticoids and then coordinate an appropriate response. Therefore, the immune system is involved in, and affected by the fight-or-flight response.[188] In return, the immune system can affect the stress response. For example, PICs can activate the HPA axis at the level of the hypothalamus, the pituitary gland, and the adrenal gland, which causes the release of CRH, ACTH, and glucocorticoids.[189] Therefore, the stress response and immune response do not function independently, but rather influence one another.

Although stress is often thought to be immunosuppressive or anti-inflammatory, growing evidence shows that psychological stress can also activate an inflammatory response.[177,190] Normally the activation of the HPA axis and the consequent release of glucocorticoids modulates further immune responses.[189] Typically, acute increases in glucocorticoid levels inhibit the production of PICs in the brain and decreases their transcription.[25] However, chronic release of PICs and activation of their signalling pathways may disrupt glucocorticoid signalling by altering the function and number of glucocorticoid receptors, thus reducing the negative feedback loop between the HPA axis and the immune system.[181-182] An example of this is

IFNγ, which can reduce the number of glucocorticoid receptors through diminishing mRNA transcription (although this response appears to be tissue-specific).[191] This results in a blunted response to glucocorticoids. The effects of this diminished glucocorticoid signalling (or glucocorticoid resistance) include altered feedback regulation of CRH, increased HPA axis activation, further release of PICs,[66,77] and it contributes to the pathophysiology of diseases including depression.[181-182] Therefore, chronic stress and HPA axis activation can result in increased inflammation, by altering responses to glucocorticoids which leads to insufficient signalling and diminished anti-inflammatory responses.[66,192]

It should be noted that glucocorticoids are not solely immunosuppressive; they also prepare the body to respond to future stimuli by facilitating inflammatory responses. These actions include the stimulation and priming of peripheral monocytes and increasing their infiltration into the brain.[25]

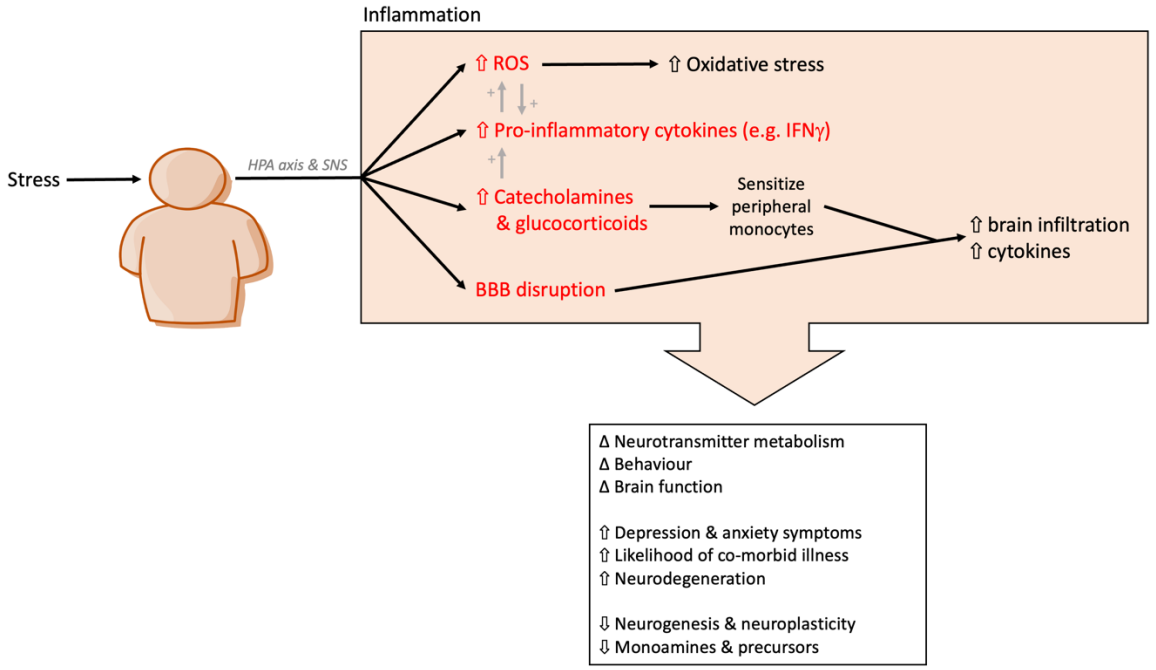

***Figure 3: Stress and inflammation**. Psychological stress can cause inflammation via the HPA axis and the SNS. The effects of stress include increased ROS, oxidative stress, pro-inflammatory cytokines, catecholamines, glucocorticoids, monocyte sensitisation & brain infiltration, and BBB disruption. Catecholamines and glucocorticoids can increase pro-inflammatory cytokines. ROS and pro-inflammatory cytokines both increase each other. These effects cause changes in neurotransmitters, brain function, behaviour, and mood. BBB – Blood-brain barrier; HPA – hypothalamic-pituitary-adrenal; IFNγ – interferon-γ; ROS – reactive oxygen species; SNS – sympathetic nervous system; Δ – change in. This diagram was adapted from Inserra et al. 2018[74] with additions from other sources.[25,77,81,167,188]*

Besides glucocorticoids, catecholamines are the other main group of molecules that are released by the stress response. Catecholamines facilitate the acute increase of cytokines in the brain and the periphery after stress exposure, via the activation of adrenergic receptors on immune cells.[25,193] They are also involved in the recruitment of monocytes into the brain

and the stimulation of microglia. Although, it should be noted that catecholamines are not exclusively pro-inflammatory; they also mediate various anti-inflammatory responses.[25]

Stress can disrupt the blood-brain barrier (BBB)[194] allowing greater infiltration of cytokines and monocytes into the brain.[195] As such, peripheral cytokines can induce central cytokines during stress.[25] In addition to PICs affecting the HPA axis, they also influence neurotransmitter metabolism, as seen in changes in serotonin, dopamine, and noradrenaline metabolism in brain areas vital for the regulation of emotion and reward, resulting in cytokine-induced behavioural changes.[77,81] Stress-induced production of cytokines (such as IFNγ), and concomitant inflammation, decrease neurotrophins, neurogenesis, neuroplasticity, and monoamines (and their precursors). These effects contribute to increased depression and anxiety, thus providing a link between stress and mental health (Figure 3).[74]

Monocytes are formed in the bone marrow. Through the effects of catecholamines and glucocorticoids on the bone marrow and lymph nodes, prolonged stress exposure can promote primed and glucocorticoid-insensitive monocytes to leave the bone marrow and enter circulation. These monocytes have an increased capacity to travel throughout the body, infiltrate the brain, and promote inflammation.[196] Supporting this is a study that used social defeat stress. Social defeat uses social conflict to generate emotional and psychological stress.[197] In this study, mice exposed to social defeat stress had higher monocyte infiltration, specifically in brain areas that are associated with anxiety.[198] In addition to the altered distribution of primed monocytes, sensitisation of microglia are also suspected to be involved in anxious behaviour. Chronic stress can also alter the activation and morphology of microglia, which increases neuroinflammation.[196]

Stress also leads to cytokine-induced production of ROS, which increases oxidative stress and activates a feedback loop which further increases cytokine production.[74] Neurons are particularly susceptible to damage caused by oxidative stress, resulting in cognitive impairment and neurodegeneration.[199] As such, increased oxidative stress may also be involved in the pathophysiology of depression (see 1.*4.3.2 Inflammation and Depression*) and increase the probability of developing comorbid illnesses, such as Alzheimer's, Parkinson's, multiple sclerosis, rheumatoid arthritis, chronic fatigue syndrome, chronic obstructive pulmonary disease, systemic lupus erythematosus, psoriasis, and diabetes.[200] As there is an association between neopterin and oxidative stress,[201] it is not surprising that neopterin may increase during times of psychological stress.[153]

In summary, PICs can be produced in response to psychological stress, which is considered to be a form of 'sterile inflammation', as it is an inflammatory response that occurs in the absence of infection.[25] In addition to increased PIC production, stress activates the HPA axis and the SNS, which leads to the disruption of the BBB and the increased production of ROS, PICs, catecholamines, and glucocorticoids. These changes have many effects including increased oxidative stress, infiltration of monocytes into the brain, and neuroinflammation. Consequently, stress leads to changes in brain metabolism and function, as well as increasing the likelihood of comorbid illness, such as depression and anxiety (Figure 3).[31-32]

1.4.3.2 Inflammation and Depression

Depression has been found to be accompanied by inflammation and changes in cell-mediated immunity.[77,202] There are associations between depression and increased leukocytes in circulation and in the brain.[198,203] In addition, increased levels of PICs are found in a subset of depressed patients and these levels correlate with symptom severity.[105,183-184] Although, this is not surprising considering the high rate of depression and inflammatory disease comorbidity.[200,204]

Stimulated increases in PICs have been associated with symptoms such as cognitive impairment, anhedonia, fatigue, depressed mood, and social withdrawal.[170,202] This is also supported by a study which found stimulated inflammation to be associated with levels of cognitive impairment and depressive symptoms in the following week.[205] Moreover, another study found that the therapeutic administration of IFNα leads to depression in up to 50% of patients[77] and a recent study found that peripheral inflammation precedes the onset of depression. In this recent study, the subsequent risk of developing depression between the ages of 9 and 18 years old was found to be related to interleukin (IL)-6 levels, whereby higher IL-6 levels increased the risk.[206] Additionally, a meta-analysis found that non-steroidal anti-inflammatory drugs (NSAIDs), particularly celecoxib, may have antidepressant effects.[207] Inflammation and cell-mediated immunity is thought to cause depressive-like behaviours through various mechanisms, such as altering neuroplasticity and neurotransmitter systems (e.g. the serotoninergic system).[188,202] Considering this, inflammation may be a cause or a contributing factor in the pathophysiology of depression.

Of particular interest are studies in which increased systemic IFNγ and neopterin production have been found in depressed patients.[74] A study using *ex vivo* peripheral blood mononuclear cells from depressed patients found that these cells displayed increased IFNγ and neopterin production.[154] Furthermore, a recent study found a correlation between elevated IFNγ and depressive-like behaviour in rats.[208] To support this, transcriptional levels of IFNγ in multiple

sclerosis patients correlated with depressive symptoms[209] and some antidepressants have been found to suppress IFNγ production.[210] Additionally, a polymorphism in the IFNγ gene has been associated with changes in key metabolites such as serotonin. This is due to IFNγ transcriptionally inducing IDO, which degrades tryptophan and thus induces serotonin depletion. Therefore, it is suggested that carriers of the aforementioned polymorphism might be more susceptible to developing depression.[80]

Oxidative stress also plays a role in the pathophysiology of depression and appears to be related to depression severity and duration.[211-213] Relating to this is the finding that some antidepressants can reduce oxidative stress, and that antioxidants have shown antidepressant properties.[214-215] As neopterin can be related to oxidative stress, it is not entirely surprising that multiple studies have found increased levels of plasma and urinary neopterin in depressed patients.[144,152,154,216] In addition, neopterin is not significantly affected by psychotropic drug therapy, namely antidepressants, therefore neopterin may be a useful marker for measuring immunological changes in depressed patients.[144]

In summary, as depression appears to be affected by inflammation, neopterin could be a potential biomarker thereof. However, it should be noted that neopterin cannot be used as a single marker for the diagnosis of mental health conditions; but should rather be combined with other measures and professional psychological assistance. This is compounded by conflicting results reported by some studies regarding neopterin concentration and mental health, thus further research is warranted into neopterin as a potential biomarker.

1.4.3.3 Inflammation and Anxiety

There is less research on the connection between anxiety and inflammation than there is on stress and depression. However, one study has found that men with anxiety disorders had low-grade systemic inflammation.[217] In addition, social stress is linked to changes in the immune system and increased incidence of anxiety-related disorders.[198] As discussed previously, stress leads to changes in the immune system and since there is an association between stress and anxiety,[31] anxiety may also present with inflammation. This is supported by increasing evidence linking anxiety to conditions in which low-grade systemic inflammation is involved in the aetiology, such as CVD and atherosclerosis.[185]

Research has mainly focused on post-traumatic stress disorder (PTSD), a type of anxiety disorder, in which higher levels of inflammatory markers have been found, including C-reactive protein,[218] IL-1b, IL-6 and lower anti-inflammatory IL-4.[185] As such, PTSD patients have been

found to show a low-grade systemic pro-inflammatory state, which relates to their PTSD symptom severity. Supporting this, a positive correlation between tumour necrosis factor-α (TNFα) and symptom severity has been found.[185] Furthermore, other research has found correlations between PTSD, cortisol, neopterin,[219] and elevated IFNγ.[186] Increased levels of the inflammatory markers have also been recorded in patients with panic disorder[186] and generalized anxiety disorder (GAD).[187]

Macrophages may provide a link between stress, anxiety, and inflammation. A murine study found that repeated social defeat, a form of social stress, primed and promoted the trafficking of peripheral macrophages into the brain, including infiltration into specific brain regions that respond to stress. These macrophages then alter the neuroinflammatory profile and promote anxiety-like behaviour.[198]

In summary, inflammation and PICs may contribute to stress, anxiety, and depression through various mechanisms, such as activating the HPA axis and the SNS, disrupting the BBB, increasing oxidative stress, and increasing the infiltration of monocytes into the brain.[195] Due to this association, neopterin was investigated in this study as a potential biomarker of inflammation in relation to mental health. In the next section, biomarkers of autonomic activity in connection to mental health aspects are discussed.

1.5 Measures of Autonomic Nervous System Activity

The main pathways activated by stressors are the SNS and HPA axis.[27] The SNS is part of the ANS, which is the primary mechanism by which most of the bodily functions are unconsciously regulated e.g. heart rate, respiratory rate, and digestion.[220] There are two branches of the ANS; the SNS and the parasympathetic nervous system (PNS). These two branches work antagonistically to regulate autonomic functions.[221] During acute stress, the SNS is activated as part of the 'fight-or-flight' response to ensure survival. The PNS is involved in 'rest-and-digest' activities, thus PNS activation is decreased during periods of stress.[221] Fluctuations between SNS and PNS dominance is part of normal and healthy responses to maintain homeostasis, however, when stress becomes chronic, an imbalance between the SNS and PNS can result in negative health outcomes. Namely, chronic stress and the imbalance of the ANS are implicated in the pathogenesis of anxiety and depression.[27] As such, measuring aspects of ANS activity and finding suitable biomarkers thereof may contribute to the management of mental health symptoms.

Heart rate variability (HRV) is a measure of ANS activity; it measures fluctuations in heartbeat intervals.[222] As stated by Shaffer and Ginsberg,[223] "a healthy heart is not a metronome; the oscillations of a healthy heart are complex and constantly changing, which allow the cardiovascular system to rapidly adjust to sudden physical and psychological challenges to homeostasis". Therefore, HRV is indicative of ANS flexibility in response to stressors and can be used to assess the links between the stress response and neuropsychological parameters.[222] Abnormalities in HRV may serve as a biomarker for various mental health disorders and stress-related variables.[222,224] For example, there is an association between reduced HRV and mental health conditions, such as anxiety and depression.[222,225] This is not entirely surprising given the high comorbidity observed between CVD and depression.[226] Conversely, higher HRV is associated with increased resilience; greater recovery from acute stressors; changes in cognitive performance and emotional regulation; and less vulnerability to depressive-like states.[222] Due to this, HRV could serve as a measure of neuropsychological parameters and ANS activity. However, HRV should not be used as a single indicator or as a diagnosis.

Other measures of ANS activity include blood-volume pulse (BVP) and electrodermal activity (EDA), which have been used as biomarkers of psychological arousal[227] and emotional states such as depression.[228] BVP is a measure of the volume of blood in the arteries, which is related to the constriction and dilation of the vessels.[228] Greater vasoconstriction leads to a lower volume of blood in the vessels, so BVP decreases. Greater vasodilation leads to a greater volume, so BVP increases. Therefore, BVP is reflective of ANS activity, as autonomic activation of adrenergic receptors on blood vessels can cause vasoconstriction.[228-230] Of relevance, ANS activity changes with emotions, thus an emotion like fear can lead to vasconstriction.[231]

Previous research using BVP as a biomarker found it to be useful in measuring anxiety levels, although it was more accurate when combined in a model with other physiological measures.[232] Another study used BVP to create a model for short-term anxiety recognition.[233] BVP might also be useful in assessing pain.[234] Pain, like mental health, is subjective, thus BVP might be a potential biomarker for the quantitative and objective evaluation of subjective experiences. Little research has been done on the use of BVP as a biomarker, particularly in the area of mental health, thus warranting further investigation.

EDA (also known as skin conductance) depends on the electrical conductivity of the skin, which is altered by sweat levels. ANS activity affects the amount of sweat on the skin due to eccrine sweat glands having sympathetic innervation. Thus, EDA can be used to measure the

sympathetic activity of the ANS.[227-228] EDA could be used as a biomarker for mental health as studies have found electrodermal hypoactivity in depression. Thus, EDA can be useful in distinguishing depressive patients from healthy patients.[228]

Blood pressure (BP) can also be used to assess ANS activity. For example, hypertension may be indicative of ANS abnormalities and imbalance.[235] In addition, chronic stress has been shown to increase heart rate and BP.[236] Given that many physiological systems influence BP, it cannot be used in isolation as a single biomarker for mental health conditions. This warrants further studies for its link and usefulness when combined with other measures.

Due to the association between the ANS and mental health aspects,[27] biomarkers of autonomic activity such as HRV, BVP, EDA, and BP may be of assistance. In addition to these markers, another method of measuring nervous system activity, particularly in the brain, is through the use of electroencephalography (EEG) and brain waves – this is discussed in the next section.

1.6 Quantitative Electroencephalography (qEEG)

1.6.1 Electric Signals

The brain works by relaying signals between neurons. Signals arrive at the dendrites of neurons, where they are processed at the soma and may result in the production of an action potential (AP). The AP is then carried along the neuronal axon and transmitted to the dendrites of other neurons via synapses. These signals produce electromagnetic fields, which can be detected through the use of EEG.[237] The electromagnetic field is formed through the production of local currents due to sodium, potassium, calcium, and chloride ion movement across neuron membranes when they are activated.[238]

Two types of potentials contribute to the formation of electric currents in the brain: post-synaptic potentials (PSPs) and APs.

PSPs are signals in the dendrites. When an AP reaches the end of an axon terminal, it causes the release of neurotransmitters which affect the post-synaptic neuron's membrane permeability. This results in the entry of ions, such as sodium and potassium ions, into the post-synaptic neuron, which increases the resting state potential for 10 milliseconds.[237]

Contrastingly, APs are signals that move along the axon. They occur if many post-synaptic potentials sum up. This results in the membrane potential of the soma reaching a threshold, which causes some voltage-gated channels to open, allowing cations to flow into the cell. This rapidly increases the potential inside the neuron and propagates the AP along the axon. However, the potential returns rapidly to its resting state in one millisecond.[237]

APs and PSPs create movements of charges that result in some very small currents within the neuron, which create electromagnetic fields. However, these electromagnetic fields are minuscule and therefore cannot be measured outside of the head with EEG. For these signals to be detectable, they need to sum up. The duration of APs is only a millisecond, which makes it difficult for them to synchronize and sum up. In contrast, PSPs have a duration of 10 milliseconds, making them more likely to sum up and produce measurable electromagnetic fields outside the head. However, the electrical currents produced by the neurons must have a common direction to sum up. The result is that about ten thousand neurons must produce field amplitudes that have a common direction to create an electric field which is detectable outside the head. The only neurons in the brain which have the organisation required to sum up PSPs are pyramidal cells.[237]

Pyramidal cells make up about 70-80% of the neocortex. They are found primarily in the grey matter of the cerebral cortex and have a thick dendrite (called the apical dendrite) that extends toward the exterior of the cortex, perpendicularly to its surface.[237]

In brief, the summed PSPs of pyramidal neuron dendrites in the cortex produce electric currents. These currents and differences in dipoles are mainly what EEG detects and records.[238]

1.6.2 Electroencephalography (EEG)

EEG is a medical imaging technique that measures the electrical activity of the brain. EEG uses electrodes that are placed on the scalp, which measure the electrical signals generated by brain structures.[239-240] Therefore, EEG can determine the position and relative strength of electrical activity in different brain regions.[240]

The presence of electrical currents in the brain was discovered by Richard Caton in 1875. He performed EEGs on the exposed brains of monkeys and rabbits. Later in 1924, Hans Berger reported that the human brain also produces electrical activity and that these currents could be measured on the scalp and thus could be recorded without opening the cranium. He noted

that electrical activity changed in the brain during different functional states (e.g. sleep, anaesthesia, hypoxia, epilepsy).[240]

An EEG can record both normal and abnormal electrical activity in the brain, which makes it a helpful tool in the field of neurology and clinical neurophysiology.[240] For example, EEG is used to diagnose and monitor a range of medical conditions including epilepsy, attention deficit hyperactivity disorder, tumours, encephalopathies, brain death, sleep disorders, and many other conditions.[241-247] EEG is also used in research areas such as neuromarketing and psychology.[239] Additionally, EEG can be used to study areas such as emotion, learning, memory, perception, attention, and language in adults and children.[240]

An advantage of using EEG is that it is a non-invasive and painless technique which can be repeatedly applied to children and adults with minimal risk.[240] Another advantage is the speed at which EEG records neural activity. Within fractions of a second after a stimulus, EEG can record complex patterns that occur in the brain.[240]

Quantitative EEG (qEEG) involves the digitalisation of raw EEG measurements. Complex algorithms then allow for the creation of brain maps using the EEG readings. These maps can be used to examine the power, amplitude, coherence, and lag phase. There are two types of power measured by qEEG: absolute power (the electrical power at each site of measurement) and relative power (the distribution of power at one site compared to other sites).[248] The electrical currents detected by an EEG are called brain waves; their functions and relevance are discussed next.

1.6.3 Brain Waves

Brain waves are measured from the peak of one wave to the peak of another and normally have an amplitude between 0.5 and 100 μV.[240] They are measured in Hertz (Hz) cycles per second. There are four main categories of brain waves: delta (1-4 Hz), theta (4-8 Hz), alpha (8-12 Hz), and beta (12-38 Hz) (Figure 4). The brain also produces gamma waves, which are the highest frequency. However, the current clinical use of gamma waves is limited due to the current EEG technology, which does not effectively measure gamma waves due to muscle contamination.[248] Different brain regions do not simultaneously produce the same frequency of brain waves, they produce varying amounts of each frequency. Therefore, signals between EEG electrodes consist of many waves that have differing characteristics. Even a single EEG recording will result in a large amount of data, which can make interpretation difficult.[240]

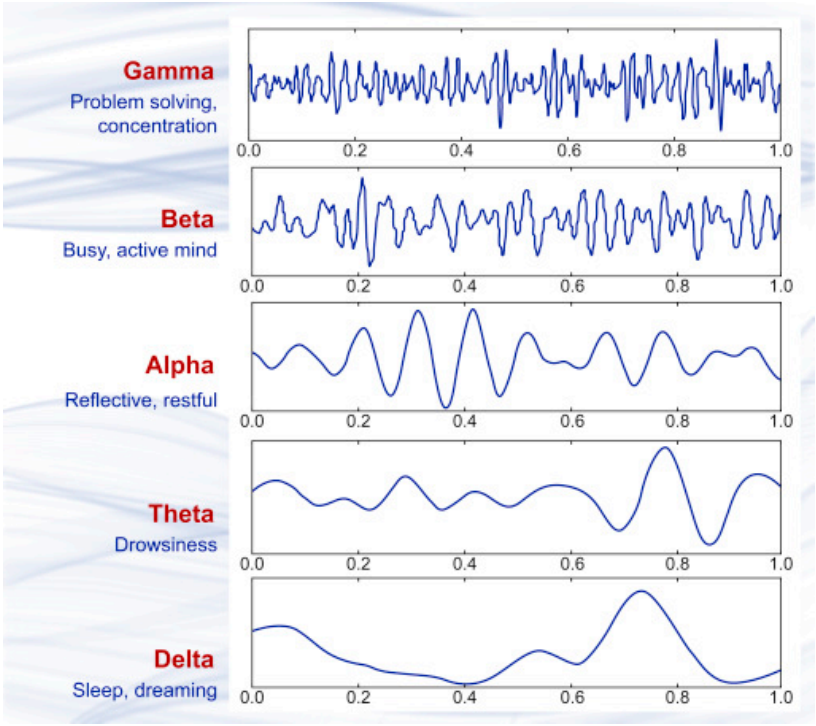

***Figure 4**: **Different brain waves**. Gamma is the highest frequency and delta is the lowest frequency.*[249]

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Different brainwaves are associated with different states. For example, when an individual's eyes are open, beta waves are usually dominant. When an individual is relaxed or drowsy, alpha activity rises. As an individual moves into a sleep state, lower frequency waves such as theta and delta increase.[240]

Alpha waves are the most studied of these waves and are usually recorded in the posterior and occipital regions of the brain,[240] and tend to be higher in the right hemisphere.[248] Alpha activity can be induced by relaxation and closing the eyes. It can be minimised by opening the eyes or by being alert e.g. thinking or calculating.[240] These waves are associated with aiding mental coordination, mind/body integration, calmness, alertness, learning, and consolidating information. Alpha waves decrease in the brain as a result of falling asleep, focusing on tasks and also with ageing.[248] Changes in normal alpha wave rhythms correlate with undesirable behaviours such as social withdrawal, depression, Parkinson's disease, and cognitive decline.[248]

Beta waves are associated with being awake, focused, conscious, alert, decision-making and problem-solving. Higher frequencies of beta are linked to integrating new experiences, very complex thoughts, and high anxiety or excitement levels.[240] Abnormally high levels of beta have been associated with anxiety, insomnia, and migraines.[248]

Theta waves are associated with daydreaming, meditation, and sleep states. Excessive theta waves in wakeful states can lead to feelings of being scattered, lack of organisation, impulsivity, or attention disorders. The level of theta activity observed in the brain changes from childhood to adulthood.[248]

Delta waves largely appear in the deepest stages of sleep. This stage of sleep is associated with being restorative, regenerative, and healing. High levels of delta waves are also associated with walking and talking whilst asleep. During wakeful hours, high delta activity makes it difficult to focus and perform conscious tasks.[248]

1.6.4 qEEG and Mental Health

Quantification of EEG recordings may give further insight into mental health and potential markers. For example, qEEG allows the identification of abnormalities, such as frontal alpha asymmetries that are often observed in depressed patients.[250] A preliminary study also found that participants with higher activity in the right anterior of the brain reported a greater tendency to feel anxious a year later. Thus, right frontal EEG activity may act as a vulnerability marker and predict the future onset of anxiety symptoms.[251] Other studies have also found greater relative right frontal EEG activity in those with anxious or depressive symptoms.[251] The power, asymmetry, communication speed, and efficiency of different brainwave frequencies can be further investigated to find potential markers for mental health conditions.

1.7 Rationale for the Study

Student populations may have elevated stress and anxiety, especially Health Science students.[15-19] Questionnaires are often used to assess symptom severity. However, in view that questionnaires do not elucidate underlying pathophysiology in individuals presenting with mental health symptoms, objective biomarkers may help to complement questionnaire scores. Therefore, the rationale for this study was to investigate the aforementioned biomarkers to complement the DASS-21 scoring system for anxiety and stress. The purpose of this was to contribute to furthering our knowledge and understanding of biomarkers in relation to mental health, thus furthering the potential benefits of using biomarkers in this field.

1.8 Aims and Objectives

The aim of this study was to investigate whether neopterin and certain neurophysiological measures could be used as complementary markers for stress and anxiety symptoms as determined by the DASS-21 questionnaire.

The objectives to accomplish the aim were to:

1. Determine anxiety and stress symptom scores using the DASS-21 and to further stratify between participants with high and normal stress and anxiety scores.
2. Compare neopterin levels between participants with high and normal stress and anxiety scores.
3. Compare each neurophysiological measurement between participants with high and normal stress and anxiety scores.
4. Identify which of the proposed markers correlate with the DASS-21 scores for stress and anxiety.

This chapter introduced the topics of mental health, inflammation, measures of ANS activity, and qEEG. As there appears to be a link between inflammation and mental health, neopterin may be a useful biomarker to assess this relationship. In addition, there appears to be a link between ANS activity and mental health, thus neurophysiological biomarkers may be of use.

The next chapter will highlight the methodology used to assess the biomarkers discussed (neopterin, HRV, BPV, EDA, BP, and qEEG) to determine if they correlate with scores of stress and anxiety.

Chapter 2

2. Methods and Materials

This chapter includes information on the participants and how they were recruited and assigned to either the Group A or B using the DASS-21 questionnaire. Furthermore, details on how each of the biomarkers was measured is discussed. A flow diagram outlining the study is shown below (Figure 5).

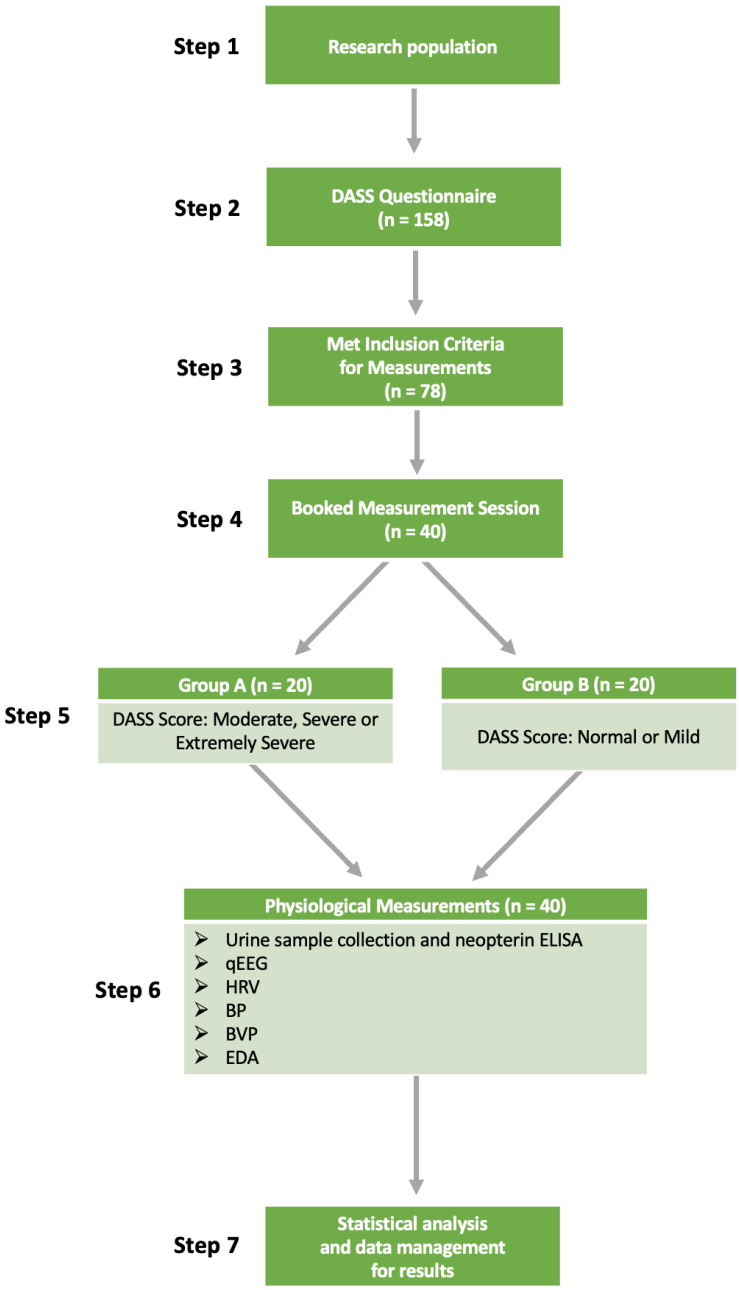


***Figure 5: Planned Research Methodology**. BP – blood pressure; BVP – blood-volume pulse; DASS – Depression, Anxiety and Stress Scale; EDA – electrodermal activity; ELISA – enzyme-linked immunosorbent assay; HRV – heart rate variability; qEEG - quantitative electroencephalograph.*

2.1 Materials

A list of equipment and materials used for this study is detailed below:

qEEG:
- Earbuds
- Nuprep Preparation gel (Weaver and Company, Aurora, United States)
- Ten20 conductive paste (Weaver and Company, Aurora, United States)
- 22 EEG grade Ag/AgCl electrodes (BrainMaster Technologies Inc., Bedford, United States)
- 24-channel DC amplifier (BrainMaster Technologies Inc., Bedford, United States)
- Brainmaster Atlantis and Discovery qEEG system (BrainMaster Technologies Inc., Bedford, United States)
- Brain Avatar 4.6.4 software (BrainMaster Technologies Inc., Bedford, United States)
- QEEG Pro program (BrainMaster Technologies Inc., Bedford, United States)

HRV:
- Zephyr BioHarness 3.0 (Medtronic PLC., Midrand, South Africa)
- ModFLEX Mini Gateway USB, ProFLEX01-R2 for Zephyr (LS Research LLC., Chesterfield, United States)
- Zephyr OmniSense Live software (Medtronic PLC., Midrand, South Africa)
- OmniSense Analysis 3.9.7 software (Medtronic PLC., Midrand, South Africa)
- Kubios HRV Standard 3.5.0 software (Kubious Oy., Kuopio, Finland)

BVP & EDA:
- Infiniti Pro biofeedback apparatus (Thought Technology Ltd., Montreal, Canada)
- SC-Flex/Pro sensor (Thought Technology Ltd., Montreal, Canada)
- BVP-Flex/Pro sensor (Thought Technology Ltd., Montreal, Canada)
- BioGraph Infiniti 3.0 software (Thought Technology Ltd., Montreal, Canada)

BP:
- Clicks Upper Arm Blood Pressure Monitor GMDN: 16157 (Clicks Retailers (Pty) Ltd., Woodstock, Cape Town)

ELISA:
- Demeditec neopterin ELISA kit, purchased via Biocom Africa (Demeditec Diagnostics GmbH., Kiel, Germany)
- 50ml urine collection vials
- Double distilled water (purified via separations filter)
- Adhesive light protection foil

- Disposable reagent reservoir, catalogue 097802
- 200µl pipette tips
- 1000µl pipette tips
- Gilson 100µl and 1000µl single channel pipettes (Gilson Inc., Middleton, United States)
- Gilson 200µl 8 x multichannel pipette Gilson Inc., Middleton, United States)
- Orbital shaker, benchtop plates (Thermo Fisher Scientific Inc., Waltham, United States)
- BioTek Epoch Photospectrometer, SN 255743 (BioTek Instruments, Winooski, United States)
- BioTek Gen5 1.11 software (BioTek Instruments, Winooski, United States)

2.2 Participants

The participants for this study were students (≥18 years old) from the Faculty of Health Sciences at the University of Pretoria. Approval for the recruitment of participants was obtained from the Research Ethics Committee (reference 210/2022, Appendix A) and the Dean of the Faculty of Health Sciences. The study was carried out in accordance with the POPI Act. These students were from any degree or year of study within the faculty. The research population was a convenience sample, and this was a non-interventional, observational, cross-sectional study. A total of 158 respondents completed the DASS questionnaire and biographical questionnaire. Of these, 78 respondents met the inclusion criteria and provided their email addresses. The inclusion criteria for this stage included signing informed consent, being ≥18 years old, completing the questionnaires, enrolled in a degree in the Faculty of Health Sciences at the University of Pretoria, not having epilepsy or an infection, not taking medication that is anti-inflammatory or would affect EEG readings, and having DASS scores that were in the high or low range.

The 78 respondents who met the inclusion criteria were invited via email to participate in the physiological measurements. The email provided information about the study, what to expect from the measurement session, and a link to book a time slot for the measurements. All recruitment and related administration were done by Rouxzan Cronjé.

The participants were divided into Group A (n = 20) and Group B (n = 20) based on their DASS questionnaire scores. Participants qualified for Group A if they scored Moderate, Severe or Extremely Severe in the Anxiety and/or Stress categories of the DASS questionnaire.

Participants qualified for Group B if they scored Normal or Mild on all three DASS categories (see *2.3 Questionnaires* for the scoring system). The reason for this division was to investigate if there were any differences in the physiological measurements between participants with high or normal DASS-21 scores. Additionally, the neopterin kit allowed for 40 samples (with two repeats). The use of 40 participants was also cleared by the biostatistician. The participants comprised the first twenty respondents who booked a slot and met the inclusion criteria for Group A and the first twenty respondents who booked and met the inclusion criteria for Group B. In other words, booking followed until a total of 40 participants, of which 20 with high DASS-21 scores and 20 with normal scores, were recruited. The sample cohort consisted of 40 participants, of whom 30 were female and 10 were male. The age range of the participants was 18-34 years old.

Participants were not included if they did not sign informed consent, complete the questionnaires, and/or withdrew from the study at any time, thus not completing the measurements (qEEG, HRV, BP, BVP, EDA, and donate a urine sample). Other exclusion criteria included epilepsy, use of recreational drugs, use of medication that may alter EEG readings (e.g. barbiturates, antidepressants, antipsychotics, antihypertensives), use of anti-inflammatory drugs, a chronic/recent infection, or an inflammatory disorder (e.g. diabetes, Crohn's disease, inflammatory bowel disease, multiple sclerosis, Parkinson's). These were determined using a biographical questionnaire (Appendix C). In line with the University of Pretoria's Survey Policy, as well as the POPI act, students were not asked other biographical questions such as disclosing additional medication and supplements they might be taking. This was also done to shorten the questionnaire thus avoiding survey fatigue[252-253] and to increase response rates.[254]

2.3 Questionnaires

The DASS was used to assess the perceived severity of depressive, anxious, and stressed symptoms. The short-form version of this scale, DASS-21, which consists of 21 questions, was used. The questions were divided into 3 scales which were designed to measure the emotional states of depression, anxiety, and stress in the participants. Each scale had 7 questions. The DASS-21 questionnaire is a dimensional rather than a categorical assessment. A categorical approach to assessment relies on diagnostic criteria to determine the presence or absence of disruptive or abnormal behaviours, such as in the DSM. Whereas, a dimensional assessment places such behaviours on a continuum of frequency and/or severity.[104] Therefore, the DASS-21 was appropriate for this research context as the aim was to assess

symptoms and their severity in relation to biomarkers, and not to diagnose participants with a mental health disorder.

Participants were asked to answer each of the questions using a rating of 0 to 3. A rating of 0 indicated “Did not apply to me at all” and a rating of 3 indicated “Applied to me very much or most of the time”. The ratings were then tallied accordingly, and the resultant scores were used to assess whether the participant could be categorised as normal, mild, moderate, severe, or extremely severe for depression, anxiety or stress symptom severity.

Participants qualified for the physiological measurements if they scored Moderate, Severe or Extremely Severe in the Anxiety (score ≥ 10) and/or Stress (score ≥ 19) categories of the DASS questionnaire. These participants comprised Group A. Participants also qualified for the physiological measurements if they scored Normal or Mild on all 3 DASS scales; Stress (score ≤ 18), Anxiety (score ≤ 9), and Depression (score ≤ 13). These participants comprised Group B. See Table 1 or Appendix D for the scoring system and more information.

Students were informed about the study via announcements from lecturers or flyers pinned to announcement boards, where they could scan a QR code which took them to an online questionnaire (Appendix E). On the first page of the online questionnaire, they were provided with informed consent (Appendix B). This was followed by a short biographical information section (Appendix C) and the DASS questionnaire (Appendix D). The questionnaire was administered using Qualtrics Software (www.qualtrics.com). If students wished to participate in the physiological measurements, they could provide their email when filling in the questionnaire. However, the provision of an email address was an optional field, and this was made clear. Information relating to the physiological measurements was also provided.

Table 1: DASS Scoring*. The scores from the questionnaire were multiplied by two to calculate the final score.*

	Depression	**Anxiety**	**Stress**
Normal	0-9	0-7	0-14
Mild	10-13	8-9	15-18
Moderate	14-20	10-14	19-25
Severe	21-27	15-19	26-33
Extremely Severe	28+	20+	34+

Taking ethical considerations into account, participants who met the inclusion criteria were anonymously referred to a qualified mental health practitioner (University of Pretoria assigned

psychologist) if their DASS scores indicated severe symptoms of depression, anxiety, or stress.

2.4 Physiological Measurements

Respondents were categorised as Group A, Group B, or neither group, based on their DASS scores (Table 1). From there, the respondents who had provided their email address while filling in the questionnaire were invited to participate in the physiological measurements. The biographical questions were also considered to identify if they met any of the exclusion criteria. Respondents who met the inclusion criteria were emailed with information about the physiological measurements as well as a link where they could book a time slot for the physiological measurements. Calendly (calendly.com) was used for the online booking software (Appendix F). Physiological measurements were recorded within a week or two after filling in the online questionnaire. Furthermore, in line with the University of Pretoria Survey Policy, the questionnaires were not administered again on the day of the physiological measurements.

The measurements were recorded in an isolated office venue on the Prinshof Campus of the University of Pretoria. The participants were invited in and were asked to be seated on a chair. The participants were then asked to first put on the Zephyr chest strap, for the measurement of HRV. Thereafter, the EEG electrodes were placed on specific regions of their head. In addition, the sensors for BVP and EDA were placed on the fingertips. BP was measured before the commencement of the recordings for the other measurements. HRV, BVP and EDA were recorded for five minutes, concurrently with the EEG measurements, which consisted of seven minutes sitting with eyes closed and seven minutes with eyes open. BP was taken again at the end of the recordings and the participants were informed on how to donate a urine sample. The chest strap, finger sensors, and EEG electrodes were removed, and the scalp was cleaned of conductive paste. The participants were then directed to the nearest bathroom. Their urine sample was wrapped in foil for light protection, as per the neopterin ELISA protocol requirements.

2.4.1 qEEG

Each participant was asked to sit on a chair and their head was cleaned in the areas where the electrodes were placed. These areas were rubbed with Nuprep preparation gel using earbuds to remove dead skin cells, which allowed for good impedance of the electrodes, as dead skin cells result in electrical resistance. Thereafter, Ten20 conductive paste was placed on each of the 21 electrodes, which allowed them to adhere to the scalp. The EEG grade

Ag/AgCl electrodes were placed according to the 10-20 system as indicated in Figure 6 on either active or reference sites. Nineteen electrode-placement sites were active and thus measured the electrical activity of the brain. The two reference electrodes were placed on the bone behind each ear (mastoid). This site had no electrical activity; therefore, it could be used as a reference site to compare with the active sites.

The placement of the electrodes for EEG measurements was standardised through the protocol dictated by the International Federation of Clinical Neurophysiology. This placement is called the 10-20 electrode placement protocol. The protocol prescribes the physical placement of 21 electrodes on the scalp. The reference points of the nasion, preauricular points and inion on the skull are used (Figure 6). These points are used to divide the skull into proportional positions which result in sufficient coverage of all the brain regions.[239] The electrodes are labelled with a number and a letter. The letter is related to the brain lobe on which the electrode is placed. The four brain lobes are the frontal, temporal, parietal and occipital lobe. As such the electrodes are lettered F (frontal), T (temporal), P (parietal), O (occipital) and C (central), although there is no central lobe and this just refers to position. The numbers refer to which cerebral hemisphere the electrode is placed on. Even numbers are on the right hemisphere, and odd numbers are on the left.[239] Z-letters instead of numbers are used for the electrodes placed on the midline or sagittal plane of the scalp.

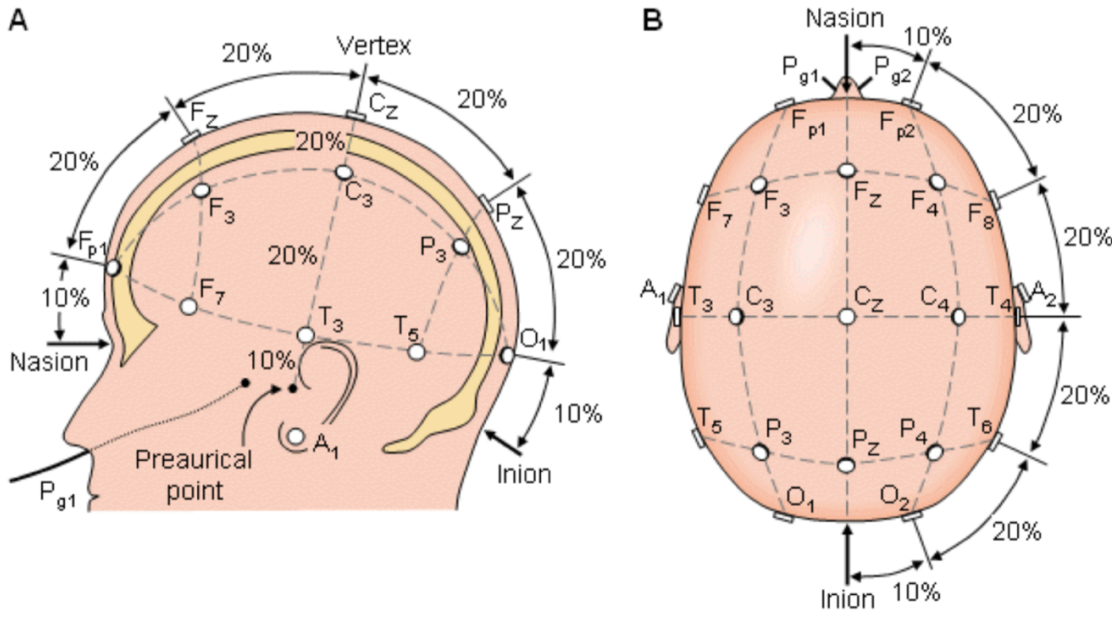


Figure 6: 10-20 Placement System. *The international 10-20 system as seen from (A) left and (B) above the head. The reference points of the nasion, preauricular points and inion on the skull are used to divide the skull into proportional positions which result in sufficient coverage of all the brain regions. A – Ear lobe; C – central; Pg – nasopharyngeal; P – parietal; F – frontal; Fp – frontal polar; O – occipital.*[255]

Once the electrodes were in place, the participant was asked to sit with their eyes closed for seven minutes (Appendix G). Thereafter, they were asked to sit for seven minutes with their eyes open and to just look at a point in the room. A 24-channel DC amplifier and Brainmaster Atlantis/Discovery system were required to measure the electrical activity. Brain Avatar software then recorded the electrical activity, which was measured as brainwaves. The Brain Avatar software (version 4.6.4) helped to remove artefacts such as muscle movement and eye blinks. The EEG data was filtered with a high-pass filter of 1 Hz and a notch filter of 50Hz. Lastly, the measurements from the 19 active electrodes (measured at a frequency of 1-45Hz) were transferred to the QEEG Pro program. This program analysed and compared the recordings to the qEEG-Pro normative database.

The normative database was created using resting-state EEGs and questionnaire data from clients that visited a Neurofeedback Institute Netherlands between 2004 and 2013; there are 10 of these institutes in the Netherlands. The participants consisted of 1696 clients who were between 4 and 82 years old. The questionnaire consisted of 292 rating-type questions that were based on the criteria of 46 DSM psychopathologies. The electrode sites were positioned according to the international 10-20 system (Figure 6).

The qEEG measurements, as well as input for interpretation of the results, were conducted with the assistance of Dr Johanni Beukes, who is a Health Professions Council of South Africa (HPCSA) registered EEG Technician and Research Psychologist. She has also been trained by the Biofeedback Certification International Alliance (BCIA). All other measurements (HRV, BP, BVP, EDA, and neopterin) were recorded by Rouxzan Cronjé.

2.4.2 Heart Rate Variability (HRV)

HRV was measured using a chest strap, namely the Zephyr BioHarness. The chest strap was sanitised between uses. Once the chest strap was in place, the participant was required to sit still, while a five-minute recording was taken. This recording was done while the qEEG measurements were taking place, during the eyes-closed phase. Thereafter, the HRV data was transferred from the Zephyr OmniSense Live software to the OmniSense Analysis V3.9.7 software. The chest strap was removed after the EEG recordings had concluded.

HRV is a measure of the variation in the time interval between individual beat detections. Normal-to-normal (NN) intervals are measured as the intervals between adjacent QRS complexes.[256] NN intervals are also known as RR intervals, as it is the time between R peaks, as seen on an electrocardiogram. NN intervals show small variations in the time interval between successive heartbeats. The standard deviation of the NN intervals (SDNN) was

measured by the chest strap, in milliseconds, which is the square root of variance. Since variance is mathematically equal to the total power of spectral analysis, SDNN reflects all the cyclic components responsible for variability in the period of recording.[256] The 'mean HRV' parameter referred to in the *Results* and *Discussion* sections is the output value from the chest strap.

The Kubios HRV Standard 3.5.0 software was also used for further HRV analysis. It produced indices in three categories: time-domain, frequency-domain, and non-linear.

Time-domain indices of HRV quantify the amount of variability in measurements between beat intervals, which is the time period between successive heartbeats. In other words, it quantifies the amount of HRV observed during the monitoring period. The time-domain measurements used were mean RR, SDNN and root mean square of successive differences between normal heartbeats (RMSSD). Mean RR is the average R-R interval duration. RMSSD is calculated using each successive time difference between heartbeats in milliseconds (ms). Then, each of the values is squared and the result is averaged before the square root of the total is obtained. The RMSSD reflects the beat-to-beat variance in heart rate and reflects short-term HRV. The RMSSD is more influenced by the PNS than SDNN.[223]

Frequency-domain indices calculate the absolute or relative power within four frequency bands. Heart rate oscillations are divided into ultra-low-frequency, very-low-frequency, low-frequency (LF), and high-frequency (HF) bands. Power is the signal energy found within a frequency band. Five frequency-domain measures were investigated: LF peak, LF power, HF peak, HF power, and LF/HF ratio. LF peak is measured in Hz and is the peak frequency of the LF band (0.04-0.15 Hz). LF power is measured in ms^2 and is the absolute power of the LF band (0.04-0.15 Hz). Respectively, HF peak and HF power are the same, except they measure the HF band (0.15-0.4 Hz). LF power may be produced by both the PNS and SNS. The HF band reflects parasympathetic activity. Finally, the LF/HF ratio is the ratio of LF to HF power, which can be used to estimate SNS or PNS activity. A low LF/HF ratio reflects parasympathetic dominance, while a high LF/HF ratio indicates sympathetic dominance, which occurs during fight-or-flight behaviours or parasympathetic withdrawal.[223]

Non-linear measurements quantify the unpredictability and complexity of a series of interbeat intervals. Non-linear relationships between variables cannot be plotted as a straight line. For HRV, a Poincaré plot can be used (Figure 7), where every RR interval is plotted against the prior interval, which creates a scatter plot. Unlike frequency-domain measurements, Poincaré plot analysis is insensitive to changes in trends in the RR intervals. In a Poincaré plot, an

ellipse is fitted to the plotted points, from which non-linear measurements are derived; SD1, and SD2. SD1 measures short-term HRV and is calculated by the standard deviation of the distance of each point from the y = x-axis. SD2 measures short- and long-term HRV and is calculated by the standard deviation of each point from the y = x + average RR interval.[223]

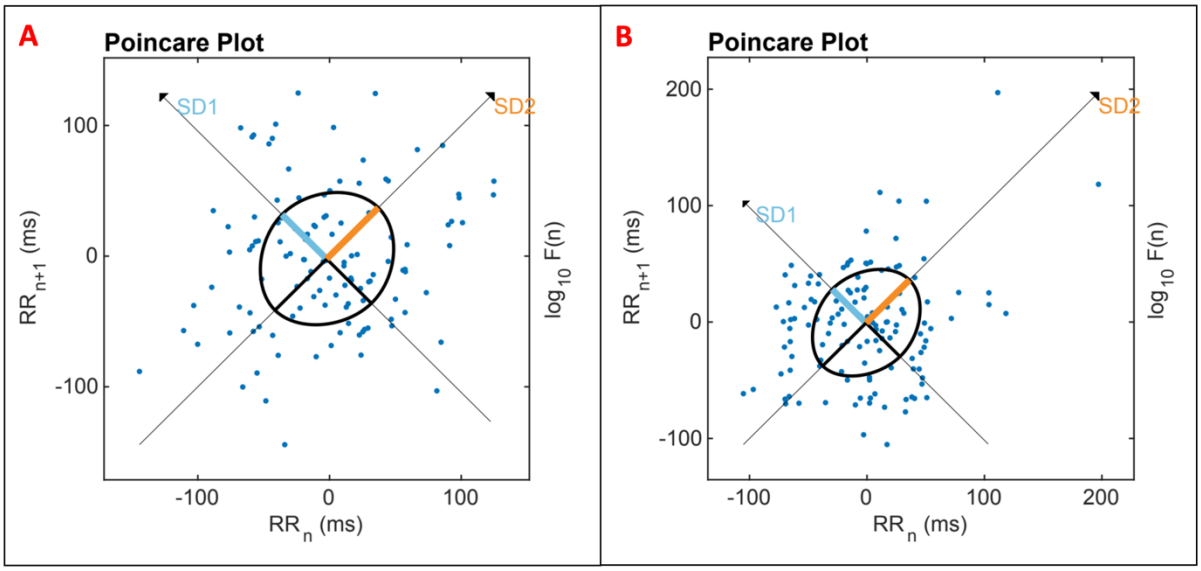


***Figure 7: Representation of typical Poincaré Plots**. The graph plots an RR interval against the preceding RR interval. Each couple of RR intervals is displayed as one dot in the graph. A spherical ellipse or an ellipse in the centre of the graph represents balance between sympathetic and parasympathetic activity (Plot A). A narrow ellipse or an ellipse in the lower quadrant represents sympathetic dominance (Plot B).[257] The images were taken from participant results in the Kubios software.*

In summary, mean HRV, mean RR, SDNN, RMSDD, LF peak, HF peak, LF power, HF power, LF/HF ratio, SD1 and SD2 were used as different measures of HRV.

2.4.3 Blood Pressure (BP)

The first BP measurement was taken when the participant was seated on the chair after the EEG electrodes were placed on their scalp and the BVP and EDA sensors were attached to their fingers. They were asked to sit without crossing their legs, as crossing their legs could increase BP.[258] Their BP was measured at this stage because the participant had been sitting still for at least 15min (while the EEG electrodes were placed), which allowed them to reach a resting state, whereby their systolic BP had mostly stabilised.[259] The BP cuff was placed on their left arm. After measuring BP, the HRV, BVP, EDA, and EEG recordings were started. BP was measured again after the conclusion of these recordings. After the second BP measurement, all sensors and electrodes were removed from the participant.

The BP of the participants was measured in mmHg using the Clicks Upper Arm Blood Pressure Monitor (Clicks GMDN: 16157), which is an automated blood pressure monitor. The systolic and diastolic BP was measured and recorded both pre- and post-EEG.

2.4.4 Blood-Volume Pulse (BVP) and Electrodermal Activity (EDA)

The Infiniti Pro biofeedback apparatus was used to measure EDA (in μSiemens) and BVP (in beats/min and best average Fast Fourier transform statistical signals). To measure EDA, the electrodes were placed on the index and ring finger of the right hand. The maximum, minimum and mean amplitude of the BVP was measured, as well as the maximum, minimum and mean Fast Fourier transform (FFT) peak frequency. FFT is an algorithm that computes discrete Fourier transformation. It is used to calculate different frequency components in time-varying signals. Thus, it is used to make a frequency-domain (spectral) representation of the signal.[260]

The BVP sensor was placed on the right middle finger, which used a non-invasive optical sensor to detect changes in light absorption density of the skin and tissue when illuminated. The light that was emitted from the sensor versus the light that returned to it was proportional to the volume of blood in the tissue.[227,229]

2.4.5 Neopterin ELISA

After the physiological recordings, participants provided a 50ml midstream urine sample. The samples were collected after the other measurements because it reduced the time between collecting the samples and storing them, which reduced the potential degradation of neopterin. Additionally, many of the participants were not ready to provide the donation upon arrival. Collecting the samples after instead of before the other recordings would not have had a great effect as the concentration of neopterin remains relatively stable.[261]

The samples were aliquoted into Eppendorf (1.5 ml) tubes. The tubes were stored in the dark and covered with foil for light protection. All samples were stored frozen at around -20°C until the ELISA was performed.

The Demeditec kit was used for the neopterin ELISA according to the manufacturer's protocol. The kit was stored at 2–8°C and kept away from heat or direct sunlight before use.
The first step of the ELISA was to pipette 20μL of the Standard, Control, and diluted urine samples into the respective wells of a microtiter plate. Samples were measured in duplicate on a 96-well plate thereby enabling the measurement of 40 samples together with six calibration standards and two quality controls. Then, 100μL of Enzyme Conjugate and 50μL of Neopterin Antiserum (rabbit) were pipetted into each well. After this, the plate was covered with black adhesive foil. The plate was incubated in the dark at room temperature (18-25°C) on an orbital shaker (500 RPM). The incubation time required was 90 minutes. Thereafter, the

adhesive foil was removed, and the plate was washed four times using 300µL of diluted Wash Buffer. The excess solution was removed by tapping the inverted plate on a paper towel. Following this, 150µL of 3,3'5,5'-Tetramethylbenzidine (TMB) Substrate Solution was pipetted using an 8-channel micropipette into each channel. Care was taken to avoid air bubble formation. The plate was incubated at room temperature for 10 minutes. Once this was done, the reaction was stopped by adding 150µL of TMB Stop Solution into each well. The content was mixed by gently shaking the plate. Within 15 minutes, the optical density of the sample was measured using a Biotek Epoch photospectrometer at 450 nm. The plate was read using Biotek Gen5 1.11 software. The intensity of the colour that developed after the plate was incubated was inversely proportional to the amount of neopterin in the sample.

Neopterin concentration was determined using the standard curve, derived from the concentration and measurements of the calibration standards. Participants' samples were extrapolated from the standard curve using the calibration curve equation, which was in the form of $y = mx + c$. The log concentration of each sample was calculated using $\log[Participant\ n] = \frac{Average\ optical\ density - c}{m}$. To find the concentration (in nM), these values were calculated as a power of 10 i.e. $10^{\log[Participant\ n]}$. Thereafter, the concentration was converted to µM/mol and multiplied by the dilution factor (ratio between urine sample and buffer solution). This gave the concentration of each sample in µmol neopterin/µmol creatinine.

2.5 Data Capture and Statistical Analysis

Data was collected for each participant using the different hardware and software mentioned. The data was then exported from the programs and captured in spreadsheets. Thereafter, IBM SPSS Statistics 28.0.1.0 software was used for analysis. The data was analysed by the biostatistician Andries Masenge and Rouxzan Cronjé.

As the total sample size for the physiological measurements was already pre-determined in terms of the maximum sample capacity (n = 40) of the neopterin ELISA kit, the group size power calculation of alpha, for various dependents, was not calculated.

The first step in statistical analysis was assessing the normality of the data, as this is a prerequisite for many statistical tests because normal data is an underlying assumption in parametric testing.[262]
Therefore, all variables were tested for normality, using the Shapiro-Wilk test, to determine if a parametric or non-parametric test should be used for analysis. The Shapiro-Wilk test was

used as it is more appropriate for small sample sizes ($n < 50$) than the Kolmogorov-Smirnov test, which is used on larger sample sizes ($n \geq 50$).

If the p-value of a test statistic for the Shapiro-Wilk test is greater than 0.05, the null hypothesis that the variable is normally distributed cannot be rejected and thus the data is considered to be normal. If the p-value is below 0.05, then the data is considered to significantly deviate from a normal distribution.

The independent samples t-test was used to determine if there was a significant difference in means between Group A and B. A p-value of less than 0.05 was considered significant. For variables which did not have a normal distribution, the Mann-Whitney U test (also known as the Wilcoxon Rank Sum Test) was also performed. The Mann-Whitney U test is the non-parametric alternative test to the independent sample t-test. It is a non-parametric test that is used to compare two sample means that come from the same population and is used to test whether two sample means are equal or not.[263] A p-value of less than 0.05 was considered significant for this test.

For variables which were not normally distributed, the median, interquartile range (IQR), and p-value for the Mann-Whitney U test were also reported. Normality was determined by having a Shapiro-Wilk p-value of less than 0.05. For variables where no median, IQR, or Mann-Whitney p-value is reported, normality was assumed.

Spearman's Rank Correlation was used as the measure of association between two variables as it can be used for data which is not normally distributed. Additionally, Spearman's Rank Correlation is less sensitive to outliers than Pearson's Correlation. As Spearman's Rank Correlation measures the monotonic association between two variables, a positive correlation coefficient indicates that as one variable increases, so does the other, although this increase does not have to be linear. A negative correlation coefficient indicates that as one variable increases, the other variable decreases.

This concludes the chapter on the methodology used in this study. Forty participants who filled in the online questionnaire and met the inclusion criteria partook in the physiological measurements: HRV, BP, BVP, EDA, and qEEG. They also donated a urine sample which was tested for neopterin via an ELISA. The next chapter presents the results of the measurements.

Chapter 3

3. Results

In this chapter, the results of the study are presented. Firstly, the results of the online questionnaire are shown. This is followed by the statistical comparison of the means of the different questionnaire scores and physiological biomarker parameters between Group A and B. Thereafter, correlations of these parameters are presented.

3.1 Online Questionnaires

There were 158 respondents who completed the online questionnaire, which consisted of biographical questions and the DASS-21. Table 2 shows the responses to the biographical questions. Of the 158 respondents, only 74 respondents met all the inclusion criteria for either Group A or B, in addition to providing their email addresses which indicated their interest in participating.

Some respondents selected 'Yes' for taking medication that may affect EEG readings but did not list their medication. Of those who did list their medication, 13 were taking antidepressants.

Table 2: Online Questionnaire Categorical Variables.

Category	Number	Percentage
Female	137	86.71%
Male	21	13.29%
Epilepsy	0	0.00%
Using recreational drugs	9	5.7%
Using EEG-affecting medication	32	20.25%
Using anti-inflammatory medication	13	8.23%
Recent or chronic infection	23	14.56%
Inflammatory disorder	3	1.90%
Provided email address	114	72.15%
Meet all inclusion criteria	74	46.84%

n = 158

As indicated by Table 3, the mean stress score for all the respondents fell just into the Moderate category, which required a score of 19-25. The mean anxiety score fell between the Moderate (score 10-14) and Severe categories (score 15-19). The mean depression score fell into the Moderate category, which required a score of 14-20. This suggests that the average respondent was experiencing moderate symptoms of stress, anxiety, and depression. The age range for the respondents was 18-34 years old.

Table 3: Online Questionnaire Continuous Variables.

	Year of study*	Age	Stress Score	Anxiety Score	Depression Score
Mean ± S.D.	3 ± 1.72	22.1 ± 2.76	19.28 ± 9.88	14.86 ± 10.25	15.61 ± 11.04
			Moderate	Moderate/Severe	Moderate

**The highest option on the questionnaire for Year of Study was "7th or more", therefore, some respondents may have been in their 8th or 9th year, and it was counted as their 7th.*
n = 158

The proportion of respondents who reported Normal, Mild, Moderate, Severe or Extremely Severe for each DASS-21 category is shown in Figures 8, 9, and 10. The Anxiety category had the highest scores, with 33% of respondents scoring Extremely Severe. When taking into account the 11% who scored Severe, a total of 44% of respondents, which is almost half, scored Severe or Extremely Severe for anxiety symptoms (Figure 9).

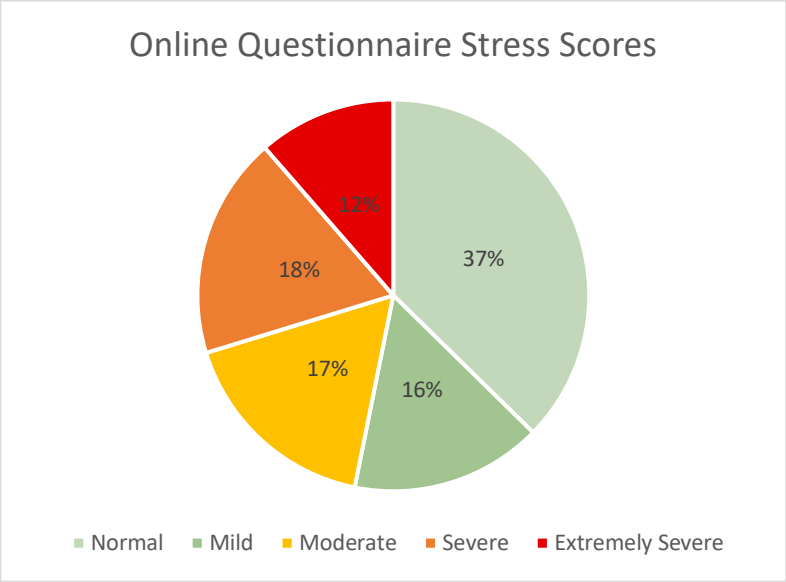


Figure 8: Online Questionnaire Stress Scores. *Severity labels were determined by the DASS-21 recommended cut-off scores (n = 158). See Appendix C for the scoring system.*

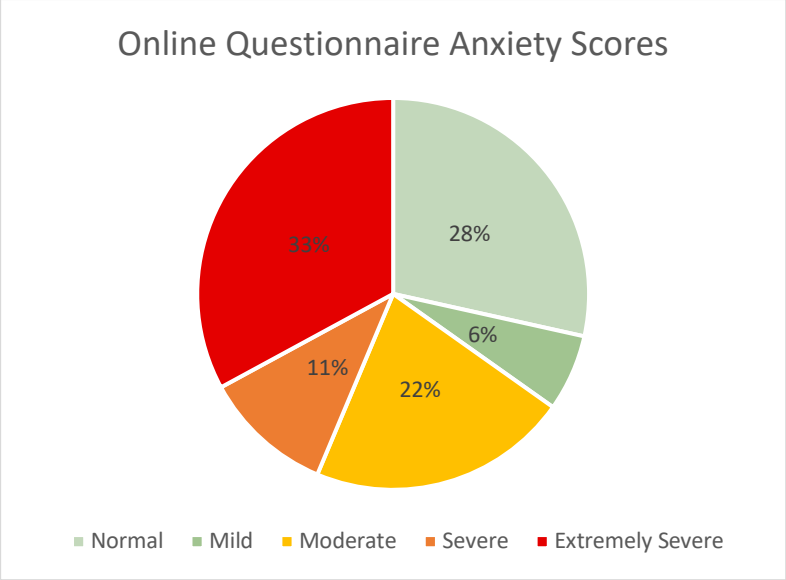


Figure 9: Online Anxiety Stress Scores. *Severity labels were determined by the DASS-21 recommended cut-off scores (n = 158). See Appendix C for the scoring system.*

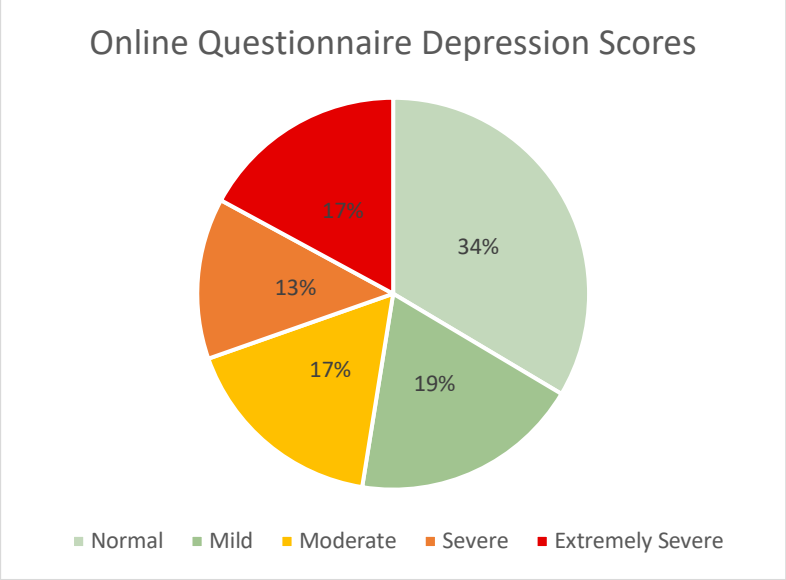
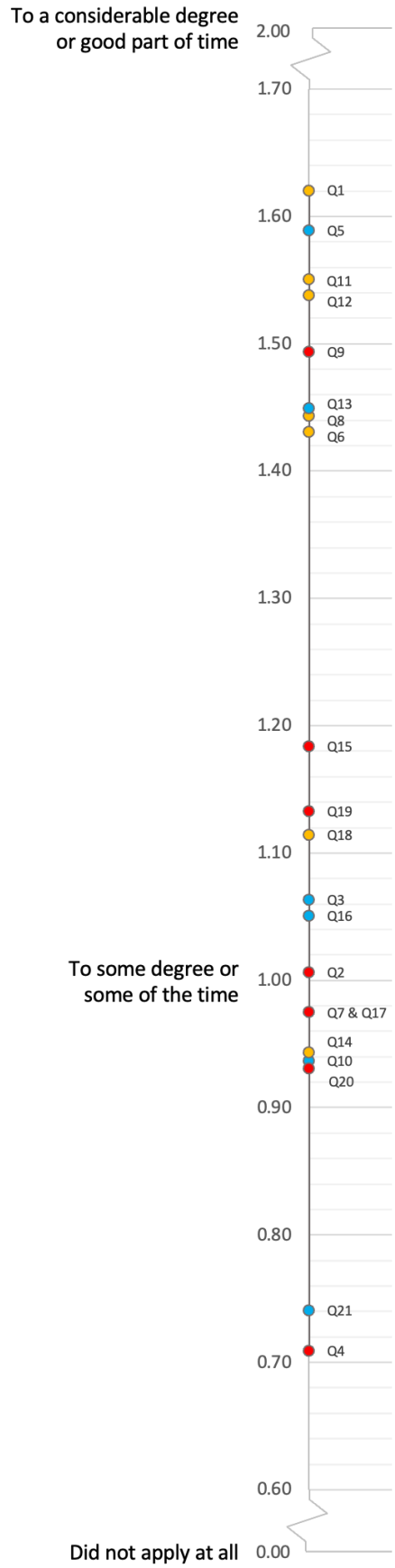


Figure 10: Online Depression Stress Scores. *Severity labels were determined by the DASS-21 recommended cut-off scores (n = 158). See Appendix C for the scoring system.*

3.1.1 Online Questionnaire Individual Questions

The mean score for each individual question in the DASS-21 questionnaire is shown in Figure 11. The question that had the highest mean (i.e. the respondents experienced it the most on average) was Question 1 “I find it hard to wind down”. This was followed by Question 5 “I found it hard to work up the initiative to do things” and Question 11 “I found myself getting agitated”. The two questions that scored the lowest mean (i.e. the respondents experienced it the least on average) were Question 21 “I felt that life was meaningless” and Question 4 “I experienced difficulty breathing”.



Key:

- Stress Question
- Anxiety Question
- Depression Question

	DASS Question
Q1	I find it hard to wind down
Q5	I found it difficult to work up the initiative to do things
Q11	I found myself getting agitated
Q12	I found it difficult to relax
Q9	I was worried about situations in which I might panic and make a fool of myself
Q13	I felt down-hearted and blue
Q8	I felt that I was using a lot of nervous energy
Q6	I tended to overreact to situations
Q15	I felt I was close to panic
Q19	I was aware of the action of my heart in the absence of physical exertion (e.g. sense of heart rate increase, heart missing a beat)
Q18	I felt that I was rather touchy
Q3	I couldn't seem to experience any positive feeling at all
Q16	I was unable to become enthusiastic about anything
Q2	I was aware of dryness in my mouth
Q7	I experienced trembling (e.g. in the hands)
Q17	I felt I wasn't worth much as a person
Q14	I was intolerant of anything that kept me from getting on with what I was doing
Q10	I felt that I had nothing to look forward to
Q20	I felt scared without any good reason
Q21	I felt that life was meaningless
Q4	I experienced breathing difficulty (e.g. excessively rapid breathing, breathlessness in the absence of physical exertion)

***Figure 11**: **Mean scores of online DASS questions**. Possible scores were between 0 (Did not apply at all) and 3 (Very much or most of the time) (n = 158).*

3.1.2 Online Questionnaire Correlations

The Spearman's Rank Correlation Coefficient showed significant correlations between the DASS categories (Table 4). The strongest correlation was between stress and anxiety with $r_s = 0.803$ ($p < 0.001$). This suggests that there is a strong positive correlation between stress and anxiety, namely as one increases, so does the other. There was also a significant positive correlation between stress and depression ($r_s = 0.650$; $p < 0.001$), and anxiety and depression ($r_s = 0.671$; $p < 0.001$). These correlations can graphically be seen in Figures 12, 13 and 14 (Note: the R^2 values displayed in these figures are based on Pearson's Correlation).

Table 4: Online Questionnaire Scores Correlations.

	Spearman's Coefficient	p-value
Stress Score and Anxiety Score	0.803	<0.001**
Stress Score and Depression Score	0.650	<0.001**
Anxiety Score and Depression Score	0.671	<0.001**

** *Correlation is significant at 0.01 level (2-tailed)*
n = 158

A positive relationship between stress and anxiety can be seen in Figure 12. The R^2 shown is based on Pearson's Correlation Coefficient. As the $R^2 = 0.659$ for this correlation, 65.9% of the variance could be accounted for by this relationship.

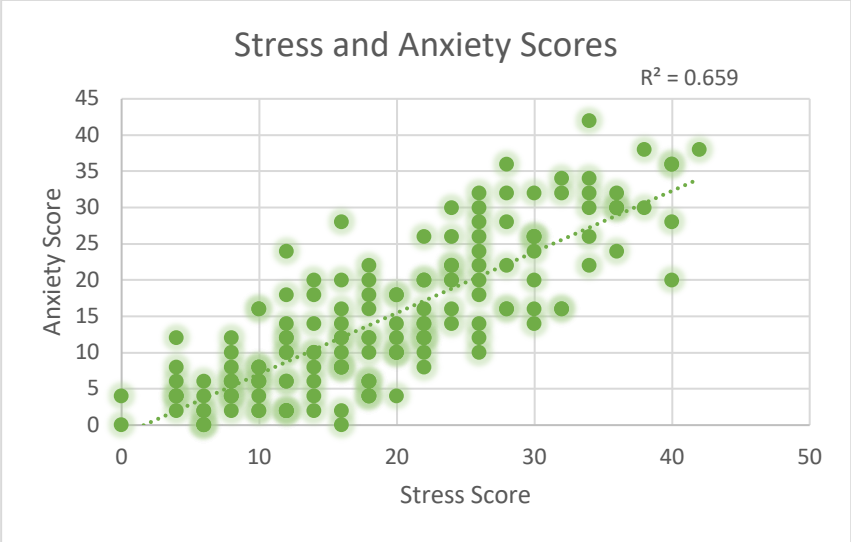


Figure 12: Online Questionnaire Stress and Anxiety Scores Scatterplot *(n = 158).*

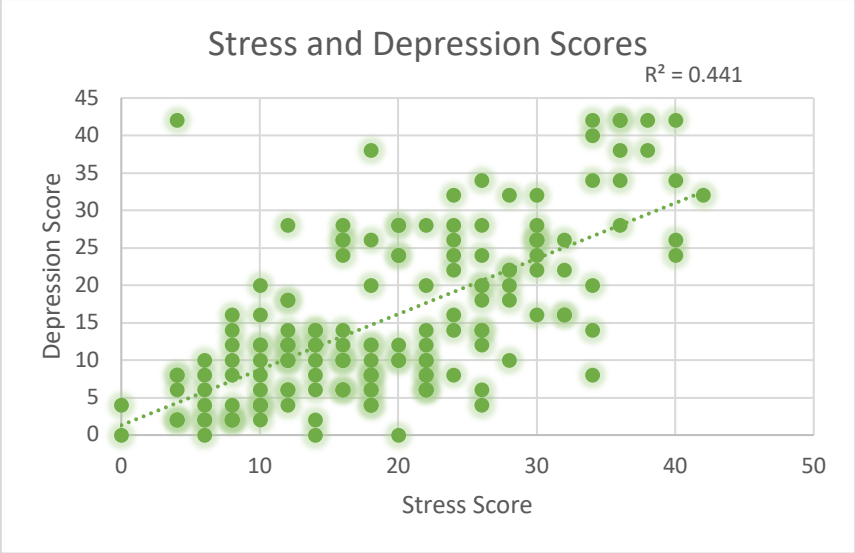


Figure 13: Online Questionnaire Stress and Depression Scores Scatterplot *(n = 158).*

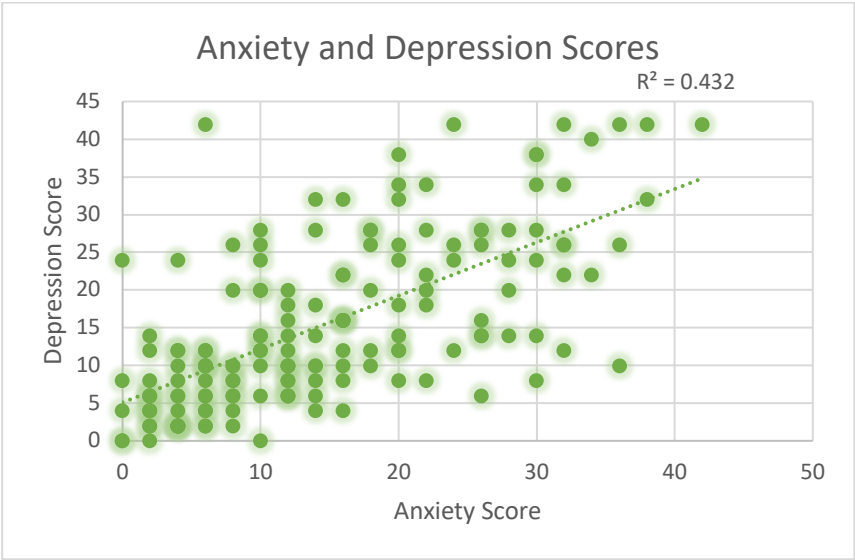


Figure 14: Online Questionnaire Anxiety and Depression Scores Scatterplot *(n = 158).*

3.2 Participant Questionnaires

The demographics and mean scores for Group A (n = 20) and Group B (n = 20) are shown in Table 5. The mean scores of Group B were in the Normal category for stress, anxiety, and depression. The mean scores of Group A were in the Moderate category for stress and depression, and in the Extremely Severe category for Anxiety.

Table 5: Participant Demographics.

	Group	Sex	Year of study*	Age	Stress score	Anxiety score	Depression score
Mean ± S.D.	A	18 Female 2 Male	3.45 ± 1.90	22.35 ± 3.27	24.10 ± 6.79	20.60 ± 8.03	15.80 ± 10.88
	B	12 Female 8 Male	4.00 ± 3.60	23.85 ± 3.60	9.20 ± 5.08	4.65 ± 3.28	5.20 ± 3.07

**The highest option on the questionnaire for Year of Study was "7th or more", therefore, some respondents may have been in their 8th or 9th year, and it was counted as their 7th.*
n = 40

3.3 Mean Tests Between Groups

3.3.1 DASS Scores

There was a significant difference between the DASS scores of the Group A and B ($p < 0.001$ for each category) (Table 6). This is not surprising considering that participants were separated based on their scores.

For Group A, the mean scores were moderate for stress (24.10 ± 6.79), extremely severe for anxiety (20.60 ± 8.03), and mild/moderate for depression (13.22 ± 7.87). Severity categories were based on the DASS scoring system, as shown in Table 1.

The mean scores for participants in Group B were Normal for the categories of stress (9.20 ± 5.09), anxiety (4.65 ± 3.28) and depression (5.20 ± 3.07). Group B had less variation in scores than Group A, as can be seen in the standard deviations.

Table 6: Mean of DASS Scores.

	Mean ± SD		t-test p-value	Median (IQR)		Mann-Whitney p-value
	Group A	Group B		Group A	Group B	
Stress Score	24.10 ± 6.79	9.20 ± 5.09	<0.001**	-	-	-
Anxiety Score	20.60 ± 8.03	4.65 ± 3.28	<0.001**	20.00 (11.00)	4.00 (7.00)	<0.001**
Depression Score	13.22 ± 7.87	5.20 ± 3.07	<0.001**	12.00 (13.00)	6.00 (6.00)	<0.001**

The two-sided independent samples t-test was performed. df = 38. n = 40.
The non-parametric Mann-Whitney U-test was also performed for variables which were not considered to be normally distributed (determined using the Shapiro-Wilk test).
***. Difference in means is significant at the 0.01 level (2-tailed).*
**. Difference in means is significant at the 0.05 level (2-tailed).*

3.3.2 Neopterin

The concentration of neopterin was calculated using the values generated by photospectrometry and a calibration curve. There was a significant difference in the concentration of neopterin between Group A and B (Table 7). Group A had a higher concentration of neopterin than Group B (33.81 ± 22.80, 95% CI [22.47,45.15] vs. 13.22 ± 10.5, 95% CI [8.29, 18.14]; $p < 0.001$). Group A also had a higher median than Group B (31.40 vs. 10.47).

Table 7: Mean of Neopterin Concentration.

	Mean ± SD		t-test p-value	Median (IQR)		Mann-Whitney p-value
	Group A	Group B		Group A	Group B	
Neopterin (µmol neopterin/ µmol creatinine)	33.81 ± 22.80	13.22 ± 10.52	<0.001	31.40 (38.39)	10.47 (15.47)	0.002**

The two-sided independent samples t-test was performed. df = 38. n = 40.
The non-parametric Mann-Whitney U-test was also performed as neopterin concentration was not considered to be normally distributed (determined using the Shapiro-Wilk test).
***. Difference in means is significant at the 0.01 level (2-tailed).*

In addition to the mean test, a difference in median concentration (Group A 33.26 vs. Group B 10.47) and distributions can be seen in the neopterin boxplot (Figure 15). Only four participants in Group B had a concentration of higher than 20 µmol neopterin/µmol creatinine, which means that 80% of Group B had a concentration less than 20 µmol neopterin/µmol creatinine. In Group A, six participants had a concentration of less than 20 µmol neopterin/µmol creatinine, which means that 70% of Group A had a concentration higher than 20 µmol neopterin/µmol creatinine. In summary, a large proportion of Group A exceeded 20 µmol neopterin/µmol creatinine, whereas a large proportion of Group B was below this value.

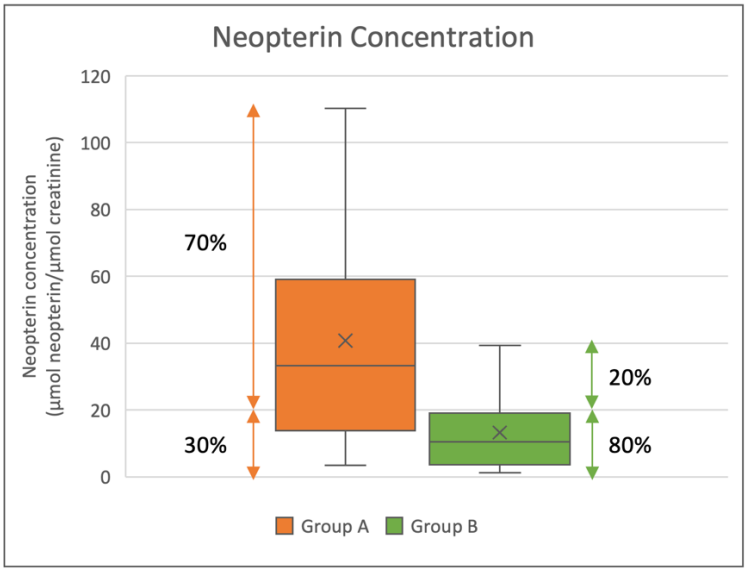


***Figure 15: Boxplot of Neopterin Concentration**. The boxplot shows the concentrations of neopterin between the Group A (n = 20) and Group B (n = 20). It also shows the percentage of participants above and below 20 µmol neopterin/µmol creatinine. A large proportion of the Group A (70%) had a concentration higher than 20 µmol neopterin/µmol creatinine, whereas a large proportion of the Group B (80%) was lower than this. The mean is represented by ×*

3.3.3 Heart Rate Variability

Multiple HRV measures were analysed. Of these, only three measures showed a significant difference in mean between the groups (Table 8). For all three of these measures, Group A had a lower value than Group B: mean HRV (57.07 ± 15.36, 95% CI [49.88, 64.26] vs. 74.21 ± 15.61, 95% CI [68.80, 100.00]; $p < 0.002$), SDNN (54.48 ± 12.42, 95% CI [48.67, 60.30] vs. 63.91 ± 14.27, 95% CI [57.23, 70.58]; $p = 0.032$), and SD2 (62.53 ± 14.66, 95% CI [55.67, 69.39] vs. 75.97± 19.40, 95% CI [66.89, 85.04]; $p = 0.018$). Group A also had a lower median value for mean HRV compared to Group B (59.74 vs. 80.68).

Table 8: Mean of Heart Rate Variability Parameters.

	Mean ± SD		t-test p-value	Median (IQR)		Mann-Whitney p-value
	Group A	Group B		Group A	Group B	
PNS Index	2.64 ± 1.09	2.12 ± 1.24	0.171	-	-	-
SNS Index	-1.80 ± 0.63	-1.49 ± 0.84	0.204	-1.83 (0.80)	-1.64 (1.38)	0.242
Mean HRV	57.07 ± 15.36	74.21 ± 15.61	0.002**	59.74 (27.14)	80.68 (33.54)	0.001**
Mean RR (ms)	1381.33 ± 196.74	1259.49 ± 235.71	0.084	-	-	-
SDNN (ms)	54.48 ± 12.42	63.91 ± 14.27	0.032*	-	-	-
RMSDD (ms)	60.92 ± 15.51	67.15 ± 14.87	0.202	-	-	-
Peak LF (Hz)	0.074 ± 0.038	0.066 ± 0.027	0.430	0.05 (0.07)	0.06 (0.03)	0.845
Peak HF (Hz)	0.186 ± 0.034	0.186 ± 0.022	0.993	0.17 (0.04)	0.18 (0.04)	0.614
LF Power (ms^2)	1601.71 ± 993.97	2031.41 ± 1306.90	0.254	1013.15 (1895.61)	2142.80 (1876.49)	0.351
HF Power (ms^2)	956.67 ± 644.01	1254.97 ± 643.07	0.151	778.82 (983.69)	1036.40 (1045.70)	0.149
LF/HF	1.79 ± 0.78	1.78 ± 1.18	0.991	1.54 (1.15)	1.68 (2.03)	0.940
SD1 (ms)	43.26 ± 11.02	47.69 ± 10.55	0.202	-	-	-
SD2 (ms)	62.53 ± 14.66	75.97 ± 19.40	0.018*	-	-	-

The two-sided independent samples t-test was performed. df = 38. n = 40.
The non-parametric Mann-Whitney U-test was also performed for variables which were not considered to be normally distributed (determined using the Shapiro-Wilk test).
***. Difference in means is significant at the 0.01 level (2-tailed).*
**. Difference in means is significant at the 0.05 level (2-tailed).*
PNS – parasympathetic nervous system; SNS – sympathetic nervous system; HRV – heart rate variability; RR – R-R interval; SDNN – standard deviation of NN intervals; RMSDD – root mean square of successive differences between normal heartbeats; LF – low frequency; HF– high frequency.

3.3.4 Blood Pressure

There was no significant difference between the mean systolic or diastolic blood pressure of Group A and B (Table 9). The mean of both groups was around normal blood pressure of $\frac{120}{80}$mmHg. Group A had a mean blood pressure of $\frac{116.08 \pm 8.45}{81.30 \pm 7.19}$ mmHg and Group B had a mean blood pressure of $\frac{121.43 \pm 13.88}{81.10 \pm 7.98}$ mmHg.

Table 9: Mean of Blood Pressure.

	Mean ± SD		t-test p-value
	Group A	Group B	
Systolic BP (mmHg)	116.08 ± 8.45	121.43 ± 13.88	0.149
Diastolic BP (mmHg)	81.30 ± 7.19	81.10 ± 7.98	0.934

A two-sided independent samples t-test was performed. df = 38. n = 40.
BP – blood pressure

Although the t-test did not show any significant difference, the spread of systolic BP values was larger for Group B than Group A, as can be seen in Figure 16 and by the greater standard deviation of Group A (± 8.45) compared to Group B (± 13.88). Both the groups had a similar value for quartile one (Group A 108.88 vs Group B 109.50), however, Group B had a higher value for quartile three (133.89mmHg) than Group A (123.13mmHg). The highest recorded mean systolic BP value for Group B was 138.5mmHg, compared to 130.5mmHg in the Group A.

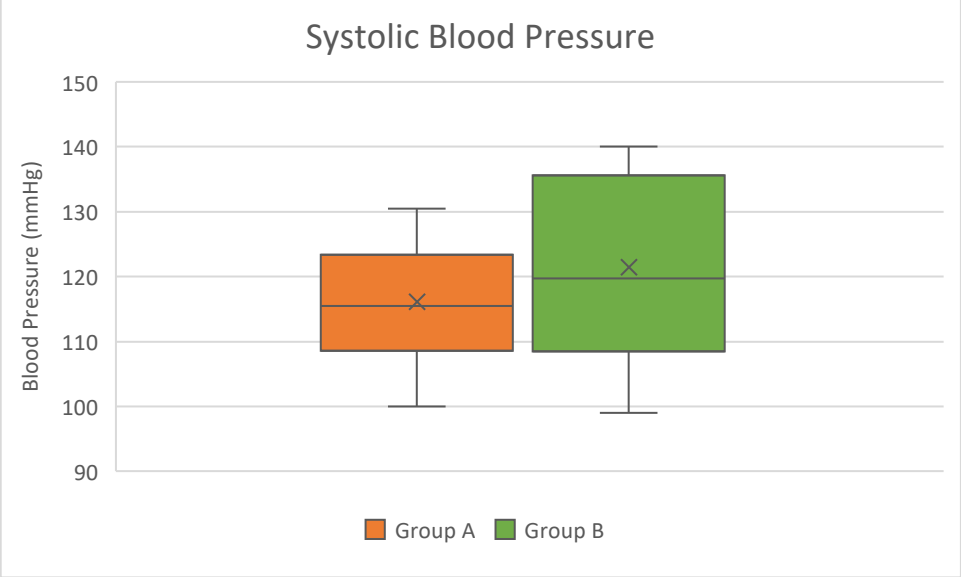


Figure 16: Boxplot of Systolic Blood Pressure *(n = 40). The mean is represented by ×*

3.3.5 Blood-Volume Pulse

There was no significant difference in the BVP measures between Group A and B (Table 10). Although the Mean BVP Amplitude of Group A (8.81 ± 4.18) was lower than Group B (11.84 ± 6.60), this difference did not reach significance (p = 0.091).

Table 10: Mean Blood-Volume Parameters.

	Mean ± SD		t-test p-value	Median (IQR)		Mann-Whitney p-value
	Group A	Group B		Group A	Group B	
Mean BVP Amplitude (%)	8.81 ± 4.18	11.84 ± 6.60	0.091	-	-	-
Min BVP Amplitude (%)	3.57 ± 2.27	4.3 ± 2.28	0.278	3.22 (3.64)	3.88 (3.69)	0.267
Max BVP Amplitude (%)	19.90 ± 10.22	18.54 ± 10.02	0.672	-	-	-
Mean BVP FFT Peak Frequency (Hz)	0.16 ± 0.07	0.13 ± 0.07	0.240	-	-	-
Min BVP FFT Peak Frequency (Hz)	0.04 ± 0.06	0.04 ± 0.05	0.775	0.02 (0.00)	0.02 (0.00)	0.820
Max BVP FFT Peak Frequency (Hz)	0.28 ± 0.08	0.25 ± 0.08	0.106	-	-	-

The two-sided independent samples t-test was performed. df = 38. n = 40
The non-parametric Mann-Whitney U-test was also performed for Min BVP Amplitude as it was not considered to be normally distributed (determined using the Shapiro-Wilk test).
BVP – blood-volume pulse; FFT – fast Fourier transform; Min – minimum; Max – maximum.

3.3.6 Electrodermal Activity

There was no significant difference in the mean EDA of Group A and B (Table 11). In fact, the means were almost the same (Group A 1.15 ± 0.86 vs. Group B 1.31 ± 1.18). This is also reflected by the Mann-Whitney p-value of 0.988.

Table 11: Mean of Electrodermal Activity.

	Mean ± SD		t-test p-value	Median (IQR)		Mann-Whitney p-value
	Group A	Group B		Group A	Group B	
Mean EDA (μSiemens)	1.15 ± 0.86	1.31 ± 1.18	0.647	0.99 (1.64)	1.15 (1.18)	0.988

The two-sided independent samples t-test was performed. df = 38. n = 40
The non-parametric Mann-Whitney U-test was also performed as EDA was not considered to be normally distributed (determined using the Shapiro-Wilk test).
EDA – electrodermal activity.

3.3.7 EEG Absolute Power

Values were recorded in μV^2. These values were then standardized using the qEEG-Pro normative database. The values for each participant were compared to the normative values of those with a similar age in the database. The z-score values for each electrode were used in the analysis.

3.3.7.1 Delta

Only two electrodes showed a significant difference in absolute power in the delta band (Table 12). The FP1 electrode showed greater mean absolute delta power in Group A than Group B (0.38 ± 0.48, 95% CI [0.12, 0.63] vs. -0.34 ± 0.72, 95% CI [-0.68, 0.01]; p = 0.002) (Figure 17). The other electrode that showed a significant difference was FP2, which also showed greater mean delta in Group A than Group B (0.63 ± 0.68, 95% CI [0.29, 0.97] vs. -0.26 ± 0.73, 95% CI [-0.60, 0.10]; p < 0.001) (Figure 18). This suggests that the absolute power of delta frequencies may be higher in the frontopolar cortex in the Group A.

Table 12: Mean of Z-Delta Absolute Power.

Absolute Power **Z-Delta**	**Mean ± SD**		**t-test p-value**	**Median (IQR)**		**Mann-Whitney p-value**
	Group A	**Group B**		**Group A**	**Group B**	
FP1	0.38 ± 0.48	-0.34 ± 0.72	0.002**	-	-	-
FP2	0.63 ± 0.68	-0.26 ± 0.73	<0.001**	-	-	-
F7	0.58 ± 1.95	0.13 ± 1.91	0.461	0.55 (1.40)	0.30 (1.60)	0.301
F3	-0.54 ± 3.19	-0.34 ± 2.44	0.829	-0.15 (1.60)	0.00 (1.60)	0.904
Fz	1.09 ± 1.34	0.16 ± 2.00	0.090	0.85 (2.05)	0.30 (1.45)	0.127
F4	-0.33 ± 3.31	-0.29 ± 2.52	0.970	0.60 (1.78)	-0.35 (1.98)	0.414
F8	0.67 ± 1.86	0.13 ± 1.78	0.354	0.95 (1.45)	0.45 (1.05)	0.134
T3	0.76 ± 2.82	0.40 ± 1.94	0.646	0.85 (1.85)	0.70 (2.10)	0.341
C3	-0.32 ± 3.37	-0.12 ± 2.59	0.838	0.15 (2.38)	0.05 (2.43)	0.925
Cz	-0.21 ± 3.90	-0.19 ± 2.94	0.985	0.25 (2.20)	-0.15 (2.55)	0.583
C4	-0.17 ± 3.46	-0.55 ± 2.73	0.706	0.10 (2.78)	-0.10 (2.33)	0.314
T4	0.62 ± 2.83	0.63 ± 1.40	0.983	1.25 (2.00)	0.50 (2.43)	0.301
T5	-1.67 ± 3.85	-0.55 ± 1.86	0.249	-0.60 (5.38)	-0.45 (2.43)	0.620
P3	-0.81 ± 3.58	-0.26 ± 2.43	0.573	0.05 (2.53)	-0.20 (2.08)	1.000
Pz	-1.03 ± 3.81	-0.95 ± 2.80	0.944	-0.40 (2.25)	-0.30 (2.80)	0.883
P4	-0.36 ± 3.49	-0.15 ± 2.62	0.835	0.35 (2.80)	-0.20 (2.55)	0.841
T6	-0.87 ± 2.76	-0.71 ± 3.14	0.869	-0.20 (2.50)	-0.35 (2.33)	0.758
O1	-1.70 ± 3.67	-0.81 ± 2.11	0.353	-1.00 (2.58)	-0.90 (2.73)	0.585
O2	-1.30 ± 2.70	-0.97 ± 1.93	0.664	-0.50 (2.28)	-1.05 (2.15)	0.947

The two-sided independent samples t-test was performed. df = 38. n = 40
The non-parametric Mann-Whitney U-test was also performed for variables which were not considered to be normally distributed (determined using the Shapiro-Wilk test).
***. Difference in means is significant at the 0.01 level.*
**. Difference in means is significant at the 0.05 level.*

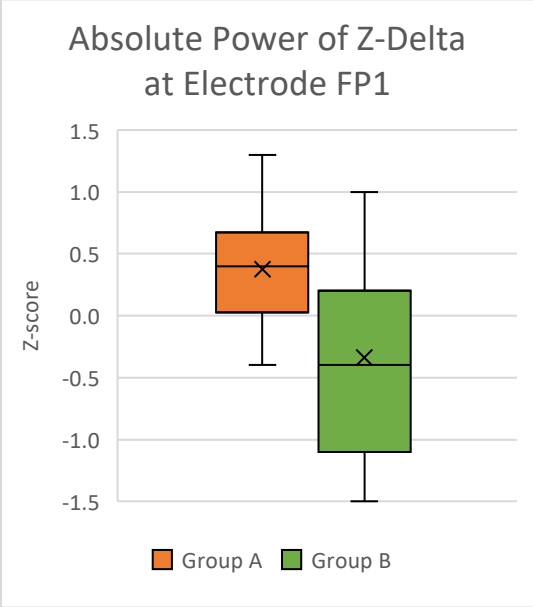


Figure 17: Boxplot of Absolute Power of Z-Delta at FP1. The mean is represented by ×

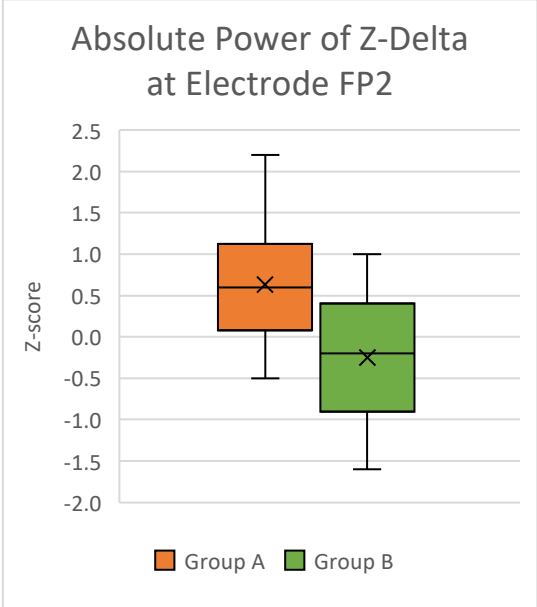


Figure 18: Boxplot of Absolute Power of Z-Delta at FP2. The mean is represented by ×

3.3.7.2 Theta

None of the electrodes showed a significant difference in mean absolute power of theta between Group A and B (Table 13).

Table 13: Mean of Z-Theta Absolute Power.

Absolute Power **Z-Theta**	**Mean ± SD**		**t-test p-value**	**Median (IQR)**		**Mann-Whitney p-value**
	Group A	**Group B**		**Group A**	**Group B**	
FP1	0.16 ± 2.42	0.70 ± 2.03	0.449	0.65 (2.48)	0.50 (1.50)	0.758
FP2	1.13 ± 1.31	0.79 ± 1.99	0.522	1.20 (2.03)	0.60 (1.45)	0.565
F7	0.97 ± 1.86	1.15 ± 2.07	0.774	1.35 (2.45)	0.85 (2.03)	0.883
F3	0.04 ± 2.38	0.48 ± 2.16	0.540	0.20 (2.78)	0.25 (1.58)	0.659
Fz	1.13 ± 1.29	1.01 ± 1.84	0.812	-	-	-
F4	0.19 ± 2.39	0.56 ± 2.21	0.614	0.50 (2.88)	0.35 (1.98)	0.947
F8	1.12 ± 1.75	1.15 ± 1.78	0.957	1.30 (2.03)	1.00 (1.15)	0.841
T3	0.34 ± 1.99	0.69 ± 1.81	0.559	0.35 (2.53)	0.55 (1.48)	0.799
C3	0.03 ± 2.27	0.37 ± 2.30	0.640	0.25 (2.68)	-0.10 (2.38)	0.883
Cz	0.07 ± 2.53	0.31 ± 2.64	0.766	0.30 (2.65)	-0.25 (3.28)	0.718
C4	0.03 ± 2.51	0.20 ± 2.29	0.824	-	-	-
T4	0.73 ± 2.07	1.06 ± 1.22	0.549	1.10 (2.63)	1.00 (1.18)	0.820
T5	-1.20 ± 2.48	-0.11 ± 1.54	0.103	-0.70 (3.35)	-0.15 (1.13)	0.157
P3	-0.27 ± 2.50	0.38 ± 2.14	0.382	-0.10 (2.48)	0.05 (1.80)	0.478
Pz	-0.43 ± 2.45	-0.14 ± 2.31	0.698	-0.15 (2.15)	-0.40 (2.48)	0.947
P4	0.13 ± 2.50	0.35 ± 2.13	0.761	-0.20 (2.33)	0.10 (2.60)	0.718

Absolute Power **Z-Theta**	**Mean ± SD**		**t-test p-value**	**Median (IQR)**		**Mann-Whitney p-value**
	Group A	**Group B**		**Group A**	**Group B**	
T6	-0.68 ± 1.73	-0.29 ± 2.03	0.517	-1.00 (2.73)	-0.10 (1.53)	0.478
O1	-1.29 ± 2.48	-0.30 ± 1.66	0.149	-0.75 (2.58)	-0.60 (1.88)	0.369
O2	-0.76 ± 1.77	-0.45 ± 1.60	0.559	-0.55 (2.55)	-0.55 (0.80)	0.947

The two-sided independent samples t-test was performed. df = 38. n = 40.
The non-parametric Mann-Whitney U-test was also performed for variables which were not considered to be normally distributed (determined using the Shapiro-Wilk test).

3.3.7.3 Alpha

None of the electrodes showed a significant difference in mean absolute power of alpha between Group A and B (Table 14). Although the T5 and O1 electrodes did have a notable difference in mean, this difference did not reach significance. Group A had a lower mean at the T5 electrode than Group B (-1.65 ± 1.38 vs. -0.95 ± 1.18; p = 0.095). This was also the case for the O1 electrode (-1.72 ± 1.61 vs. -0.86 ± 1.23; p = 0.067).

Table 14: Mean of Z-Alpha Absolute Power.

Absolute Power **Z-Alpha**	**Mean ± SD**		**t-test p-value**	**Median (IQR)**		**Mann-Whitney p-value**
	Group A	**Group B**		**Group A**	**Group B**	
FP1	-0.33 ± 1.52	0.35 ± 1.60	0.180	-	-	-
FP2	0.17 ± 1.12	0.38 ± 1.60	0.633	-	-	-
F7	0.09 ± 1.23	0.50 ± 1.60	0.369	-	-	-
F3	-0.50 ± 1.54	0.07 ± 1.54	0.257	-	-	-
Fz	0.03 ± 1.10	0.42 ± 1.58	0.377	-	-	-
F4	-0.48 ± 1.56	0.11 ± 1.59	0.247	-	-	-
F8	0.09 ± 1.28	0.46 ± 1.44	0.389	-	-	-
T3	-0.42 ± 1.25	0.09 ± 1.31	0.219	-	-	-
C3	-0.50 ± 1.50	0.04 ± 1.66	0.291	-	-	-
Cz	-0.41 ± 1.70	-0.03 ± 1.79	0.496	-	-	-
C4	-0.65 ± 1.65	-0.23 ± 1.67	0.429	-	-	-
T4	-0.31 ± 1.32	0.13 ± 1.08	0.255	-	-	-
T5	-1.65 ± 1.38	-0.95 ± 1.18	0.095	-	-	-
P3	-0.76 ± 1.71	-0.18 ± 1.54	0.266	-	-	-
Pz	-0.74 ± 1.75	-0.37 ± 1.60	0.490	-	-	-
P4	-0.49 ± 1.64	-0.21 ± 1.59	0.580	-	-	-
T6	-1.59 ± 1.05	-1.10 ± 1.39	0.221	-	-	-
O1	-1.72 ± 1.61	-0.86 ± 1.23	0.067	-	-	-
O2	-1.41 ± 1.34	-0.99 ± 1.24	0.316	-	-	-

The two-sided independent samples t-test was performed. df = 38. n = 40.
The non-parametric Mann-Whitney U-test was also performed for variables which were not considered to be normally distributed (determined using the Shapiro-Wilk test).

3.3.7.4 Beta

None of the electrodes showed a significant difference in mean absolute power of beta between Group A and B (Table 15).

Table 15: Mean of Z-Beta Absolute Power.

Absolute Power **Z-Beta**	**Mean ± SD**		**t-test p-value**	**Median (IQR)**		**Mann-Whitney p-value**
	Group A	**Group B**		**Group A**	**Group B**	
FP1	1.25 ± 1.48	1.53 ± 1.37	0.539	1.50 (1.55)	1.75 (1.53)	0.369
FP2	1.70 ± 1.08	1.58 ± 1.34	0.757	-	-	-
F7	1.67 ± 1.33	1.68 ± 1.47	0.982	-	-	-
F3	0.78 ± 1.42	0.99 ± 1.36	0.644	-	-	-
Fz	1.11 ± 0.85	1.62 ± 2.17	0.335	1.05 (1.03)	1.55 (2.03)	0.445
F4	0.64 ± 1.38	1.10 ± 1.34	0.293	0.65 (1.48)	1.10 (1.68)	0.183
F8	1.69 ± 1.32	1.84 ± 1.31	0.720	-	-	-
T3	1.03 ± 1.45	0.91 ± 1.14	0.773	-	-	-
C3	0.54 ± 1.31	0.97 ± 1.42	0.331	-	-	-
Cz	0.46 ± 1.40	1.00 ± 2.10	0.349	0.65 (1.75)	1.10 (2.43)	0.529
C4	0.38 ± 1.52	0.84 ± 1.50	0.342	-	-	-
T4	1.16 ± 1.33	1.33 ± 1.30	0.677	-	-	-
T5	-0.20 ± 1.83	0.10 ± 1.33	0.562	-0.20 (1.45)	0.35 (1.60)	0.429
P3	0.28 ± 1.58	0.92 ± 1.61	0.216	-	-	-
Pz	0.16 ± 1.56	0.53 ± 1.36	0.429	-	-	-
P4	0.75 ± 1.64	0.86 ± 1.53	0.827	-	-	-
T6	-0.18 ± 1.29	0.13 ± 1.77	0.536	-	-	-
O1	-0.74 ± 1.92	0.04 ± 1.29	0.142	-0.40 (2.10)	0.30 (1.75)	0.165
O2	-0.30 ± 1.39	-0.10 ± 1.15	0.623	-	-	-

The two-sided independent samples t-test was performed. df = 38. n = 40.
The non-parametric Mann-Whitney U-test was also performed for variables which were not considered to be normally distributed (determined using the Shapiro-Wilk test).

3.3.7.5 HiBeta

None of the electrodes showed a significant difference in mean absolute power of hibeta between Group A and B (Table 16).

Table 16: Mean of Z-HiBeta Absolute Power.

Absolute Power **Z-HiBeta**	**Mean ± SD**		**t-test p-value**	**Median (IQR)**		**Mann-Whitney p-value**
	Group A	**Group B**		**Group A**	**Group B**	
FP1	2.38 ± 1.95	2.24 ± 1.42	0.797	-	-	-
FP2	2.88 ± 1.63	2.42 ± 1.40	0.344	-	-	-
F7	2.78 ± 1.82	2.27 ± 1.59	0.351	-	-	-
F3	2.11 ± 1.81	1.94 ± 1.42	0.743	-	-	-
Fz	2.29 ± 1.49	2.38 ± 1.54	0.852	2.15 (1.73)	2.40 (1.38)	0.529
F4	1.96 ± 1.77	1.93 ± 1.39	0.945	-	-	-
F8	2.81 ± 1.79	2.52 ± 1.50	0.582	-	-	-
T3	1.88 ± 1.47	1.43 ± 1.18	0.292	-	-	-
C3	1.68 ± 1.74	1.73 ± 1.63	0.926	-	-	-
Cz	1.46 ± 1.77	1.40 ± 1.75	0.915	-	-	-
C4	1.53 ± 1.80	1.45 ± 1.49	0.879	-	-	-
T4	1.90 ± 1.30	1.75 ± 1.47	0.735	-	-	-
T5	1.09 ± 2.21	1.06 ± 1.56	0.961	-	-	-
P3	1.54 ± 1.82	1.84 ± 2.03	0.625	1.50 (1.78)	1.60 (1.25)	0.678
Pz	1.42 ± 1.80	1.37 ± 1.35	0.921	1.60 (1.90)	1.30 (1.23)	0.841
P4	1.96 ± 1.70	1.61 ± 1.50	0.494	1.60 (1.85)	1.95 (1.35)	0.947
T6	1.00 ± 1.66	0.98 ± 1.88	0.979	-	-	-
O1	0.70 ± 2.09	0.81 ± 1.35	0.851	-	-	-
O2	0.91 ± 1.55	0.70 ± 1.24	0.647	-	-	-

The two-sided independent samples t-test was performed. df = 38. n = 40.
The non-parametric Mann-Whitney U-test was also performed for variables which were not considered to be normally distributed (determined using the Shapiro-Wilk test).

3.3.8 EEG Relative Power

Values were recorded in μV^2. These values were then standardised using the qEEG-Pro normative database. The values for each participant were compared to the normative values of those with a similar age in the database. The z-score values for each electrode were used in the analysis.

3.3.8.1 Delta

The only electrodes that showed a significant difference in the mean relative power of delta were FP2 (p = 0.005) and Fz (p = 0.038) (Table 17). The mean at FP2 was significantly higher in Group A (-2.00 ± 1.82, 95% CI [-1.89, -0.88]) than in Group B (-2.81 ± 1.77, 95% CI [-3.53, -1.93]) (Figure 19). The mean at Fz was also significantly higher in Group A (-1.74 ± 2.11, 95% CI [-2.72, -0.75]) than Group B (-3.98 ± 3.75, 95% CI [-5.73, -2.23]) (Figure 20). More

importantly for Fz, the median of Group A (-1.65), was higher than the median of Group B (-4.15).

Table 17: Mean of Z-Delta Relative Power.

Relative Power **Z-Delta**	**Mean ± SD**		**t-test p-value**	**Median (IQR)**		**Mann-Whitney p-value**
	Group A	**Group B**		**Group A**	**Group B**	
FP1	-2.00 ± 1.82	-2.81 ± 1.77	0.164	-	-	-
FP2	-1.39 ± 1.08	-2.73 ± 1.71	0.005**	-	-	-
F7	-1.49 ± 1.45	-2.10 ± 1.61	0.216	-	-	-
F3	-2.65 ± 2.41	-3.19 ± 2.50	0.491	-	-	-
Fz	-1.74 ± 2.11	-3.98 ± 3.75	0.025*	-1.65 (2.80)	-4.15 (4.38)	0.038*
F4	-2.41 ± 2.52	-3.34 ± 2.44	0.243	-	-	-
F8	-1.37 ± 1.38	-2.13 ± 1.63	0.118	-	-	-
T3	-1.57 ± 2.44	-2.13 ± 2.05	0.433	-	-	-
C3	-2.37 ± 2.64	-3.43 ± 2.92	0.233	-	-	-
Cz	-2.36 ± 3.13	-3.51 ± 3.22	0.259	-	-	-
C4	-1.78 ± 2.54	-3.07 ± 2.62	0.123	-	-	-
T4	-2.03 ± 2.67	-2.35 ± 2.83	0.715	-1.30 (2.85)	-1.50 (2.85)	0.820
T5	-2.22 ± 3.42	-1.55 ± 2.39	0.480	-	-	-
P3	-2.73 ± 3.29	-3.71 ± 3.84	0.389	-	-	-
Pz	-2.82 ± 3.36	-3.94 ± 3.62	0.317	-	-	-
P4	-3.00 ± 3.54	-3.26 ± 3.70	0.818	-	-	-
T6	-0.87 ± 2.31	-1.46 ± 2.77	0.469	-	-	-
O1	-1.45 ± 2.85	-2.00 ± 2.88	0.547	-	-	-
O2	-1.69 ± 2.83	-1.86 ± 2.66	0.841	-	-	-

The two-sided independent samples t-test was performed. df = 38. n= 40.
The non-parametric Mann-Whitney U-test was also performed for variables which were not considered to be normally distributed (determined using the Shapiro-Wilk test).
***. Difference in means is significant at the 0.01 level.*
**. Difference in means is significant at the 0.05 level.*

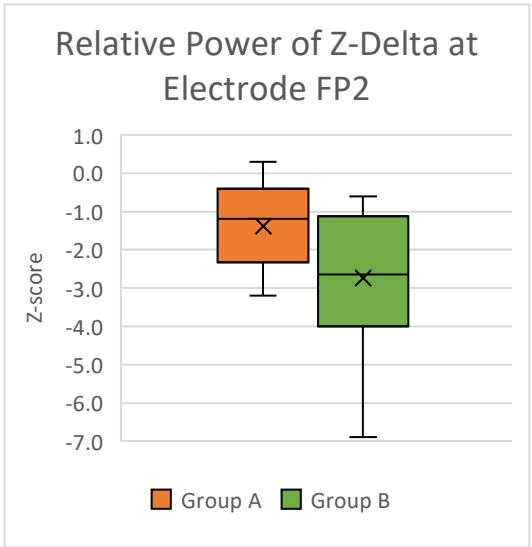


Figure 19: Boxplot of Relative Power of Z-Delta at FP2. *The mean is represented by ×*

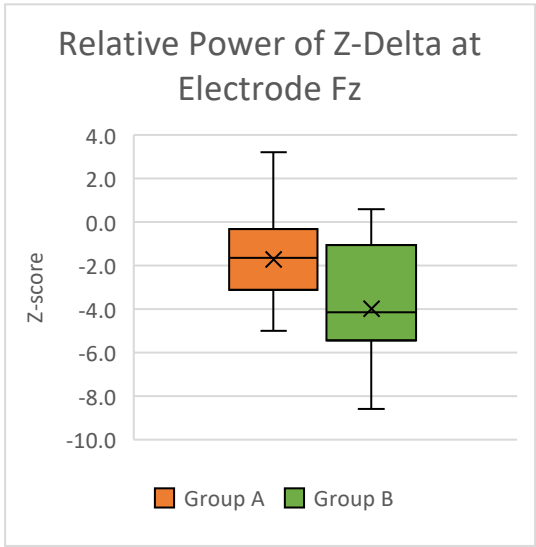


Figure 20: Boxplot of Relative Power of Z-Delta at Fz. *The mean is represented by ×*

3.3.8.2 Theta

The mean values for the relative power of theta at each electrode were mostly similar between the groups (Table 18). None of the means were found to be significantly different.

Table 18: Mean of Z-Theta Relative Power.

Relative Power **Z-Theta**	**Mean ± SD**		**t-test p-value**	**Median (IQR)**		**Mann-Whitney p-value**
	Group A	**Group B**		**Group A**	**Group B**	
FP1	-1.21 ± 1.09	-0.71 ± 1.53	0.242	-	-	-
FP2	-0.98 ± 1.06	-0.73 ± 1.44	0.544	-	-	-
F7	-1.42 ± 1.19	-0.99 ± 1.49	0.320	-	-	-
F3	-1.39 ± 1.39	-1.32 ± 1.51	0.871	-	-	-
Fz	-0.81 ± 1.03	-1.44 ± 2.01	0.220	-0.75 (1.43)	-0.80 (2.25)	0.398
F4	-1.17 ± 1.18	-1.32 ± 1.59	0.728	-	-	-
F8	-1.12 ± 0.95	-1.01 ± 1.26	0.757	-	-	-
T3	-2.02 ± 1.33	-1.38 ± 1.59	0.176	-	-	-
C3	-1.31 ± 1.18	-1.63 ± 1.32	0.424	-	-	-
Cz	-1.32 ± 1.19	-1.52 ± 1.45	0.635	-	-	-
C4	-1.05 ± 1.21	-1.17 ± 1.25	0.750	-	-	-
T4	-1.56 ± 1.71	-1.25 ± 1.68	0.567	-1.10 (2.43)	-1.15 (1.98)	0.698
T5	-1.47 ± 2.02	-0.65 ± 1.18	0.128	-1.05 (1.65)	-0.70 (1.45)	0.341
P3	-1.36 ± 1.28	-1.60 ± 1.62	0.599	-	-	-
Pz	-1.42 ± 1.47	-1.60 ± 1.65	0.710	-	-	-
P4	-1.50 ± 1.78	-1.40 ± 1.58	0.845	-	-	-
T6	-0.65 ± 1.11	-0.62 ± 1.18	0.935	-	-	-
O1	-0.92 ± 1.33	-0.88 ± 1.45	0.928	-	-	-
O2	-0.80 ± 1.31	-0.83 ± 1.54	0.947	-	-	-

The two-sided independent samples t-test was performed. df = 38. n = 40.
The non-parametric Mann-Whitney U-test was also performed for variables which were not considered to be normally distributed (determined using the Shapiro-Wilk test).

3.3.8.3 Alpha

There were quite a few electrodes that showed a difference in the mean relative power of alpha, although these did not reach significance (Table 19). Of these electrodes, all of them had a lower mean in Group A compared to Group B: FP1 (-1.40 ± 1.37 vs. -0.48 ± 1.53; p = 0.051) (Figure 21), FP2 (-1.45 ± 1.45 vs. -0.55 ± 1.54; p = 0.066), F7 (-1.54 ± 1.32 vs. -0.78 ± 1.46; p = 0.092), T5 (-2.47 ± 1.32 vs. -1.81 ± 1.11; p = 0.096), and O1 (-2.49 ± 1.21 vs. -1.82 ± 1.12; p = 0.079). This is suggestive of Group A having lower alpha relative to other brain areas in the front and the left back of the brain, although these differences were not significant. The only electrode with a significant difference was T3 (p = 0.033); Group A had a lower mean (-2.03 ± 1.09, 95% CI [-2.54, -1.52]) than Group B (-1.20 ± 1.29, 95% CI [-1.80, -0.59]) (Figure 22). This suggests reduced relative alpha activity in the temporal lobe, near the left ear.

Table 19: Mean of Z-Alpha Relative Power.

Relative Power **Z-Alpha**	**Mean ± SD**		**t-test p-value**	**Median (IQR)**		**Mann-Whitney p-value**
	Group A	**Group B**		**Group A**	**Group B**	
FP1	-1.40 ± 1.37	-0.48 ± 1.53	0.051	-	-	-
FP2	-1.45 ± 1.45	-0.55 ± 1.54	0.066	-	-	-
F7	-1.54 ± 1.32	-0.78 ± 1.46	0.092	-	-	-
F3	-1.68 ± 1.35	-1.02 ± 1.39	0.135	-	-	-
Fz	-1.51 ± 1.29	-1.02 ± 1.32	0.239	-	-	-
F4	-1.69 ± 1.37	-1.05 ± 1.42	0.153	-	-	-
F8	-1.55 ± 1.31	-0.86 ± 1.40	0.115	-	-	-
T3	-2.03 ± 1.09	-1.20 ± 1.29	0.033*	-	-	-
C3	-1.57 ± 1.27	-1.15 ± 1.26	0.300	-	-	-
Cz	-1.54 ± 1.39	-1.16 ± 1.36	0.395	-	-	-
C4	-1.76 ± 1.26	-1.24 ± 1.19	0.192	-	-	-
T4	-1.99 ± 1.23	-1.45 ± 1.20	0.167	-	-	-
T5	-2.47 ± 1.32	-1.81 ± 1.11	0.096	-	-	-
P3	-1.78 ± 1.30	-1.41 ± 1.14	0.350	-1.80 (2.48)	-1.65 (2.25)	0.221
Pz	-1.68 ± 1.28	-1.36 ± 1.19	0.416	-1.35 (2.35)	-1.25 (2.40)	0.369
P4	-1.75 ± 1.35	-1.38 ± 1.16	0.360	-1.55 (2.00)	-1.20 (2.18)	0.414
T6	-2.45 ± 0.94	-1.96 ± 1.07	0.133	-	-	-
O1	-2.49 ± 1.21	-1.82 ± 1.12	0.079	-2.45 (1.70)	-1.75 (2.43)	0.096
O2	-2.30 ± 1.15	-1.84 ± 1.16	0.216	-2.30 (2.00)	-1.45 (2.30)	0.157

The two-sided independent samples t-test was performed. df = 38. n = 40.
The non-parametric Mann-Whitney U-test was also performed for variables which were not considered to be normally distributed (determined using the Shapiro-Wilk test).

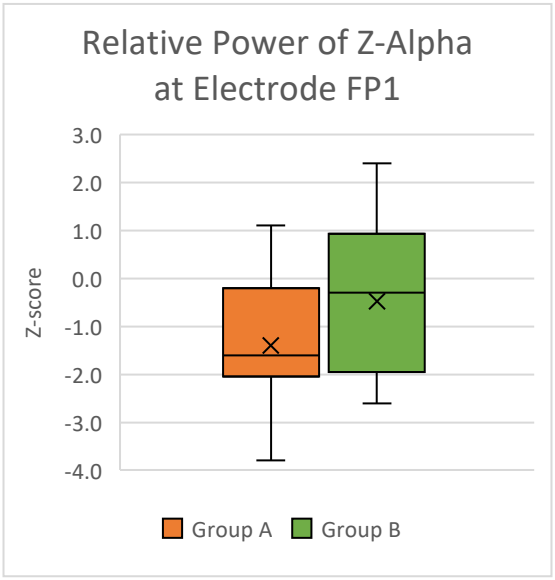


Figure 21: Boxplot of Relative Power of Z-Alpha at FP1.
The mean is represented by ×

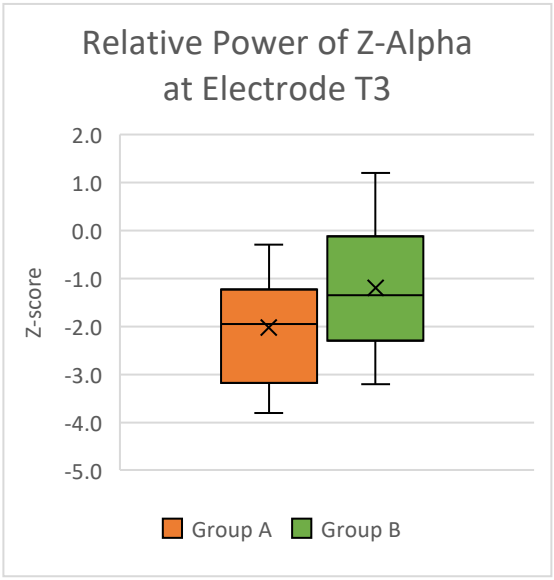


Figure 22: Boxplot of Relative Power of Z-Alpha at T3.
The mean is represented by ×

3.3.8.4 Beta

None of the electrodes showed a significant difference in mean relative power of beta between Group A and B (Table 20).

Table 20: Mean of Z-Beta Relative Power.

Relative Power **Z-Beta**	**Mean ± SD**		***t*-test *p*-value**	**Median (IQR)**		**Mann-Whitney *p*-value**
	Group A	**Group B**		**Group A**	**Group B**	
FP1	0.76 ± 1.64	0.81 ± 1.55	0.922	-	-	-
FP2	0.07 ± 0.99	0.75 ± 1.61	0.115	-	-	-
F7	-0.08 ± 1.19	0.03 ± 1.38	0.798	-	-	-
F3	0.16 ± 1.29	-0.07 ± 1.27	0.591	-	-	-
Fz	-0.44 ± 0.93	0.16 ± 1.72	0.177	-0.25 (0.83)	0.05 (1.88)	0.301
F4	-0.21 ± 1.24	-0.09 ± 1.46	0.781	-	-	-
F8	-0.09 ± 1.25	0.21 ± 1.23	0.457	-	-	-
T3	-0.45 ± 1.12	-0.53 ± 1.00	0.812	-	-	-
C3	-0.23 ± 1.23	-0.25 ± 1.25	0.970	-	-	-
Cz	-0.37 ± 1.54	0.11 ± 1.76	0.370	-	-	-
C4	-0.32 ± 1.33	0.10 ± 1.39	0.341	-	-	-
T4	-0.41 ± 1.04	-0.31 ± 0.79	0.734	-0.30 (1.25)	-0.15 (1.28)	0.883
T5	0.19 ± 1.33	-0.23 ± 1.12	0.288	-	-	-
P3	-0.22 ± 1.56	-0.34 ± 1.40	0.799	-	-	-
Pz	-0.21 ± 1.69	-0.18 ± 1.53	0.946	-	-	-
P4	-0.23 ± 1.38	-0.27 ± 1.42	0.920	-	-	-
T6	0.18 ± 1.09	0.14 ± 1.19	0.924	-	-	-
O1	0.01 ± 1.59	-0.19 ± 1.34	0.669	-	-	-
O2	0.11 ± 1.47	-0.13 ± 1.04	0.571	-	-	-

The two-sided independent samples t-test was performed. df = 38. n = 40.
The non-parametric Mann-Whitney U-test was also performed for variables which were not considered to be normally distributed (determined using the Shapiro-Wilk test).

3.3.8.4 HiBeta

None of the electrodes showed a significant difference in mean relative power of beta between Group A and B (Table 21).

Table 21: Mean of Z-HiBeta Relative Power.

Relative Power **Z-HiBeta**	**Mean ± SD**		***t*-test *p*-value**	**Median (IQR)**		**Mann-Whitney *p*-value**
	Group A	**Group B**		**Group A**	**Group B**	
FP1	2.63 ± 2.37	1.96 ± 2.51	0.388	-	-	-
FP2	1.87 ± 1.99	2.10 ± 2.47	0.753	-	-	-
F7	1.58 ± 2.03	0.86 ± 2.26	0.298	-	-	-
F3	2.12 ± 2.29	1.33 ± 2.18	0.274	-	-	-
Fz	1.32 ± 2.21	1.17 ± 2.20	0.825	-	-	-
F4	1.82 ± 2.31	1.19 ± 2.48	0.410	-	-	-
F8	1.54 ± 1.90	1.16 ± 2.11	0.549	-	-	-
T3	1.01 ± 1.32	0.55 ± 1.41	0.300	-	-	-
C3	1.45 ± 2.40	0.87 ± 2.62	0.465	-	-	-
Cz	1.22 ± 2.77	0.75 ± 2.54	0.579	-	-	-
C4	1.43 ± 2.46	1.10 ± 2.49	0.671	-	-	-
T4	0.94 ± 1.22	0.71 ± 1.22	0.547	-	-	-
T5	2.28 ± 2.31	1.38 ± 1.70	0.166	-	-	-
P3	1.54 ± 2.73	1.10 ± 2.81	0.618	-	-	-
Pz	1.81 ± 2.89	1.35 ± 3.08	0.629	-	-	-
P4	1.56 ± 2.63	0.96 ± 2.47	0.458	-	-	-
T6	2.15 ± 2.00	1.58 ± 1.92	0.368	2.45 (3.05)	1.35 (3.88)	0.414
O1	2.19 ± 2.04	1.25 ± 2.23	0.172	2.35 (2.90)	1.30 (2.63)	0.149
O2	2.01 ± 1.99	1.36 ± 2.14	0.327	-	-	-

The two-sided independent samples t-test was performed. df = 38
The non-parametric Mann-Whitney U-test was also performed for variables which were not considered to be normally distributed (determined using the Shapiro-Wilk test).
n = 40

3.3.9 Summary of EEG Results

In terms of a difference in mean or correlation with DASS score (see *3.4 Correlations*), the only notable electrodes were FP1, FP2, F7, F3, Fz, and T3, and the only notable frequencies were delta, alpha, and hibeta. No significant correlation or difference in mean was found at any of the other electrodes or frequencies (theta and beta). The significant results regarding the qEEG measures are summarised in Table 22 and Figure 23.

Table 22: Summary of EEG Results.

Electrode	Area	Frequency & Power	Significance
FP1	Anterior prefrontal cortex	Delta Absolute Power	Higher mean in Group A Positive correlation with stress score
		Alpha Relative Power	Lower mean in Group A *(Note: p = 0.051)*
FP2	Anterior prefrontal cortex	Delta Absolute Power	Higher mean in Group A Positive correlation with stress score Positive correlation with anxiety score
		Delta Relative Power	Higher mean in Group A
F7	Prefrontal association cortex	Alpha Relative Power	Negative correlation with anxiety score
		HiBeta Relative Power	Positive correlation with anxiety score
F3	Intermediate prefrontal gyrus	HiBeta Relative Power	Positive correlation with stress score Positive correlation with anxiety score
Fz	Intermediate prefrontal gyrus	Delta Relative Power	Higher mean in Group A
T3	Auditory cortex	Alpha Relative Power	Lower mean in Group A Negative correlation with stress score Negative correlation with anxiety score
		HiBeta Relative Power	Positive correlation with stress score

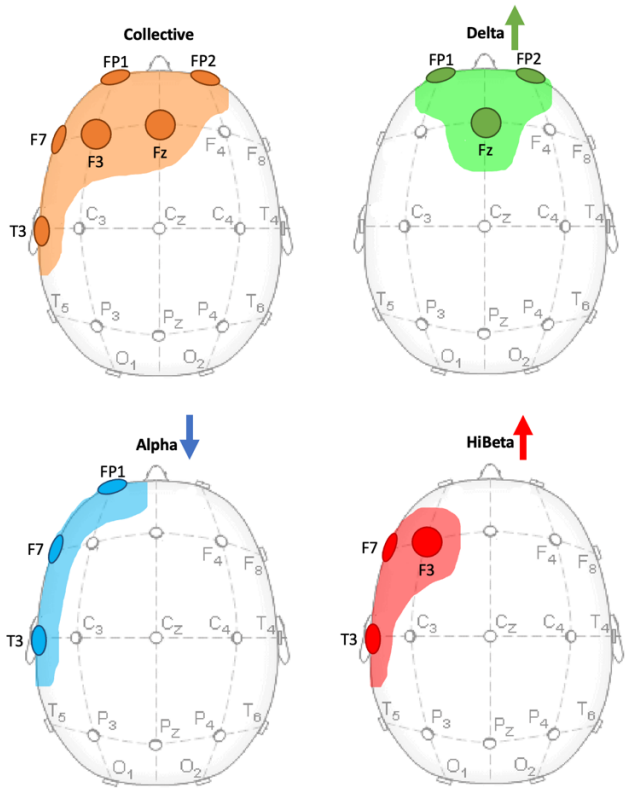


Figure 23: Summary of EEG Results. *Electrodes at which there were a significant correlation with DASS score or difference in mean were included. The first image (Collective, in orange) shows all the electrodes with a significant association. The results suggest that delta power increased, alpha power decreased, and hibeta power increased with stress and anxiety symptoms.*

Note: The inclusion of FP1 in the alpha frequency should be treated with caution given that the p-value for the difference in means was 0.051 and not p < 0.05

3.3.8 Summary of Mean Tests

The difference in means between Group A and B are summarised in Figure 24.

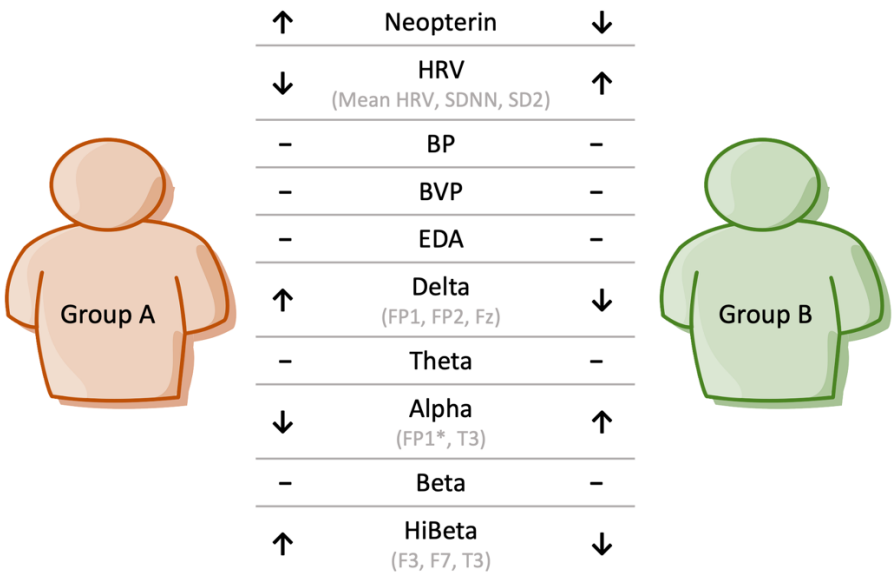


Figure 24: Summary of Mean Tests between Group A and B. *↑ higher mean; ↓ lower mean; − no significant difference. BP – blood pressure; BVP – blood-volume pulse; EDA – electrodermal activity; HRV – heart rate variability. *p = 0.051*

3.4 Correlations

Due to the large number of measurements, only the correlations which reached significance ($p < 0.05$) are displayed in the tables below. If a variable does not appear in the table, it is an indication that it did not have a significant correlation with the other variable.

3.4.1 Correlations with Stress Score

The variable that had the strongest correlation with the stress score was the anxiety score. There was a significant strong positive correlation between stress and anxiety ($r_s = 0.829$; $p < 0.001$). There was also a strong positive correlation between stress and depression ($r_s = 0.732$; $p < 0.001$) (Table 23). The correlations are summarized in Figure 25.

The stress score also positively correlated with neopterin concentration ($r_s = 0.588$; $p < 0.001$), the absolute power of delta at the FP1 ($r_s = 0.372$; $p = 0.027$) and FP2 electrodes ($r_s = 0.344$; $p = 0.03$), and the relative power of hibeta at the F3 ($r_s = 0.323$; $p = 0.042$) and T3 electrodes ($r_s = 0.333$; $p = 0.036$).

The stress score negatively correlated with mean HRV ($r_s = -0.433$; $p = 0.005$) and the relative power of alpha at the T3 electrode ($r_s = -0.379$; $p = 0.016$).

Table 23: Correlations with Stress Score.

			Spearman's Correlation Coefficient	p-value
Stress Score	and	**Anxiety Score**	0.829	<0.001**
	and	**Depression Score**	0.732	<0.001**
	and	**Neopterin**	0.588	<0.001**
	and	**Mean HRV**	-0.433	0.005**
	and	**FP1 Z-Delta Absolute Power**	0.373	0.027*
	and	**FP2 Z-Delta Absolute Power**	0.344	0.030*
	and	**T3 Z-Alpha Relative Power**	-0.379	0.016*
	and	**F3 Z-HiBeta Relative Power**	0.323	0.042*
	and	**T3 Z-HiBeta Relative Power**	0.333	0.036*

***. Correlation is significant at the 0.01 level (2-tailed).*
**. Correlation is significant at the 0.05 level (2-tailed).*
HRV – heart rate variability.
n = 40

As Stress Score Increases:					
↑	Anxiety	↓	HRV	−	BP
↑	Depression	↓	Alpha (T3)	−	BVP
↑	Neopterin			−	EDA
↑	Delta (FP1, FP2)			−	Theta
↑	HiBeta (F3, T3)			−	Beta

Figure 25: Summary of Stress Score Correlations. *↑ increases; ↓ decreases; − no change. BP – blood pressure; BVP – blood-volume pulse; EDA – electrodermal activity; HRV – heart rate variability.*

3.4.2 Correlations with Anxiety Score

The variable that had the strongest correlation with the anxiety score was the stress score. There was a significant strong positive correlation between anxiety and stress (r_s = 0.823; p < 0.001). There was also a strong positive correlation between anxiety and depression (r_s = 0.642; p < 0.001) (Table 24). The correlations are summarized in Figure 26.

Anxiety score also positively correlated with neopterin concentration (r_s = 0.426; p = 0.006), the absolute power of delta at the FP2 electrode (r_s = 0.374; p = 0.018), and the relative power of hibeta at the F7 (r_s = 0.325; p = 0.041) and F3 electrodes (r_s = 0.359; p = 0.023).

The anxiety score negatively correlated with mean HRV (r_s = -0.365; p = 0.021), minimum BVP amplitude (r_s = -0.366; p = 0.02), mean BVP amplitude (r_s = -0.344; p = 0.03), and the relative power of alpha at the F7 (r_s = -0.324; p = 0.042) and T3 electrodes (r_s = -0.399; p = 0.011).

Table 24: Correlations with Anxiety Score

			Spearman's Correlation Coefficient	p-value
Anxiety Score	and	**Stress Score**	0.823	<0.001**
	and	**Depression Score**	0.642	<0.001**
	and	**Neopterin**	0.426	0.006**
	and	**Mean HRV**	-0.365	0.021*
	and	**Min BVP Amplitude**	-0.366	0.020*
	and	**Mean BVP Amplitude**	-0.344	0.030*
	and	**FP2 Z-Delta Absolute Power**	0.374	0.018*
	and	**F7 Z-Alpha Relative Power**	-0.324	0.042*
	and	**T3 Z-Alpha Relative Power**	-0.399	0.011*
	and	**F7 Z-HiBeta Relative Power**	0.325	0.041*
	and	**F3 Z-HiBeta Relative Power**	0.359	0.023*

***. Correlation is significant at the 0.01 level (2-tailed).*
**. Correlation is significant at the 0.05 level (2-tailed).*
BVP – blood-volume pulse; HRV – heart rate variability; Min – minimum.
n = 40

As Anxiety Score Increases:					
↑	Stress	↓	HRV	−	BP
↑	Depression	↓	Alpha (F7, T3)	−	EDA
↑	Neopterin	↓	BVP	−	Theta
↑	Delta (FP2)			−	Beta
↑	HiBeta (F7, F3)				

Figure 26: Summary of Anxiety Score Correlations. *↑ increases; ↓ decreases; − no change. BP – blood pressure; BVP – blood-volume pulse; EDA – electrodermal activity; HRV – heart rate variability.*

3.4.3 Correlations with Depression Score

The variable that had the strongest correlation with the depression score was the stress score. There was a significant strong positive correlation between depression and stress (r_s = 0.732; $p < 0.001$). There was also a strong positive correlation between depression and anxiety (r_s = 0.642; $p < 0.001$) (Table 25). The correlations are summarized in Figure 27.

Depression score also positively correlated with neopterin concentration (r_s = 0.451; p = 0.003) and Max BVP FFT Peak Frequency (r_s = 0.387; p = 0.014).

The depression score negatively correlated with age (r_s = -0.315; p = 0.048), mean HRV (r_s = -0.383; p = 0.15), minimum BVP amplitude (r_s = -0.400; p = 0.011), and mean BVP amplitude (r_s = -0.368; p = 0.019).

Table 25: Correlations with Depression Score.

			Spearman's Correlation Coefficient	p-value
Depression Score	and	**Stress Score**	0.732	<0.001**
	and	**Anxiety Score**	0.642	<0.001**
	and	**Neopterin**	0.451	0.003**
	and	**Age**	-0.315	0.048*
	and	**Mean HRV**	-0.383	0.015*
	and	**BVP Min Amplitude**	-0.400	0.011*
	and	**BVP Mean Amplitude**	-0.368	0.019*
	and	**BVP Max FFT Peak Frequency**	0.387	0.014*

***. Correlation is significant at the 0.01 level (2-tailed).*
**. Correlation is significant at the 0.05 level (2-tailed).*
BVP – blood-volume pulse; FFT – fast Fourier transform; HRV – heart rate variability; Max – maximum; Min – minimum.
n = 40

As Depression Score Increases:					
↑	Stress	↓	HRV	−	BP
↑	Anxiety	↓	BVP	−	BVP
↑	Neopterin			−	EDA
				−	Delta
				−	Theta
				−	Alpha
				−	Beta
				−	HiBeta

Figure 27: Summary of Depression Score Correlations. *↑ increases; ↓ decreases; − no change. BP – blood pressure; BVP – blood-volume pulse; EDA – electrodermal activity; HRV – heart rate variability.*

This concludes the chapter on the results of the questionnaires and physiological measurements. The next chapter will discuss the meaning and implications of the results.

Chapter 4

4. Discussion

As previously discussed, mental health is an increasing global crisis.[2] It has large social, economic, and health costs, with stress and anxiety disorders accounting for a large portion of the impact.[4-9,11] This is of particular concern in Health Science students who have a high incidence of mental health struggles.[15-19] Subjectivity, misdiagnosis and social stigma can interfere with the detection, prevention, and treatment of mental health problems.[83,113,120] Therefore, more objective measures such as biomarkers are necessitated to aid in the identification and treatment of mental health disorders.[107-108,110,128] As the ANS is implicated in stress and anxiety,[27-28,46-48] biomarkers measuring ANS activity were investigated in this study, namely HRV, BP, BVP, EDA, and qEEG. Additionally, inflammation appears to have a bidirectional relationship with stress and anxiety.[169,177,185,188-190] Therefore, the inflammatory marker neopterin was also investigated as a possible biomarker of stress and anxiety scores. The study aimed to determine whether the aforementioned biomarkers could be used as complementary markers for stress and anxiety scores as determined by the DASS-21 questionnaire. Thus, the DASS-21 scoring system was used to stratify participants between Group A (who were experiencing high levels of symptoms) and Group B (who were experiencing normal levels). Subsequently, the concentration of neopterin and each neurophysiological measurement were compared between Group A and B. Finally, taking into consideration the difference in means between the groups and any correlations with the DASS scores, the most suitable biomarkers are discussed.

4.1 Questionnaires

To recruit participants for this study, an online questionnaire was used. Those who completed the online questionnaire are referred to as respondents, and those who completed the questionnaire and partook in physiological measurements are referred to as participants. The results of the online questionnaire are discussed first.

According to the DASS-21 scores obtained, both the mean stress and the mean depression scores for the respondents were in the Moderate category, while the mean anxiety score was between the Moderate and Severe categories. This suggests that the average Health Science student may have been experiencing moderate symptoms of stress, anxiety, and depression. Of these, 37% of respondents had Normal scores for stress, 28% Normal for anxiety, and 34% Normal for depression symptoms. On the other hand, 30% of respondents had Severe or Extremely Severe scores for stress and depression symptoms. The Anxiety category had the

highest scores, with 33% of respondents scoring Extremely Severe. When considering the 11% who scored Severe, a total of 44% of the respondents, which is almost half, scored Severe or Extremely Severe for anxiety symptoms. Therefore, anxiety symptoms are a major concern for these Faculty of Health Sciences students.

The results of the online DASS-21 questionnaire equate to approximately one in three respondents having normal levels, while approximately one in three respondents have severe or extremely severe stress, anxiety, or depression symptoms. A nationally representative study found the prevalence of major depression to be 9.7% in South Africa[10] which is lower than the ~30% found in this study. A possible reason for this is that university students may have a higher prevalence of anxiety and depression than the general population. A review of multiple countries (including middle- and low-income countries) found the mean prevalence of depression in university students to be 30.6%,[264] which is similar to the value obtained in the present study. Other studies have also found that the prevalence of depression in university students is higher than in the general population and is around 30%.[265-266] In terms of anxiety, a different study found the lifetime prevalence to be 15.8% and the 12-month prevalence to be 8.1% in South Africa.[4] A survey conducted in 2020, which used more than 28,000 students from 17 universities in South Africa, found the prevalence of any anxiety disorder to be 37.1% in students,[267] which is similar to the proportion found in the present study. This follows a similar pattern as with depression, whereby anxiety had a higher prevalence among university students than the general population. It should be noted that as the DASS-21 is not a diagnostic tool, it complicates the comparison between the present study's findings of symptom severity and rates of diagnosed disorders. However, the DASS-21 findings are still of importance as they highlight the high rate of individuals experiencing mental health symptoms and it emphasises the need for attention in dealing with these struggles. In addition, the DASS-21 questionnaire has shown to be useful in identifying individuals with potential mental health conditions, therefore clinicians can use it as a screening tool and then later confirm the diagnosis with a professional evaluation.[268-269]

A component to consider when evaluating the present study's results is the COVID-19 pandemic, which increased cases of anxiety and depression.[270] Therefore, the post-COVID prevalence of mental health disorders may be higher than previous counts and estimates. Another aspect is the potential for response bias, as students who were interested in mental health issues may have been more likely to fill out the questionnaire. In addition, the present study used a greater proportion of female respondents than males. This may have influenced the results as it has been reported that females may have a higher risk for and increased severity of common mental disorders.[4,267] It has also been found that females are 1.75 times

more likely to experience depression in their lifetime than males.[10] This inequality should be noted when interpreting the results of the present questionnaire, given that 87% of the respondents were female. As such, the prevalence of mental health symptoms presented could be an overestimation. Nevertheless, the findings of the present study show that a large proportion of female students are struggling with mental health, irrespective of whether the study is representative of males, which needs to be addressed.

Other than the potentially higher rate of mental health symptoms in females, another reason for a greater proportion of female participation could be that male students are usually less willing to seek support.[264] Thus, it could be postulated that the higher percentage of female respondents was due to females being more willing to participate in an activity related to mental health than males. This is also supported by the higher percentage of females who partook in the physiological measurements. Although further research is required to establish motivational differences between sexes in research participation.

Only 8% of the respondents reported using antidepressants compared to the 30% of participants who scored severe or extremely severe for depression symptoms. This suggests that students in general may struggle to seek or ask for help, not just males. However, antidepressants are not the only form of treatment; students may be pursuing other avenues of assistance that are not reflected in the questionnaire. Therefore, an investigation into which services students are using to help support their mental health struggles, and the rate at which they seek assistance would be of interest.

The findings of the present study suggest that Health Science students have high levels of anxiety and depression, which is in juxtaposition with the idea that poor education is a risk factor for developing mental illness. This idea is supported by a study which found the prevalence of depressive episodes to be significantly higher in South Africans with a low average level of education.[10] In addition, university students are typically socio-economically advantaged, which is considered protective against depression, yet the incidence of mental health appears to be higher in this population.[264] Many factors may increase the vulnerability to depression and exacerbate or precipitate mental health problems in students, such as financial stressors, family relationship alterations, new social environments, academic pressure, preoccupation with post-graduation life, and changes in sleeping and eating habits.[267,271] In addition to this, there are increased opportunities for the use of substances, such as drugs which can increase or trigger mental health symptoms. The prevalence of drug abuse disorder has been found to be 5.1% in students,[267] which is noteworthy given that 5.7% of the respondents in the present questionnaire said they used recreational drugs.

Furthermore, particularly in the South African context, socio-economic factors can diminish students' mental health, such as poverty, inequality, crime, and gender-based violence.[267] Collectively, these factors may help to explain the high proportion of students struggling with mental health symptoms.

The high incidence of mental health symptoms has important financial, social, health, and academic implications. For example, anxiety disorders are estimated to be responsible for about 28.68 million disability-adjusted life years globally (the sum of the years of life lost due to premature mortality and the years lived with a disability).[272] As mentioned in *Chapter 1*, stress, anxiety, and depression are costly and have adverse impacts on health. Additionally, poor mental health can reduce energy levels, concentration, cognition, and knowledge acquisition, which impacts academic performance.[273] Furthering this, a study carried out in 2022 at a different South African university found that higher levels of depression symptoms among first-year students were associated with a greater likelihood of progression delay (not meeting the academic requirements to proceed from one academic year to the next). The probability of progression delay increased with the severity of depression symptoms, with moderate-severe and severe depression symptoms nearly tripling the probability of progression delay.[273] Other studies carried out in South African universities have also found associations between depression and academic failure.[273] A study found that students experiencing MDD were almost four times more likely to perform poorly.[274] This is a concern given that MDD is one of the leading causes of disability worldwide, and its incidence is increasing.[2] Additionally, depression contributes to significant morbidity and mortality, increasing the risk of diabetes,[62] CVD,[63] and cognitive impairment.[64]

The findings that anxiety and depression symptoms impact academic performance are not limited to South Africa as studies carried out in other countries also found similar results, particularly in medical students. These countries include the United Arab Emirates,[265] Syria,[275], England,[276] and the United States of America.[277]

Not only do anxiety and depression have implications for health and well-being, but they also have economic consequences which are of particular importance in the South African context, where more than half of the population lives in poverty and most young people are unemployed.[273,278] As higher education is vital in the development and economic growth of developing countries,[267] mental health should be a priority in these institutions due to its impact on academic performance and future well-being. Compounding this and possible progression delay, anxiety disorders increase dropping-out from academic institutions,[278] which has

particularly negative consequences for economically-disadvantaged students, especially those who may be expected to support their families financially upon graduation.[273]

When looking at the mean score for each individual DASS-21 question, the question with the highest mean, in that it was experienced the most often, was Question 1 “I found it hard to wind down”. This was followed by Question 5 “I found it hard to work up the initiative to do things” and Question 11 “I found myself getting agitated”. Although speculation, this could be interpreted as the average Health Science student finding it difficult to stop working and relax, but when they do stop, they may experience difficulty finding the motivation to start tasks again. This might lead to agitation, as they find it difficult to stop doing tasks and take breaks, but also to start doing tasks again once they do take a break. This is supported by the next highest-rated question, Question 12 “I found it difficult to relax”. As such, a possible intervention could involve teaching relaxation techniques to students.

The individual DASS-21 questions with the lowest scores were Question 21 “I felt that life was meaningless”, followed by Question 4 “I experienced difficulty breathing”, which was experienced the least and the average respondent felt those questions did not often apply to them. A potential reason why Question 21 had the lowest rating could be linked to the students’ motivation for studying a Health Science degree, such as choosing to study their particular course in order to help people or doing so because of their family and friends.[279-280] As a result, the respondents might feel like their life has meaning and that they are pursuing a worthy cause, although further research is required to confirm this assumption.

Three of the four highest-rated questions formed part of the Stress scale of the DASS-21. In contrast, Question 4, which also formed part of the Stress scale, ranked lowly. This suggests that the manifestations of stress symptoms in the respondents were more closely related to feelings of agitation and difficulty relaxing, rather than somatic feelings such as difficulty breathing.

Correlations between stress, anxiety, and depression scores were found from the results of the online questionnaire. The strongest of these correlations was between stress and anxiety ($r_s = 0.829$). This finding shows that there is a strong positive relationship between stress and anxiety; as stress increases, so does anxiety. This is not surprising given that stress is associated with the onset and severity of a variety of psychiatric disorders[281] and it has been suggested that there is an influential relationship between stress and anxiety.[40] As anxiety disorders are among the most prevalent and disabling mental disorders,[9] identifying this relationship may be useful in preventing the development and treatment of anxiety disorders.

Mental health is of particular concern in a faculty such as Health Sciences, as several studies suggest that these students have a high incidence of mental health struggles.[15-19] Decline in mental health has a negative impact on a student's academic performance and their ability to care for their patients, as well as contributing to other negative professional and personal aspects.[20-22] As such, mental health struggles not only affect the students but may also affect future patients. The cost of anxiety and depression can be particularly high in young adults because they represent the future of a community, its workforce, and potential leaders. Experiencing mental health conditions early on can have negative consequences that persist throughout adult life, such as impacting career prospects, social relationships, and work performance.[264] Therefore, steps need to be taken to treat and prevent symptoms of stress, anxiety, and depression in university students – a group who are particularly vulnerable to mental health struggles.[273]

Universities have an important role to play given that 75% of mental health disorders emerge by age 25,[267] and that the mean age of onset of a major depressive episode is between 25 and 26 years old,[10] a time when most students are finishing studies and transitioning into employment. In addition, mental health problems often increase during the first year of university. Younger students are more likely to experience mental health struggles than older students as transitioning into university life has many related stressors and their prefrontal cortex is still undergoing development in relation to impulse control, planning, problem-solving, and regulation skills.[267] This is supported by the present study which found a negative correlation between depression scores and age (r_s = -0.315) in the physiological measurement participants. Furthermore, the onset of many mental health problems manifest during late adolescence, which means that many younger students may not have experienced any mental health problems before, and therefore they have either not learnt how to manage them or have yet to receive a diagnosis and treatment.[267] As a result, universities should help to better prepare students for this fundamental period and should consider mental health to be a key component in academic performance and future success.

A common feature between mental health and academic performance is class attendance; those with mental health disorders have higher levels of absenteeism, while class attendance is an important contributor to academic success.[273] As such, monitoring class attendance could be useful in identifying at-risk students. Additionally, making classes compulsory could also be beneficial and could be part of a solution that universities could employ.

Regarding the questionnaires filled in by the students who participated in the physiological measurements, there was also a correlation between stress, anxiety, and depression. The strongest of these correlations was between stress and anxiety (r_s = 0.829), which was similar to the results of the online questionnaire. The correlation between anxiety and depression was also similar for both the online questionnaire respondents and the study participants' questionnaire (r_s = 0.671 vs. r_s = 0.642). There was a stronger positive correlation between stress and depression in the study participants (r_s = 0.732) than in the respondents to the online questionnaire (r_s = 0.650). The implications of these correlations are discussed above.

In summary, symptoms of stress, anxiety, and depression, appear to be highly prevalent in university students, namely in Health Science students. Struggles with mental health have important economic, social, health, and academic consequences. Thus, this is an issue that needs to be addressed, especially in the South African context. Universities have an important role to play in aiding the reduction of mental health struggles in their students, by building institutional cultures that promote good mental health, provide required resources, and teach coping strategies.

4.2 Neopterin

Neopterin is a marker of immune activation during inflammation.[165] This study found a significantly higher concentration of neopterin in Group A, compared to Group B ($p < 0.01$). In addition, neopterin was found to be positively correlated with scores for stress (r_s = 0.588), anxiety (r_s = 0.426), and depression (r_s = 0.451). Therefore, neopterin concentration could potentially be used as a biomarker of stress, anxiety, and depression scores. This is in line with other studies which found increased neopterin in depression,[144,152,154,216] PTSD,[219] and psychological stress.[153] Increased inflammatory markers have also been found in panic disorder[186] and GAD.[187] It should be noted that the neopterin concentrations found in this study correlated with symptom severity and not diagnosed disorders.

Apart from studies that directly investigate neopterin and mental health disorders, other studies have also found inflammation to be connected to stress,[77,177] anxiety,[177,186-187,217] and depression.[74,77,183,184] These studies support the results of the present study, which suggests that participants with high mental health scores may have increased inflammation. Additionally, there is increasing evidence for a bidirectional causal link between inflammation and mental health problems.[169,177,185,188-190] This is supported by studies which found increased PICs predating the occurrence of depression[77,206] and that anti-inflammatory medication (i.e.

NSAIDs) may have antidepressant effects.[207] As such, anti-inflammatory medication or diets may be of use in the treatment of mental health disorders.

Mental health disorders are complex and influenced by many factors. Here, the association between mental health symptoms and neopterin concentration provides insight into potential factors. Neopterin is reflective of the levels of macrophage activation, ROS, and the pro-inflammatory cytokine IFNγ.[141,165,201] All of these have been found to be elevated in mental health patients or involved in the onset of symptoms.

The relationship between neopterin and mental health scores could be due to macrophage activation. Greater infiltration of macrophages into the brain during stress alters the neuroinflammatory profile and promotes anxiety-like behaviour.[195,198] In addition, increased macrophages have been found in post-mortem brain samples from individuals who died as a result of depressed suicide.[282] Elevated macrophages are not just found in the brain; a significant positive relationship has been found between monocytosis (increased monocytes, the precursor of macrophages, circulating in the blood) and the severity of depression.[203] Once in the brain, increased macrophages can create an inflammatory state and increase PICs, which have emotional, cognitive, and behavioural effects (Figure 3).[77,81,173,175]

Neopterin levels can be considered an indirect marker of the amount of ROS and oxidative stress induced by the immune system.[145,165] ROS are produced by some immune cells, such as macrophages, to kill pathogens.[129,283] However, an imbalance between the production and accumulation of ROS can result in oxidative stress, which can damage several cellular structures, such as membranes, lipids, proteins, and DNA.[284] Oxidative stress has been found to increase the risk of cancer, atherosclerosis, rheumatoid arthritis, CVD, respiratory diseases, kidney diseases, and neurological diseases including Parkinson's disease, Alzheimer's disease, multiple sclerosis, and depression.[284] Additionally, psychological stress can increase oxidative stress, especially in students on the day of an exam.[285] There is also a link between anxiety and oxidative stress.[286-287] A causal role of oxidative stress in anxiety-like behaviours in rats has been established, however, causal roles rather than associative links require further research in humans.[287] Considering this, an imbalance of ROS, leading to oxidative stress, may provide a link between neopterin concentration and mental health symptoms.

The role of oxidative stress in anxiety, depression, and psychological stress may generate interest in using antioxidants in symptom prevention or reduction. One study has already found an inverse relationship in postmenopausal women between dietary antioxidant intake with DASS scores and some oxidative stress biomarkers.[288] Although, changes in hormone levels

due to menopause should be considered as postmenopausal women often present with chronic low-grade inflammation, perhaps due to reduced oestrogen, a potent anti-inflammatory.[289] Therefore, the effectiveness of using antioxidants as part of therapy in a student population or in males may vary and further research is required.

Neopterin concentration is also reflective of the levels of IFNγ as this cytokine stimulates neopterin production. In the present study, neopterin was found to correlate with the stress score, which complements a study that found IFNγ to increase after psychological stress.[190] As such, the concentrations and effects of IFNγ and neopterin may be linked. Research suggests systemic IFNγ and neopterin production is increased in depression, which may be due to activated macrophages.[74] The present study also found neopterin to correlate with the depression score. Furthermore, several studies have found IFNγ to impact brain function, thus this cytokine has been identified as a regulator of cognitive performance.[290] IFNγ can also impact levels of serotonin, dopamine, and glutamate by stimulating the activity of the enzymes IDO and GTP-cyclohydrolase I.[72,140,159,164,170,179-180] Additionally, the absence of IFNγ in the hippocampus, a region involved in memory and learning, has positive neuronal effects. These neuroplastic changes have been associated with improved performance in learning and memory tasks,[290] which is of particular importance in the context of university students. Therefore, IFNγ could be a therapeutic target for treating or preventing cognitive dysfunction associated with inflammation.[290] Another neuroplastic consequence of IFNγ is its effect on synaptic plasticity in the hippocampus,[290] which has been implicated in vulnerability to stress and mental disorders.[291] Consequently, IFNγ may be involved in some mental disorders and could be a potential therapeutic target to reduce symptoms related to cognition and inflammation. As neopterin is easier to measure than other cytokines like IFNγ,[143,145] it could be used to indirectly measure the effects of IFNγ and potential IFNγ antagonists on mental health.

As inflammation and mental health have a bidirectional relationship, not only does inflammation increase mental health symptoms as discussed, but mental health struggles can increase inflammation. For example, stress can cause inflammation via increased ROS,[74] PICs,[25,77] sensitisation and brain infiltration of macrophages,[25,195] glucocorticoid insensitivity,[66] and BBB disruption,[194] which in turn influence mental health symptoms. The idea that there is a relationship between stress and inflammation is supported by the results of the present study, whereby a correlation between stress and neopterin was found, which was the strongest correlation between neopterin and the DASS scores. But correlation does not mean causation; further research is required to establish the exact nature of the relationship between

neopterin and mental health facets. Neopterin may provide a connection between inflammation and mental health by linking immune system activation and neurotransmitter abnormalities.[144,152] Additionally, IFNγ has been associated with disturbances in serotonergic signalling[290] and tryptophan metabolism, which play a role in the pathophysiology of depression.[80]

An intriguing result from the present study is that Group B had a much smaller neopterin concentration range than Group A. There were also no outliers or large values in Group B (Figure 15). This may suggest that Group B was healthier than Group A in terms of inflammation, which might be related to their mental health. Although this cannot be proven from these results, other studies support such findings. Research has found that increased inflammation and cytokine activity in PTSD and other anxiety disorders can lead to a plethora of health conditions, including CVD, fibromyalgia, diabetes, chronic fatigue syndrome, musculoskeletal disorders, autoimmune disorders (e.g. rheumatoid arthritis), gastrointestinal disease, and irritable bowel disease.[292-293] Therefore, a pro-inflammatory state may provide a mechanism by which anxiety disorders are highly co-morbid with other diseases.[292] Depression also increases the likelihood of many of these diseases.[74] Furthermore, recent discoveries have found that the immune system and inflammation are involved in a wide variety of mental and physical health problems. Chronic inflammatory diseases constitute a large part of present-day morbidity and mortality and are the most significant cause of death worldwide – more than 50% of all deaths are attributable to inflammation-related diseases such as depression, CVD, stroke, cancer, diabetes mellitus, chronic kidney disease, autoimmune disorders, and neurodegenerative conditions. Chronic systemic inflammation is believed to be involved in the aetiology of these diseases.[132] Unlike healthy and necessary acute inflammation, chronic systemic inflammation typically occurs in the absence of an acute infectious insult. As chronic inflammation is low-grade and persistent, it causes collateral damage to tissues and organs over time; one such method is by inducing oxidative stress.[132] Essentially, there appears to be a high comorbidity between mental health, inflammation, and disease. Therefore, persons without chronic systemic inflammation and mental health conditions may have a lower risk of developing other diseases, such as inflammatory diseases which are a significant concern worldwide.

Another curious result regarding neopterin concentrations was the difference in distribution between Group A and B (Figure 15). Most of Group A (70%) had a concentration higher than 20 µmol neopterin/µmol creatinine, whereas most of Group B (80%) had a concentration lower than this. This is suggestive of the development of a potential threshold value for neopterin in

terms of mental health symptoms, as most of the participants with moderate to extremely severe symptoms were above 20 µmol neopterin/µmol creatinine and a large proportion of Group B was below this value.

In summary, there appears to be an association between neopterin and mental health symptom severity. The exact nature of the relationship requires further research. Several potential factors could be involved, including macrophage activation, IFNγ, oxidative stress, and neurotransmitter abnormalities. The correlation between neopterin and mental health scores may provide treatment ideas for mental health patients who present with elevated neopterin, such as using anti-inflammatory medication and antioxidants as potential adjunct therapies. However, since neopterin can be influenced by other factors, it should not be used solely as a marker or to discriminate severity but should be combined with other mental health measures.

4.3 Neurophysiological (ANS) Measurements

4.3.1 Heart Rate Variability

In this study, various parameters were used to measure HRV: mean RR, SDNN, RMSDD, LF peak, HF peak, LF power, HF power, LF/HF ratio, SD1 and SD2. Of these, only mean HRV, SDNN and SD2 showed a significant difference in means between Group A and B. This is suggestive of Group A having reduced ANS flexibility and resilience to stress[294] and that HRV is decreased among those with high mental health scores.

Group A had a significantly lower SDNN (the standard deviation of NN intervals) than Group B ($p < 0.05$). As SDNN increases, so does variability; therefore, Group A had reduced variability. Higher variability in heart rate has been associated with better health, self-regulation, adaptability, and resilience. Although, the 'normal' range for an individual is based on age and sex. It should be noted that females tend to have a higher mean heart rate, which means smaller NN intervals, and lower SDNN when compared to males. In addition, HRV time-domain measurements decrease with age.[223] These should be considered when interpreting the results of the present study given the difference between the oldest and youngest participant was 16 years and the sample was predominately female.

Group A had a significantly lower mean SD2 compared to Group B ($p < 0.05$). SD2 is similar to SDNN, so it is not surprising that both of these measures were significantly different between the groups. SD2 is calculated as the standard deviation of the points parallel to the line of identity in a Poincaré plot. It represents long-term variability in NN intervals and

sympathetic modulation.[295] Lower SDNN and SD2 in Group A suggest increased SNS activity (or reduced PNS activity) in individuals with mental health struggles. This is supported by other studies that found increased sympathetic activity in depression[296] and anxiety,[47] and reduced autonomic flexibility in patients with anxiety disorders.[297] Furthermore, it has been suggested that ANS imbalance is involved in the pathogenesis of anxiety and depression.[27]

The only HRV parameter that correlated with DASS scores was mean HRV, which correlated with stress, anxiety, and depression scores. This finding is supported by meta-analyses which also found that both anxiety[298] and depression[299] are associated with decreased HRV. Furthering this, HRV has been found to be even more reduced in patients with comorbid anxiety and depression.[300] However, the previously mentioned meta-analyses used studies that focused on RMSSD, LF, and HF, yet no significant differences or correlations were found in the current study with these parameters. The small sample size, age difference, recording time, measuring apparatus, or predominately female sample in the present study may provide an explanation for discrepancies with other studies. Additionally, measures like RMSSD and HF reflect parasympathetic activity, and the present study only found differences in parameters that reflect sympathetic activity. Yet, neither SNS Index nor LF/HF ratio was significantly higher in Group A, which would indicate sympathetic dominance.[223] Thus, the involvement of sympathetic and parasympathetic activity in HRV parameters and mental health needs to be further investigated.

In addition to being a potential marker for mental health, reduced HRV has also been associated with other negative health outcomes, such as diabetes and obesity.[301-302] More importantly, HRV has been associated with CVD and the risk thereof, as such HRV could be a predictor of CVD.[303-304] This provides a link between mental disorders and the high rate of comorbid CVD observed, in that both anxiety and depression increase the risk of CVD. In fact, anxiety can be considered a predictor or early marker of CVD risk.[298] Furthermore, comorbid anxiety and depression increase the risk of mortality and CVD by two- to threefold.[305] Given that CVD is the leading global cause of death,[306] this relationship is important to note. Worldwide, CVDs account for almost a third of deaths and comprise 20% of the total 'Years of Life Lost', a measure of premature death and the sum of each death multiplied by the standard life expectancy at each age.[306] Addressing mental health problems, particularly in student populations, not only impacts the present but may also help prevent future health problems. Furthermore, immune cells express receptors that can respond to ANS signals[188] and HRV is affected by the ANS. Thus, it is not entirely surprising that there could also be a link between HRV and inflammation. Indeed, correlations between inflammatory and oxidative stress markers and HRV have been found.[307] A meta-analysis of 51 studies found an inverse

relationship between HRV and inflammation, in which SDNN has the strongest association.[308] Therefore, reduced HRV could also be an indicator of inflammation.

The present study only found significant associations between mean HRV, SDNN, SD2, and mental health, which is inconsistent with previous findings. This highlights that there is some kind of relationship between HRV and mental health, but that further research is required. In addition, the relationship between sympathetic and parasympathetic balance in HRV parameters needs to be further investigated. Despite the discrepancies in the results between the present study and other studies, HRV should not be discounted as a viable biomarker as there is a large body of evidence to suggest otherwise. However, further research is required to determine the best parameters and to reduce the ambiguity regarding ANS balance in relation to HRV. Additionally, given the involvement of HRV in CVD, an advantage of using this biomarker could be monitoring cardiovascular health, which is a major concern. Possible interventions for those with reduced HRV and mental health struggles could include changes in diet and exercise, as these positively affect both.[298,309]

4.3.2 Blood Pressure

There were no significant differences in mean systolic or diastolic BP between Group A and B. In addition, BP did not correlate with stress, anxiety, or depression scores. The mean of each group was very close to the normal BP value (for the age group) of $\frac{120}{80}$ mmHg.

In terms of distribution, diastolic BP was similar for both groups. In contrast, Group B had much greater variation in systolic BP than Group A. The mean, median, minimum, and quartile 1, were similar between the groups, but Group B had a greater maximum and quartile 3. Quartile 3 was 10mmHg higher in Group B than in Group A (Figure 16). This is a puzzling finding as higher blood pressure is often associated with stress and anxiety.[310-311] A possible explanation for this result is that the participant sample may not have been representative of the greater population in terms of BP, and coincidentally, some members of Group B had high blood pressure for reasons other than mental health, such as high salt or alcohol intake, sedentary lifestyle, insulin resistance, or had a low potassium or calcium intake.[312]

As there was no significant correlation or difference in mean, the results suggest that BP would not make a good biomarker for stress and anxiety scores. This is compounded by the multiple other factors that can affect BP, other than mental health.

4.3.3 Blood-Volume Pulse

BVP sensors measure the vasodilation of microvessels in the finger; thus BVP can be considered an indirect marker of autonomic activity because vasodilation is affected by the ANS. Curiously, none of the BVP parameters showed a significant difference in mean between Group A and B, yet significant correlations with two of the parameters were found with the DASS scores: mean BVP amplitude negatively correlated with the anxiety (r_s = -0.344, $p < 0.05$) and depression scores (r_s = -0.368, $p < 0.05$), but not with the stress score. Additionally, the minimum BVP amplitude was also negatively correlated with the anxiety (r_s = -0.366, $p < 0.05$) and depression scores (r_s = -0.400, $p < 0.05$), but not with the stress score. It is thought that BVP decreases with increasing sympathetic activity, and increases with decreasing parasympathetic activity. [228-230] Therefore, the results of the present study suggest that BVP may decrease (and sympathetic activity may increase) as symptoms of anxiety and depression increase. This is supported by the correlations found with minimum BVP amplitude, which suggests that the lower the minimum value recorded, the greater the anxiety and depression symptoms. This is intriguing considering that stress is usually more acute and is regularly associated with activation of the sympathetic activity, yet BVP only correlated with anxiety and depression scores and not stress.

Unfortunately, not much research has been done concerning BVP and mental health. However, BVP reflects heart rate, which more studies have investigated and found that heart rate is often elevated in those with mental health conditions. For example, elevated resting heart rate is often found in psychiatric patients.[313] Additionally, a longitudinal study that used more than one million men found that resting heart rate during late adolescence could be a marker of subsequent psychiatric disorder development and that a higher resting heart rate was associated with an increased risk of developing anxiety disorders.[314] Since increased sympathetic activity (or decreased parasympathetic activity) increases heart rate and decreased BVP is associated with increased sympathetic activity, it is not surprising that there might be an inverse relationship between heart rate and BVP, hence their correlation with mental health. Additionally, as with HRV, BVP can be considered to be a marker of CVD and myocardial infarction.[315] As previously discussed, CVD is related to anxiety and depression, so it could be plausible to assume that BVP could also be related to these conditions.

The results from the present study suggest that BVP could be a potential biomarker for anxiety and depression scores, with the added benefit of reflecting aspects of cardiovascular health.

4.3.4 Electrodermal Activity

EDA is a basic and indirect measure of SNS activity. This is due to the sympathetic innervation of eccrine sweat glands, which affects EDA. EDA has been associated with both emotion and attention.[316] However, in the present study no significant difference was found in mean EDA between Group A and B. In fact, the central tendency and dispersion of mean EDA were similar between the groups. In addition, EDA did not correlate with the stress, anxiety, or depression scores.

The HRV and BVP results from the present study suggest increased sympathetic activation in participants with higher DASS scores, but the EDA results contrast this. A reason why EDA might not corroborate the implication of increased sympathetic activation in those with high mental health symptoms is because the SNS does not produce a uniform and simultaneous response in all sympathetically innervated systems, i.e. sweat glands might not be influenced by mental stress to the same magnitude as heart rate.[297] Another factor to consider is the environment; increases in temperature, relative humidity, and physical exertion can increase EDA.[317] The measurements from the participants were not taken on the same day, therefore changes in weather parameters could have had a confounding effect. Considering this, if EDA is sensitive to weather changes it might not be a good biomarker. An alternative solution could be to incorporate temperature into the EDA indices or having longer recording times to obtain EDA under a variety of conditions.

The lack of correlation between EDA and DASS scores is in contrast with the results of a systematic review which found EDA to be hypoactive in depression and that EDA could be useful in discerning depressive patients from healthy controls.[228] Another study also found that EDA could be used to classify stress into categories of Mild, Moderate, or Severe with ~95% accuracy.[318] However, the aforementioned study used a small sample (n = 18) and only healthy participants, therefore results from a larger sample of participants with mental health struggles could vary. Additionally, the Stroop test was used to induce periods of stress, which is in opposition to the present study where participants may have had chronic rather than acute stress and where EDA was measured at rest without any form of intervention. Similarly, studies used in the systematic review of EDA in depression[228] involved some form of stimulation of participants, such as pharmacotherapy, auditory or visual stimuli, or other activities that could induce changes in mental or emotional states. Therefore, the contrast in the results of the present study with those of other studies may be due to the measurement of EDA at rest rather than in response to a stimulus. As such, EDA might only be viable as a biomarker when measuring reactions rather than baseline values. In addition, the stimuli used in the aforementioned studies are not reflective of real-life situations and thus may produce

impractical results. Furthermore, the lack of correlation or difference in mean EDA in the present study could be due to comorbid symptoms of anxiety and depression, which have not been thoroughly investigated in relation to EDA. In summary, the results of the present study suggest that EDA is not a good biomarker for stress and anxiety scores, especially at rest.

4.3.5 EEG Power

Of the 19 electrodes used in the EEG, only six electrodes showed a significant association with the DASS scores. These electrodes resided in the frontal and left side of the brain, five of which were on the frontal lobe and the other one was located at the left mid-temporal lobe. These findings suggest altered function in the prefrontal cortex, which is involved in emotion, cognitive function, and motivation.[319]

The power of delta frequencies showed the greatest associations. The absolute power of delta at the FP1 ($p = 0.002$) and FP2 ($p < 0.001$) electrodes was significantly higher in Group A than in Group B. In addition, the relative power of delta at the FP2 ($p = 0.005$) and Fz ($p = 0.038$) electrodes were significantly higher in Group A than in Group B. The FP1 and FP2 electrodes measure activity from the left and right Brodmann area 10 respectively, and the Fz electrode measures Brodmann area 8 on the left. As such, the results are indicative of an increase in delta power in the prefrontal cortex of students with mental health problems. The location of FP2 appears to be largely involved and could be a predictor of the severity of the symptoms, given that the absolute power of delta at FP2 had a significant positive correlation with both the stress score and the anxiety score. This is suggestive of delta power in the prefrontal cortex increasing as stress or anxiety symptoms increase, which might make focusing and performing tasks difficult as the FP1, FP2, and Fz electrodes are associated with executive function (e.g. planning, decision-making, working memory), self-regulation, regulation of emotions, and social behaviour.[320] Delta waves were originally characterised as dominating during deep and restorative sleep. However, they can also largely appear during wakeful hours[321] increasing drowsiness while decreasing focus, attention, and awareness of the physical world.[320,322] A study found increased delta activity at rest in the prefrontal cortex of those practising Zen meditation, compared to controls. This is peculiar given the association between meditation and good mental health. However, the increased activity was interpreted as inhibition of the prefrontal cortex, which resulted in reduced cognitive and emotional engagement, described by the Zen meditators as detachment.[321] As such, the increased delta activity observed in the present study may increase the feelings of drowsiness and detachment in Group A. Additionally, Brodmann area 8 includes the frontal eye field, which is involved in visual attention and control of eye movements, therefore visual disturbances may also be present, exacerbating feelings of detachment from the surroundings.[323]

When looking at the mean relative power of delta, the mean was higher in Group A than in Group B at each electrode. This could be suggestive of a global delta increase, although only two of the means had a significant difference. Therefore, the trend observed could just be coincidental and the difference may not be large enough to be noteworthy. It would be curious to see if a similar trend could be found in other populations and if the difference would be significant.

Significant associations were also found in the alpha frequency band. In terms of the difference in mean between Group A and B, the relative power of alpha was significantly lower at the T3 electrode in Group A ($p = 0.033$). The mean was also lower at the FP1 electrode in Group A, although this difference did not reach significance ($p = 0.051$). This is suggestive of a relative decrease in alpha activity on the left side of the brain in relation to DASS scores. As alpha waves are associated with alertness and relaxation, low alpha can be indicative of anxiety.[320]

The relative power at the T3 electrode provided the most interesting findings in the alpha band, as it also negatively correlated with both the stress score ($r_s = -0.379$, $p = 0.016$) and the anxiety score ($r_s = -0.399$, $p = 0.011$). The T3 electrode records activity from the left temporal lobe, which is usually the dominant side in most people. It is involved in memory, learning, perception, hearing, speech, and understanding language.[324] Damage to this area can result in impaired memory, executive function, learning, speech and understanding thereof. Other effects include apathy, memory loss, and poor impulse control.[324] Specifically, the T3 electrode records activity from Brodmann areas 41 and 42 which form part of the primary auditory cortex and are found in the left anterior transverse temporal gyrus (also known as Heschl's gyrus). This area is involved in speech perception, sound intensity, pitch, auditory working memory, and the processing of auditory information. There is sparse information on the effect of alpha oscillations in the auditory cortex, however, some research suggests that alpha waves are involved in selective auditory attention, speech processing, and tinnitus.[325] Multiple studies have found reduced alpha activity in the temporal area of tinnitus patients; as alpha activity reflects inhibition in sensory regions, reduced alpha activity suggests enhanced excitability, resulting in tinnitus.[326-327] Although the alpha activity of the aforementioned study's patients was measured using magnetoencephalography instead of EEG, the measurements were taken in a resting state without any auditory stimulation, similar to the environment in which the measurements of the present study were recorded. Thus, it would have been interesting to know if the participants in the present study experienced any auditory abnormalities; unfortunately, no such information was obtained. Further highlighting the curiosity of this finding is that almost half of tinnitus patients also have a mental disorder, mostly anxiety and

depression, which correlates with the severity of tinnitus symptoms.[328-329] Perhaps reduced alpha at the T3 location could provide an explanation for the association between tinnitus and mental health. This is supported by studies which found that tinnitus is often subjective and associated with cognitive and emotional problems, rather than a physical auditory problem.[330] Furthermore, it has been postulated that stress can cause tinnitus. Tinnitus might work as an alarm signal, warning of potential danger.[331] Thus tinnitus only becomes disabling when people who are chronically stressed cannot cope with the effect of stressors and switch off the alarm signal.[331] As such, patients with tinnitus might have a higher tendency to respond to stress and have less effective coping strategies than healthy controls.[331] Tinnitus might be indicative of other auditory processing abnormalities occurring during mental distress. For example, another hearing phenomenon related to stress and anxiety is auditory exclusion, a form of temporary hearing loss that can occur in highly stressful situations. During fight-or-flight, priority is given to visual stimuli over auditory stimuli – hearing decreases as stress increases.[332] This phenomenon can be compared to 'tunnel vision'. These and other auditory aberrations might be related to difficulties experienced with dissociation, 'zoning-out', concentration, and social withdrawal in mental health conditions. Therefore, the relationship between decreased alpha at the T3 electrode and increasing stress and anxiety scores might be due to abnormalities in auditory processing contributing to symptoms and reduced functioning, however, further research would be required to substantiate this theory.

In terms of both delta and alpha waves, a study found increased delta power and decreased alpha power in elderly patients with mild cognitive impairment.[322] Therefore, it could be interpreted that participants in the present study with high DASS scores, which were associated with increased delta and decreased alpha, might be suffering from cognitive impairment, a symptom of mental health struggles.[333] An alternative interpretation is that changes in brain waves could help explain the cognitive impairment observed in patients with mental health problems. If cognitive impairment is related to the increased delta and decreased alpha, then this might negatively impact the academic performance of those with mental health symptoms; as discussed mental health struggles impact academic achievement.[265,273,275-277] However, caution should be exercised with these interpretations given the age difference between the present study and the study with elderly patients.

Furthering the idea of potential cognitive impairment in individuals with mental health struggles is the finding that there is a relationship between higher brain cortisol and cognitive impairment, and that there is an association between altered frontal EEG activity and increased cortisol.[322] Therefore, cortisol, especially chronically high levels, can alter frontal lobe activity and induce cognitive impairment.[322] Chronic stress and high cortisol can also

damage brain areas, reduce motivation, and increase the risk of mental health conditions such as depression.[322] Another study also suggested that cortisol can cause changes in the brain that increase activity patterns associated with anxiety.[334] As a result, further investigation into the relationship between cortisol, brain waves, and mental health symptoms would be of interest. Since high cortisol has been related to mental health conditions, it supports the idea of utilising frontal alpha and delta power as biomarkers for stress and anxiety scores.

The beta frequency is usually considered to be from 13 to 30Hz; hibeta is the upper part of this range, from 20 to 30Hz. Although no significant differences in the mean frequency of hibeta were found between the groups in the present study, hibeta in some locations was correlated with stress and anxiety scores. The relative power of hibeta at the F3 electrode positively correlated with both the stress ($r_s = 0.323$, $p = 0.042$) and anxiety scores ($r_s = 0.359$, $p = 0.023$). Two other electrodes also correlated: the relative power at the T3 electrode positively correlated with the stress score ($r_s = 0.333$, $p = 0.036$) and the F7 electrode positively correlated with the anxiety score ($r_s = 0.325$, $p = 0.041$). This suggests that hibeta increases in the position of F3 and T3 as stress increases, and that hibeta increases in the position of F3 and F7 as anxiety scores increase.

Excess hibeta has been associated with being tense and anxious, and it can be indicative of inefficient frontal alpha activity in areas associated with emotional control.[320] Considering that decreased alpha and increased hibeta were found at both the T3 and F7 positions in Group A, difficulties with emotional control could be associated with their mental health scores. Furthering this, decreased frontal alpha and increased hibeta is thought to be indicative of agitation, anxiety, feeling overwhelmed, and impulsivity.[320] As there may be an inverse relation between alpha and hibeta, increased hibeta and concomitantly decreased alpha at T3 may produce alterations in auditory processing as discussed.

The F7 electrode measures activity from Brodmann area 47, which is in the orbitofrontal cortex. The function of this area involves motivation, social behaviour, and emotional reactions. Interestingly, the orbitofrontal cortex has been implicated in disorders involving thinking, feeling, or fear, with altered activity during sadness, depression, and distress.[335] In conjunction with F3, activity at F7 is thought to regulate engagement, mood, processing of positive emotions, and conscious awareness. Alterations in alpha and hibeta activity in these areas may be linked to aberrations in these processes in mental health conditions. Specifically, increased hibeta at the F3 electrode is thought to indicate that a patient is hiding emotions and feelings, although this effect occurs with a simultaneous increase at FP2.[320]

The activity at three electrodes (FP2, F3, and T3) correlated with both stress and anxiety scores. This suggests that these regions are involved in both stress and anxiety symptoms. This is supported by previous research which found that brain regions involved in anxiety, such as the prefrontal cortex, are also implicated in the stress response.[40] The present study suggests that hibeta increases in the prefrontal gyrus with increasing stress and anxiety scores. It also suggests that hibeta increases, while alpha decreases in the left temporal lobe (near the ear), as either stress or anxiety score increases. Finally, findings from the present study showed an increase in the power of delta in the anterior prefrontal cortex with increasing stress or anxiety scores. See Table 22 for a summary.

No significant correlations were found between EEG power and depression scores. Therefore, one could speculate that elevated depression scores were a confounding factor and if it had been excluded from the study and only participants with high stress and anxiety scores were included, then more differences and stronger correlations could have been found. Alternatively, depression symptoms may not significantly affect the power at each electrode. However, these suggestions are unlikely to be true given that other studies have found differences in EEG patterns in depressed patients compared to healthy controls. Further research would need to be conducted to confirm the influence of depression on EEG recordings.

In summary, people with mental health struggles, particularly those with high stress and anxiety scores, might present with increased delta and hibeta, and decreased alpha activity, in the frontal and left side of the brain. Differences at the T3 and F7 locations occurred in more than one frequency, which might be related to difficulties with attention, focus, cognition, emotional regulation, and visual and auditory processing. Therefore, delta, alpha, and hibeta frequencies could potentially be used as biomarkers for stress and anxiety scores. See Figure 23 for a visual summary of the EEG findings.

4.4 Potential Biomarkers

The most promising biomarker in this study was neopterin concentration, as it showed a significant difference between Group A and B, and crucially, neopterin showed a strong positive correlation with all three of the DASS score categories. Therefore, neopterin could be of use in aiding the measurement of stress, anxiety, and depression scores. An advantage of using neopterin as a biomarker is that it can be obtained simply and non-invasively via a urine sample. Although neopterin concentration was determined in this study using an ELISA (considered to be costly for research purposes), other faster and more efficient methods such

as high-performance liquid chromatography can be used.[336] Chromatography may help to improve the speed and practicality of measuring neopterin. Although chromatography can be very costly, when implemented routinely (other than for research purposes) it may become more cost-effective.

There appears to be a negative relationship between mental health and HRV (mean HRV, SDNN, and SD2). Although the present study did not find the same associations in HRV parameters as other studies, HRV should not be discounted as a biomarker. More research is required to define the best parameters and ranges thereof that can be considered normal or at-risk. These definitions will need to produce consistent, reliable, and reproducible results. HRV may be a particularly important marker in students as it has been related to attention, emotional processing, and executive function,[337] which are important for university success. Additionally, monitoring HRV could help to negate the effects of mental health conditions and the development of CVD and other diseases later in life. As such, HRV not only represents a potential marker for aiding immediate mental health struggles but could help to potentially reduce future costs thereof. Lower BVP could also be a marker for mental health scores and potential risks thereof, such as CVD. As BVP and HRV both measure activity of the heart, they could easily be used in conjunction.

Due to the number of frequencies and electrodes that showed a difference with DASS scores, facets of qEEG readings could be used as viable biomarkers. However, there are some factors to consider such as recordings can vary depending on the state of the individual and EEG can be quite sensitive. Additionally, EEG requires a trained technician to perform. Further research is required to validate the findings of the present study and to verify changes in brain activity in relation to mental health scores.

In summary, the findings from the present study suggest that neopterin and aspects of HRV, BVP, and qEEG measurements could be potential biomarkers for mental health scores. This concludes the chapter discussing the results of the present study and the interpretation thereof. In the next chapter, the findings are summarised, and the limitations of the study and the future directions are considered.

Chapter 5

5. Conclusion

In this chapter, the findings and implications of the study are summarised. The limitations and future directions are also discussed.

5.1 Conclusion

This study found that neopterin and certain neurophysiological measures could be used as complementary markers for stress and anxiety symptom scores as determined by the DASS-21 questionnaire. This interpretation is supported by the statistical findings of significant correlations between some of the parameters tested and the DASS scores. It is also supported by significant differences found in the means of some parameters between Group A and B.

It was found that BP and EDA did not show a significant difference in mean between Group A and B nor did they correlate with scores of stress and anxiety. Whereas neopterin and certain parameters of HRV, BVP, and qEEG did correlate with stress and anxiety scores and showed a significant difference in mean between Group A and B. Neopterin concentration positively correlated with stress and anxiety scores, while HRV and BVP negatively correlated with these scores. Delta and hibeta activity increased in the left and frontal brain regions in participants with high DASS scores, whereas alpha activity decreased in these regions.

Therefore, HRV, BVP, qEEG, and neopterin may have potential to be used as biomarkers in conjunction with existing measures (e.g. questionnaires). These findings also indicate that there may be inflammatory and neurophysiology changes associated with increased stress and anxiety, which contributes to our understanding of mental health. Identifying physical changes associated with mental health conditions could be useful in the prevention, identification, and treatment of these struggles. This is prudent considering that anxiety is the leading mental health disorder, and that stress and anxiety are associated with inflammation, another major contributor to disease.

This study also found that the prevalence of stress and anxiety is high in this Health Sciences student population. Suggesting that more treatment, prevention measures, and self-regulation techniques are required to minimise the incidence of mental health struggles in these students. The university has a responsibility to implement measures to help reduce and prevent mental health struggles and to mitigate possible negative personal and professional consequences thereof. Possible interventions that could be employed include mindfulness and relaxation

techniques, anti-inflammatory medications, antioxidants, educational workshops, monitoring class attendance, and changes in exercise and diet.

5.2 Limitations

There are a couple of limitations to the study that need consideration. Firstly, as the measurements in the present study were taken from one moment in time, they may not reflect the average of the lives of the participants. The recording time was relatively short, and no follow-up measurements were taken at a later stage, as such the findings of the first set of measurements cannot be corroborated. Taking multiple measurements would have increased the credibility of the findings, which may have helped to explain the inconsistencies found in this study compared to others. However, the inconsistencies may also have been due to the small sample size used for the physiological measurements. In addition, as the total sample size for the physiological measurements was already pre-determined in terms of the maximum sample capacity (n = 40) of the neopterin ELISA kit, the group size power calculation of alpha, for various dependents, was not calculated. This was a limitation and can be improved on in future studies.

As the participant sample was from one faculty at a particular university, it raises the question of the generalisability of the findings to other universities and populations, as well as to other age groups, and those with different levels of education. These limitations should be taken into account when interpreting the results of the study. For example, the participants were between 18 and 34 years old, therefore applying the findings to older or younger groups would constitute extrapolation, as such the correlations and differences in the markers may not follow the same patterns at other ages. Additionally, the majority of the participants were female, which leads to potential sex bias – males and females may display significantly different values for each marker. Further, the ethnicity of the participants was not recorded. Although a diverse group was used, the ratios cannot be determined. A future study that stratifies participants would be interesting to determine if there are racial differences in markers.

Other lifestyle factors were also not recorded, such as exercise, all medications (e.g. oral contraceptives), supplements, sleep, diet, and alcohol consumption. Participants were also not asked if they had participated in intense exercise on the day or if they had an upcoming exam, which could have affected perceived anxiety. Extra questions were not incorporated into the survey and biographical questionnaires were not administered on the day of participation in order to avoid survey fatigue[252-253] and to be in line with the UP survey policy and the POPI act. However, these factors should be considered as a limitation of the study.

The research was not isolated to those with only anxiety or only stress; most of the participants in Group A shared moderate to extremely severe symptoms for at least two categories (stress, anxiety, or depression). This complicates the interpretation of the results due to possible confounding comorbidity.

Only the DASS-21 questionnaire was used for assessment. The participants were not evaluated by mental health professionals and no other questionnaire or measure was used to confirm the symptom ratings. In addition, the participants were a convenience sample of willing students and not randomly selected, which adds potential selection bias. There may also have been response bias in the questionnaire, due to respondents answering inaccurately. This may happen for several reasons including lack of self-awareness, fear of judgement, or lack of taking the questions seriously.

Most importantly, it would be erroneous to interpret that a single biomarker could be used to diagnose a mental health disorder. All the biomarkers used in this study can be affected by biological factors other than mental health, therefore they should be interpreted with caution and with professional psychological assistance.

5.3 Future Directions

Future directions should focus on improving the accuracy and convenience of biomarkers. A large number of studies have found potential biomarkers for mental health and other disorders, however, these biomarkers cannot be used yet in clinical settings due to conflicting results, small sample sizes, short-term measurements, and difficult-to-measure parameters. The present study also contributes to these obstacles, such as presenting results that conflict with previous study findings.

However, as we are living in an age of technology and Big Data, the feasibility of using biomarkers in clinical practice is greatly increased. The development of technologies such as artificial intelligence, particularly machine learning, allows for the analysis of large data sets through the use of complex algorithms. Machine learning models learn from data sets by finding patterns and making decisions which improve analysis. The accuracy of a model increases as more data is inputted, which allows for the continual updating and improvement of biomarker parameters and ‘normal’ and ‘at-risk’ values. This makes the use of biomarkers considerably more plausible and accessible than it has ever been. As such, future studies should incorporate these technologies in order to move away from the rudimentary values and

correlations that exist in previous biomarker research. For example, the incorporation of BVP into a computer model increased its accuracy as a biomarker.[232-233]

One way in which this data can be collected is through the use of wearable devices, such as smartwatches. They can provide a non-invasive, easy, cheap, and long-term method to improve biomarker research. They are also widely available and can easily be used by the public. The coalescence of the data from these devices into one database would allow for the use of machine learning and long-term measurements. However, policies regarding the storage and sharing of data and the ethics thereof should be considered. The combination of wearable devices and machine learning may be a viable future direction for research as some studies have already combined these to develop a model for stress detection.[338-340] Additionally, long-term, continuous measurements could help to improve the accuracy and validity of selected biomarkers as well as helping to account for the complexities, patterns, and effects of daily life, rather than being derived from artificial laboratory-based measures. Ideally, these measures could help to identify at-risk people and monitor the efficacy of treatments, which could aid an overburdened and strained public health sector. For example, the present study found HRV to be correlated with mental health scores and HRV is also related to the risk of CVD.[303-304] Thus, more research to improve the validity and accuracy of this biomarker may be of use. In addition, wearable devices could obtain other measurements such as sleep and exercise[341-342] which can be studied to determine if they influence the biomarker.

Another consideration for future research involving biomarkers is to focus on personalisation and inclusivity, especially due to the heterogeneity of mental disorders,[110,343] which means they should not be treated homogeneously. The consideration of age, sex, race, and other variables in biomarker research would allow for the stratification and collection of data from underrepresented groups, thus potentially reducing the gender and racial biases present in existing medical guidelines and practices, which are mainly based on measurements from Caucasians and men.[344-348] Furthermore, the heterogeneity of mental health struggles is worsened by the effects of intersectionality, whereby patients may belong to more than one group who are at higher risk of mental health conditions due to socio-economic status, environment, ethnicity, gender, and sexual orientation, compounding their risk.[267] Thus, the discovery and use of representative values for biomarker thresholds is vital in accurate application and the prediction of outcomes, especially in individuals with compounded risk.

In terms of the present study, wearable devices would help measure HRV, BVP and EEG. Although, the accuracy of wearing a headband to monitor EEG has been questioned,[349]

therefore measurement of EEG might still require visiting a professional. However, technology is developing to help improve the reliability of wearable EEG devices. For example, a wireless, low-cost EEG device has been used for emotion recognition.[350] In addition, machine learning can be used to improve emotion recognition using EEG.[351-352] Future directions involving EEG should focus on using machine learning to develop more accurate biomarkers for both clinical and personal use.

There is currently no wearable device or sensor available to measure neopterin. Therefore, future research with regard to neopterin should focus on determining whether oxidative stress, IFNγ, macrophages, the neopterin molecule itself, or inflammation in general are the cause/causes of correlation with mental health scores. Such findings may aid in developing new therapeutic targets. Perhaps in the future, neopterin quantification could become more convenient by using a non-invasive fibre laser, such as the laser that is currently available for the detection of glucose in the blood.[353] Alternatively, an electrochemical aptamer-based (E-AB) sensor could be used.[354-355] In theory, measuring neopterin using an E-AB sensor could become as convenient as current blood glucose measurements using a finger prick, if an appropriate aptamer for neopterin is discovered.[354,356] Alternatively, other inflammatory markers should be investigated to determine their relationship with mental health aspects, as well as establishing which inflammatory markers are the most reliable, convenient, and easiest to measure. However, the use of one biomarker should be cautioned against; as mentioned previously, models and algorithms which combine more than one measurement appear to be more accurate when assessing mental and emotional parameters.

Despite the appeal of widely using wearable devices and sensors as convenient ways of measuring biomarkers, there are negative aspects and difficulties to consider. Apart from privacy and information regulation concerns, wearable devices may contribute to negative outcomes, such as excessive monitoring, reduced enjoyment of beneficial activities, eating disorder symptoms, and hypochondriac behaviours.[357-359] Although, other research has found that wearable trackers can have positive effects on users, such as increasing motivation.[360] Therefore, the potential positive and negative consequences of such devices on the user should be carefully considered.

There are some factors to consider in terms of future directions and the feasibility of technological endeavours in the South African context. The wide-scale application of wearable devices to the South African population is greatly hindered by an overburdened public healthcare system, economic constraints, and a lack of infrastructure. These deficits in infrastructure relate to various aspects including education, connectivity, and information

technology.[361-363] Collectively, without these services it is difficult to access or utilise the necessary technologies, complicating the large-scale implementation of wearable devices in South Africa. In addition, there are other unique factors to consider when developing sensors for use in South Africa. If a finger-prick sensor for neopterin or other measurement involving blood was to be developed, the risk of spreading human immunodeficiency virus (HIV) needs to be taken into account, as HIV is highly prevalent in the South African population.[364] It then appears that the proposed future directions will be difficult to widely apply in South Africa and other methods should be considered.

As more people in South Africa have access to a smartphone than medical aid,[365-366] a possible solution may lie there. For example, a small pilot study used a smartphone camera for thermographic analysis in order to detect stress,[367] although more research is required, especially on how these measurements will be affected by environmental factors. Alternatively, as the present study used Health Science students, perhaps the focus should be on improving biomarker measurements for this population, which would be more feasible. However, in doing so there are ethical considerations, such as potentially widening inequality gaps and the potential misuse of smart devices in academic environments.

Although important considerations are required, technology, artificial intelligence, and Big Data are probably the most promising future direction for biomarkers. They are fast, relatively cheap, allow for long-term measurements, reduce problems associated with small sample sizes, increase gender and racial diversity in scientific values, and allow for the collection and analysis of large amounts of data and variables. In terms of the present study, further investigation into the differences in neopterin, HRV, BVP, and qEEG parameters in comparison to the mental health scores of different groups, different ages, and different sexes is required. Furthermore, studies that consider comorbidity and lifestyle should also be conducted to consolidate associations. Finally, studies that investigate the exact mechanisms that are involved are necessary, to establish causation and not just correlation.

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Appendix A: Ethical Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: 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 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0278 Approved for use through August 31, 2023.

Faculty of Health Sciences **Research Ethics Committee**

1 June 2022

Approval Certificate
New Application

Dear Miss RM Cronje

Ethics Reference No.: 210/2022
Title: Neopterin and neurophysiological measurements as markers of anxiety and stress

The **New Application** as supported by documents received between 2022-04-28 and 2022-06-01 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-06-01 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-06-01.
- Please remember to use your protocol number (210/2022) 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, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- 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

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, 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)

Research Ethics Committee
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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Appendix B: Informed Consent

ICD 1A

PARTICIPANT'S INFORMATION & INFORMED CONSENT DOCUMENT

STUDY TITLE: NEOPTERIN AND NEUROPHYSIOLOGICAL MEASUREMENTS AS MARKERS OF ANXIETY AND STRESS

Principal Investigators: Rouxzan Cronjé and Dr Priyesh Bipath

Institution: University of Pretoria

DAYTIME AND AFTER-HOURS TELEPHONE NUMBER(S):

Daytime number/s: 0123192424

Afterhours number: 0123192424

DATE AND TIME OF FIRST INFORMED CONSENT DISCUSSION:

			:	
Date	**month**	**year**	**Time**	

Dear Prospective Participant

Dear Mr/Ms/Mx. .....

1) INTRODUCTION

You are invited to volunteer for a research study. I am doing research for a MSc Human Physiology Degree purpose at the University of Pretoria. The information in this document is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this document, do not hesitate to ask the researcher. You should not agree to take part unless you are completely happy about all the procedures involved.

2) THE NATURE AND PURPOSE OF THIS STUDY

The aim of this study is to determine whether neopterin (a biomarker of inflammation) and certain non-invasive neurophysiological measures can be used as markers to identify persons at risk of anxiety and stress. Neurophysiological measures refer to the functioning of the nervous system; brain waves, heart rate, blood pressure and pulse will be the measurements used in this study. Identifying suitable biomarkers may contribute to the scientific understanding of mental well-being and help with the better management thereof.

3) EXPLANATION OF PROCEDURES AND WHAT WILL BE EXPECTED FROM PARTICIPANTS.

The first step in your participation will be the completion of a mental health questionnaire. This questionnaire consists of 21 questions and may take about 5-10 minutes to complete. The questionnaire will be administered online. It will be kept on a secure database to ensure confidentiality. You do not need to answer any questions that are of a sensitive nature to you.
You will be confidentially informed should the outcome of the questionnaire necessitate referral to a mental health practitioner. The Faculty of Health Sciences has a support system in place to assist students who are struggling with a psychiatric/psychological disorder or are experiencing stress or emotional problems. Services are offered free of charge to all students and therefore as a participant in this study, you may be informed about or directed to these support services of your own free will.

You may be invited to participate in the collection of physiological measurements. Your participation in this is not compulsory and you may opt-out at any time. The measurements we would like to take are quantitative encephalography (qEEG), heart rate variability (HRV), blood-volume pulse (BVP), electrodermal activity (EDA), and blood pressure (BP). EDA is a non-invasive measure of skin conductance. These measurements will require you to sit still for about 10 minutes. The electrodes for the qEEG will be placed on your scalp and a heart rate monitor around your chest. Sensors will also be placed on your ring, middle, and index fingers of your left hand. You may opt out of these measurements at any time. Finally, a urine sample donation will also be required. The urine will only be tested for neopterin concentrations (a biomarker of inflammation).

4) POSSIBLE RISKS AND DISCOMFORTS INVOLVED

There are no medical risks associated with the study.
The only possible discomfort that may occur is during the placement of the blood pressure monitor, heart rate chest strap and/or EEG electrodes.

5) POSSIBLE BENEFITS OF THIS STUDY

Although you may not benefit directly, the study results may help to improve our understanding of biomarkers and their potential use in diagnosis and therapeutic interventions relating to mental health and overall well-being.
Your results can be made available to you upon request. However, the data collected cannot be used or serve in any form as a diagnosis.

6) COMPENSATION

You will not be paid to take part in the study. There are no costs involved for you to be part of the study.

7) YOUR RIGHTS AS A RESEARCH PARTICIPANT

Your participation in this study is entirely voluntary and you can refuse to participate or stop at any time without stating any reason.

8) ETHICS APPROVAL

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 356 3084 / 012 356 3085 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving humans/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

9) INFORMATION

If I have any questions concerning this study, I should contact:

Rouxzan Cronjé Email: u17027617@tuks.co.za

10) CONFIDENTIALITY

All information obtained during the course of this study will be regarded as confidential. Each participant that is taking part will be provided with an alphanumeric coded number e.g. A001. This will ensure the confidentiality of the information so collected. Only the researcher will be able to identify you as a participant. Results will be published or presented in such a fashion that patients remain unidentifiable. The hard copies of all your records will be kept in a locked facility at the Faculty of Health Sciences, University of Pretoria.

11) CONSENT TO PARTICIPATE IN THIS STUDY

- I confirm that the person requesting my consent to take part in this study has told me about the nature and process, any risks or discomforts, and the benefits of the study.
- I have also received, read, and understood the above-written information about the study.
- I have had adequate time to ask questions and I have no objections to participating in this study.
- I am aware that the information obtained in the study, including personal details, will be anonymously processed and presented in the reporting of results.
- I understand that I will not be penalised in any way should I wish to discontinue the study and that withdrawal will not affect my further treatments.
- I am participating willingly.
- I have received a signed copy of this informed consent agreement.
- I confirm that I may be contacted and invited to participate in the physiological measurement aspects of the study.
- If any adverse health issues are detected, I may be contacted by the researcher and opt (or decline) to be anonymously referred to an appropriate health care provider.

_____	_____
Participant's name (Please print)	Date
_____	_____
Participant's signature	Date
_____	_____
Researcher's name (Please print)	Date
_____	_____
Researcher's signature	Date

Appendix C: Biographical Questionnaire

Age: _____

Year of study: _____

Degree: _____

Sex: Female Male Other

Please read the following carefully and respond truthfully.

1. Do you have epilepsy? Yes ☐ No ☐
2. Do you use recreational drugs? (regularly or recently) Yes ☐ No ☐
3. Are you using any medication that may alter EEG readings e.g. barbiturates, antidepressants, antipsychotics, antihypertensives, etc? Yes ☐ No ☐
4. **If you are unsure** whether your medication may alter EEG readings, please list your medication _____ _____
5. Are you taking anti-inflammatory medication? Yes ☐ No ☐
6. Have you had a chronic or recent infection (e.g. bacterial or viral)? Yes ☐ No ☐
7. Do you have an inflammatory disorder e.g. diabetes, Crohn's disease, inflammatory bowel disease (IBD), multiple sclerosis, Parkinson's, etc? Yes ☐ No ☐
8. **If you are unsure** whether your medication is anti-inflammatory or whether you have an inflammatory condition, please list here _____ _____

Appendix D: DASS-21 Questionnaire

DASS21

Name: Date:

Please read each statement and circle a number 0, 1, 2 or 3 which indicates how much the statement applied to you **over the past week**. There are no right or wrong answers. Do not spend too much time on any statement.

The rating scale is as follows:

0 Did not apply to me at all
1 Applied to me to some degree, or some of the time
2 Applied to me to a considerable degree or a good part of time
3 Applied to me very much or most of the time

1 (s)	I found it hard to wind down	0	1	2	3
2 (a)	I was aware of dryness of my mouth	0	1	2	3
3 (d)	I couldn't seem to experience any positive feeling at all	0	1	2	3
4 (a)	I experienced breathing difficulty (e.g. excessively rapid breathing, breathlessness in the absence of physical exertion)	0	1	2	3
5 (d)	I found it difficult to work up the initiative to do things	0	1	2	3
6 (s)	I tended to over-react to situations	0	1	2	3
7 (a)	I experienced trembling (e.g. in the hands)	0	1	2	3
8 (s)	I felt that I was using a lot of nervous energy	0	1	2	3
9 (a)	I was worried about situations in which I might panic and make a fool of myself	0	1	2	3
10 (d)	I felt that I had nothing to look forward to	0	1	2	3
11 (s)	I found myself getting agitated	0	1	2	3
12 (s)	I found it difficult to relax	0	1	2	3
13 (d)	I felt down-hearted and blue	0	1	2	3
14 (s)	I was intolerant of anything that kept me from getting on with what I was doing	0	1	2	3
15 (a)	I felt I was close to panic	0	1	2	3
16 (d)	I was unable to become enthusiastic about anything	0	1	2	3
17 (d)	I felt I wasn't worth much as a person	0	1	2	3
18 (s)	I felt that I was rather touchy	0	1	2	3
19 (a)	I was aware of the action of my heart in the absence of physical exertion (e.g. sense of heart rate increase, heart missing a beat)	0	1	2	3
20 (a)	I felt scared without any good reason	0	1	2	3
21 (d)	I felt that life was meaningless	0	1	2	3

DASS-21 Scoring Instructions

The DASS-21 should not be used to replace a face to face clinical interview. If you are experiencing significant emotional difficulties you should contact your GP for a referral to a qualified professional.

Depression, Anxiety and Stress Scale - 21 Items (DASS-21)

The Depression, Anxiety and Stress Scale - 21 Items (DASS-21) is a set of three self-report scales designed to measure the emotional states of depression, anxiety and stress.

Each of the three DASS-21 scales contains 7 items, divided into subscales with similar content. The depression scale assesses dysphoria, hopelessness, devaluation of life, self-deprecation, lack of interest / involvement, anhedonia and inertia. The anxiety scale assesses autonomic arousal, skeletal muscle effects, situational anxiety, and subjective experience of anxious affect. The stress scale is sensitive to levels of chronic non-specific arousal. It assesses difficulty relaxing, nervous arousal, and being easily upset / agitated, irritable / over-reactive and impatient. Scores for depression, anxiety and stress are calculated by summing the scores for the relevant items.

The DASS-21 is based on a dimensional rather than a categorical conception of psychological disorder. The assumption on which the DASS-21 development was based (and which was confirmed by the research data) is that the differences between the depression, anxiety and the stress experienced by normal subjects and clinical populations are essentially differences of degree. The DASS-21 therefore has no direct implications for the allocation of patients to discrete diagnostic categories postulated in classificatory systems such as the DSM and ICD.

Recommended cut-off scores for conventional severity labels (normal, moderate, severe) are as follows:

NB Scores on the DASS-21 will need to be multiplied by 2 to calculate the final score.

	Depression	Anxiety	Stress
Normal	0-9	0-7	0-14
Mild	10-13	8-9	15-18
Moderate	14-20	10-14	19-25
Severe	21-27	15-19	26-33
Extremely Severe	28+	20+	34+

Lovibond, S.H. & Lovibond, P.F. (1995). Manual for the Depression Anxiety & Stress Scales. (2nd Ed.)Sydney: Psychology Foundation.

Appendix E: Participant Flyer

Figure 28: Participant Recruitment Flyer. *The QR code is a placeholder and not the actual QR code that was used.*

Appendix F: Booking System

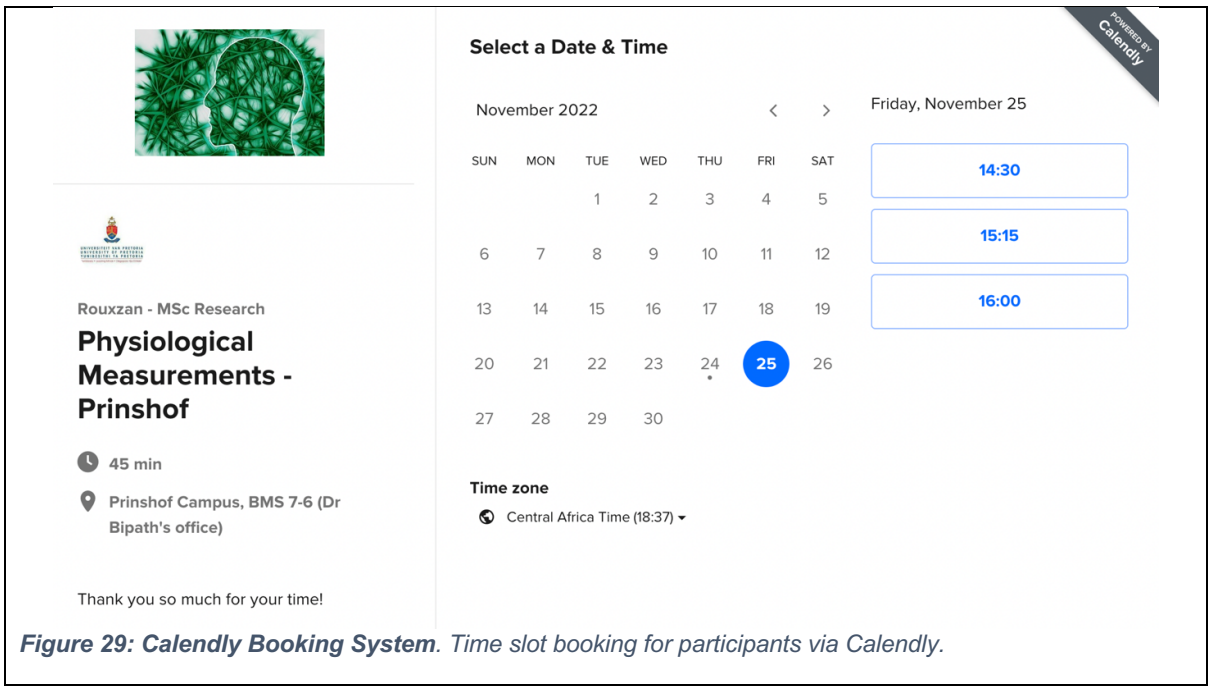


Figure 29: Calendly Booking System*. Time slot booking for participants via Calendly.*

Appendix G: Data Collection Setup

***Figure 30: Screenshot of EEG Activity**. A screenshot of the Brain Avatar software collecting raw data from the EEG electrodes.*

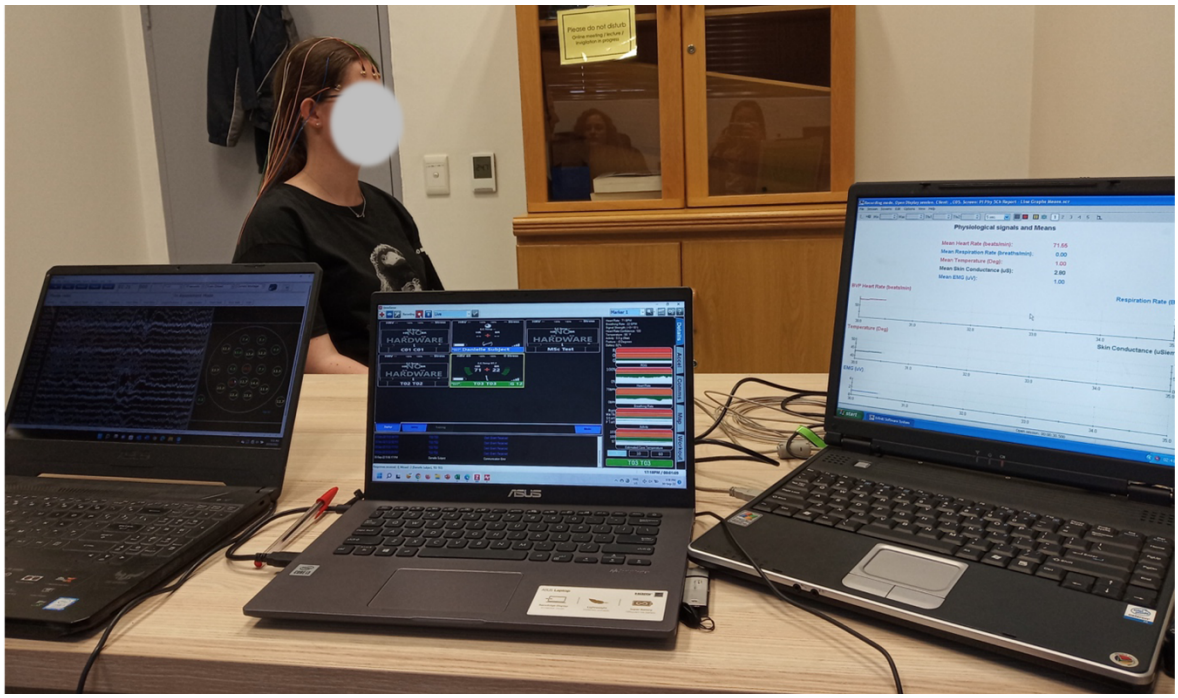
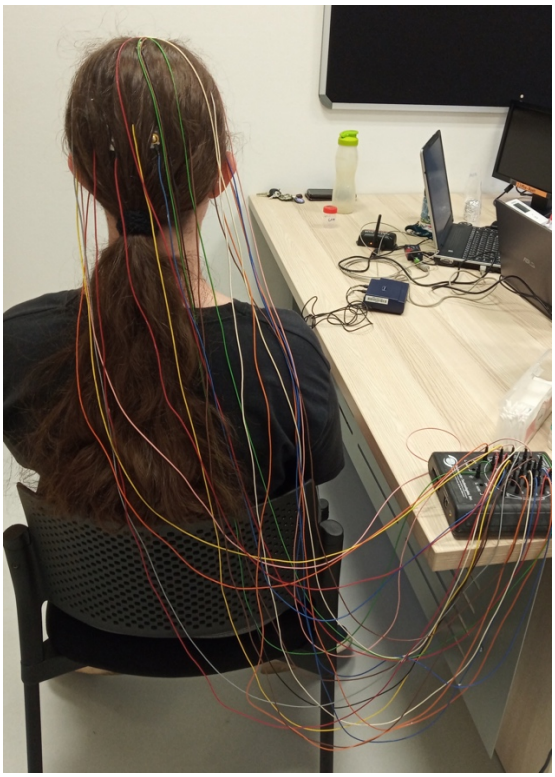
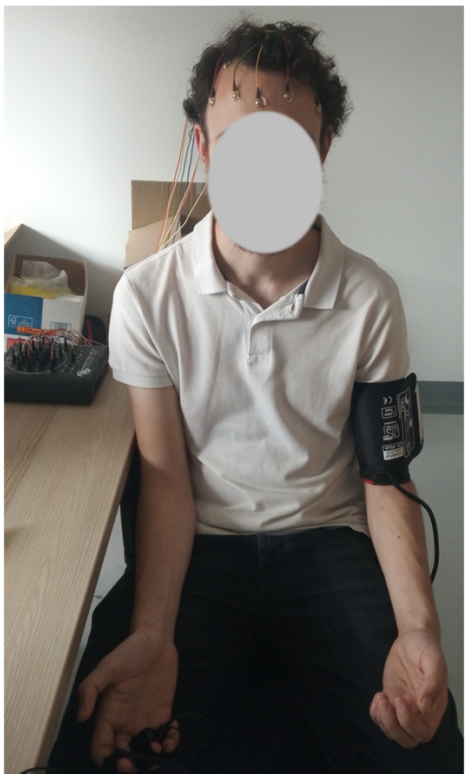


***Figure 31: Data Collection Setup.** The EEG, HRV and BVP software were each run on a separate computer.*

A



B



C

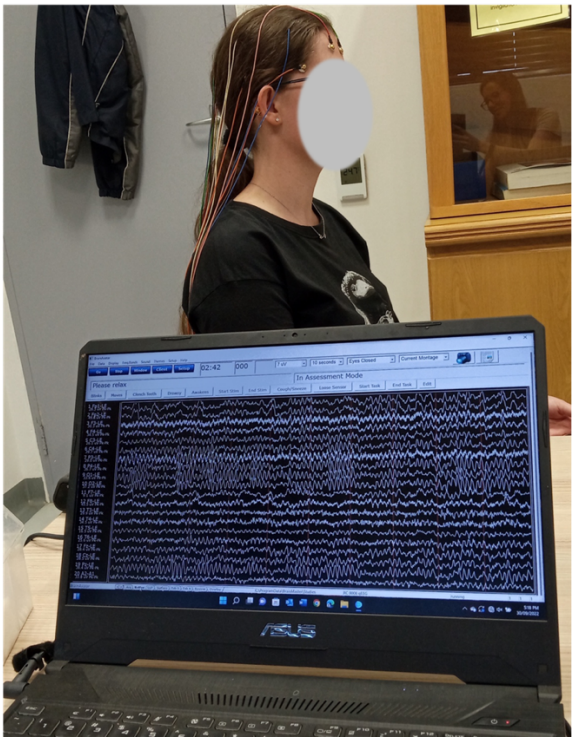


Figure 32: EEG Setup. *Picture A shows a view from behind all the electrodes. Picture B shows a view from the front and Picture C shows a view from the side and the computer screen recording the EEG.*