# Observing the Effects of Anxiety Levels on Male Reproductive Parameters

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# Declaration

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# Abstract

Literature presents a complex and often contradictory picture of the link between psychological stress and male fertility. Very limited information is available on the specific relationship between anxiety level and male fecundity. University students, when compared to the general population, exhibit signs of decreased mental health, with students having increased prevalence of depression, anxiety, psychosis and addictions. The aim of the study was to determine if there is a relationship between anxiety and reproductive parameters in a male student model and what that relationship is.

Twenty-one male participants between the ages of 20 and 25 were recruited as a part of this study. Participants were asked to donate at 4 different time-points. At each time-point, a semen sample and blood sample were collected and a State Trait Anxiety Inventory questionnaire was filled in. A semen analysis, presented in a spermiogram report, was performed using computer-aided sperm analysis to gather information about the basic semen parameters e.g. sperm concentration, sperm motility and sperm kinematics. The percentage fragmented sperm DNA, sperm nitric oxide level and semen reactive oxygen species levels were investigated. The cytokine profile and cortisol level were investigated in both the seminal plasma and blood plasma.

The student population observed in this study displayed increased anxiety levels when considering their state (39.0, SD=13.69, n=83) and trait (43.0, SD=12.55, n=83) anxiety scores, respectively. Blood plasma cortisol levels were noted to increase as state and trait scores increased. Cortisol was thought to have an overall immuno-suppressant effect both locally, in the reproductive tract, as well as systemically. Round cells were positively correlated to blood plasma cortisol levels and trait anxiety levels. Throughout the study it was observed that average path velocity (VAP) and straight-line index (STR) kinematic parameters decreased as state anxiety scores, trait anxiety scores and blood plasma cortisol levels increased. An inverse relationship between total motility and trait anxiety was

observed. The inverse relationship observed between elevated blood plasma cortisol and decreased spermatozoa viability and spermatozoa motility parameters is possibly a function of the increased number of round cells observed during this study.

These findings suggest a possible role for increased anxiety or psychological stress to elucidate idiopathic infertility by means of affecting the cytokine profile. The increase in number of round cells in this study could therefore be a result of increased release of immature germ cells from a compromised blood-testis barrier. The changes in the cytokine profile, round cells in the semen and sperm motility often correspond to values observed in men presenting with idiopathic infertility.

It can be concluded from this study that an increase in anxiety levels were found to have a negative relationship with male reproductive parameters.

# Opsomming

Die literatuur bied 'n komplekse en dikwels teenstrydige prentjie van die verhouding tussen sielkundige stres en manlike vrugbaarheid. Beperkte inligting is tans beskikbaar oor die spesifieke verband tussen angsvlak en manlike fekunditeit. In vergelyking met die algemene bevolking toon universiteitstudente tekens van verminderde geestesgesondheid, wat die voorkoms van depressie, angs, psigose en verslawing verhoog. Die doel van die studie was om vas te stel óf daar 'n verband bestaan tussen angs en voortplantingsparameters in 'n manlike studentemodel, en wat hierdie verband is.

Een-en-twintig manlike deelnemers tussen die ouderdomme van 20 en 25 is as deel van hierdie studie gewerf. Deelnemers is gevra om een semenmonster en een bloedmonster op 4 verskillende tydpunte te skenk. By elke tydpunt was 'n staats-trek angs inventaris (STAI) vraelys ingevul. 'n Semenalise, aangebied in spermiogram formaat is uitgevoer met behulp van rekenaargesteunde semenanalise om inligting oor die basiese semenparameters in te samel, bv. konsentrasie, motiliteit en spermkinematika. Die persentasie sperme met gefragmenteerde DNA, sperm stikstofoksiedvlakke en semen reaktiewe suurstofspesies vlakke is ondersoek. Die sitokienprofiel en kortisolvlak is in beide die seminale plasma en bloedplasma ondersoek.

Die studentepopulasie wat in hierdie studie ondersoek is, het verhoogde angsvlakke met betrekking tot beide hulle staat-angs (“state”) (39.0, SD = 13.69, n = 83) en trek-angs (“trait”) (43.0, SD = 12.55, n = 83) tellings, getoon. Daar is opgemerk dat bloedplasma-kortisolvlakke toeneem namate die staat-angs (“state”) en trek-angs (“trait”) tellings verhoog. Daar word vermoed dat kortisol 'n algehele immuunonderdrukkende effek het, beide plaaslik, in die voortplantingskanaal, sowel as sistemies. Ronde sel voorkoms het positief gekorreleer met bloedplasma-kortisolvlakke en trek-angs (“trait”) telling. Gedurende die studie is daar waargeneem dat die kinematiese parameters naamlik, gemiddelde pad snelheid (VAP) en

reguit-lyn-indeks (STR), afneem soos die staat-angs (“state”) en trek-angs (“trait”) tellings, sowel as bloedkortisolvlakke, verhoog. 'n Omgekeerde verband is waargeneem tussen totale spermmotiliteit en trek-angs (“trait”) telling. Die omgekeerde verband wat waargeneem is tussen verhoogde bloedplasma-kortisol en verminderde sperm lewensvatbaarheid en spermmotiliteit parameters is moontlik 'n funksie van die toenemende aantal ronde selle wat tydens hierdie studie waargeneem is.

Hierdie bevindinge dui op 'n moontlike rol vir verhoogde angs of sielkundige spanning om idiopatiese onvrugbaarheid toe te lig deur die sitokienprofiel te beïnvloed. Die toename in die aantal ronde selle in hierdie studie kan dus die gevolg wees van 'n verhoogde vrystelling van onvolwasse kiemselle van 'n beskadigde bloed-testis-skans. Die veranderinge in die sitokienprofiel, ronde selle in die semen en spermmotiliteit stem dikwels ooreen met waardes wat waargeneem word by mans met idiopatiese onvrugbaarheid.

Ten slotte, in hierdie studie is dit waargeneem dat 'n toename in angsvlakke 'n negatiewe verwantskap met manlike voortplantingsparameters gehad het.

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# Dedication

This body of work is dedicated to my exceptional grandparents, the late Albert de Jager and his wife Thelma de Jager. All the sacrifices you have made have allowed me to be here and my heart is overwhelmed with gratitude. I hope I can always make you proud.

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# List of Abbreviations

| | |
|---|---|
| % | Percent |
| °C | Degrees Celsius |
| µl | Microlitre |
| µm/s | Micrometres/second |
| $10^6$/ml | Million per millilitre |
| 5HT | 5-hydroxytryptamine (serotonin) |
| 5HTR | 5-hydroxytryptamine receptor |
| 5HTT | 5-hydroxytryptamine transporter |
| ACTH | Adrenocorticotropic hormone |
| ALH | Amplitude of Lateral Head Displacement |
| ANOVA | Analysis of variance |
| AR | Androgen receptor |
| BCF | Beat cross frequency |
| BD | Becton Dickinson |
| BDS | Bachelor of Dentistry |
| BIS | Septo-hippocampal behavioural inhibition system |
| BMI | Body mass index |
| BNST | Bed nucleus of the stria terminalis |
| BOH | Bachelor of Oral Health |
| BrdU | Bromodeoxyuridine/ 5-bromo-2'-deoxyuridine |
| Br-dUTP | Bromolated deoxyuridine triphosphates |
| BSA | Bovine serum albumin |
| CAF | Central Analytical Facility |
| CASA | Computer-aided sperm analysis |

| | |
|---|---|
| CNS | Central nervous system |
| CRF | Corticotropin-releasing factor |
| CRH | Corticotropin-releasing hormone |
| CST | Cytometer setup and tracking |
| DAF-2-DA | 4,5-diaminofluorescein-2/diacetate |
| DAF-2T | Triazolofluorescien |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| DSM | Diagnostic and Statistical Manual of Mental Disorders |
| dUTP | Deoxyuridine triphosphate |
| EDTA | Ethylendiaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FITC | Fluorescein isothiocyanate |
| FSC | Forward scatter |
| FSH | Follicle stimulating hormone |
| GABA | Gamma-aminobutyric acid |
| GB | Gigabytes |
| GHz | Gigahertz |
| GnRH | Gonadotropin-releasing hormone |
| $H_2O_2$ | Hydrogen peroxide |
| $H_2SO_4$ | Sulfuric acid |
| HPA | Hypothalamic pituitary adrenal |
| HPG | Hypothalamic pituitary gonadal |
| HRP | Horseradish peroxidase |
| Hz | Hertz |

| | |
|---|---|
| ICD | International Classification of Diseases |
| IFN-γ | Interferon gamma |
| IL-1β | Interleukin- 1 beta |
| IL-6 | Interleukin- 6 |
| IVF | *in vitro* fertilization |
| Kg | Kilogram |
| LH | Luteinizing hormone |
| LIN | Linearity index |
| M | Meter |
| max | Maximum |
| MBChB | Bachelor of Medicine, Bachelor of Surgery |
| MFI | Mean fluorescence intensity |
| Min | Minimum |
| ml | Millilitre |
| mM | Millimolar |
| ng/ml | Nanogram/millilitre |
| NO | Nitric oxide |
| no. | Number |
| $O_2^{\cdot -}$ | Superoxide |
| PBS | Phosphate-buffered saline |
| Pc | Personal computer |
| pg/ml | Picogram/millilitre |
| PI | Propidium iodide |
| PMT | Photomultiplier tubes |
| PVN | Paraventricular nucleus |
| RLU | Relative light units |

| | |
|---|---|
| RLU/sec/$10^6$ | Relative light units/second/million |
| RNase | Ribonuclease |
| ROS | Reactive oxygen species |
| rSD | System electronic noise |
| SASH | South African Stress and Health |
| SCA | Sperm Class Analyser ® |
| SD | Standard deviation |
| Sdv | Single dependent variables |
| SSC | Side scatter |
| STAI | State trait anxiety inventory |
| STR | Straight-line index |
| Streptavidin -PE | Streptavidin-phycoerythrin |
| SURRG | Stellenbosch University Reproductive Research Group |
| TdT | Deoxynucleotidyl-transferase |
| TMB | 3,3',5,5'- tetramethylbenzidine |
| TNF-α | Tumour necrosis factor- alpha |
| TSC | Total sperm count |
| US | Unstained |
| VAP | Average path velocity |
| VCL | Curvilinear velocity |
| VSL | Straight-line velocity |
| WHO | World Health Organization |
| WOB | Oscillation index |

# Chapter 1

# Introduction

## 1.1. Background

Semen analysis remains the standard to evaluate male factor infertility, the factor responsible for half of the couples presenting with infertility. A variety of factors influence sperm quality, leading to variability between individuals and within an individual over time. The factors could be a result of environmental effects, congenital defects or even psychological in nature. Psychological factors have been associated with cases of idiopathic infertility in both sexes (Vellani, et al., 2013).

One of these psychological factors is anxiety. Anxiety disorders were found to be the most prevalent lifetime disorder (15.8%) and present as the most prevalent class of anxiety disorders with an annual prevalence rate of 8.1% in the South African population (Herman, et al., 2009). University students, when compared to the general population, exhibit signs of decreased mental health. Students have been shown to have increased prevalence of depression, anxiety, psychosis and addictions (Aldiabat, et al., 2014). Risk factors leading to mental health disorders in university students specifically include: academic pressure, financial burden, the limited accessibility for minority groups to higher education, the imbalance between gender groups at the tertiary level, the detrimental effects of technology and the drastic life style change students experience while at university (Aldiabat, et al., 2014). Anxiety is noted as one of the most common mental health disorders amongst university students.

## 1.2. Motivation for study

The student population is one that is often sampled for research at tertiary institutions. Yet the effects of anxiety on male reproductive parameters have not been extensively studied in

a student population. Given the prevalence of increased anxiety levels in the student population, it is possible that the increased anxiety levels can negatively affect the reproductive parameters in this population. Insights into how self-reported psychological stress, and measured biological stress can affect semen samples on a basic and biochemical level can lead to researchers taking this into account when designing their future research protocols. Furthering the understanding of the effects of anxiety on the male reproductive system will assist in the improved management of pre-conceptual stress should conception be of interest (Virtanen, et al., 2017). For this reason, the relationship between anxiety levels and reproductive parameters will be investigated.

### 1.3. Thesis Layout

This thesis consists of an overview on the literature pertaining to the concept of anxiety, the biology and physiology of anxiety and its effects on the male reproductive system forming chapter 2. Chapter 3 provides a comprehensive breakdown of the materials and methods utilized in the study. The results and the discussion form chapter 4 and chapter 5, respectively. The conclusion forms chapter 6.

### 1.4. Study Setting

The study was performed at the Stellenbosch University Reproductive Research Group (SURRG) laboratory in the Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg.

### 1.5. Research Question

Will an increase in reported anxiety levels negatively affect male reproductive parameters?

### 1.6. Aim and Objectives

Therefore, the aim of this study was: to determine *if* there is a relationship between anxiety and reproductive parameters and *what* that relationship is.

This was achieved by completing the following objectives:

1. To determine the reported level of anxiety of the male students by means of the State Trait Anxiety Index (STAI) questionnaire.
2. To investigate the relationship between reported anxiety (STAI questionnaire) and the single dependent variables such as: basic sperm parameters, reactive oxygen species levels, nitric oxide levels, percentage DNA fragmentation, blood and seminal plasma cytokine profile and blood and seminal plasma cortisol levels.
3. To investigate blood and seminal plasma cortisol levels as a biological indicator of anxiety level.
4. To investigate the relationship between measured anxiety (blood and seminal plasma cortisol levels) and the single dependent variables such as: basic sperm parameters, reactive oxygen species levels, nitric oxide levels, percentage DNA fragmentation and blood and seminal plasma cytokine profile.

# Chapter 2

# Literature Review

## 2.1. Introduction

Semen quality is generally considered the measure of male fertility. Factors that affect the male's ability to impregnate a female can vary from endocrinological issues, anatomical variations, environmental and occupational stressors, as well as lifestyle factors, diseases and even something as unsuspecting as your state of mental health.

It is generally accepted that male factor infertility is responsible for half of all reported infertility incidences and refers directly to the male partner's inability to successfully impregnate a fertile female partner even after 12 months of unprotected intercourse (WHO, 2010). Clinically, the inability to produce a successful pregnancy after a couple has unprotected intercourse for a period of 12 months is defined as infertility (Zegers-Hoschshild, et al., 2009). Semen quality is merely a surrogate marker of male factor infertility and semen quality alone is not able to explain all instances of infertility. However, the semen quality is still a critical component to measure. The quality of the semen is quantified by assessing seminal parameters such as sperm concentration, motility, and morphology. With the advances in technology, different techniques such as the DNA fragmentation or reactive oxygen species are being investigated more regularly. These parameters are able to shed more light on possible causes of idiopathic, or unexplained, infertility.

Due to the complexity of the nature of fertilization and reproduction it is often difficult to pinpoint difficulties in conception. The possible effects of a decline in mental health, particularly increased psychological stress, has been investigated in literature as a possible factor involved in the reduction in male fertility. While there are differing opinions, the general consensus is that increased psychological stress has negative effects on the male

reproductive system. However, there is a very small pool of literature that focuses on the relationship between anxiety and the male reproductive system.

This literature review aims to elaborate on this relationship. First, mental health will be defined, and its relevance discussed. The concept of anxiety will be discussed as well as the biology, physiology, and pathophysiology of anxiety. The reader will then be provided with an overview of the male reproductive system followed by delving into what is known about how anxiety affects the reproductive system.

## 2.2. Defining the Relevance and Importance of Mental Health

Defining mental health is imperative to the understanding of mental illness. It is however an arduous task considering the differences in values, ideals and principles across countries, class, cultures, and genders. The World Health Organisation (WHO) has suggested that mental health be defined, in a broad sense, as a state of well-being in which the individual is able to realize his or her own abilities, is able to cope with the stresses of everyday life, is able to work productively and fruitfully and produce a contribution to society (WHO, 2004). Mental health forms the basis for the welfare and efficient functioning of an individual and for a community and, consists of more than just the absence of mental illness (WHO, 2004). Mental health and physical health are not mutually exclusive. Instead the mental health status, physical well-being and social functioning of an individual or community are integrated and interdependent. Mental, physical and social functioning are only considered mutually exclusive in the event that health is defined in a restrictive way i.e. as the absence of disease (Sartorious, 1990; WHO, 2004).

Individual factors and experiences, social-, psychological-, and biological factors influence the mental health of an individual. An individual's mental health influences other aspects of the individual's life and eventually influences the mental health status of the community or

population. Mental illness can be defined as a health condition in which an individual's thinking, feelings and/or behaviour changes negatively impacting the way in which the individual copes with the stresses of daily life, this causes the individual distress and results in difficulty in functioning (National Institutes of Health, 2007).

Mental illnesses are universally present, according to WHO, one in four people across the globe will be affected by mental or neurological disorders. Approximately 450 million people globally currently suffer from such conditions, making mental health disorders one of the leading causes of ill-health and disability worldwide (WHO, 2001). Depression is expected to be the second largest disease burden after ischaemic heart disease by the year 2020 (WHO, 2004). The global prevalence of anxiety disorders was 7.3% (4.8-10.9%) (Baxter, et al., 2013). A portion of South Africans (16.5%) suffered from common mental disorders like depression and anxiety in the year 2007, with 17% of those consisting of children and adolescents (Williams, et al., 2008). According to Herman et al. (2009), in South Africa anxiety disorders are one of the most common mental disorders with a 12-month prevalence rate of 8.1% specifically. Interestingly, the Western Cape, has the highest life-time prevalence of common mental disorders according to data collected by the South African Stress and Health (SASH) study at 42% (Herman, et al., 2009). The SASH study is the first large-scale study of the descriptive epidemiology of mental health in South Africa and although the study has limitations in its exclusion criteria and that the interviews were lay based, it produced valuable insights into the state of mental health in South Africa.

Despite the prevalence of mental health diseases, it is often regarded as a taboo topic for both governments and members of society. This is often to the detriment of society, as the mental health status of an individual is associated with the behaviour of said individual, throughout all stages of life. This is of relevance to university students who when compared to the general population, exhibit signs of decreased mental health, with students having

increased prevalence of depression, anxiety, psychosis and addictions (Aldiabat, et al., 2014).

The social factors associated with impaired mental health are related to increased drug and alcohol consumption, crime and drop out of school and risk behaviours such as unsafe sexual practices and physical inactivity (WHO, 2004). Poor health outcomes and physical morbidity are increased in instances of impaired mental health with poor general health occurring more often in individuals who report emotional distress e.g. evidence supports the link between depression and anxiety, and cardiovascular and cerebrovascular diseases (Carson, 2002; Kuper, et al., 2002; New York City Department of Health and Mental Hygiene, 2003; WHO, 2004).

Determinants of health are defined as factors that can either improve or threaten an individual's or community's health status (WHO, 2004). Whether these factors are a matter of individual choice or extend to socioeconomic or environmental factors outside of the individual's control; when the individual feels they are unable to cope with the demands of life it could lead to the individual perceiving themselves as 'feeling stressed'. Stress, although not classified as a mental illness, is a key risk factor that can lead to -and is associated with- the development or progression of mental illness (Aldiabat, et al., 2014).

Anxiety is one of the most common mental health disorders amongst university students. Risk factors leading to mental health disorders in university students specifically include: academic pressure, financial burden, the limited accessibility for minority groups to higher education, the imbalance between gender groups at the tertiary level, the detrimental effects of technology and the drastic life style change students experience while at university (Aldiabat, et al., 2014).

## 2.3. Defining the Concept of Anxiety

The concept of anxiety finds its origins in the Classical Greek period (McReynolds, 1975; Endler & Kocovski, 2001). Anxiety can appear as a relatively ambiguous concept because it has been conceptualized in various ways. Aubrey Lewis defined the term anxiety "as an emotional state, with the subjectively experienced quality of fear as a closely related emotion", he further elaborates that the emotion experienced is unpleasant, out of proportion to the perceived threat, is future directed and the emotion experienced involves subjective aspects and manifest bodily disturbances (Endler & Kocovski, 2001). Therefore, two crucial features to consider when defining anxiety, is the strong negative emotion and the element of fear experienced, and that anxiety is experienced psychologically, physiologically and behaviourally (Steimer, 2002; Hartley, 2008).

It is also imperative that the positive features of anxiety are also considered. The feeling of anxiety acts as a warning signal for imminent danger or harm and is able to induce motivation; therefore, some anxiety is adaptive and is usually at the low end of the anxiety continuum (Endler & Kocovski, 2001). Anxiety disorders, however, fall on the opposite end of the anxiety continuum and result in the individual experiencing a severe amount of anxiety that impairs daily functioning and is considered maladaptive.

There is very little consensus on the global prevalence of anxiety disorders. Current prevalence estimates varied between the ranges of 0.9% and 28.3% in a systematic review and meta-regression analysis conducted by Baxter et al. (2013). Studies (87) from 44 different countries conducted between 1980 and 2009 were analyzed and the past year prevalence ranged between 2.4% and 29.8%. The suggested explanations for the large variation in data include factors such as gender, age, and culture. These factors have been suggested to account for most of the variability, and methodological factors such as prevalence period and diagnostic instruments are thought to explain the remaining variability (Baxter, et al., 2013). When methodological differences were adjusted for the global current

prevalence of anxiety disorders was 7.3% (4.8-10.9%). Interestingly, anxiety prevalence was found to be higher in Euro/Anglo cultures at a rate of 10.4% (7.0-15.5%) compared to African cultures at a rate of 5.3% (3.5-8.1%) (Baxter, et al., 2013).

Anxiety disorders were found to be the most prevalent lifetime disorder (15.8%) and present as the most predominant class of anxiety disorders with an annual prevalence rate of 8.1% in the South African population (Herman, et al., 2009). In an American study it was found that of the 2843 participants an estimated 15.6% of undergraduates and 13.0% graduate students presented with a depressive or anxiety disorder (Eisenberg, et al., 2007). In a more local setting 17.8% of 214 first-year students from a historically black university in South Africa presented in the severe range of the Beck Anxiety Inventory (Pillay, et al., 2001).

There are many ways to measure anxiety. The method by which anxiety is measured ultimately depends on what the investigator aims to achieve. The four main subtypes of anxiety that are usually differentiated in literature include post-traumatic stress disorder (PSD), phobic disorders, panic disorders and general anxiety disorder (Rose & Devine, 2014). Each subtype presents with different symptoms, therefore when picking an appropriate tool to measure anxiety, it should be decided whether the focus will be on the more generic symptoms of anxiety that exist between several anxiety disorders, or to focus on symptoms of a particular disorder. Once a tool has been picked, it became evident that cut-off values need to be defined in order to separate pathologies from natural phenomena (Rose & Devine, 2014). Documents such as the Diagnostic and Statistical Manual of Mental Disorders (DSM) or International Classification of Diseases (ICD) serve to aid in the diagnosis of anxiety disorders. Typically, a screening tool should supply good sensitivity i.e. the likelihood that patients suffering from a particular disorder are identified. As well as specificity i.e., the likelihood that the individuals found to be differing from a disorder are in fact suffering from said disorder. Anxiety measurement tools, therefore, can be grouped into specific tools and generic tools with specific tools measuring specific traits of an anxiety

disorder and generic tools measuring the shared characteristics that appear in multiple anxiety disorders. Some of the latter include the Beck Anxiety Inventory and the anxiety subscale of the Hospital Anxiety and Depression Scale (Julian , 2011). One of the generic tools is the State Trait Anxiety Inventory.

Cattel and Schleier are often credited with introducing the terms trait anxiety and state anxiety (Cattell & Schleier, 1960). While in the year 1966, Spielberger popularized the concept of multifaceted definitions of the term anxiety by introducing the State Trait Anxiety Inventory (STAI) questionnaire. Trait anxiety is defined as an individual's predisposition to respond to a perceived threat. State anxiety referred to the transient emotion characterized by physiological changes accompanied with consciously perceived feelings of dread, tension, and a sense of apprehensiveness. The level of state anxiety is dependent on both the person (trait anxiety) and the situation causing stress (Endler & Kocovski, 2001). The STAI questionnaire consists of twenty state questions that enquire as to how you feel 'right now' and 20 trait questions enquiring how you feel 'generally'. It has good psychometric properties, displays good retest reliability and has proven validity. The test has a sensitivity of 0.82 and a specificity of 0.88. There are independently created short versions available as well as normative data for various age groups or in various conditions e.g. rheumatoid conditions (Rose & Devine, 2014).

Biologically when an individual is experiencing anxiety, they experience increased mental arousal and expectancy as well as autonomic and neuroendocrine activation (Steimer, 2002). Another phenomenon that causes a very similar biological response is the phenomenon known as fear. Steimer (2002) alluded to anxiety as being a more elaborate form of fear as it allows the individual to adapt and prepare for the future. However, two of the main differences in the definitions of fear and anxiety, is that in the case of anxiety the threat is often unknown or internal while in the case of fear, the object is real and often external.  It is difficult to distinctly distinguish fear from anxiety because many definitions of

anxiety refer to an element of fear and biologically there is overlap between the brain and behavioural mechanisms of the two however for the purposes of this review they will be considered as two distinct emotional states. Both these emotional states culminate into an array of adaptive or defensive behavioural responses (Steimer, 2002).

## 2.4. The Biology and Physiology of Anxiety

Anxiety is experienced in a myriad of ways. The experience can be separated into affecting four dimensions of life: behaviourally, emotionally, in thinking and attitude patterns and in the physiology of the body. Each person suffering from anxiety has their own distinctive pattern which displays elements from all four dimensions but usually one dimension is particularly afflicted.

### 2.4.1. Psychological Effects of Anxiety

#### 2.4.1.1. Behavioural

The way the person who is undergoing an anxiety episode behaves is often dependent on where the symptoms are occurring and the severity of the symptoms. Some behaviours can alleviate the symptoms e.g. when experiencing a panic attack in a crowded area leaving the area can make the individual feel much better. Other behaviours are known to actually make the symptoms experienced worse e.g. trying to breathe more during a panic attack and instead hyperventilating. These behavioural changes can result in an individual constructing their life so as to avoid triggering the unpleasant symptoms.

#### 2.4.1.2. Emotional

This aspect of anxiety can be very difficult to describe. Often descriptors such as ‘general uneasiness’ or ‘panicky feelings’ are used, and they fail to depict the depersonalization and derealization experienced by the individual. The feeling of ‘unreality’ mixed with fear, a sense of sadness and hopelessness, shame, frustration, and the inability to fully explain the

emotions experienced render the emotional aspect of an anxious state particularly unpleasant.

#### 2.4.1.3. Thought patterns

The physical manifestations of anxiety are often accompanied by anxiety inducing thoughts. Although it may be difficult to recall all the thoughts or images flooding the mind in the anxious state, these thoughts often contain sentiments of personal failure or uncertainty. 'I am going to die', 'I am making a fool out of myself again', 'I cannot do this' or 'nobody can help me, I have to pull it together' are typical examples of these thoughts. Thoughts like these can alter the individual's perceptions of themselves and the world.

#### 2.4.1.4. Physical

During the anxious state, the body can be affected in various ways. Common complaints include increased heart rate, shakiness, dry mouth, increased sweating, dizziness, and weakness. Pain, muscle tension and tension headaches are also reported. Less frequently the individual will be overwhelmed with nausea and vomit or in certain cases simply be frozen on the spot (Cobb, 1982).

The physical afflictions described above result as a consequence of activation of the autonomic system. The 'anxious response' often resembles the 'stress response'. Stress can be defined as the body's response to demands (Koob , 1999). During the stress response the hypothalamic pituitary adrenal (HPA) axis is activated. The sympathetic response from the autonomic branch is also activated resulting in the physical changes we experience when stressed regardless of whether the stressor is systemic or processive (Maier & Watkins, 1998).

An anxiety response can result in two possible coping strategies. During the active coping strategy, the 'flight-or-flight' response is activated as a result of sympathetic system activation (Steimer, 2002). Passive coping strategies are a result of the less significant

occurrence of activation of the parasympathetic nervous system and culminate in immobilization of the individual as a result of autonomic inhibition, increased neuroendocrine response, marked HPA-axis activation and increased glucocorticoid secretion (Steimer, 2002). The distinct coping reactions to different stressors appear to be mediated by specific brain circuits (Keay & Bandler, 2001; Steimer, 2002). The behavioural and neuroendocrine patterns and not just the contextual clues of the anxiety i.e. if there is an exit available for the individual to leave the crowded venue, also explain the coping style utilized.

When exploring the biology of anxiety more in depth it is only natural to investigate aspects of the functional neuroanatomy involved in the anxious response.

### 2.4.2. Effect of Anxiety on the Structure and Function of the Brain

#### 2.4.2.1. Locus Coeruleus

The locus coeruleus is defined as 'the nucleus of the hindbrain that gives rise to a massive noradrenaline containing projection throughout the neuroaxis' (Dajas, et al., 1992). This major source of noradrenaline/ norepinephrine plays a crucial role in modulating arousal (Counts & Mufson, 2012). Increased arousal and autonomic activation are synonymous with the anxious state. The locus coeruleus has been shown to be highly responsive during the anxious state and it has been suggested that the ascending noradrenergic system originating from the locus coeruleus is where the anxious feelings are organized (Steimer, 2002).

#### 2.4.2.2. The Septo-hippocampal System

The septum and the hippocampus are anatomically strongly linked and are separated from the rest of the brain by ventricles. The septo-hippocampal behavioural inhibition system's (BIS) primary function is to compare actual stimuli with expected stimuli (Gray, 1972). If discrepancies arise between the actual and expected stimuli the BIS is activated (Grey , 1994). Therefore, it was hypothesized that anticipatory anxiety is mediated by the activation

of BIS and that this activation of the BIS is usually a result of uncertainty or novelty (Grey, 1987; Steimer, 2002). Literature argues that the role of the BIS activation is not causal to anxious response but that it is rather correlated or supportive thereof (Panksepp, 1990). The septo-hippocampal system is still however thought to yield a fundamental role in the creation of the emotional state of anxiety.

#### 2.4.2.3. The Amygdala:

Electrical stimulation in three different areas of the brain will elicit a full fear response: the anterior and medial hypothalamus, specific areas of the periaqueductal gray, and the central and lateral zones of the amygdala (Steimer, 2002). The amygdala is defined as 'an almond shape set of neurons located deep in the brain's medial temporal lobe' (Williams, 2018). Although the amygdala's role in the perception of emotions such as anger and fear are well known, literature seems to find no solid basis of support for the amygdala's role in the anxious state. Instead it is noted that activation of bed nucleus of stria terminalis, accepted to not be a part of the extended amygdala, by corticotropin releasing factor (CRF), is more specific for anxiety (Aggleton, 2000).

#### 2.4.2.4. The Prefrontal Cortex:

The prefrontal cortex is involved in the analysis of complex stimuli and the control of emotional responses (Steimer, 2002). It was suggested that the septo-hippocampal activity is influenced by the prefrontal cortex by Grey (1987) upon revising his BIS model. This has since been confirmed in studies involving both primates and humans. The dorsolateral, ventromedial and orbital sectors have been identified to have a role in the control of emotional responses, although there appear to be functional differences between the left and right sides of these specific sectors (Davidson & Irwin, 1999; Davidson, 2002; Steimer, 2002). Activation of the prefrontal cortex in an asymmetric fashion is possibly associated with vulnerability to mood and anxiety disorders (Gainotti & Caltagirone, 1989). In a study on rats it was found that lesions on the right of the prefrontal cortex were shown to have

anxiolytic effects and were better at the suppression of neuroendocrine and autonomic stress response (Sullivan & Gratton, 2002). Therefore, the role of the prefrontal cortex in modulating anxiety consequently seems to deem cerebral laterality and hemisphere location as an important feature.

### 2.4.3. Effect of Anxiety on the Sympathetic Nervous System

There are a myriad of neurotransmitters, neuromodulators, hormones, and peptides with roles in the anxious response. A few examples will be highlighted below:

#### 2.4.3.1. Noradrenergic System:

Noradrenaline, also known as norepinephrine, is a hormone that is released by the adrenal medulla and the sympathetic nerves. In the body its primary function is to serve as a neurotransmitter, particularly during the fight-or-flight response (Encyclopaedia Britannica Inc, 2018). Literature has shown that both the stressed and anxious states can culminate in a significant increase in noradrenaline release in multiple regions of the rat brain including the amygdala, locus coeruleus and the hypothalamus. However, literature is conflicted on the true role of the noradrenergic system in the role of anxiety (Steimer, 2002).

#### 2.4.3.2. Serotonergic System:

It is very difficult to discern whether a neurochemical plays an anxiolytic or anxiogenic role without first considering which receptor subtypes the compound binds to and where in the brain this is taking place (Steimer, 2002). 5-hydroxytryptamine (5HT) better known as serotonin is a neurotransmitter/hormone that is involved in various neurological and physiological processes (Dictionary.com, 2018). 5HT receptors (5HTR) are grouped into 7 different groups depending on distribution, molecular structure, function and cell response. The 7 receptor families can be individually subdivided into subtypes (Štrac, et al., 2016). It has been reported that $5HTR_{1A}$ knockout mice exhibit an ‘anxious’ phenotype at a behavioural and autonomic response level (Gingrich & Hen, 2001; Pattlj, et al., 2002). Mice without the serotonin transporter (5HTT) gene also displayed an increased level of anxiety

in behavioural tests, as well as increased ACTH compared to the 5HTT heterozygote population (Nutt & Mallzia, 2001).There is therefore evidence supporting the possible roles of both $5HTR_{1A}$ and 5HTT gene expression in the modulation of anxiety.

#### 2.4.3.3. GABAergic System:

Gamma-aminobutyric acid (GABA) is known to be the most abundant inhibitory transmitter in the central nervous system (CNS) with a third of all CNS neurons thought to be GABAergic (Lydiard, 2003). GABA works to offset the excitatory action of glutamate to achieve homeostasis and subsequently prevent hyperexcitement of the neurons and CNS arousal. The receptor $GABA_A$-benzodiazepine is a target for anxiolytic drugs (Steimer, 2002). Individuals presenting with various anxiety disorders have been shown to display downregulated GABA systems. These individuals were shown across literature to exhibit diminished benzodiazepine binding compared to control subjects as well as decreased brain levels of GABA compared to healthy individuals (Lydiard, 2003). Multiple $GABA_A$ receptor subtypes have been defined. Receptors that have the $\alpha_1$ subunit make up the majority of the $GABA_A$ receptors and are largely expressed in the cerebellum and thalamus whereas the $\alpha_2$ subtype are expressed predominantly in the amygdala, striatum and hippocampus (Steimer, 2002; Lydiard, 2003). The $\alpha_2$- $GABA_A$ subtype is thought to be involved in anxiolysis, as a point knock-in mutation of the subunit in a study on mice suppressed the anxiolytic action of diazepam (Rudolph, et al., 2001; Möhler, et al., 2002).

The HPA-axis, so often mentioned, is the meeting of two vital body systems: the central nervous system and the endocrine system, to form the core stress response for our bodies and is shown in *Figure 1*. Simply explained, when the body experiences a stressor, it responds by the release of CRF from the hypothalamus. CRF is also known as corticotropin-releasing hormone (CRH). Upon CRF's binding to CRF receptors situated on the anterior pituitary gland a hormone known as adrenocorticotropic hormone (ACTH) is released. Receptors for ACTH are found on the adrenal cortex and when ACTH binds to these

receptors it stimulates the adrenal release of cortisol. The release of cortisol is continued even once the stressor has passed until it reaches a circulatory cortisol level enough to achieve a protective effect and activate the negative feedback loop of decreasing the release of CRF and ACTH from the hypothalamus and pituitary respectively, until homeostasis is achieved.

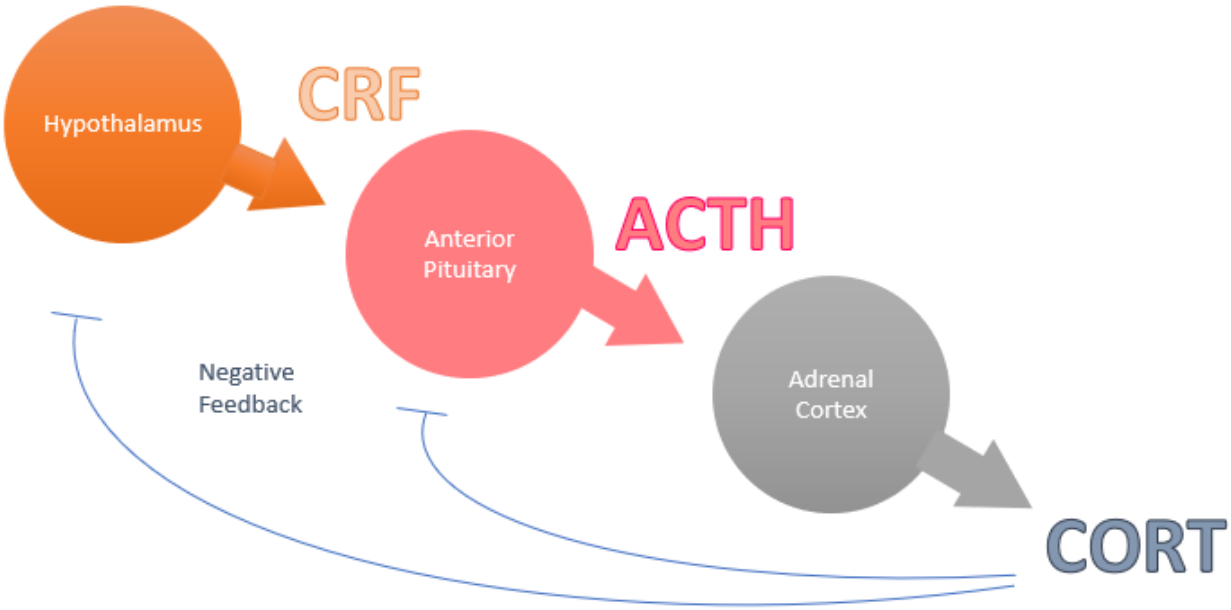



Figure 1 Simplified diagram describing the HPA-axis adapted from (Xiao, 2015).

During the anxious response, a similar increase in cortisol is observed. Similarly, a neuroendocrine response results which culminates in the release of elevated levels of norepinephrine and epinephrine. The hormone prolactin as well as growth hormone are also released (Hoehn-Saric & McLeod, 2000). Corticotropin-releasing factor administered intracerebrally to rats has shown to cause them to display anxious-like behaviour (Dunn & Berridge, 1990). Two CRF systems exist in the brain, one consisting of neuroendocrine system the paraventricular nucleus (PVN) of the hypothalamus and the other of CRF cells found in the bed nucleus of the stria terminalis (BNST) and amygdala (Steimer, 2002). The latter of which is more directly associated with the physiological responses accompanying the anxious response. Glucocorticoids are known to decrease CRF production via the

neuroendocrine negative feedback loop in the PVN, conversely glucocorticoids seem to increase the expression of CRF in the BNST and the amygdala and subsequently increasing the anxious response (Shulkin, et al., 1998). Overexpression of CRF in transgenic mice display a neuroendocrine and behavioural profile comparable to elevated anxiety levels including increased ACTH and corticosterone levels (Stenzel-Poore, et al., 1994; Bakshi & Kalin , 2000).

Mice deficient in the CRF receptor 1 gene show an impaired anxious and stress response, while the CRF receptor 2 deficient male mice have the opposite response. This is indicative of the possible role of CRF receptor 1 in the anxiogenic effects of CRF and of CRF receptor 2's possible role in anxiolysis.

From the above it is quite clear that the biology and physiology of anxious response is quite a complex phenomenon that is tightly regulated multi-systemically and because of this it is often difficult to quantify. Anxiety is a normal biological response, but we must ask the question: at what point does it become pathological?

## 2.5. Normal versus Pathological Anxiety

As previously mentioned, the feeling of anxiety acts as a warning signal for imminent danger or harm and is able to induce motivation; therefore, some anxiety is adaptive and is usually at the low end of the anxiety continuum (Endler & Kocovski, 2001). The tendency for animal models to display increased anxiety is dependent on two main factors: a genetic predisposition and environmental influence. For humans Barlow (2000) defined three interrelating sets of 'vulnerability factors' that can be described as

1. A generalized biological vulnerability
2. A generalized psychological vulnerability
3. A specific psychological vulnerability.

Generalized biological vulnerabilities are mainly of a genetic origin. Generalized psychological vulnerability refer to experiences in early life particularly of a traumatic nature that leaves the individual predisposed to the anxious state. Particular events or environments that form a part of specific psychological vulnerability play a role in the culmination of specific anxiety disorder (Barlow, 2000).

When excessive anxiety is experienced for prolonged periods of time it can have numerous negative effects on the mind and body of the individual experiencing the symptoms, and cause disruption to their daily activities. In cases like these a disorder can be diagnosed. The diagnostic criteria for diagnosing generalized anxiety disorder is a 6-part list. In summary, it states that the anxiety or apprehensive expectative feeling should have been occurring for at least 6 months and that the individual finds the feelings hard to control. The individual should also present with three or more of the symptoms listed, some of which include restlessness, fatigue, sleep disturbance and muscle tension. The symptoms experienced should be severe enough to impair important areas of functioning and that these symptoms should not attributable to any other substances. Lastly, if the disorder cannot be better explained by any other mental disorder known then a diagnosis can be made (American Psychiatric Association, 2013).

Anxiety disorders are maladaptive and occur when a severe amount of anxiety impairs daily functioning. One would expect individuals with chronic anxiety disorders to exhibit physiological hyperarousal in a rest state or to exhibit intensified responses to stressors. However, a study noted that patients suffering from chronic anxiety disorders presented with ‘diminished physiological flexibility’. This means that they reacted with a weaker physiological response to laboratory stressors than the control group (Hoehn-Saric & McLeod, 2000). It was only upon the presentation of phobic stimuli that a strong physiological response was noted in these individuals (Hoehn-Saric & McLeod, 2000). This phenomenon is often associated with a hindered return of physiological activity to baseline

levels once the stressor is removed in the chronically anxious individuals. The discrepancies between self-reported anxiety levels, and physiological changes do not mean the individual is not able to detect the differences in physiological changes due to stress or anxiety (McLeod, et al., 1986).

## 2.6. The Male Reproductive System at a Glance

The reproductive systems are purposed to enable the union of genetic material. The male reproductive system consists of various organs and structures designed for reproductive success. The penis, scrotum, testes, and epididymis are located outside of the abdominal cavity. The position of these components of the male reproductive system allows for optimum temperature control during spermatogenesis as well as optimizing copulation. Internally the vas deferens, ejaculatory ducts, urethra, seminal vesicles, prostate gland and bulbourethral glands are located to ensure sperm transportation and protection (Hirsch, 2018). The spermatozoon is the product of this highly organized system. It is the haploid cell designed to traverse the depths of the female reproductive tract to meet with its haploid mate, the ovum, to form the diploid zygote. The zygote is equipped with all the information to form life itself.

The development of the testes takes place at the urogenital ridge. At birth, the testis descends into the scrotum through the inguinal canal at birth (Bennet Jr., 2010). The pair of testes are encapsulated in a durable outer fibrous capsule that encloses masses of coiled seminiferous tubules.

The seminiferous tubules are the location of sperm production and contain spermatogonia in various developmental stages as well as Sertoli cells (Silverthorn, 2010). Layers of increasingly mature cells are found toward the lumen of the seminiferous tubule. The cells progressively mature from the primary spermatocytes, secondary spermatocytes to the spermatids and spermatozoa (Bennet Jr., 2010). Sertoli cells linked by tight junctions form

the blood-testis barrier that restricts molecule movement between compartments. Additional functions of the Sertoli cells are to support developing spermatogenic cells by providing nourishment, controlling the movements of spermatogenic cells, releasing sperm into the seminiferous tubule lumen, removing excess spermatid cytoplasm by phagocytosis. The Sertoli cell also produces fluid necessary for sperm transport and the secretion of inhibin. It is vital in the regulation of FSH and testosterone (Bennet Jr., 2010). The interior of the testis is compartmentalized and contains both seminiferous tubules and interstitial tissue (Bennet Jr., 2010). The interstitial tissue, comprising of blood vessels and testosterone producing Leydig cells, are found between the seminiferous tubules. The Leydig cells are also responsible for the aromatization of oestrogens. Oestrogens are thought to modulate gonadotropin secretion from the pituitary in response to Gonadotropin-releasing hormone (GnRH) (Nargund, 2015). The seminiferous tubules exit the testes and join the epididymis which is described as forming a tightly coiled cord on the surface of the testicular capsule (Silverthorn, 2010).

Anatomically, the 6-meter-long epididymis is comprised of three sections: the head (caput), the body (corpus) and the tail (cauda). Functionally, the epididymis has three broadly defined roles. It is necessary for storage, maturation, and transport of sperm (Bennet Jr., 2010). The epididymis is innervated by sympathetic fibers, the pelvic plexus and hypogastric plexus that form the inferior and intermediate spermatic nerves (Bennet Jr., 2010). Blood is supplied to the epididymis by the testicular artery, and venous blood drains by means of the pampiniform plexus to the right and left testicular veins (Lumley, et al., 1980). The epididymis forms the vas deferens, which passes into the abdomen and enters the urethra (Silverthorn, 2010).

In males, the urethra serves as the pathway for sperm and urine, although not at once. The prostate gland, seminal vesicles and the bulbourethral (Cowper's) glands form the accessory glands and ducts found in the male reproductive system. The bulbourethral glands are pea sized glands with ducts that open into the spongy urethra. This gland

secretes a slightly alkaline fluid thought to be involved in the neutralization of the urethral urine during sexual arousal (Bennet Jr., 2010). The seminal vesicles secrete fluid that can comprise up to 80% of the seminal volume. The fluid is viscous and alkaline, to ensure the survival of spermatozoa in the acidic environment of the vaginal tract. Seminal vesicle fluid contains fructose, prostaglandins and clotting proteins that ensure semen is able to coagulate after ejaculation (Bennet Jr., 2010). The prostate is found at the base of the bladder. The prostatic secretions are a milky, slightly acidic fluid that comprises of several substances including citric acid, pepsinogen, amylase, and acid phosphatase. Prostatic proteases are responsible for the liquefaction of semen.

Three major subdivisions: the hypothalamus, the pituitary gland and the testis form the functional reproductive axis. Multiple hormones and signaling molecules are involved in the functioning of the male reproductive axis (Bennet Jr., 2010). Peptide hormones secreted by the hypothalamus and anterior pituitary form the basis of the male reproductive system regulation. These hormones control gonadal secretion of the steroid sex hormones. Steroid sex hormones arise from the same steroid precursors and include oestrogens, progesterones and androgens. Androgens are predominant in males; however, males also naturally produce estrogens, but the feminizing effects of estrogens are usually not obvious in males (Silverthorn, 2010).

The hypothalamus-anterior pituitary-peripheral gland pattern is utilized for hormonal control of reproduction. Control of male sexual and reproductive function begins with GnRH that finds its origin in the hypothalamus and mediates the release of gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary as shown in *Figure 2* (Bennet Jr., 2010; Silverthorn, 2010). FSH primarily stimulates spermatogenesis while LH is the major stimulus for testicular testosterone secretion. GnRH output is influenced by season, circadian rhythm and pulsality, with peaks occurring approximately every 90-120 minutes (Bennet Jr., 2010). LH follows the pulsatile rhythm of GnRH release,

while FSH secretion progresses slowly. Both LH and FSH are glycoproteins that use the cyclic adenosine monophosphate second messenger system to affect their target organs. For example, LH stimulates the Leydig cells to secrete testosterone in a directly proportional manner i.e. the more LH is released the more testosterone is released. Therefore, if testosterone exceeds homeostatic levels a negative feedback cycle is initiated that acts on both the hypothalamus and pituitary to ultimately decrease LH levels and subsequently the testosterone levels (Bennet Jr., 2010). Inhibin, mentioned earlier, is also a glycoprotein. It is secreted by the Sertoli cells in the seminiferous tubules in response to rapid spermatogenesis to create a negative feedback cycle and decrease secretion of FSH by the anterior pituitary (Bennet Jr., 2010). This system is defined as the hypothalamic-pituitary-gonadal (HPG) axis and is depicted in *Figure 2*.

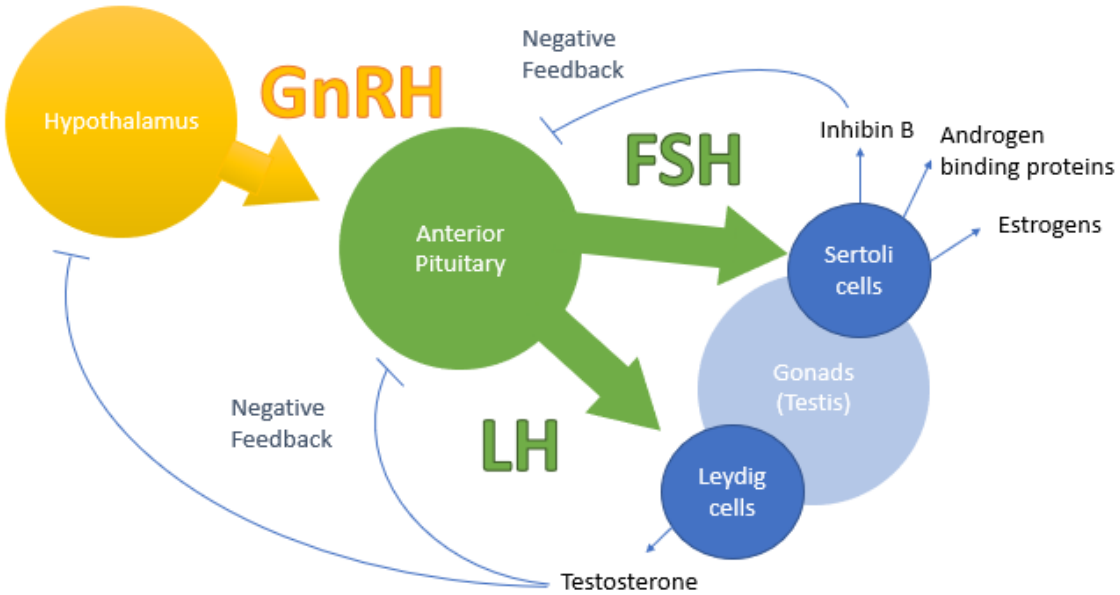



Figure 2 Simplified diagram describing the HPG-axis adapted from (Bennet Jr., 2010; Nargund, 2015)

Elevated serum testosterone levels lead to the inhibition of LH through a negative-feedback mechanism that affects both the hypothalamus and anterior pituitary (Nargund, 2015). Both endogenous testosterone and its functional receptor play a crucial role in spermatogenesis. The receptor is known as the androgen receptor (AR) and it can be found in both Sertoli and Leydig cells (Kotitschke, et al., 2009; Nargund, 2015). Although Leydig cells produce testosterone, the Sertoli cells are the most important target cells for testosterone because of their involvement in spermatogenesis. Decreased levels of testosterone or absence of AR's is known to lead to a malfunctioning blood-testis barrier. The implications of this include immature spermatozoa being released prematurely from the Sertoli cells while mature spermatozoa fail to release. This defect in the blood-testis barrier can also lead to exposure of spermatozoa in various stages of development being exposed to gonadotoxic agents and possible autoimmune attack (Smith & Walker, 2014). Stress is one possible cause of testosterone decline.

## 2.7. Anxiety and the male reproductive system

Physiologically, psychological stress can be described as the domino effect leading to the disruption of homeostasis as a consequence of stress on the mind (Rivier & Rivest, 1991). Psychological factors have been associated with cases of idiopathic infertility in both sexes (Vellani, et al., 2013). However, literature presents a complex and often contradictory picture of the relationship between psychological stress, anxiety and male infertility. In the years 1992-1994 the effects of job stress (job demand and job control) were investigated in Danish couples looking to get pregnant. It was observed that male job demand and job control was not associated with fertilization ability in this study (Hjollund, et al., 2004). Similarly, a study performed by Fenster et.al. (1997) found no association between psychological job stress and seminal quality (Fenster, et al., 1997). The discrepancies in studies investigating the relationship between psychological stress and male reproductive

parameters can be attributed to different instruments to measure stress, the methods employed to measure semen quality and participant selection. Often results obtained from participants selected from infertility clinics differed from instances where the student population was observed. Students were shown to exhibit impaired semen quality, specifically a decline in sperm concentration and a decrease in the linearity of the average path of sperm. This decline was attributed to the proximity of the sample donation to the exam period, no method of measuring the stress level other than proximity to the exam date was mentioned (Lampiao, 2009). In another study, psychological stress in a group of students was measured by means of the STAI questionnaire (Eskiocak, et al., 2006). In this study it similarly found that sperm concentration and rapid motility were significantly decreased during the reported stress period (shortly before exams). They also reported significantly higher state scores during the stress period than the non-stress period (Eskiocak, et al., 2006). In a controlled study the relationship between semen quality and state/trait anxiety was investigated (Vellani, et al., 2013). Patients enrolled in an *in vitro* fertilization program (ages 29 to 49 years, mean age 38.91 $\pm$ 4.54) and control subjects were used (ages 31 to 48 years, 37.71 $\pm$ 3.76). It was found that increased state and trait anxiety scores were associated with a decrease in semen volume, a decline in sperm concentration, count, motility and increased sperm DNA fragmentation in the patients enrolled in the *in vitro* fertilization program (Vellani, et al., 2013). Similar results were reported for the control subjects of this study, although it more pronounced in the patient's group.

Psychological stress and anxiety affect the male reproductive system in various ways. It is well accepted and known that the activation of the HPA-axis during the stress response inhibits the HPG-axis by inhibiting GnRH at the hypothalamic level. Glucocorticoids, such as cortisol, affect testicular function on multiple levels (Nargund, 2015). There are glucocorticoid receptors present in the testes, the pituitary, and the hypothalamus.

Therefore, at a hypothalamic level GnRH can be downregulated by increased glucocorticoids. This can lead to the impaired pulsatile release of the gonadotropic hormone's: LH and FSH. The presence of glucocorticoids reduces the testicular response to LH, ultimately leading to reduced testosterone secretion (Hu & et al., 2008; Whirledge & Cidlowski, 2010). Glucocorticoids could also possibly directly inhibit testosterone production through genomic and non-genomic mechanisms resulting from the activation of the Leydig cells (Hu & et al., 2008). The connection between the HPA- and HPG-axis is demonstrated in *Figure 3.* Stress and the activation of the HPA-axis involves various compounds such as growth hormone and prolactin that will not be discussed in this literature review.

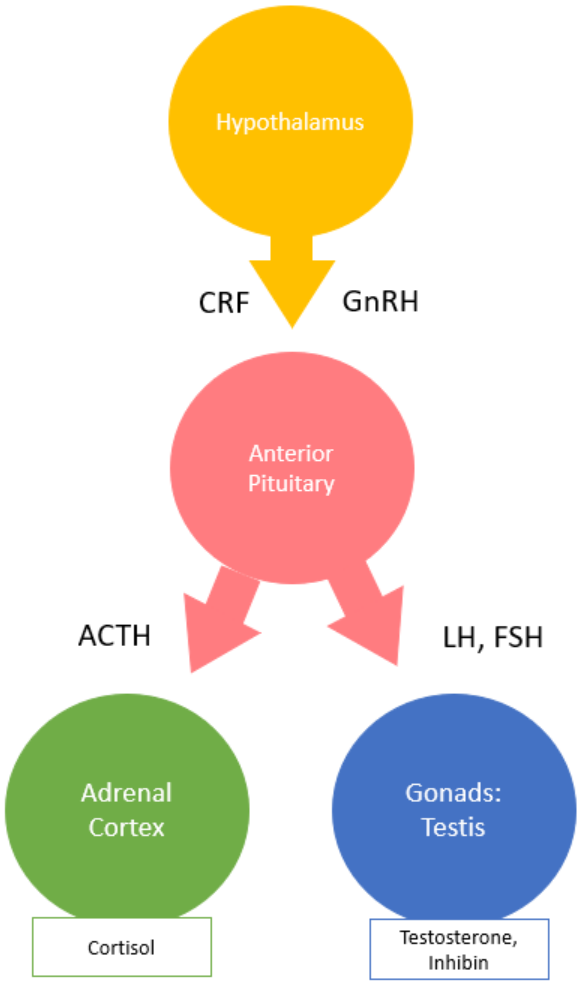



Figure 3 Simple diagram depicting the HPA- and the HPG-Axis

However, the next segment of this literature review will report on the relationship between common male reproductive parameters and psychological stress and anxiety.

### 2.7.1. Effect of Anxiety on the Male Reproductive System

The spermiogram or seminogram is a collection of quantitative parameters that forms the basis of andrological investigation. The ejaculate provides a snapshot representation of three crucial biological events: production of spermatocytes, the maturation of spermatocytes to spermatozoa in the epididymis, and the correct working of the neurophysiological mechanisms leading to ejaculation of the sample (La Vignera, et al., 2007). Parameters of routine semen analysis include volume, pH, sperm count, motility, morphology, and vitality.

#### 2.7.1.1. Volume

In a routine semen analysis, the volume of the ejaculate is a parameter that is important to quantify. An accurate measurement of volume is vital in any evaluation of semen, because it allows the total number of spermatozoa and non-sperm cells in the ejaculate to be calculated. In literature it has been observed that increased psychological stress, even in healthy volunteers, leads to decreased seminal volume (Vellani, et al., 2013; Nordkap, et al., 2016). It was also found that the average semen volume remained unchanged during periods of stress and non-stress (Clarke, et al., 1999; Eskiocak, et al., 2006).

#### 2.7.1.2. Concentration and motility

Sperm concentration and motility are part of the initial microscopic analysis of a semen sample that forms part of the spermiogram. Increased state and trait anxiety were found to be associated with decreased sperm concentration (Vellani, et al., 2013). Similar observations have been noted in other studies (Eskiocak, et al., 2006; Zorn, et al., 2007; Janevic, et al., 2014). However, Hjollund and his colleagues deem psychological stress to be inconsequential on many parameters including sperm concentration (Hjollund, et al., 2004).

Decreased motility was noted in both the *in vitro* fertilization patients and controls in the study investigating the association of state and trait anxiety to semen quality (Vellani, et al., 2013). Rapid progressive motility was found to be significantly lower in individuals during a high stress period compared to a low stress period (Eskiocak, et al., 2006). This is supported by (Janevic, et al., 2014) and (Clarke, et al., 1999) who also found the lateral head displacement velocity parameter to be significantly reduced during a stressful event.

#### 2.7.1.3. Viability/Vitality

Although viability/vitality of sperm is often mentioned as a part of a spermiogram, no studies have reported on the relationship between viability/vitality and psychological stress/ anxiety.

#### 2.7.1.4. Morphology

The large variation in the morphology of human spermatozoa combined with the number of difficulties associated with lack of objectivity and variation in interpretation of results makes assessment of morphology quite challenging (WHO, 2010). However, it still forms a part of the initial microscopic analysis of the semen sample. No statistical differences in normal morphology between IVF patients and controls were reported by (Vellani, et al., 2013). This is agreement with literature where it was found that the percentage of spermatozoa with abnormal morphology increased with stress although not significantly (Eskiocak, et al., 2006; Zorn, et al., 2007).

#### 2.7.1.5. Biochemical analysis

There are limitations to the basic semen analysis, as men have been known to present with normal WHO spermiogram values and still struggle to achieve pregnancy despite their partner having normal reproductive parameters. This is known as idiopathic infertility. This has prompted researchers to investigate biochemical parameters such as (reactive oxygen species) ROS, (nitric oxide) NO and DNA fragmentation. The biochemical parameters can provide insight into the mechanisms in which various stressors such as psychological stress and anxiety affect male reproductive parameters.

#### 2.7.1.6. ROS

An imbalance of ROS and antioxidant defenses can result in a phenomenon known as oxidative stress. The lipid-rich, high oxygen consuming brain is highly susceptible to redox imbalances. The link between oxidative stress metabolic pathways, and anxiety phenotypes and behaviors are discussed in-depth in an article by Bouayed et al. (Bouayed, et al., 2009). They conclude the article by reviewing the limitations to defining an anxiogenic effect of oxidative stress and stress that the data available supports a causal relationship between oxidative stress and anxiety.

The spermatozoon too, is also particularly susceptible to oxidative damage due to its limited antioxidant capacity and lipid rich membrane.

#### 2.7.1.7. NO

The role of NO in anxiety has not yet been fully elucidated. However, in a study performed on male mice lacking the gene that encodes for Nitric Oxide Synthase 1, these mice were found to have abnormal anxiety levels compared to their counterparts (Bilbo, et al., 2003; Pitsikas, 2018). NO levels in seminal plasma were higher in individuals who scored higher on the State Anxiety Index questionnaire (Eskiocak, et al., 2006).

#### 2.7.1.8. DNA Fragmentation

Following a regression analysis, state anxiety was found to have a strong association with increased level of DNA fragmentation of the spermatozoa (Vellani, et al., 2013).

DNA fragmentation is one of the later steps of apoptosis. Sperm biological structure is affected by impaired DNA integrity. This can result in poor pregnancy outcomes. ROS and DNA fragmentation are techniques that are used to investigate sperm biological structure (Jungwirth, et al., 2012).

#### 2.7.1.9. Cytokines and anxiety

Cytokines are secreted by different parts of the male genital tract and are thought to exert effects on steroidogenesis, spermatogenesis and sperm function (Dousset, et al., 1997). Literature has reported a positive correlation between anxiety and inflammatory markers such as (tumor necrosis factor- alpha) TNF-α and (interleukin -6) IL-6 in humans in other body systems. In semen, cytokines such as (interleukin-1 beta) IL-1β, IL-6 and (interferon gamma) IFN-γ are found in the semen of infertile men (Havrylyuk, et al., 2015).

The existing pool of literature describing the relationship between psychological stress and anxiety is quite vast and yields a variety of results, owing to the differences in tools to measure psychological stress, study designs and differences in sample populations. Adding to this comprehensive literature set and observing the effects of anxiety on male reproductive parameters in the student population at a South African university adds great value. The student population is one that is often sampled for research at tertiary institutions and having insights into how self-reported psychological stress, and measured biological stress is affecting their semen sample on a basic and biochemical level can lead to researchers taking this into account when designing their future research protocols.

# Chapter 3

# Materials and Methods

## 3.1. Introduction

The materials and methods will be discussed in this chapter. A brief description of the rationale for each method will be provided and accompanied by detailed protocols. *Figure 4* provides a brief outline of the experimental procedure. A total of 21 participants were recruited for this study and each completed 4 donation sessions in total, with the exception of one donor who was only able to complete 3 donation sessions. In each donation session each donor completed a questionnaire, provided a semen sample and provided a blood sample. It should be noted that not all participants were able to donate blood samples and those who were selected to donate blood were selected at random. A total of 83 samples were collected and analyzed using various techniques.

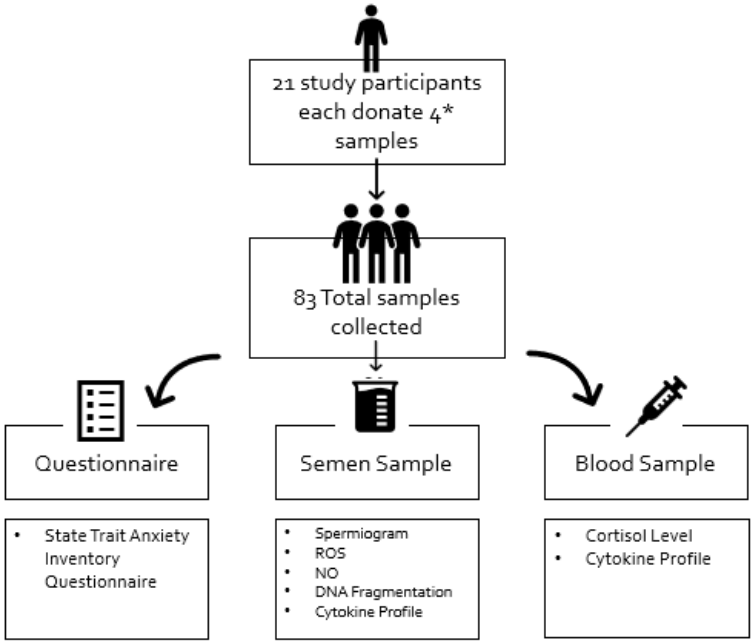



Figure 4 Brief description of the experimental design. * One participant was only able to attend three of the four scheduled donation sessions.

## 3.2. Ethical Clearance

Institutional permission (service desk ID: IRPSD 593) and ethical clearance (ethics clearance number: S17/08/137) were granted by the University of Stellenbosch Institutional Review Board. The study participants were recruited and informed of the research protocol by means of consent forms. The participants were ensured anonymity and that their samples would be used solely for research purposes. By signing the consent form, they selected the disposal option for their samples following experimental use.

## 3.3. Participant Recruitment

In total, 83 samples were collected from 21 participants. Study participants consisted of students from the Faculty of Medicine and Health Science (n=17), Stellenbosch University and from the Faculty of Dentistry (n=3) and Faculty of Law (n=1), University of the Western Cape. The participants were all within the age range of 18-25 and either formed part of the sperm donor programs at the Stellenbosch University Reproductive Research Group (SURRG) laboratory, the Medical Bioscience Laboratory at the University of the Western Cape or were recruited by means of posters. No exclusion criteria were applied. As part of the recruitment process donors were asked to complete consent forms as well as a donor profile questionnaire. The donor profile questionnaire is a laboratory standardized questionnaire that collects demographic data for each participant.

## 3.4. Semen Collection

Participants were asked to donate 4 samples at 4 different timepoints, they were encouraged to deliver two samples during a period of “high stress” and two more samples during a period of “low stress”. Periods of high stress could refer to any stressor that the participant felt was particularly stressful to them e.g. financial stress but predominantly periods of academic stress. Low stress periods referred to whenever the participant felt the stress in their life was lower than typically perceived i.e. non-examination period. The semen

samples were collected according to WHO guidelines (WHO, 2010), following a 3-5-day abstinence period. Semen acquisition was by means of masturbation into a wide mouth sterile plastic container. The containers were placed in an incubator (37°C, 5% $CO_2$), where the samples could liquefy for a period of 30-60 minutes prior to analysis. Semen was used for the spermiogram and analyses that were to be completed within the same day i.e. measuring ROS levels and NO levels. Aliquots of semen and seminal plasma were also flash frozen and stored in liquid nitrogen (-196° C) for further analysis.

### 3.5. Blood Collection

In total, 68 samples were collected from 17 participants chosen at random to donate blood. Study participants consisted of students from the Faculty of Medicine and Health Science (n=17), Stellenbosch University and from the Faculty of Dentistry (n=3) and Faculty of Law (n=1), University of the Western Cape. The participants were all within the age range of 18-25 and either formed part of the sperm donor programs at the Stellenbosch University Reproductive Research Group (SURRG) laboratory and Medical Bioscience Laboratory at the University of the Western Cape or were recruited by means of posters. No exclusion criteria were applied. Blood was drawn from the participants at 4 different timepoints, they were encouraged to deliver two samples during a period of “high stress” and the other two samples during a period of “low stress”. Study clinicians from the Molecular Biology Unit assisted in acquiring the blood samples. During each session 10ml of blood was acquired in a lavender top EDTA blood tube. Each blood sample was centrifuged at 2000rpm for 10 minutes at 18° C for separation into the blood plasma, buffy coat consisting of the white blood cells and the remainder of the blood sample consisting of the erythrocytes as depicted in *Figure 5* (Theron, 2018). The buffy coat and blood plasma were collected, and aliquots were flash frozen and stored in liquid nitrogen (-196° C) for further analysis. The remainder of the blood sample was discarded of in the biomedical waste bins to be collected for incineration.

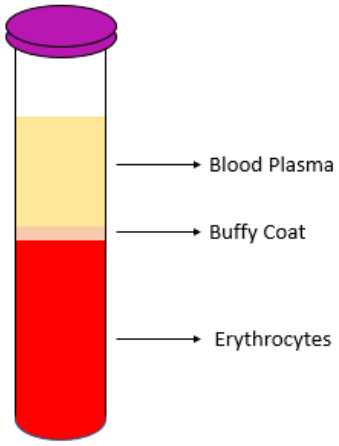



Figure 5 A depiction of the 10 ml lavender topped EDTA tubes used for blood collection after the whole blood sample has been centrifuged. The colours used are purely representative of the different components that the blood separates into after centrifugation.

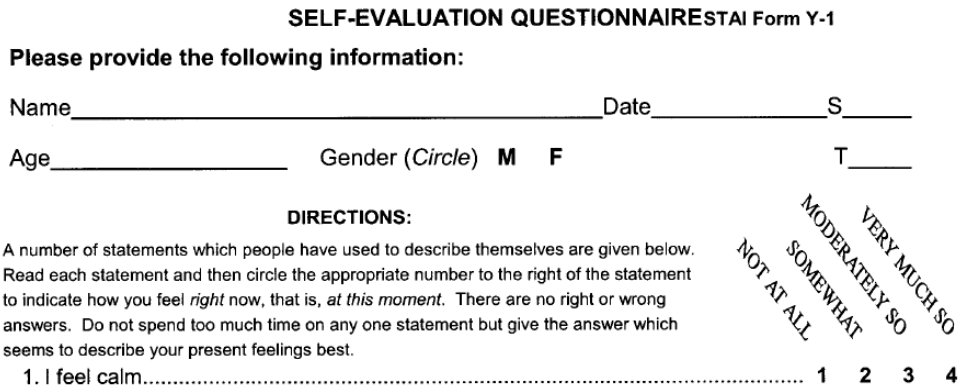
## 3.6. State Trait Anxiety Index Questionnaire

Anxiety was measured using the State Trait Anxiety Index (STAI) questionnaire. The purpose of this questionnaire was to measure the presence and severity of anxiety, as well as a general tendency to be anxious. The questionnaire was self-reported and not conducted in an interview-like fashion. Participants were briefed on how to complete the questionnaire which was completed on paper and they were instructed to circle the answer that is most correct for the specific question. Directions were also clearly and concisely described on the questionnaire. The participants were instructed to complete both STAI Form Y-1 and Form Y-2. Form Y-1 encouraged the participants to indicate how they feel "at this moment". Statements were provided e.g. I feel calm and the participant is instructed to circle a number corresponding to the answer that is most appropriate for them ranging from 'not at all', 'somewhat', 'moderately so' or 'very much so'. The participant is encouraged not to linger on a question and rather provide the answer as quickly and as truthfully as possible. The STAI questionnaire consists of two subscales within the questionnaire. Form Y-1 forms

the state anxiety scale which measures the more transient type of anxiety and is depicted in *Figure 6*. The trait anxiety scale which measures the general propensity to the anxious state forms Form Y-2 (Julian, 2011). Once again participants are encouraged not to linger to long on any statement. The participants were asked to consider each statement as how they generally feel and circle the number corresponding to the answer most appropriate to them. An example of a statement listed is 'I feel nervous and restless' and participants could choose a number corresponding to the answers ranging from 'almost never', 'sometimes', 'often' to 'almost always'.

The questionnaire comes with a scoring key for both forms. The scoring key stencil was folded in half and lined up with the fitting test side, either Form Y-1 or Form Y-2. Each answer chosen by the participant has a scoring weight on the scoring key stencil. The scoring weights are totaled for each category. The results are a score between the range of 20-80 for both Form Y-1 and Form-Y-2.

An increased score on the STAI questionnaire correlates to an increase in anxiety (Spielberger, 1968). Anxiety scores were stratified into 'high anxiety' and 'low anxiety', with scores of 40 and above classified as 'high anxiety' and scores below 40 were classified as 'low anxiety'.



**SELF-EVALUATION QUESTIONNAIRE** STAI Form Y-1

**Please provide the following information:**

Name_____Date_____S_____

Age_____ Gender (*Circle*) **M** **F** T_____

**DIRECTIONS:**

A number of statements which people have used to describe themselves are given below. Read each statement and then circle the appropriate number to the right of the statement to indicate how you feel *right* now, that is, *at this moment*. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe your present feelings best.

NOT AT ALL | SOMEWHAT | MODERATELY SO | VERY MUCH SO

1. I feel calm..... 1 2 3 4

Figure 6 A screenshot of the first portion of the STAI questionnaire that the participants received. The instructions are clearly communicated to them and they can circle the answer most applicable to them. Using these circles, it is possible to use the scoring key to allocate a score to their answer.

## 3.7. Spermiogram

The spermiogram or seminogram is a collection of quantitative parameters that forms the basis of andrological investigation. The spermiogram is also known as a neat analysis and is performed before any treatments or manipulation to a semen sample- it provides bassline information of seminal quantitative parameters. It consists of macroscopic and microscopic analysis.

### 3.7.1. Macroscopic Analysis

The macroscopic analysis is the portion of the spermiogram that is conducted primarily with the naked eye or apparatus that does not include a microscope. Volume, pH, colour and odour form part of the macroscopic analysis. Only volume and pH will be included as data in this thesis as they are quantifiable parameters.

### 3.7.2. Volume

In a routine semen analysis, the volume of the ejaculate is a parameter that is important to quantify. An accurate measurement of volume is vital in any evaluation of semen, because it allows the total number of spermatozoa and non-sperm cells in the ejaculate to be calculated. After the post-ejaculate liquefaction period, semen samples were decanted from the plastic collection container into graduated Falcon tubes using Pasteur pipettes. The volume of the collected sample was weighed on the laboratory scale and recorded in milliliters as per WHO guidelines (WHO, 2010). The WHO lower reference limits ($5^{th}$ centiles and their 95% confidence intervals) for semen volume is 1.5 (1.4-1.7) ml (WHO, 2010).

### 3.7.3. pH

The pH of the semen sample was measured during a routine spermiogram, as its value is a reflection of various accessory sex gland secretions (WHO, 2010). This parameter was analysed by submerging a strip of litmus paper into the semen sample and then comparing the colour of the litmus paper to the pH colour wheel (pH- indicator paper, Merck Millipore)

to obtain a pH reading. An acidic ejaculate is a potential indication of blocked seminal vesicles while a basic ejaculate is an indication of a possible infection. The pH scale is between 1 and 14, however the WHO criteria specify the normal pH range for a semen sample between 7.2- 7.8 (WHO, 2010) . The pH-indicator paper used is a special indicator that is equipped to measure pH values within the range of pH 6.4-8.0. This means that the pH paper is able to detect colorimetric pH values of the normal semen sample as well as the abnormal sample within the range of the paper. The pH data will not be reported statistically because the indicator paper results are subject to being subjective and the test does not provide great sensitivity.

## 3.8. Microscopic Analysis

The microscopic analysis involves using microscopes to ascertain more detail about the sperm, whereas macroscopic analysis primarily allows us to obtain information about the semen. During microscopic analysis, sometimes with the assistance of staining techniques or specialized software, we can measure parameters such as concentration, motility and kinematics, morphology and the viability of the sperm present in a sample. Computer-aided sperm analysis (CASA) is how concentration, motility and kinematics was measured. CASA can also be used to analyse viability, however in this case a stand-alone light microscope was used, and viability was manually assessed. First the methodology for CASA acquired data will be discussed. It will be followed by the methodology for viability analysis.

### 3.8.1. CASA- related Analysis

#### 3.8.1.1. Description of Instrumentation and Software

CASA is a term used to describe instruments that have the joined purpose to analyse spermatozoa. The following components are usually found in a CASA system: a computer loaded with appropriate software, a video camera and a video-frame grabber card installed into the computer.

The software used is the Sperm Class Analyser® (SCA®) software system version 6.3.0.59 (Microptic, S.L., Barcelona, Spain). The software is what enables the identification and tracking of the spermatozoa in the video images. SCA® is also programmed to perform all the data calculations that generate valuable data such as the kinematics of a spermatozoa.

The camera used in this CASA system is the Basler A312fc digital colour camera fitted to a Nikon Eclipse 50i Microscope (IMP, Cape Town, South Africa). The HP EliteDesk 800 G3 TWR was the computer model associated with this CASA system. Windows 10 Pro, version 1703 was installed on the pc. The processor specifications the computer was equipped with was: Intel® Core™i7-7700 Central Processing Unit at 3.60Ghz as well as a 64-bit operating system (x64-based processor) with 16 GB of installed random-access memory.

**Concentration**

Concentration of spermatozoa can be defined as the number of spermatozoa per milliliter in a semen sample.

An unwashed sample (1.5µL) was pipetted into warmed, specialized, disposable 2-micron Leja® (Leja Products B.V., The Netherlands) chamber slides post-liquefaction.

The samples were observed under the microscope employing the following settings: phase contrast, 435; brightness, 100; objective 10x; 50 images per second; temperature 37ºC, green filter.

At least 500 spermatozoa were examined by photo-microscopic capturing by randomly selected representative fields. In poor samples, where analyzing 500 spermatozoa was not possible, 10 randomly selected representative fields were chosen. The concentration of spermatozoa was expressed as $x10^6$ per ml. Total sperm number, or total sperm count, is calculated by multiplying sperm concentration of a sample by the semen volume of the sample and is expressed as $x10^6$ per ejaculate. The WHO lower reference limits for sperm

concentration are 15 (12-16) $10^6$ per ml and for total sperm number is 39 (33-46) $10^6$ per ejaculate (WHO, 2010).

**Motility**

Sperm motility is a parameter that describes the movement of the spermatozoa. It is considered an important functional parameter of spermatozoa as appropriate motility is a crucial component of the fertilization process.

Motility is analyzed in conjunction with the concentration analysis. The motility of the sample was expressed as a percentage and the velocity parameters of the spermatozoa were subsequently classified as well. The “Human 50” setting was picked on the SCA® software. It is pre-assigned settings that have been optimized for human spermatozoa.

The system analyzed motility parameters according to the WHO (2010) criteria: total motility (Type A+B+C), progressive motility (Type A+B) and immotile spermatozoa (Type C). The velocity (kinematic) parameters were also observed such as: VCL, curvilinear velocity (µm/s); VS.L, straight line velocity (µm/s); VAP, average path velocity (µm/s) and ALH, amplitude of lateral head displacement (µm) among others such as LIN (linearity), WOB (wobble), STR (straightness), BCF (beat-cross frequency, Hz) and MAD (mean angular displacement, degrees). The WHO lower reference limits for total motility is 40 (38-42) % and for progressive motility the lower reference limit is 32 (31-34) % (WHO, 2010).

### 3.8.2. Viability Assay Procedure

#### 3.8.2.1. Description of the Instrument

As previously mentioned, we did not make use of CASA to analyze the viability (vitality) of the spermatozoa. Instead we used the Leica CME compound microscope system (Meyer Instruments, Houston, Texas) and a manual counter. The Leica CME microscope has been designed to be durable and user friendly (Meyer Instruments, 2010) and is shown in *Figure 7.*

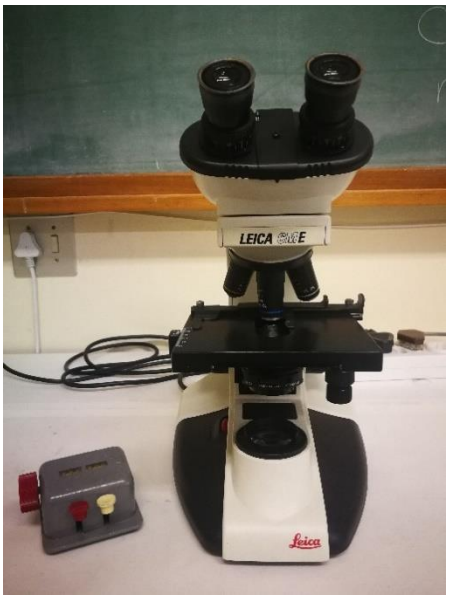



Figure 7 The Leica CME microscope and manual counter used to manually count the number of viable spermatozoa for each sample

#### 3.8.2.2. Assessment of Viability

Viability is a parameter that provides an indication of how many spermatozoa are ‘alive’ and ‘dead’. The principle is that spermatozoa with structurally intact cell membranes are not stained and therefore representative of the live population in the semen sample, and the opposite is also true.– A dye-exclusion staining method (Eosin and Nigrosin), was employed as the method to assess the viability of each spermatozoon. A mixture of 10 µl sperm, 20 µl Eosin (Sigma-Aldrich, St Louis, MO) and 30 µl Nigrosin (Sigma-Aldrich, St Louis, MO) was prepared in an 2ml Eppendorf tube. A smear was created using 10 µl of the prepared mixture

and allowed to air dry before being mounted for analysis as depicted in *Figure 8*. Eosin penetrates cells with a damaged membrane and is therefore an indicator of the non-viable cells. Nigrosin provides the background stain. Both non- Viable (red-stained) and viable (white or unstained) cells are to be counted using a light microscope at 40X magnification. A minimum of 100 cells were counted; this was repeated for all samples and the results were expressed as percentage viability (% viable vs. % non-viable). The WHO lower reference limit for viability is 58 (55-63) % viable (WHO, 2010).

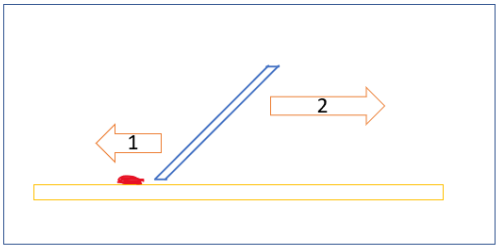



Figure 8 A depiction of the smearing technique. The red dot represents the 10 µl sperm-eosin-nigrosin mixture placed on the frosted slide. A plain slide was gently pushed toward the sample as indicated with arrow 1 at a 45° angle. The sample spread along the edge of the plain slide touching the frosted slide. The plain slide gently pulled the sample in the direction of arrow 2, spreading the sample evenly across the frosted slide.

## 3.9. Reactive Oxygen Species

It was noted that there is a link between oxidative stress and anxiety in the body (Bouayed, et al., 2009). Measuring the reactive oxygen species level by chemiluminescence is the gold standard for measuring ROS in sperm and is used in clinical andrology laboratories across the world.

### 3.9.1. Description of the Instrumentation and software

The AutoLumat Plus LB 953 (Berthold Technologies, Wildbad, Germany) automatic tube luminometer was used for the ROS analysis. Up to 180 samples can be placed in a chain and measured once or more depending on the protocol. The luminometer is equipped with an ultra-fast single photon counter that has a very large dynamic range. Spectral sensitivity

of this instrument ranges from 380 nm to 630 nm. The detection unit makes use of Peltier cooling to ensure low background noise and increased sensitivity. To keep conditions ideal for analysis, the temperature in the measuring chamber of the luminometer can be controlled.

The Berthold TubeMaster version 1.0 built-in software (Copyright © Celsis International, plc 1997-1999, under license to Berthold Technologies, GmbH & Co. KG, Wildbad, Germany) used, allows for operation and control of the luminometer via the attached computer. It also allowed for data to be easily transferred to Excel. Windows 10 Home was installed on the pc. The processor specifications the computer was equipped with was: Intel® Pentium® Central Processing Unit at 3.30Ghz as well as a 32-bit operating system (x32-based processor) with 4 GB of installed random-access memory.

### 3.9.2. Principle of Chemiluminescence

Chemiluminescence is based on the principle of the measurement of light emitted in the reaction of the reagents that are added to a human semen sample (Benjamin, et al., 2012). Measuring ROS by chemiluminescence with membrane- permeable luminol as the probe measures both (hydrogen peroxide) $H_2O_2$ and (superoxide) $O_2^{\bullet-}$ and others by reacting as luminol or as univalently oxidized luminol radical (Benjamin, et al., 2012). A luminescent signal is generated through a one-electron oxidative event by $H_2O_2$ endogenous peroxidase. A radical species is created that interacts with ground state oxygen to produce $O_2^{\bullet-}$. This $O_2^{\bullet-}$ participates in the oxygenation of luminol radical species, creating an unstable endoperoxide that degrades and leads to light emission (Benjamin, et al., 2012). The redox cycling activity associated with luminol permits for significant amplification of the signal and easy measurement of $H_2O_2$.

### 3.9.3. ROS Assay Procedure

#### 3.9.3.1. Reagent Preparation

Luminol Stock Solution (100 mM) was prepared by weighing out 177.09 mg of luminol (Sigma-Aldrich, St Louis, MO) and adding it to 10 ml Dimethyl Sulfoxide (DMSO) solution (Sigma-Aldrich, St Louis, MO) in a polystyrene tube that was covered in foil. This solution was able to be stored at room temperature until expiration date.

The luminol working solution (5 mM) was prepared by mixing 20 µL of the luminol stock solution with 380 µL DMSO in a foil covered polystyrene tube. A fresh solution was made prior to every use and the solution was stored at room temperature until needed. The solution is known to be stable for 24 hours if not exposed to light.

#### 3.9.3.2. Instrument Setup

The ‘Berthold tube master’ icon was selected from the desktop to start the program. The ‘measurement definition’ option was selected from the ‘setup menu’ and then a ‘new measurement’ was created. A pop-up screen then prompted the insertion of the ‘Measurement Name’ (Initials_Date_Patient Initials_Measurement_Donation session e.g. TLDJ_19-04-18_A537_ROS_1). The measurement name that was created was copied and after clicking ‘OK’ proceeded to the next step. The ‘tool bar’ prompt allowed us to select ‘luminometer measurement’ followed by ‘rep.assay’ from the drop down menu.

The following parameters were then defined:

i. Read time 1 sec.
ii. Background read time 0 sec.
iii. Total time 900 sec.
iv. Cycle time 30 sec.
v. Delay ‘Inj M read 9 (s)’ 0 sec.
vi. Injector M (µL) 0 sec.
vii. Temperature (°C) 37°C
viii. Temperature control (0 = OFF) 1=ON

Once that was complete from the ‘setup menu’, ‘assay definition’ was selected and then ‘new assay’. We then pasted the measurement name we had copied earlier and selected the corresponding name from the ‘measurement method’ drop-down menu. We were then able to access the ‘Column Menu’ option and hid all the parameters except:

i. Sample ID
ii. Status
iii. RLU Mean
iv. Read date
v. Read time

Normal sample type was selected under the sample type drop-down menu. Thereafter, we created a new workload from the ‘File’ option and opted to save it in an appropriate folder as a workload file. The instrument was then ready to proceed with analysis

#### 3.9.3.3. Sample Preparation

Once the instrument was ready to proceed with analysis, we then labeled 11 Falcon tubes and added the reagents as listed in *Table 1.* The labeled tubes were then placed into the luminometer in the following order: Blank (tubes 1-3), Negative control (tubes 4-6), Test sample (tubes 7-8) and positive control (tubes 9-11).

Table 1 A Table containing the reagents that were added to each respective tube for the ROS analysis

| Tube No. | Tube | Phosphate-buffered saline (PBS) (µL) | Test Sample (µL) | Hydrogen peroxide (30%) (µL) | Probe luminol (5mM) (µL) |
|---|---|---|---|---|---|
| **1-3** | **Blank** | 400 | - | - | - |
| **4-6** | **Negative control** | 400 | - | - | 10 |
| **7-8** | **Test (sample)** | - | 400 | - | 10 |
| **9-11** | **Positive control** | 400 | - | 50 | 10 |

### 3.9.3.4. Analysis of the samples

The luminometer scanned for the tubes after 'Start' was selected. After following a series of prompts, 'Finish' was selected, and the Excel Spreadsheet opened, the tubes were measured by the luminometer using the pre-determined protocol from the instrument set up. Once complete the Excel Spreadsheet was saved and a pdf containing the analysis data was saved for each luminometer run.

### 3.9.3.5. Post Analysis

Levels of ROS results were expressed as Relative Light Units per second (RLU/sec/$10^6$) and an example of data generation is shown in *Figure 9* (Homa, et al., 2015).

We were then able to calculate the corrected ROS that is used for all further statistical analysis by first calculating the 'average RLU' for the negative control, samples, and positive controls. Sample ROS was calculated by subtracting the 'negative control average RLU' from the 'sample average RLU' as depicted in *Equation 1*.

$$Calculated\ sample\ ROS = average\ 'RLU\ mean'\ sample - average\ 'RLU\ mean'\ negative\ control$$

Equation 1 Equation used to calculate sample ROS

The sample ROS was then corrected by dividing it by the sperm concentration as shown in *Equation 2.*

$$Corrected\ sample\ ROS = Calculated\ sample\ ROS \div Sperm\ concentration$$

Equation 2 Equation used to calculate corrected sample ROS

A score of 93 RLU/sec/$10^6$ and above is considered critical and indicative of a state of being ROS positive. Anything below 93 RLU/sec/$10^6$ falls within the normal range for semen.

| Sample | Sample_ID | Status | RLU_Mean | Read_Date | Read_Time |
|---|---|---|---|---|---|
| 1 | Blank | Done | 15823 | 11/04/2018 | 10:58:29 |
| 2 | | Done | 17610 | 11/04/2018 | 10:58:30 |
| 3 | | Done | 15455 | 11/04/2018 | 10:58:32 |
| 4 | Negative Control | Done | 24641 | 11/04/2018 | 10:58:33 |
| 5 | | Done | 25542 | 11/04/2018 | 10:58:35 |
| 6 | | Done | 15605 | 11/04/2018 | 10:58:36 |
| 7 | Sample | Done | 23291 | 11/04/2018 | 10:58:38 |
| 8 | | Done | 21927 | 11/04/2018 | 10:58:40 |
| 9 | Positive Control | Done | 40031249 | 11/04/2018 | 10:58:41 |
| 10 | | Done | 35123432 | 11/04/2018 | 10:58:43 |
| 11 | | Done | 33081230 | 11/04/2018 | 10:58:44 |

Figure 9 An example of the data generated by the Berthold TubeMaster software for ROS analysis

### 3.10. Flow Cytometry: Nitric Oxide and DNA Fragmentation

In a study performed by (Eskiocak, et al., 2006) they found elevated levels of NO in individuals who scored higher on the State Anxiety Index questionnaire. DNA fragmentation is one of the later steps of apoptosis. The activation of endonucleases during apoptosis results in chromatin structure degradation. Following a regression analysis state anxiety was found to have a strong association with increased level of DNA fragmentation (Vellani, et al., 2013).

### 3.10.1. Description of instrumentation

The BD FACSCanto II flow cytometer is a fixed-alignment benchtop analyser, equipped with blue (488-nm), red (635-nm) and violet (405-nm) solid state lasers for excitation of up to 9 fluorescent parameters and two scatter parameters, forward scatter (FSC) and side scatter (SSC).

The analyser employs detector arrays with photomultiplier tubes (PMTs) and a photodiode detector to detect and amplify emitted photons from excited fluorochromes. The blue laser has an octagon detector array with six PMTs that detect SSC and the red and violet lasers each have a trigon array with two PMTs to detect fluorescence signals from the lasers. The FSC signal is detected by the photodiode detector. The PMTs convert photons into electrical pulses, which are subsequently processed by the electronics system and converted into visual data. All acquisition and instrument functions are controlled by BD FACSDiva v5 software.

### 3.10.2. Methodology for flow cytometric analysis

The acquisition and analysis of samples was performed by the investigator at the Becton Dickinson/Central Analytical Facilities Fluorescent (BD-CAF) Flow Cytometry Unit, Stellenbosch University. Prepared samples were acquired on the BD FACSCanto II (BD Biosciences, USA). As we have two separate flow cytometry experiments, we have described the methodology for each separately.

### 3.10.3. Nitric Oxide analysis

#### 3.10.3.1. Principle of Analysis

The fluorescent probe 4,5-diaminofluorescein-2/diacetate or DAF-2-DA (EMD Millipore, Darmstadt, Germany) was used in the detection of the free radical, nitric oxide (NO). DAF-2-DA is described as a cell-permeable NO indicator. Once inside lumen of the cell, DAF-2-DA is deacetylated by intracellular esterase's to form DAF-2. It is DAF-2 that is able to react

with NO to produce the extremely fluorescent triazolofluorescien (DAF-2T) (Abcam plc, 2019). Research grade DAF-2-DA has a stock concentration of 2.2 mg/ml. Aliquots of 2 µl were made in brown 2ml Eppendorf tubes to protect the light sensitive DAF-2-DA.

#### 3.10.3.2. Assay Procedure

Using a protocol developed in our laboratory, that was modified from a protocol for cardiomyocytes, an aliquot of semen sample was double washed by adding an equal volume of Hams-BSA to the semen sample and centrifuging for 10 minutes at 1.5rpm (Strijdom, et al., 2004; Lampiao, et al., 2006). The supernatant was then discarded, and the pellet resuspended in 2ml Hams-BSA and centrifuged again at the same settings. Once again, the supernatant was discarded, and the pellet was then resuspended in 1ml of Hams-BSA. The concentration of the sample suspension was adjusted to $2x10^6$ cells/ml.

The samples (998 µl) were then loaded with 2 µl of DAF-2-DA, which is the equivalent of 10 µM treatment, and allowed to incubate for 120 minutes at 37° C in the dark. After the incubation the samples were analysed using flow cytometry.

#### 3.10.3.3. Instrument setup

To determine optimal voltage settings for the experiment, a stained cell control was employed for the fluorochrome, as well as an unstained cell control. The applications settings were saved and applied with each experiment in BD FACSDiva 8.0.1 software, to standardize our experimental data. To determine optimal experimental voltage settings as shown in *Table 2*, the unstained cells were adjusted to at least 2.5 times higher than the system the electronic noise (rSD).

Table 2 The voltages that were used in the NO analysis. FSC= forward scatter, SSC= side scatter, A= area, H=height and W=width

| | Voltage | Log | A | H | W |
|---|---|---|---|---|---|
| **FSC** | 360 | | ✓ | ✓ | ✓ |
| **SSC** | 540 | | ✓ | ✓ | ✓ |
| **FITC** | 460 | ✓ | ✓ | | |

### 3.10.3.4. Sample acquisition and analysis

Sample tubes were resuspended by vortexing for 5 seconds before acquisition. For data acquisition, a minimum of 30 000 single events were collected for each sample tube. All samples were run on the application settings. For further analysis, all files were exported as FCS 3.1 files and further analysed in FlowJo™ v10.5.2

### 3.10.3.5. Gating

To separate spermatozoa from debris, forward scatter (FSC-A) vs. side scatter (SSC-A) plot was used and spermatozoa were gated as shown in *Figure 10*. Within this population, a time gate (FITC vs. Time) was created to exclude any erroneous data that may have resulted from the instrument performance. Thereafter, a histogram was created (Count vs. FITC) depicting captured fluorescence. This was applied to all samples and exported to Microsoft Excel for further analysis.

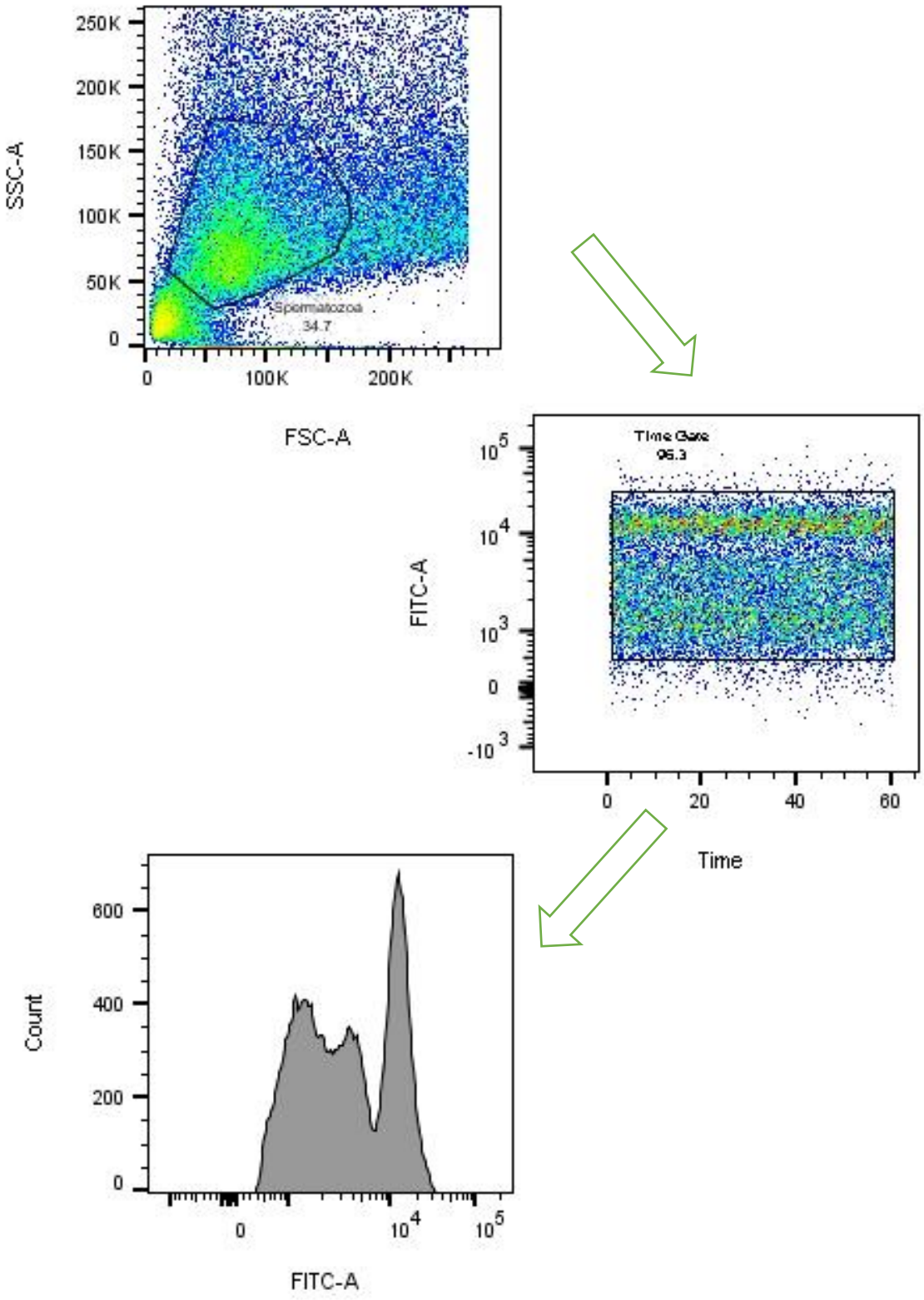



Figure 10 Gating Strategy employed for NO analysis

### 3.10.4. DNA Fragmentation analysis on Spermatozoa

#### 3.10.4.1. Principle of Analysis

The BD APO-DIRECT™ Kit (BD Biosciences, USA) was used for the DNA fragmentation assay. This flow cytometric assay is a method for labelling DNA breaks with FITC-labelled dUTP, in combination with prodium iodide (PI). In this method, terminal deoxynucleotidal transferase (TdT) catalyses an addition of bromolated deoxyuridine triphosphates (Br-dUTP) to the 3'-hydroxyl (OH) termini of the double-stranded and single-stranded DNA. The DNA strands, usually 50 base pairs in length when fragmented, will have the BR-dUTP incorporated to these fragmented sites, which will bind the FITC-labelled anti-BrdU monoclonal antibody. This FITC fluorescence is then identified by flow cytometry.

For fixation and staining, all the reagents were prepared as per manufacturer's instructions. Briefly, cells were washed twice with Hams-BSA and resuspended in a 1% (w/v) paraformaldehyde solution at a concentration of 10 million spermatozoa/ml. The suspension was kept at 4°C for 30 minutes. Thereafter the spermatozoa were centrifuged for 5 minutes at 300 x g, the supernatant was discarded, and the spermatozoa were suspended in 70% (v/v) ice cold ethanol. The collected samples were stored at -20°C for further analysis.

#### 3.10.4.2. Assay Procedure

The samples were batched for processing and 14 samples were analyzed at a time. To prepare the samples for staining, the tubes were gently swirled to resuspend the sperm-ethanol solution. Thereafter, the spermatozoa were centrifuged for 5 minutes at 300 x g and the ethanol supernatant was discarded. The sample was then resuspended in Wash Buffer and the centrifugation step was repeated. An additional interim step was added where 1ml of 0.1% Triton was added to the pellet which was gently disturbed and left to sit for 5 minutes to increase the permeability of the cells. The wash buffer step was then repeated. DNA labeling solution was prepared as per kit instructions and 50 µl of the solution was added to each tube, which was then allowed to incubate at 37°C for 60 minutes. After the incubation

period, 1 ml of the Rinse Buffer was added to each tube and the centrifugation step repeated. Thereafter, the pellet was resuspended in 0.5 ml of PI/ RNase Staining Buffer and incubated for 30 minutes in the dark before analysis on the flow cytometer commenced.

#### 3.10.4.3. Instrument setup and Compensation

To determine optimal voltage settings for the experiment, single stain cell controls were employed for each fluorochrome, also with an unstained cell control. Applications settings were saved and applied with each experiment in BD FACSDiva 8.0.1 software, to standardize our experimental data. To determine optimal experimental voltage settings, the unstained cells were adjusted to 2.5 times higher than the system the electronic noise (rSD) and the dUTP and PI single stained positive controls was used to adjust the positive populations to be within the maximum linearity range for channel. The settings were saved as Application Settings, which is linked to the daily quality control Cytometer Setup and Tracking (CST) check. The application settings for voltage changes, using the CST as its measure automatically adjusts the voltage settings, accordingly, ensuring the reproducibility of the data. As a second control, we included a lot specific 8-peak bead control as the with the daily run to ensure that application settings were valid and reproducible.

For the compensation, single stained cell controls were employed, an ethanol fixed sample was used for the PI positive control and a DNAse treated cells sample was used for the dUTP-FITC positive control. The compensation matrix values are shown in *Table 3*.

The samples were acquired on pre-determined voltages, and the compensation matrix was then applied to the experiment for the acquisition and analysis of the samples.

Table 3 Representative Compensation Matrix for DNA Fragmentation analysis. This is depicted as a percentage of spillover subtraction.

| | **FITC-A** | **PERCP-A** |
|---|---|---|
| **FITC-A** | 100 | 1,526 |
| **PERCP-A** | 0,2878 | 100 |

### 3.10.4.4. Sample acquisition and analysis

Sample tubes were resuspended by vortexing for 5 seconds before acquisition. For data acquisition, a minimum of 20 000 singlet events were collected for each sample tube. All samples were run on compensated application settings, as previously described in the optimisation and compensation was performed with every run. For further analysis, all files were exported as FCS 3.1 files and further analysed in FlowJo™ v10.5.2

### 3.10.4.5. Gating

The gating strategy is illustrated in *Figure 11*. To separate spermatozoa from debris, forward scatter (FSC-A) vs. side scatter (SSC-A) was plot was used and spermatozoa were gated. Within this population, a time gate (dUTP FITC vs. Time) was created to exclude any erroneous data that may have resulted from the instrument performance. The following 4 populations were gated using a dUTP FITC-A vs. PI-A plot: unstained (US), dUTP FITC+, PI PerCP+ only and the dUTP FITC + PI PerCP double positive cells. This gating was determined with single stains and applied all samples and was exported to Microsoft Excel for further analysis.

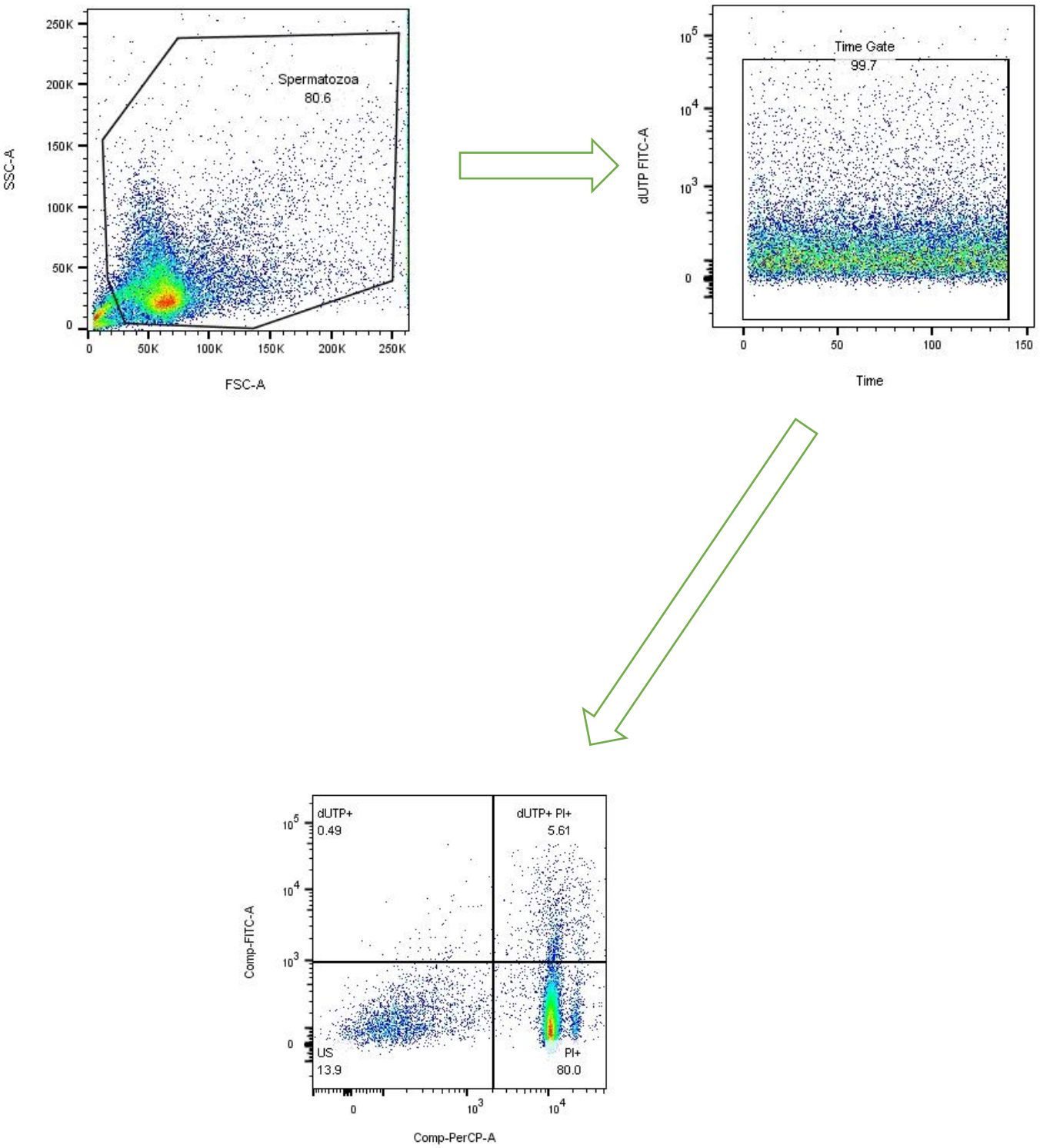



Figure 11 Gating strategy used for DNA Fragmentation analysis

## 3.11. Cytokine Profile

Cytokines are secreted by different parts of the male genital tract and are thought to exert effects on steroidogenesis, spermatogenesis and sperm function (Dousset, et al., 1997). Literature has reported a positive correlation between anxiety and inflammatory markers such as TNF-α and IL-6 in humans in other body systems. In semen cytokines such as IL-1β, IL-6 and IFN-γ are found in the semen of infertile men (Havrylyuk, et al., 2015).

### 3.11.1. Description of the instrument

The Magnetic Luminex® Assay multiplex kit was used for this analysis. The kit is designed for use with the Bio-Rad® platform. The Bio-Rad® Bio-Plex® is a dual laser, flow-based sorting and detecting platform. The analyser makes use of a single laser to excite the dyes inside each microparticle. This then identifies each microparticle region. A second laser excites the PE and subsequently measures the amount of analyte bound to each microparticle. The emitted excitation as each microparticle passes through the flow cell is analysed to differentiate between excitation levels using a PMT and Avalanche Photodiode.

### 3.11.2. Principle of the assay

Magnetic microparticles are pre-coated with analyte-specific antibodies. The magnetic microparticles are embedded with fluorophores at predetermined ratios for each microparticle region. Samples, standards and microparticles were pipetted into the 96 well plate. The analytes of interest are then bound by the immobilized antibodies. Once the unbound substances are washed away, a biotinylated antibody cocktail specific to the analytes of interest, was added to each well. A wash step removed any unbound biotinylated antibody. Streptavidin-phycoerythrin conjugate (Streptavidin-PE) is able to bind to biotinylated antibody and was added to each well. A final wash step removed unbound Streptavidin-PE. The microparticles were resuspended in a kit specific buffer and read using the Bio-Rad® Bio-Plex® Analyzer.

### 3.11.3. Assay Procedure

All the reagents were prepared as per manufacturer's instructions. Briefly, all reagents and samples were brought to room temperature before use. All standards and samples were assayed in duplicate and prepared using a plate layout provided. The diluted microparticle cocktail was resuspended by inversion or vortexing. The cocktail (50 µl) was added to the well of each microplate. The microplate was then securely covered with a foil plate sealer and incubated for 2 hours at room temperature on a horizontal microplate shaker. A wash step procedure was performed three times. Biotin-antibody cocktail (50 µl) was added to each well, covered with a foil plate and incubated for hour on the microplate shaker. The wash step was repeated three times. Streptavidin-PE (50 µl) was added to each well, the plate sealed and then incubated for 30 minutes on the shaker. The wash step was repeated as before. The microparticles were resuspended in 100 µl of wash buffer and incubated for 2 minutes before reading on the Bio-Rad® analyser.

The following proinflammatory cytokines and cytokine-soluble receptors are to be examined in the semen and blood plasma: IL-1β, IL-6, and IFN-Y (R&D Systems, Minneapolis, MN). Data was expressed in pg/mL.

### 3.11.4. Instrument Settings

The instrument flow rate is set to 60 µl/minute (fast) and the sample volume was set to 50 µl. Bio-Plex MagPlex Beads (magnetic) were the bead types used for this assay. The doublet discriminator gates were set at 8000 and 23 000. The low RP1 target value for the CAL2 setting was used for the reporter gain setting. The microparticle region for each analyte measured was assigned. The median fluorescence intensity was collected.

## 3.12. Cortisol Determination

Human plasma cortisol was measured directly using a competitive immunoenzymatical colourmetric technique. Cortisol is an important component of the stress response and was thus measured as a representation of biological stress that can be compared to the self-reported stress measured using the STAI questionnaire. A cortisol ELISA kit was used (Diametra, Italy).

### 3.12.1.1. Principle of the Assay

Cortisol present in the sample competes with the antigenic cortisol that is conjugated to horseradish peroxidase (HRP) for binding to the limited quantity of anti-cortisol antibodies that are coated on the microplate. The bound/free separation is performed, after incubation, by a simple wash step. Thereafter, HRP in the bound fraction reacts with $H_2O_2$ (substrate) and the (3,3',5,5'- tetramethylbenzidine) TMB substrate to develop a blue colour. Once the stop solution ($H_2SO_4$) is added the solution turns to a yellow colour. The cortisol intensity is inversely proportional to the colour intensity in the sample. The cortisol intensity is calculated through a calibration curve.

### 3.12.1.2. Assay Procedure

All reagents and samples were brought to room temperature for at least 30 minutes and the procedure followed as in *Table 4.* All reagents and samples were added as per the plate layout.

Table 4 A Table describing the analysis procedure

| **Reagent** | **Calibrator** | **Sample** | **Blank** |
|---|---|---|---|
| Calibrator ($C_0$-$C_4$) | 20 µl | | |
| Sample | | 20 µl | |
| Conjugate | 200 µl | 200 µl | |
| The microplate was incubated for 1 hour at 37°C<br>The microplate was washed 6 times using an automatic washer and the wash buffer prepared per kit instructions. | | | |
| TMB Substrate | 100 µl | 100 µl | 100 µl |
| The microplate was then incubated for 15 minutes in the dark at room temperature | | | |
| Stop Solution | 100 µl | 100 µl | 100 µl |

The microplate was then gently shaken, and the absorbance was read on a microplate reader at 450 nm against a reference wavelength of 620-630 nm within 5 minutes.

The mean absorbance for each point of the calibration curve was calculated. The mean value of absorbance of the calibrators was plotted against the concentration. A best-fit curve was drawn between the plotted points. The results were calculated by interpolating the values of the samples on the calibration curve to obtain the corresponding concentrations expressed in ng/ml. The reference range for cortisol between the hours of 08:00 and 10:00

were 60-230 ng/ml. At 16:00 the anticipated cortisol reference values range between 30-150 ng/ml.

### 3.13. Statistical Approach and Analysis

GraphPad™ Prism version 5.00 (San Diego, USA) and TIBCO Statistica® version 13.5.0 (INRIA, France) were used for all statistical analysis as per the statistical strategies employed in *Figure 12*. Descriptive results are reported as mean ± standard deviation (SD). Statistical significance was set to $p \leq 0.05$. The structure of this project has allowed for a complex statistical approach. As depicted in *Figure 12* initially the co-relationship of the independent variables (state and trait anxiety) to the single dependent variables (sdv's) such as total motility or ROS level was investigated using Spearman and Pearson correlations.

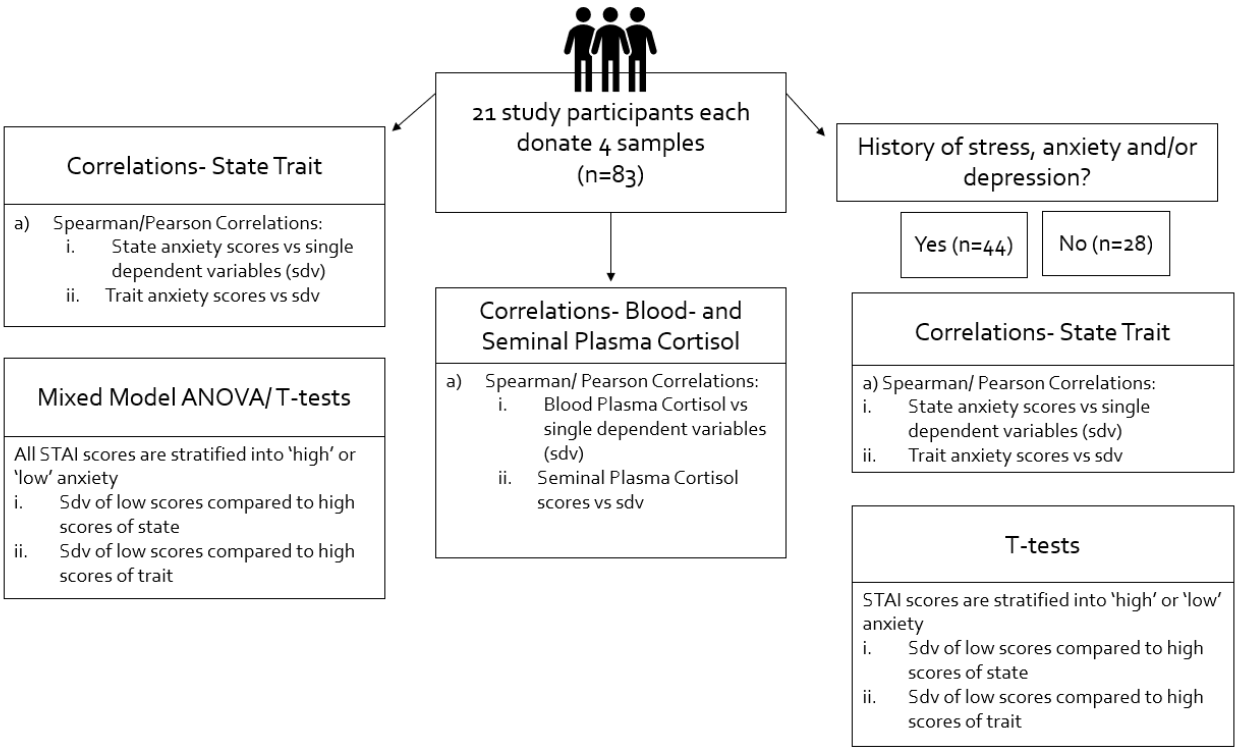



Figure 12 The statistical strategy employed throughout this study. One participant was only able to donate 3 samples. Scores of ≥40 considered 'high' anxiety, scores <40 considered 'low' anxiety.

Using the Shapiro-Wilke test for normality and prevalence of outliers in the data, it determined whether Spearman or Pearson results were reported.

Given the data set repeats for each participant (each participant had four donation sessions), it called into question whether the single independent variables were truly statistically independent. A Mixed Model ANOVA (variation of repeated measure ANOVA) was performed on the stratified anxiety scores. This analysis of variance allows us to possibly verify the results from the correlations and accounted for the repeated measures and handles missing data sets effectively. The least significant difference post-hoc test was used where the p-value was significant.

A possible biological indicator of anxiety is cortisol. Both blood and seminal plasma cortisol were investigated in this study. To examine the co-relationship between a possible biological indictor of anxiety and the other biological variables measured Spearman/Pearson correlations were investigated as it was for the State- Trait Anxiety.

The ‘yes’ vs. ‘no’ strategy provides insights into the possible difference that having a history of stress, anxiety and/or depression may have on the results obtained. Spearman and Pearson correlations were performed to assess the relationship between state and trait anxiety, and the sdv’s. The STAI scores were stratified into high and low as before and t-tests were performed for each sdv to verify the correlation results. Though participants were encouraged to donate within periods of “high stress” and “low stress”, participants were not always able to adhere. Thus, periods of “high stress” and “low stress” were not factored into the grouping strategies.

# Chapter 4

# Results

## 4.1. Introduction

This chapter will present the findings of this study, using the statistical strategies discussed in the methods and materials chapter. The robustness of the data has allowed for various statistical approaches in order to fully investigate if the aims and objectives stipulated have been met.

## 4.2. State- Trait Anxiety Inventory: State/Anxiety vs. Single Dependent Variables

First the STAI results will be presented which includes the descriptive data of the participant population, the correlations found using Spearman/ Pearson correlation and the STAI data stratified into ‘high’ or ‘low’ anxiety scores to allow us to verify the correlations by means of a mixed model ANOVA.

## 4.3. Descriptive Statistics: Demographic Data

A total of 18 participants, of the 21 participants recruited into this study, were compliant in completing the donor profile questionnaire that provided the demographic data that will be illustrated in this chapter.

### 4.3.1. Age

The average age of the participant population is 22.7222 (SD=1.4874). With the youngest participant being 20 years old and the oldest participant being 25 years old as depicted in *Figure 13*.

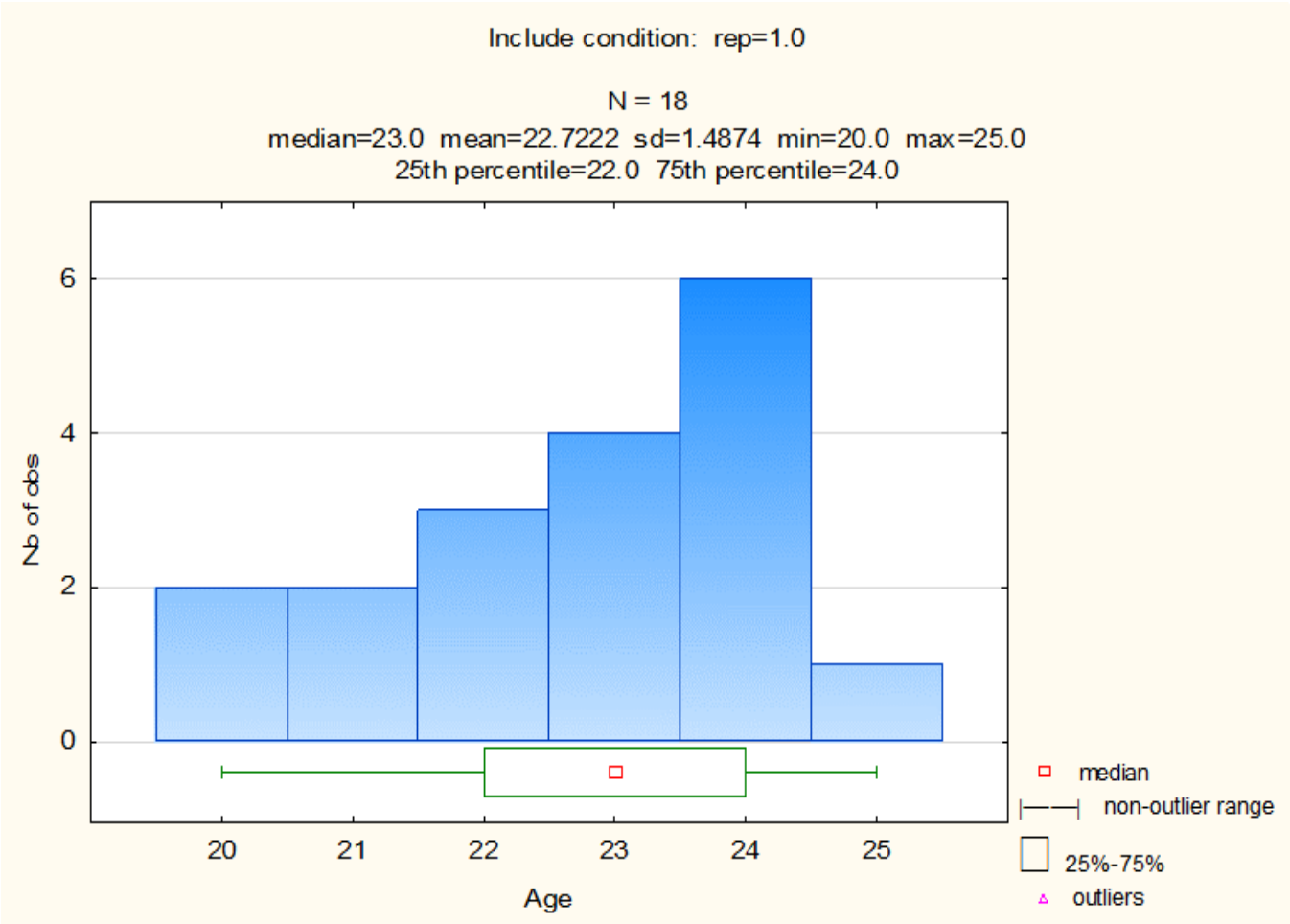



Figure 13 Histogram depicting the number of observations and distribution of age of the participant population

### 4.3.2. Academic Program

The participants in this study were enrolled in a number of different programs: 11 participants were registered for a medical degree (MBChB), 2 in postgraduate programs, 2 in dentistry (BDS) and 3 participants were enrolled in BSc Physiotherapy, BSc Applied Geology and Bachelor of Oral Health (BOH) respectively as depicted in *Figure 14*.

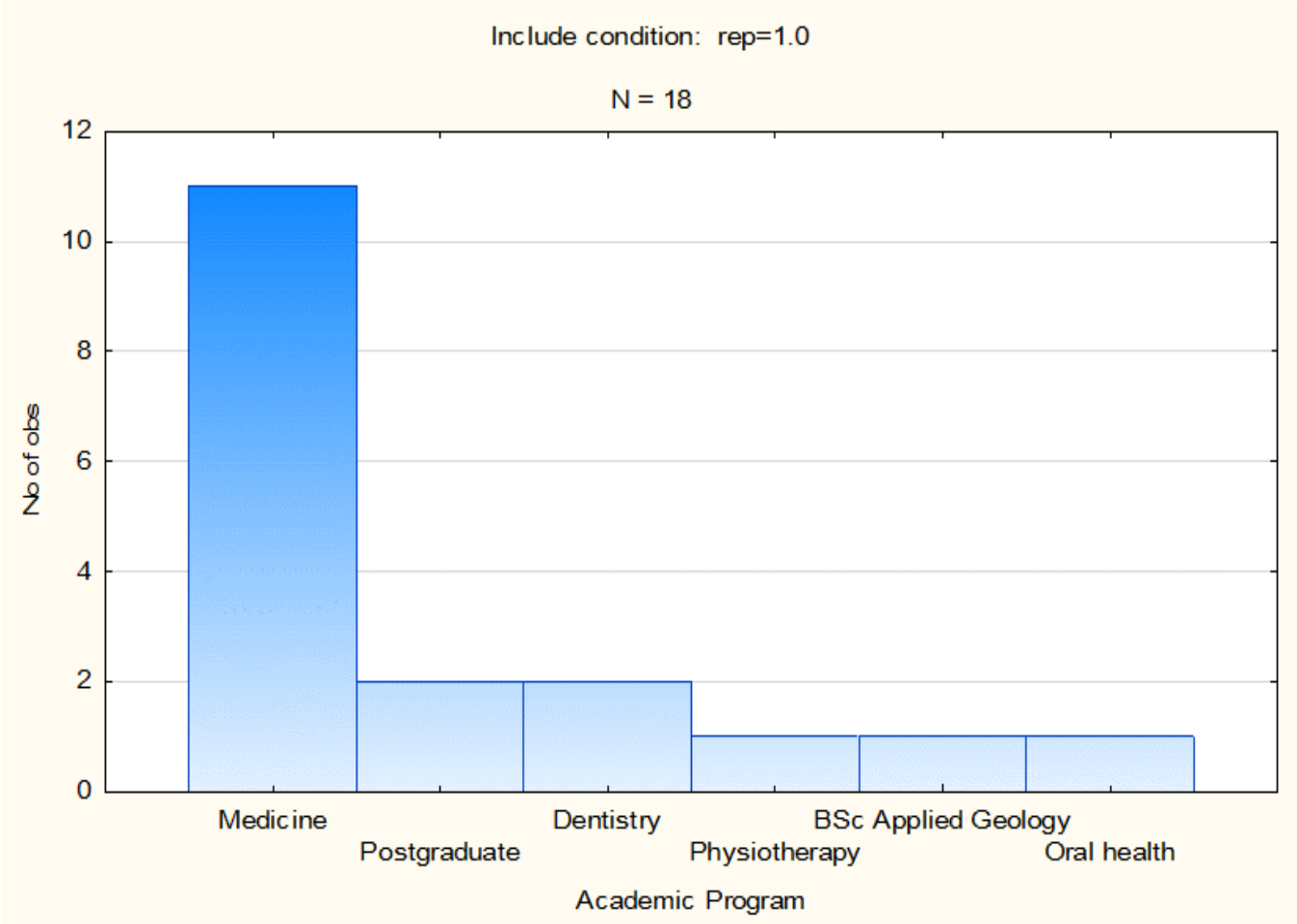



Figure 14 Histogram depicting the number of observations and distribution of academic programs of the participant population

### 4.3.3. Height

The average height of the participants was 1.7806m (SD=0.0919). The shortest height recorded was 1.63m and the tallest height recorded was 1.95m as depicted in *Figure 15*.

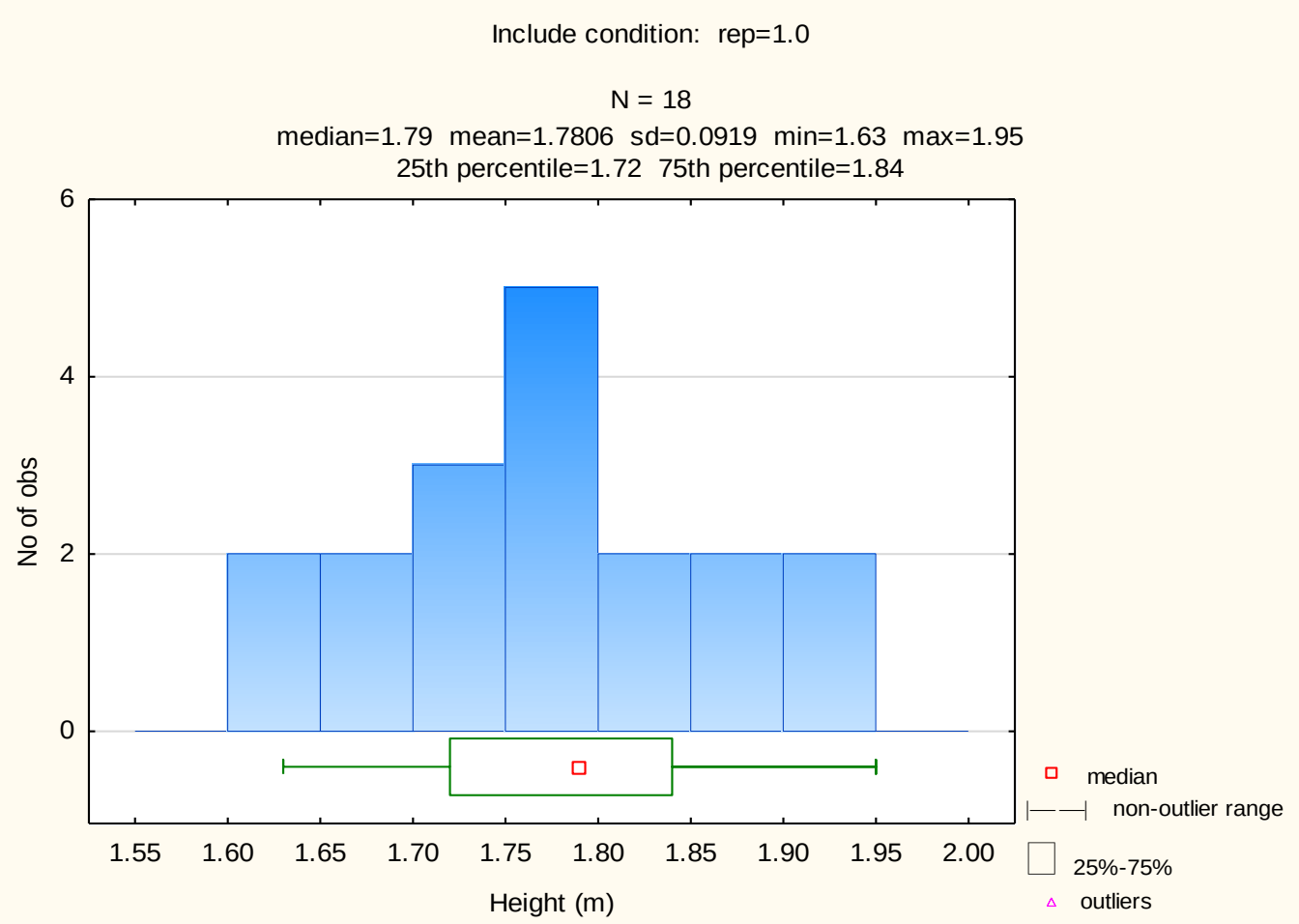



Figure 15 Histogram depicting the number of observations and distribution of the height of the participant population

### 4.3.4. Weight

The lowest weight measured in this study was 57.0 kg while the heaviest weight recorded was 90.0 kg. The average weight recorded throughout this study was 73.7444 kg (SD=9.8816) as depicted in *Figure 16*.

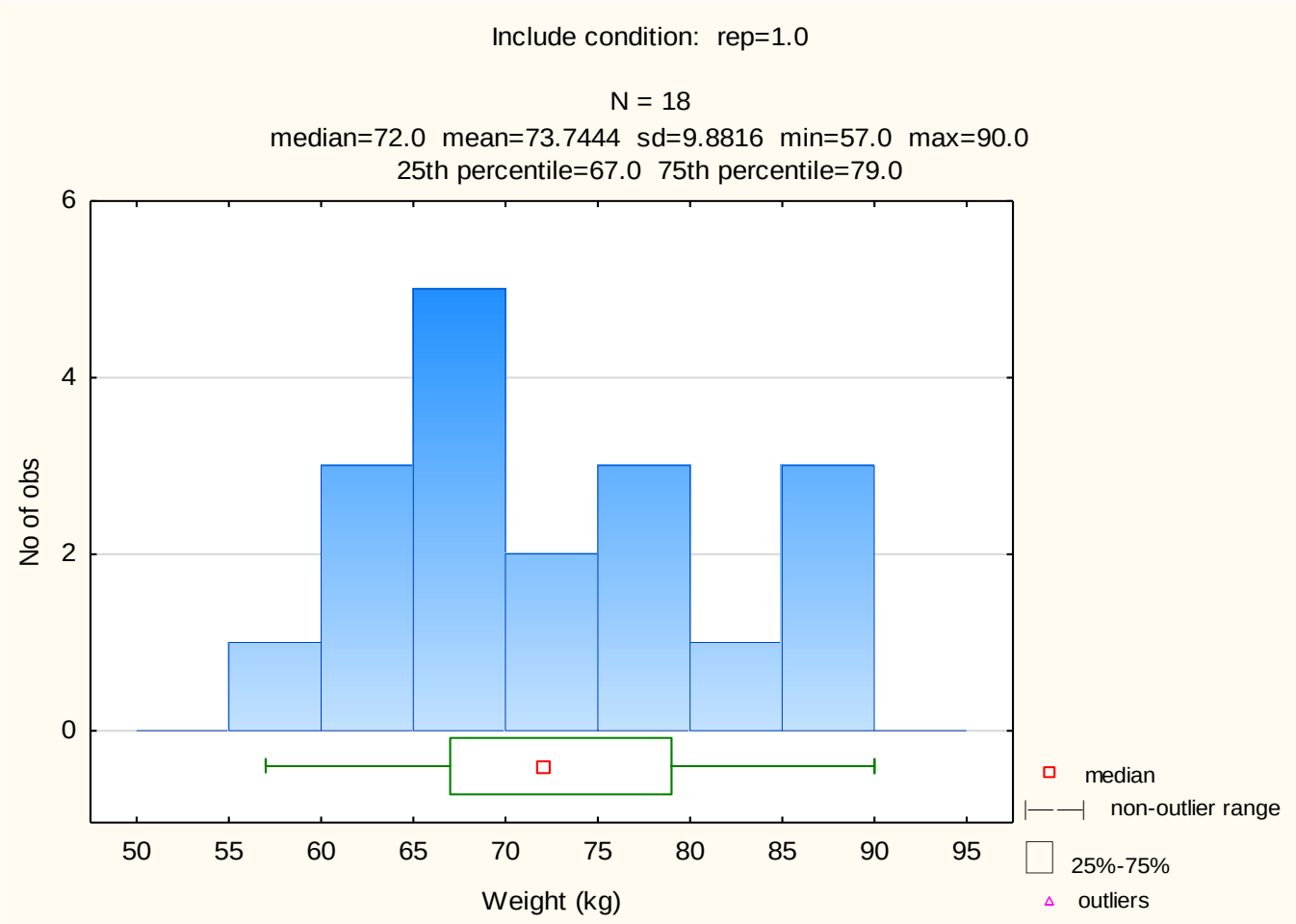



Figure 16 Histogram depicting the number of observations and distribution of the weight of the participant population

### 4.3.5. Body Mass Index (BMI)

The average BMI recorded in this study was 23.2837 (SD=2.8571). The maximum BMI recorded was 29.8707 while the lowest BMI recorded was 19.1598 as depicted in *Figure 17*.

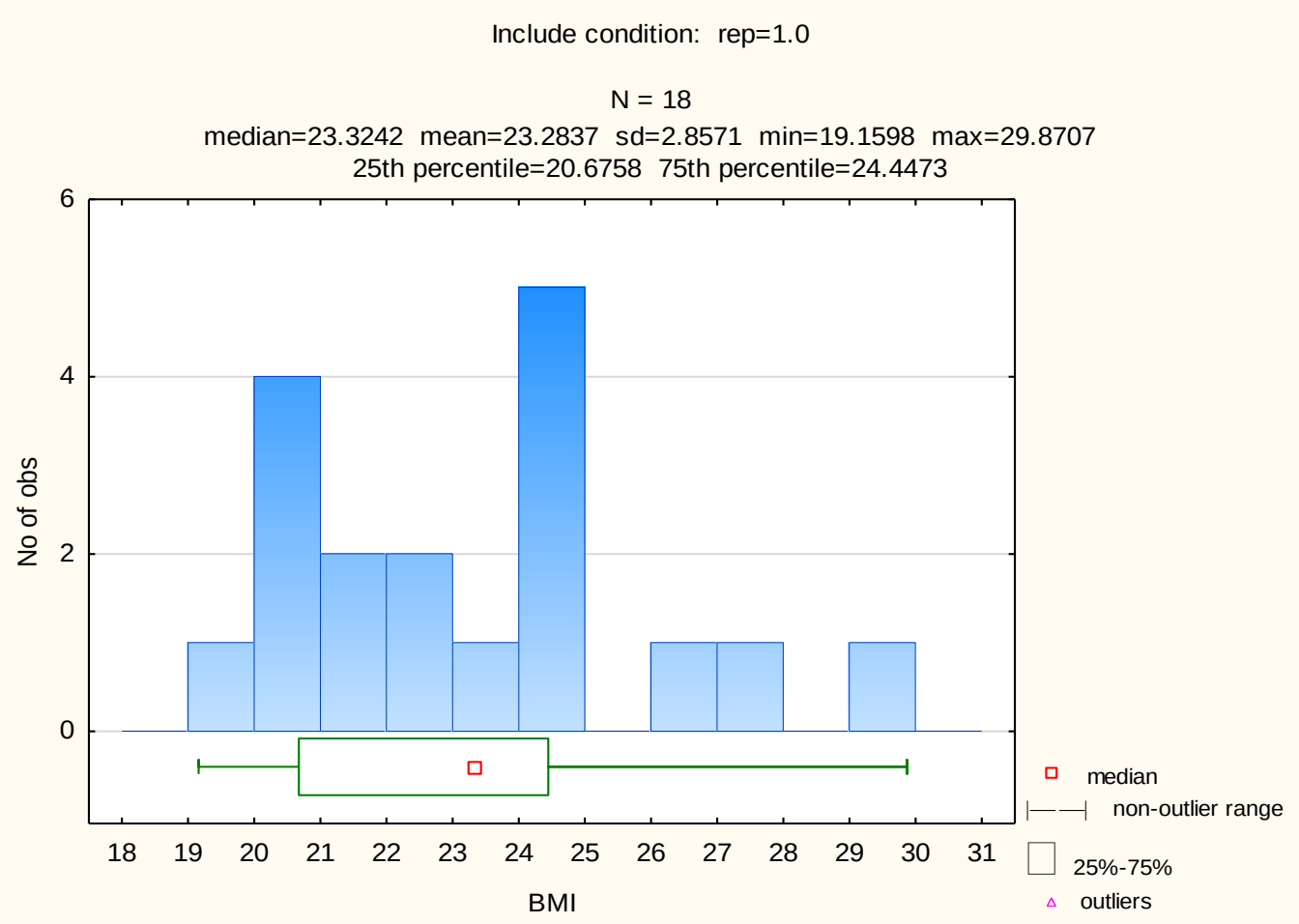



Figure 17 Histogram depicting the number of observations and distribution of the BMI of the participant population

### 4.3.6. Smoking Habits

A total of 13 participants (72%) who partook in this study do not smoke. A further 5 participants were smokers. Of these 5 participants, 1 did not indicate the frequency of their smoking habit, while 1 indicated that they smoked habitually and 3 indicated that they only smoked in social situations as indicated in *Figure 18*.

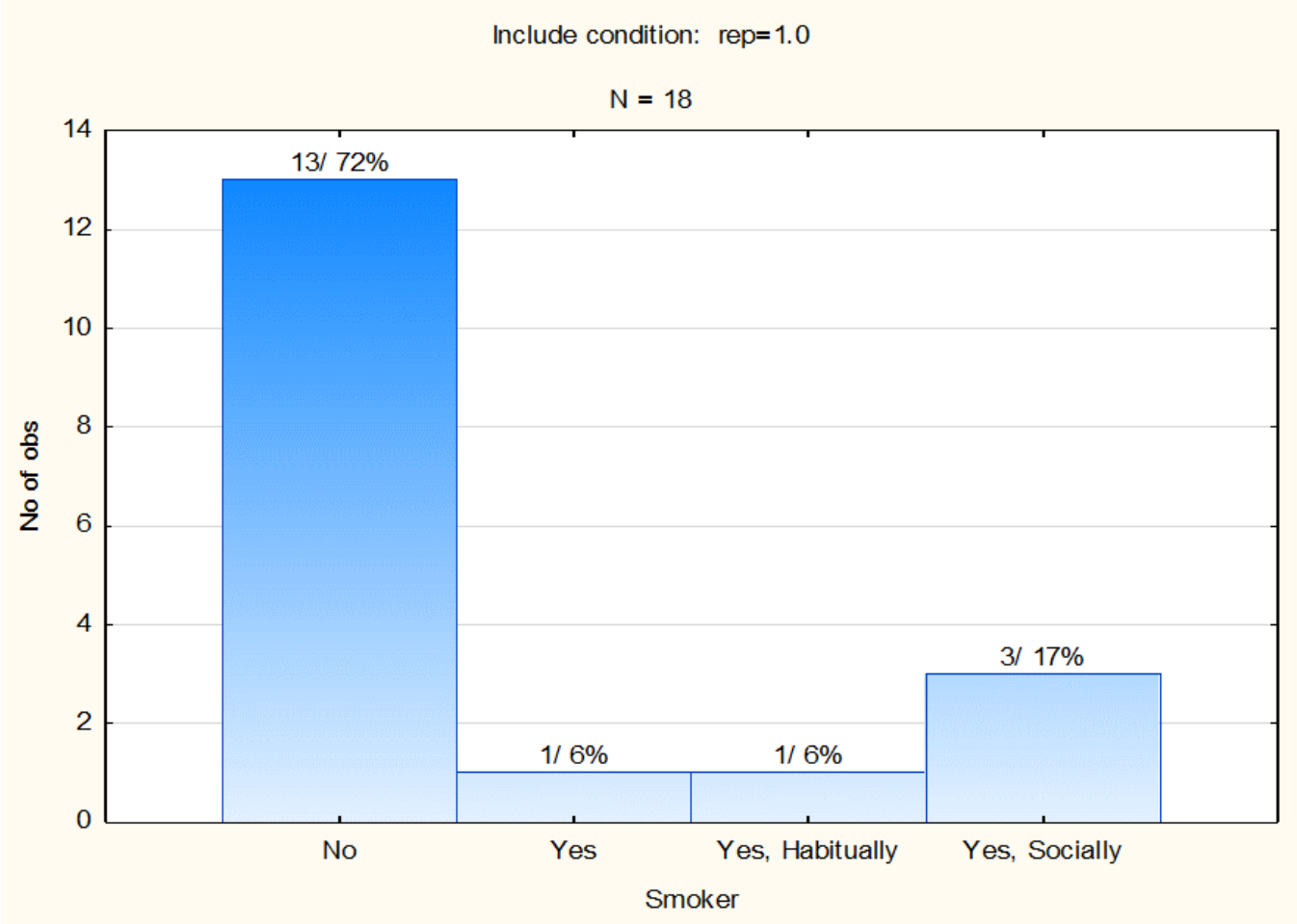



Figure 18 Histogram depicting the number of observations and distribution of the smoking habits of the participant population

### 4.3.7. Vaping Habits

Vaping is often used as an alternative to smoking, 15 of the 18 participants indicated that they do not vape. The remainder of the participants (17%) indicated that they do vape as indicated in *Figure 19*.

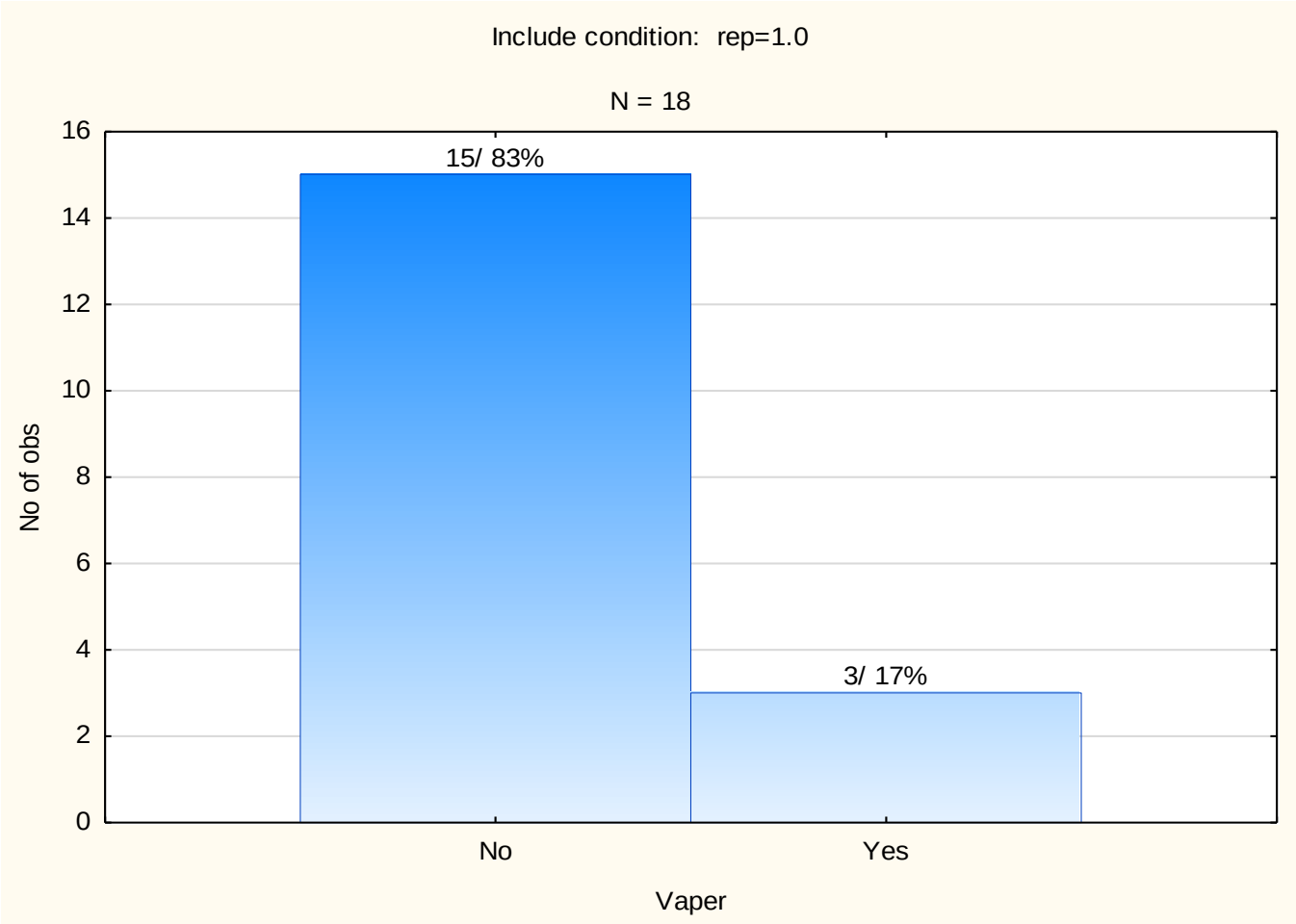



Figure 19 Histogram depicting the number of observations and distribution of the vaping habits of the participant population

### 4.3.8. Alcohol Consumption

A total of 72% of the participant population consume alcohol as depicted in *Figure 20*. The remaining 5 participants indicated that they do not consume alcohol.

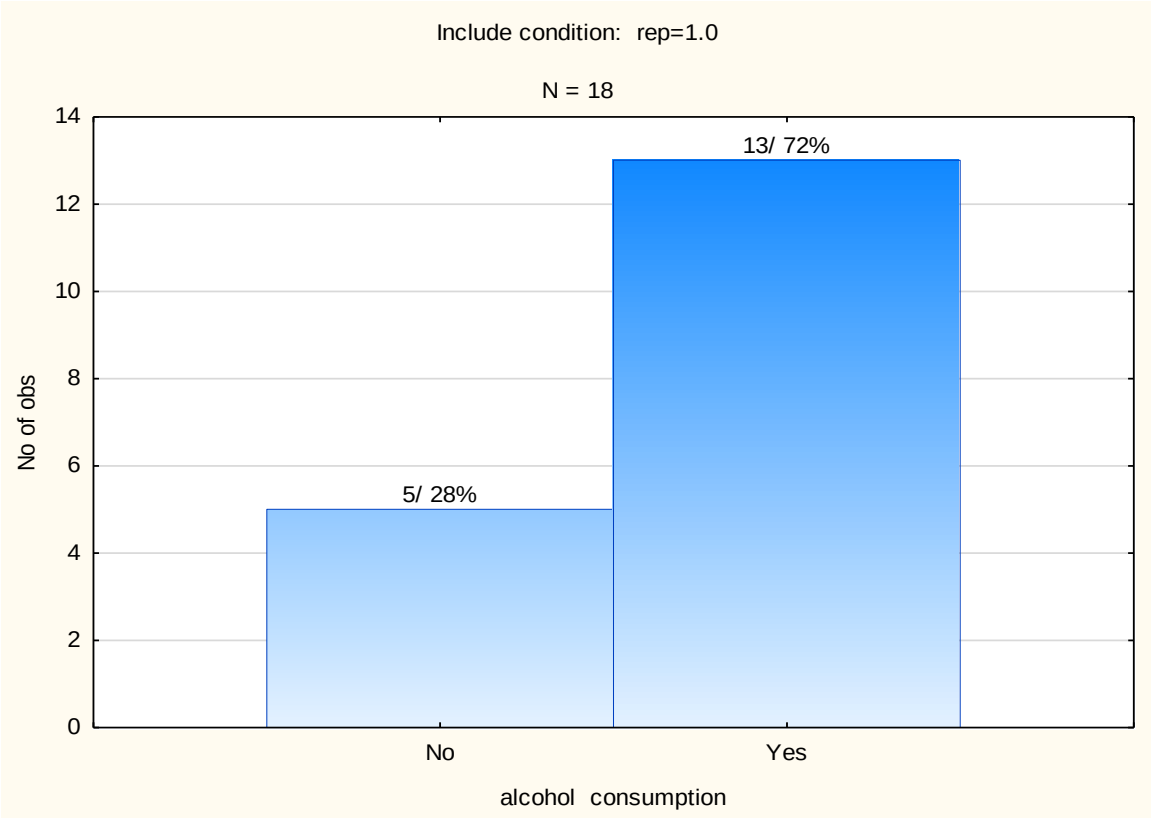



Figure 20 Histogram depicting the number of observations and distribution of the alcohol consumption of the participant population

### 4.3.9. Average Sleep Quantity

The average number of hours of sleep that the participants indicated was 6.6111 hours (SD=0.867). The lowest hours of sleep recorded was 5.0 hours and the maximum amount of sleep recorded was 8.5 hours as indicated in *Figure 21*.

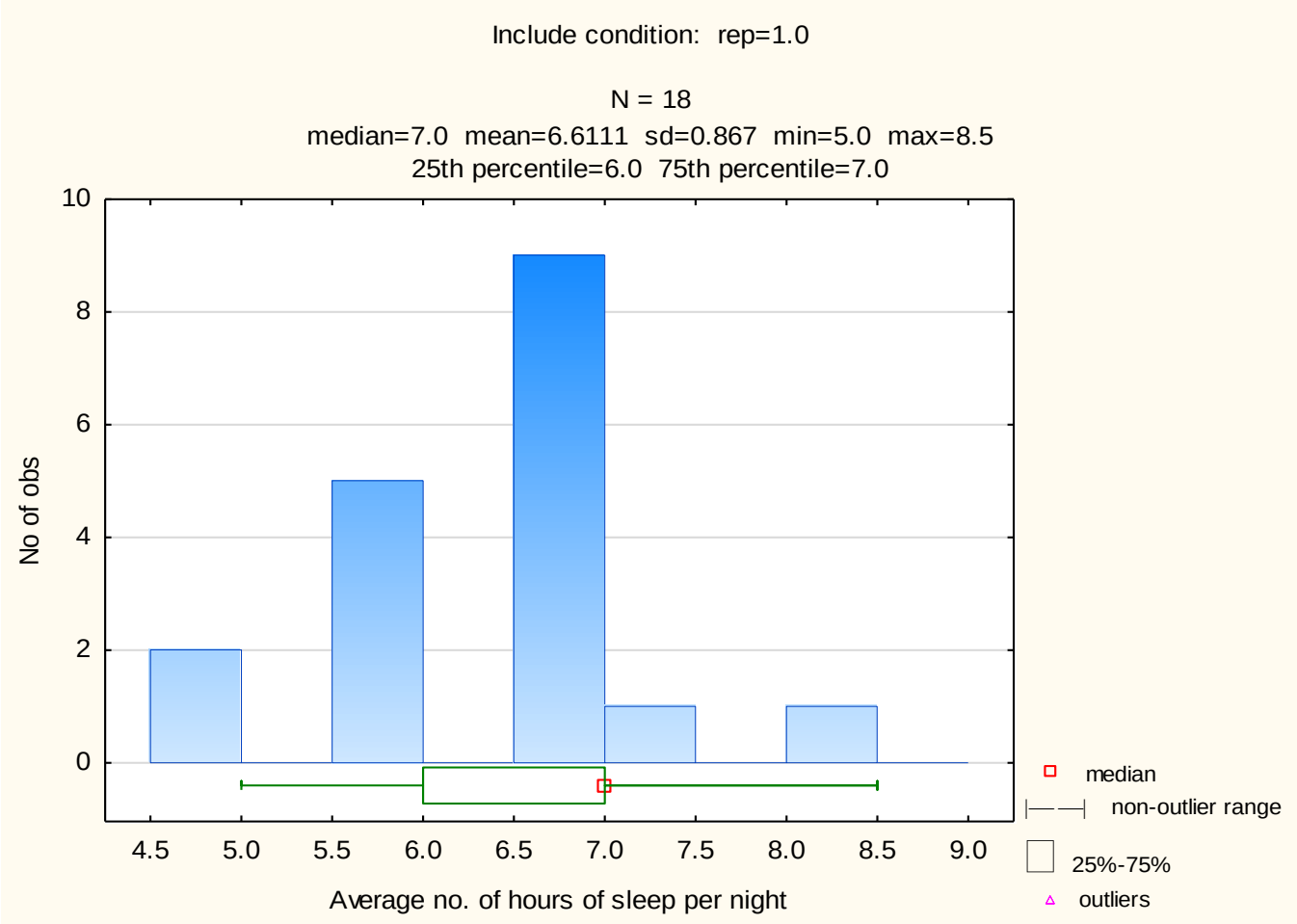



Figure 21 Histogram depicting the number of observations and distribution of the average no. of hours of sleep per night of the participant population

### 4.3.10. Exercise

A large portion of the participants enrolled in this study lead quite healthy lives, with 78% of the participants indicating that they exercise. The remainder of the participant population indicated that they do not exercise as depicted in *Figure 22*.

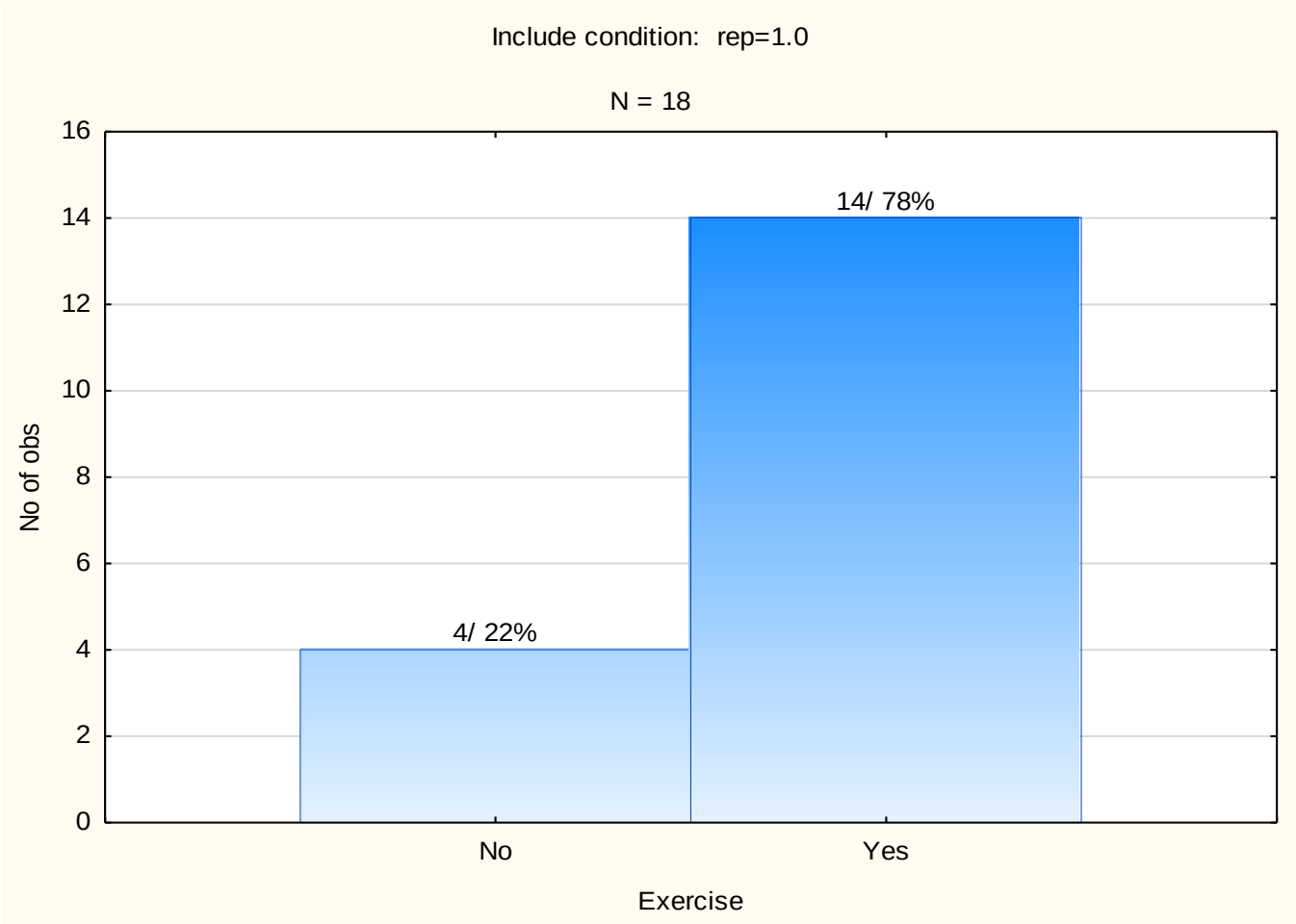



Figure 22 Histogram depicting the number of observations and distribution of exercise of the participant population

### 4.3.11. Frequency of Exercise

Of the 14 individuals who indicated that they exercised, 6 individuals indicated that they exercised 2-3 times a week. While 4 individuals indicated that they exercised 4-5 times a week and 3 individuals exercised every day. One of the participants did not mention the frequency of their exercise as indicated in *Figure 23*.

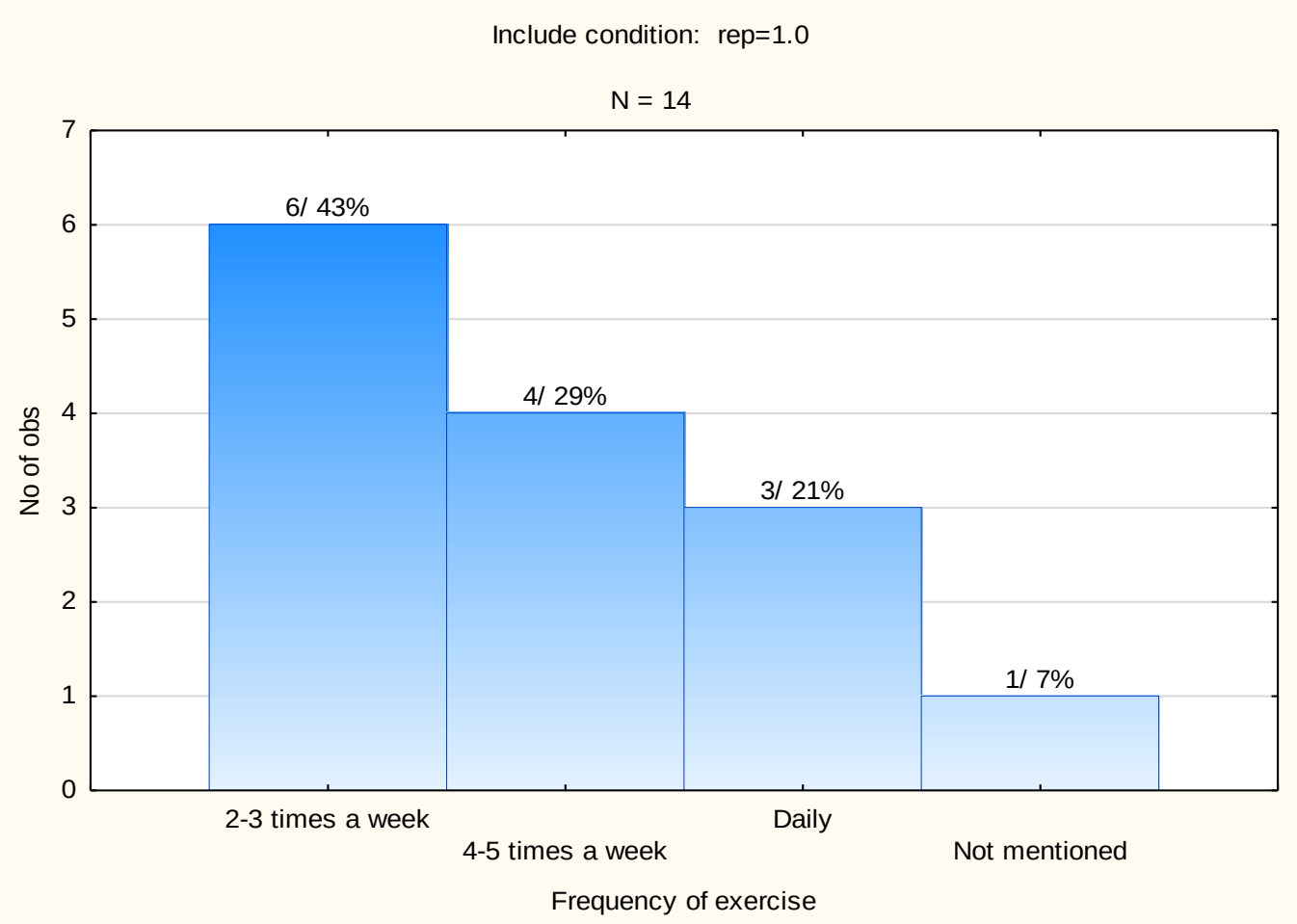



Figure 23 Histogram depicting the number of observations and distribution of frequency of exercise of the participant population

### 4.3.12. History of Stress, Anxiety and/or Depression

The participants were asked if they have history of stress, anxiety or depression. More than half of the participant population (61%) indicated that they have a history of stress, anxiety or depression as indicated in *Figure 24*. Of the 11 participants who answered 'yes', 2 of those participants indicated that they suffered from anxiety, 1 participant suffered from depression and 2 more participants suffered from an anxiety-depression comorbidity. The remaining 6 participants did not indicate whether they suffered from stress, anxiety or depression. Some of the treatments mentioned for those who answered 'yes' included: Fluoxetine, Serdep, Sertraline and Venlaflexin. Cognitive behavioural therapy, therapy and breathing exercises as well as exercise were also mentioned as possible treatments. The remaining participants (39%) indicated that they do not have a history of stress, anxiety, or depression.

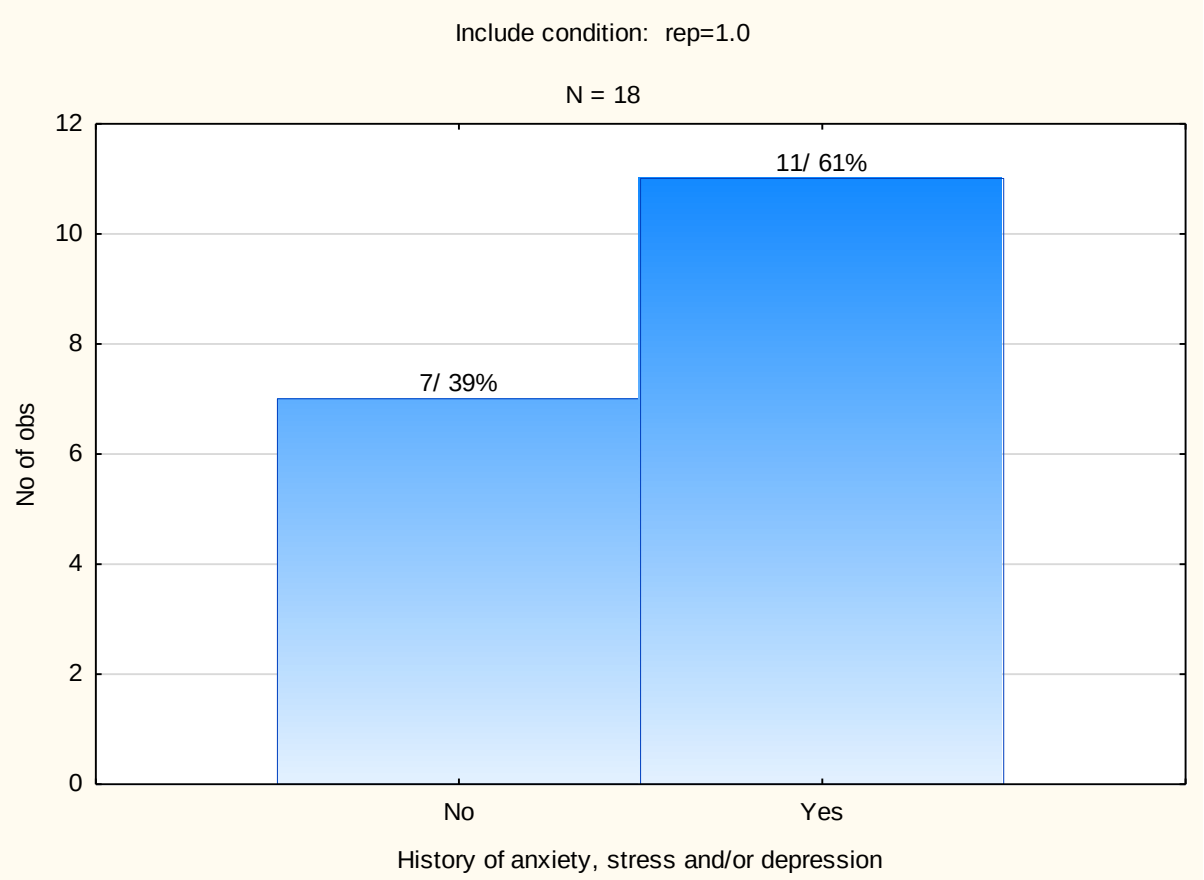



Figure 24 Histogram depicting the number of observations and distribution of answers to donor profile questionnaire asking about the history of stress, anxiety and/or depression of the participant population

## 4.4. Descriptive statistics: Biometric Data

A total of 21 participants participated in the study, each participant attended four scheduled donation sessions apart from one participant. The participant was only compliant in attending 3 of the 4 scheduled donation sessions. Therefore, the biometric data sample size is 83. Any differences in the sample size will be discussed accordingly.

### 4.4.1. STAI Questionnaire Results

The average state score observed was 40.6627 (SD=13.6938) out of a possible score of 80. The lowest state score reported was 20.0 and the highest reported score was 69.0 as indicated in *Figure 25*. While the median score collected was not far from it at 39.0 (SD=13.6938).

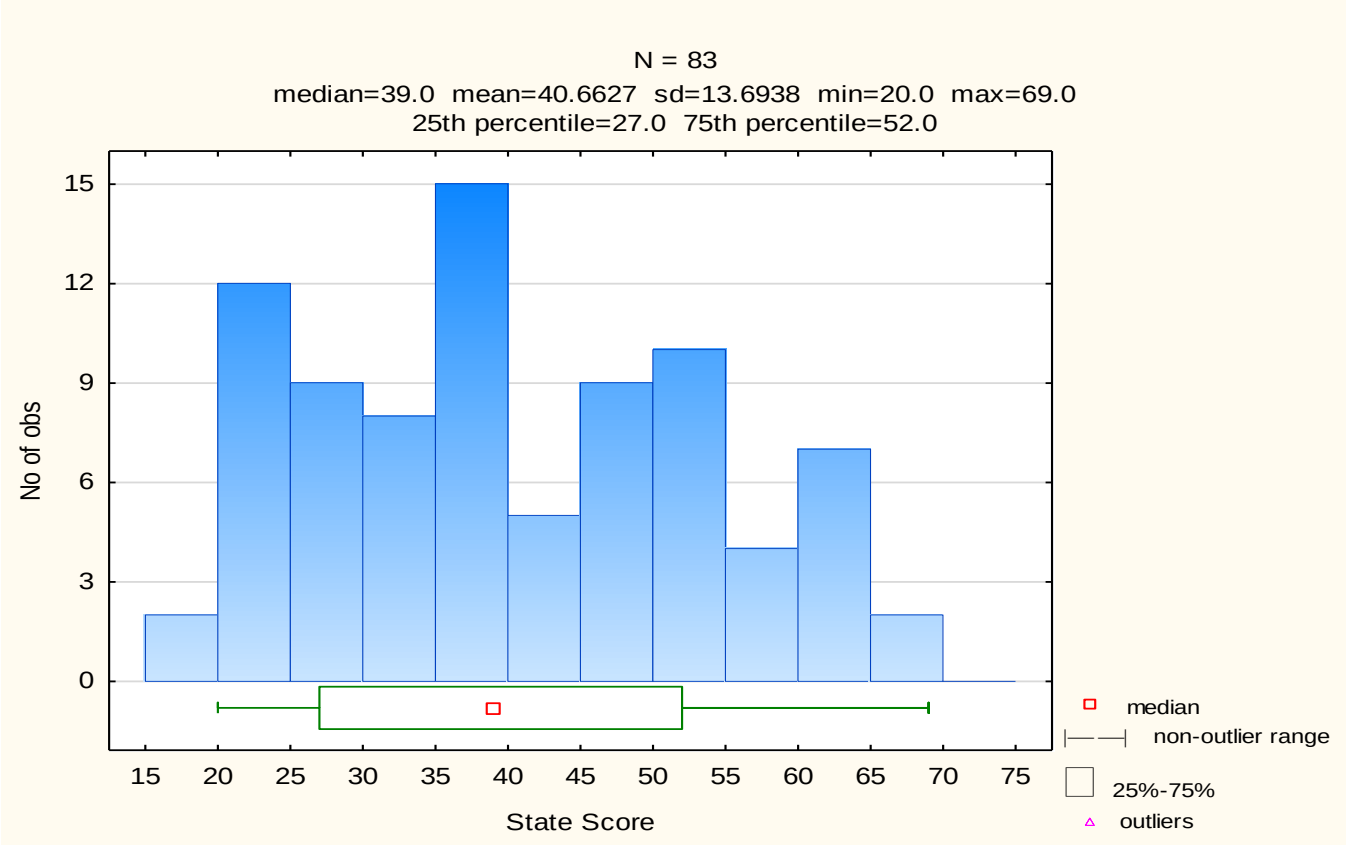



Figure 25 Histogram depicting the number of observations and distribution of the state score of the participant population

Comparatively, the average trait anxiety recorded was 42.0602 (SD=12.5468) as indicated in *Figure 26*. While the median score collected 43.00 (SD=12.5468). The highest score reported was 71.0 out of a possible score of 80, while the lowest score reported was 21.0

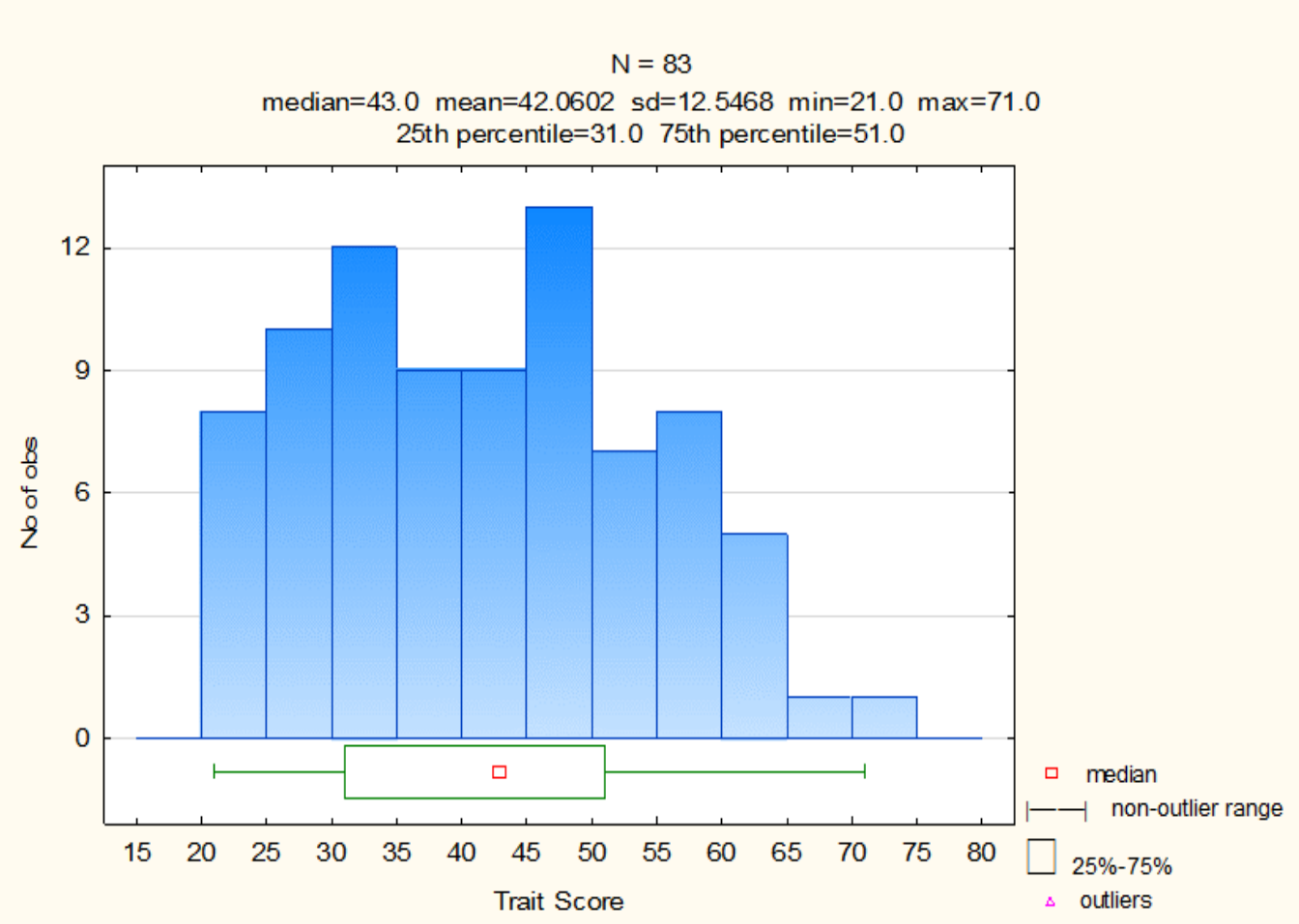



Figure 26 Histogram depicting the number of observations and distribution of trait score of the participant population

### 4.4.2. Abstinence Period

The recommended abstinence period is between 3-5 days (WHO, 2010). However, older sources accept 2-7 days. The average number of days abstained before a donation period was 3.8049 days (SD=1.6588), this is expected as half the data (25%-50%) consists of 3- and 4-day abstinences as shown in *Figure 27*. An abstinence period of two-days was the shortest abstinence period noted, while longer abstinence periods of 6, 7, 9 and 14 days were also observed.

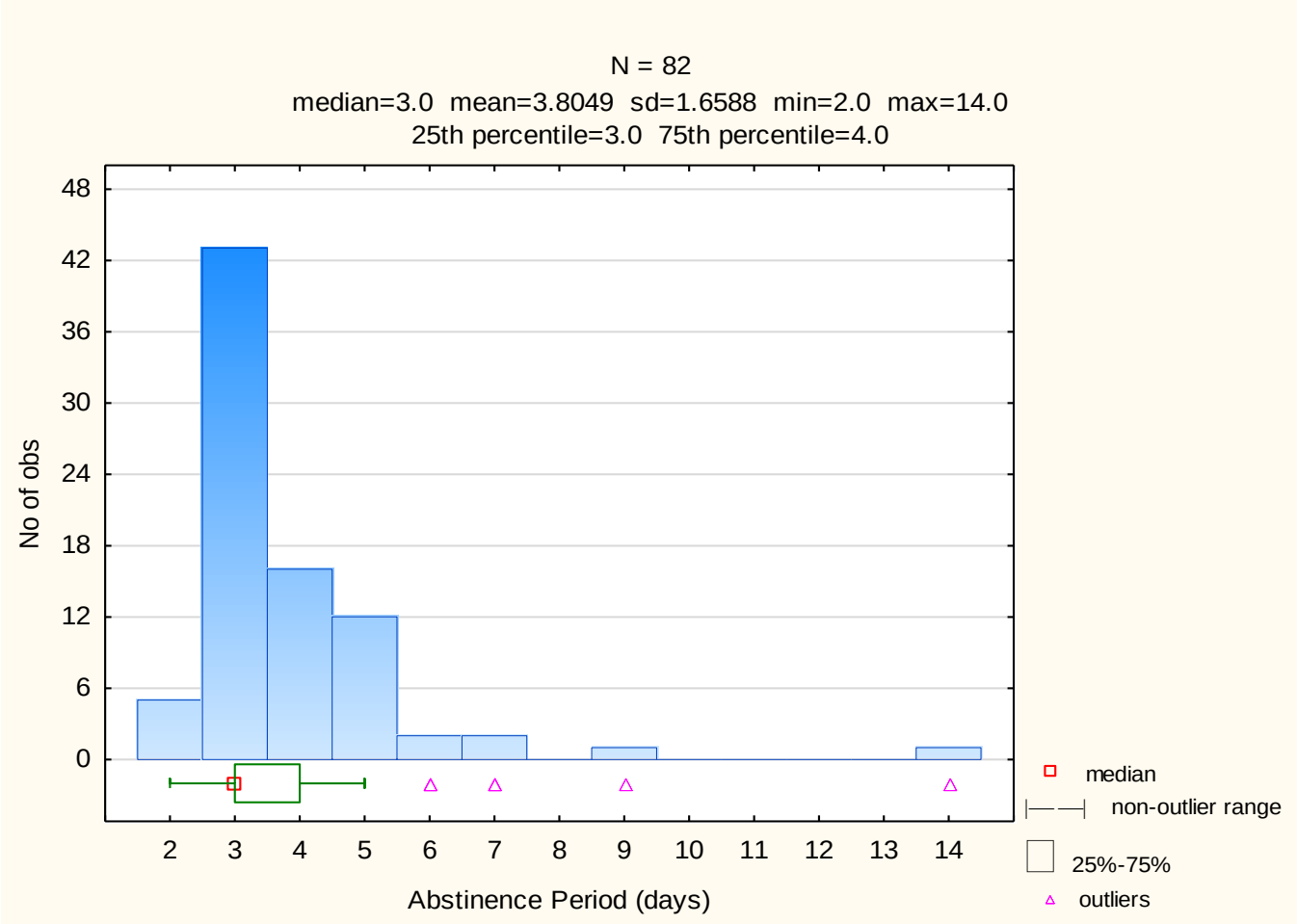



Figure 27 Histogram depicting the number of observations and distribution of abstinence period (days) of the participant population

### 4.4.3. Semen Volume

The average semen volume observed throughout this study was 3.7439 ml (SD=1.4877). The lowest value observed was 1.11 ml which is lower than the WHO lower reference value of 1.5 ml (WHO, 2010). While the highest value observed was 8.36 ml as depicted in Figure *28*.

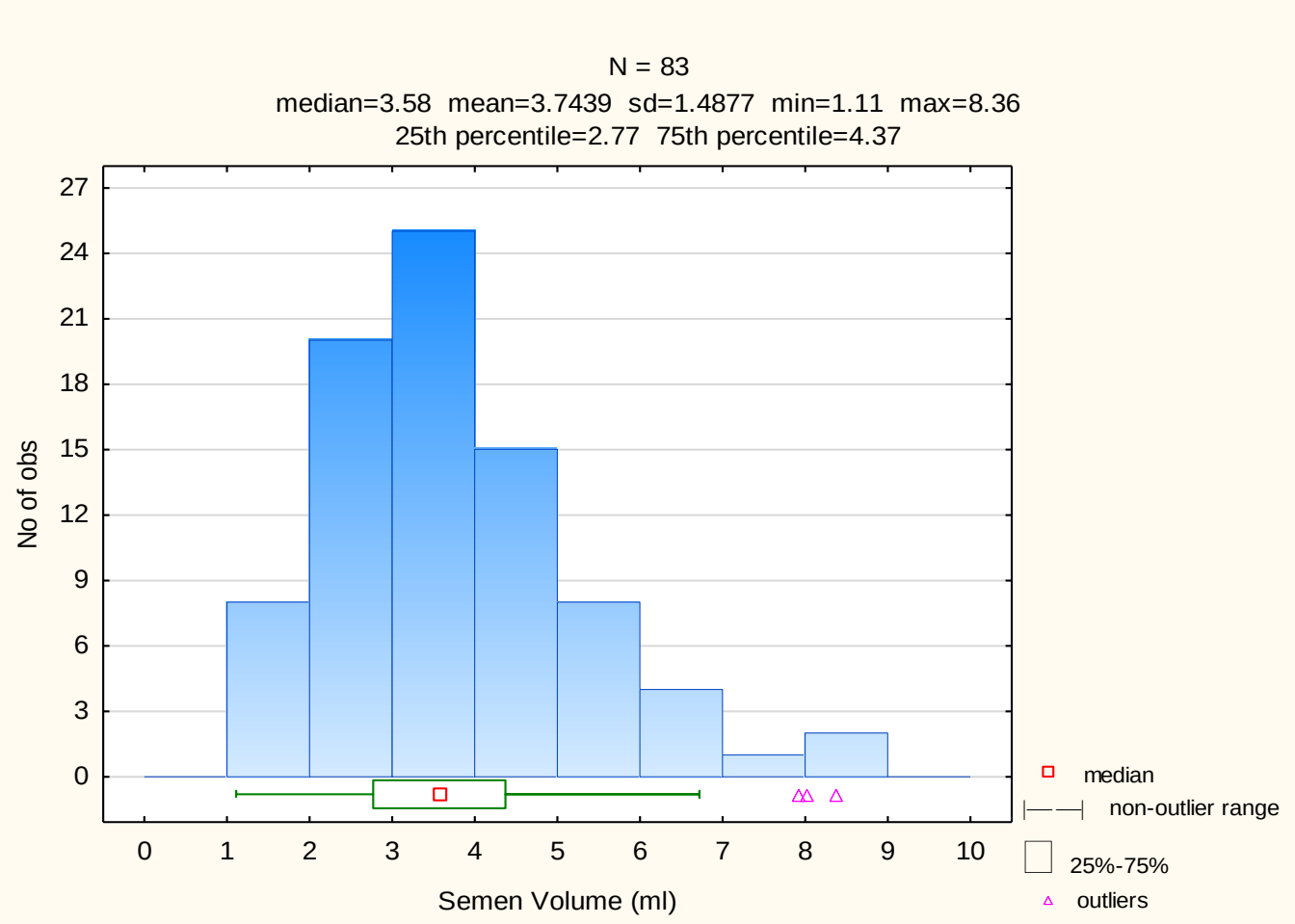



Figure 28 Histogram depicting the number of observations and distribution of semen volume (ml) of the participant population

### 4.4.4. Concentration

The concentration of spermatozoa is measured in million spermatozoa per milliliter. The WHO 5th Edition lower reference value for concentration is 15x10$^6$/ml, while the average concentration observed in this study was 57.2253 x10$^6$/ml (SD=35.6577). The lowest observed value was 4.64 x10$^6$/ml which is much lower than the WHO recommendations (WHO, 2010). The maximum observed concentration was 183.77 x10$^6$/ml as depicted in *Figure 29*.

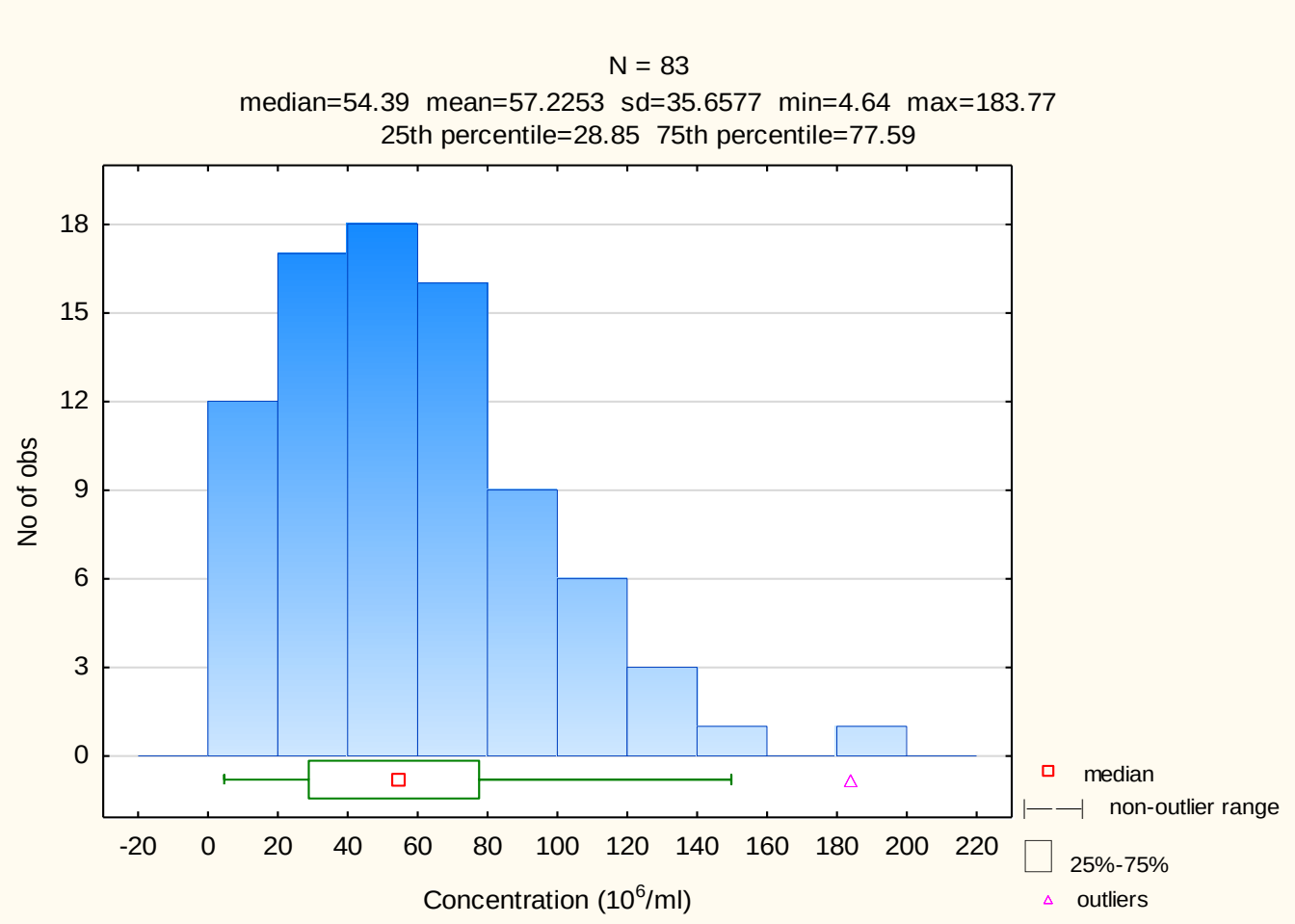



Figure 29 Histogram depicting the number of observations and distribution of concentration (10$^6$/ml) of the participant population

### 4.4.5. TSC

As mentioned, TSC is calculated by multiplying concentration and volume. The WHO lower reference value for TSC is 39.00 x$10^6$ (WHO, 2010). In the participant population, the average observed TSC value was 213.8184 x$10^6$ (SD=185.2906), with a median value observed at 155.93 x$10^6$ as depicted in *Figure 30*. The highest observed TSC value was 1146.32 x$10^6$and the lowest value observed was 15.58 x$10^6$.

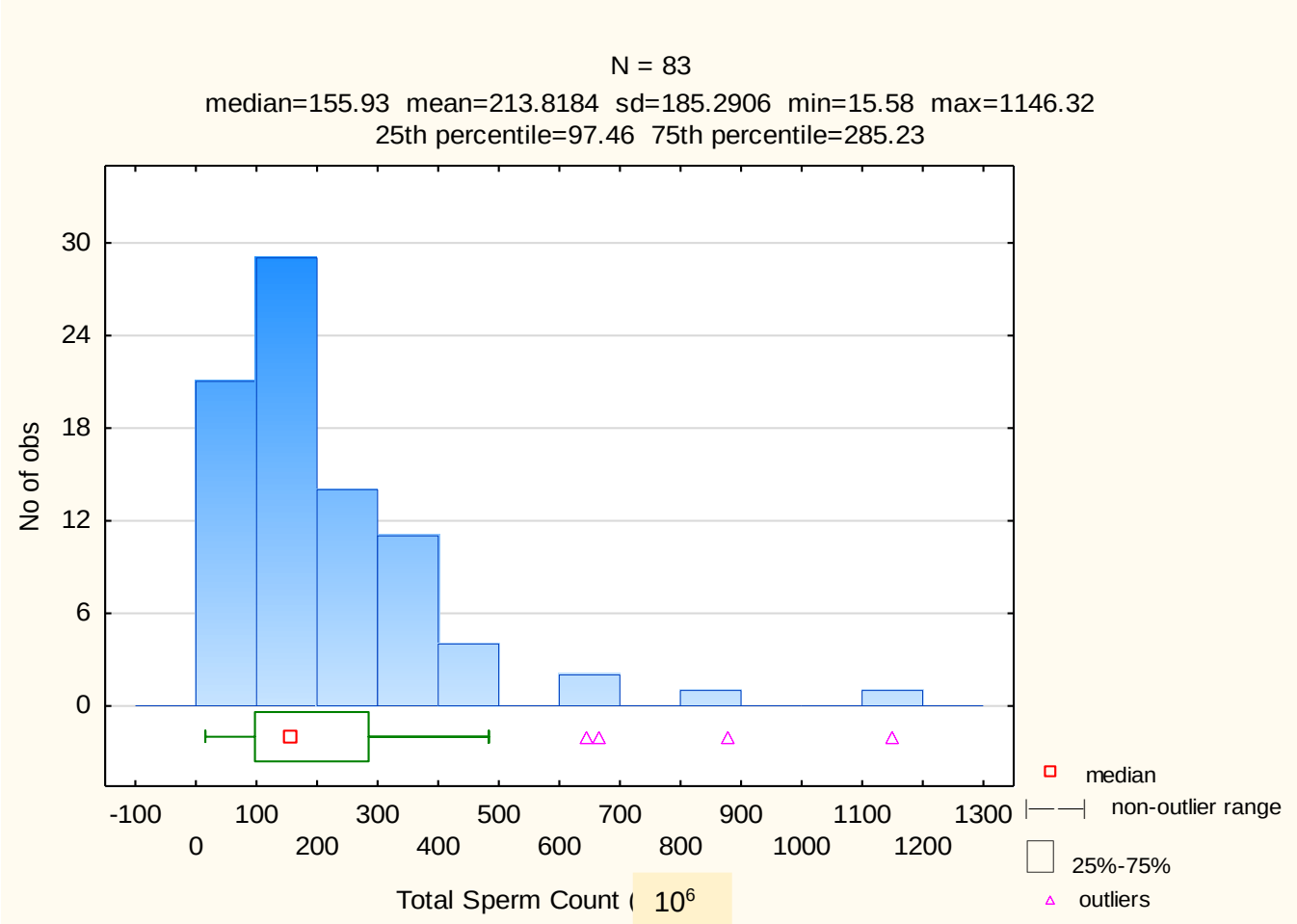



Figure 30 Histogram depicting the number of observations and distribution of total sperm count (M) of the participant population

### 4.4.6. Total motility

Sperm total motility can be split into total motility, progressive motility, non-progressive motility, and immotile cells. Total motility encompasses progressive and non-progressive motility, however, for the purpose of this study only total motility and progressive motility will be reported. The WHO lower reference value for total motility is 40% (WHO, 2010). For the participant population, the average total motility value observed was 55.5029% (SD=20.6019) with a median value of 57.75 as depicted in *Figure 31*. The lowest value observed was 4.61% and the highest value observed was 93.67%.

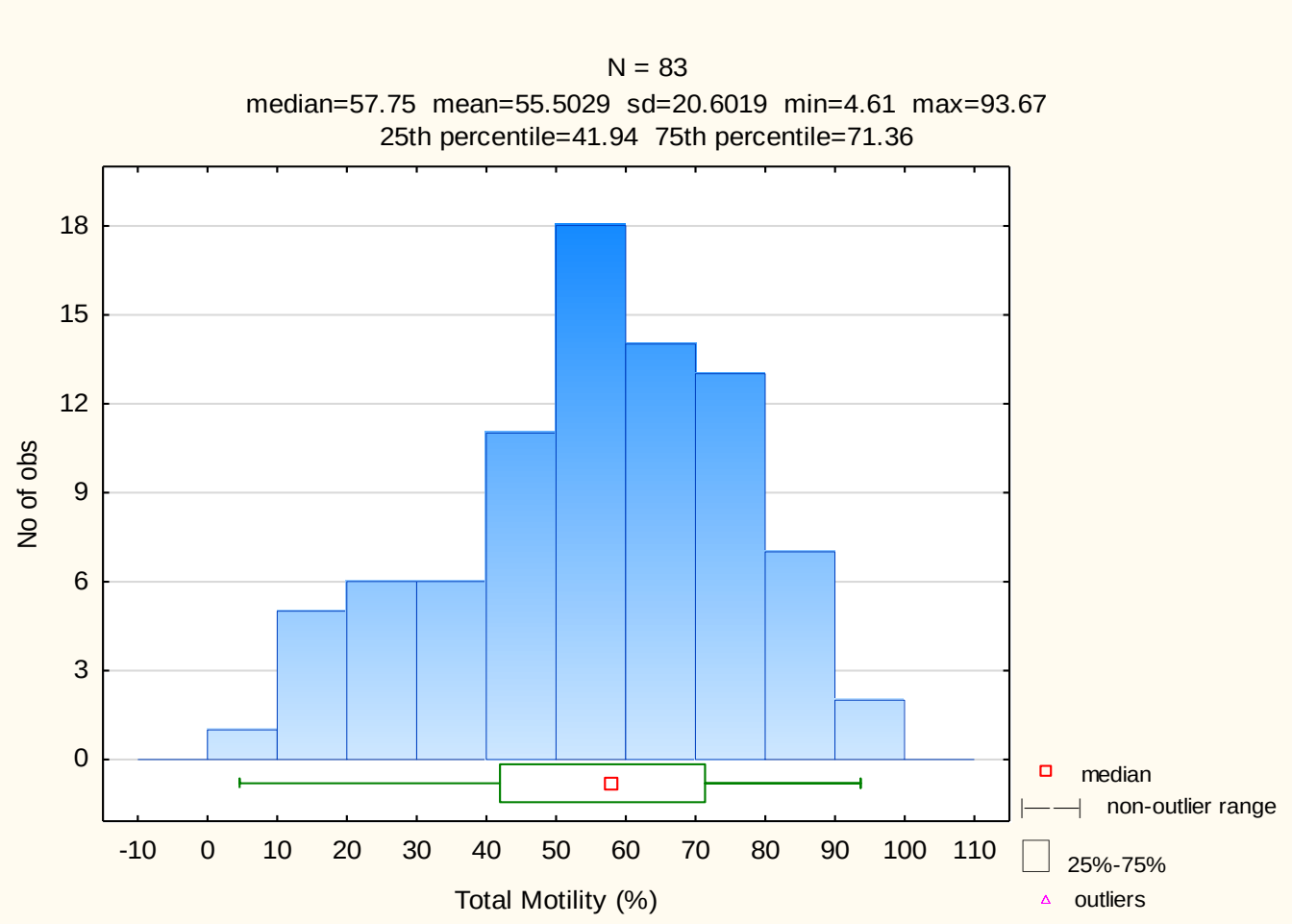



Figure 31 Histogram depicting the number of observations and distribution of total motility (%) of the spermatozoa of the participant population

### 4.4.7. Progressive Motility

Progressive motility has a WHO lower reference value of 32% (WHO, 2010). The average progressive motility observed was 39.562% (SD=19.1496), with a median of 38.91% as depicted in *Figure 32*. The minimum observed progressive motility value was 1.96%, which is far below the WHO recommendations and the highest observed value was 83.63%.

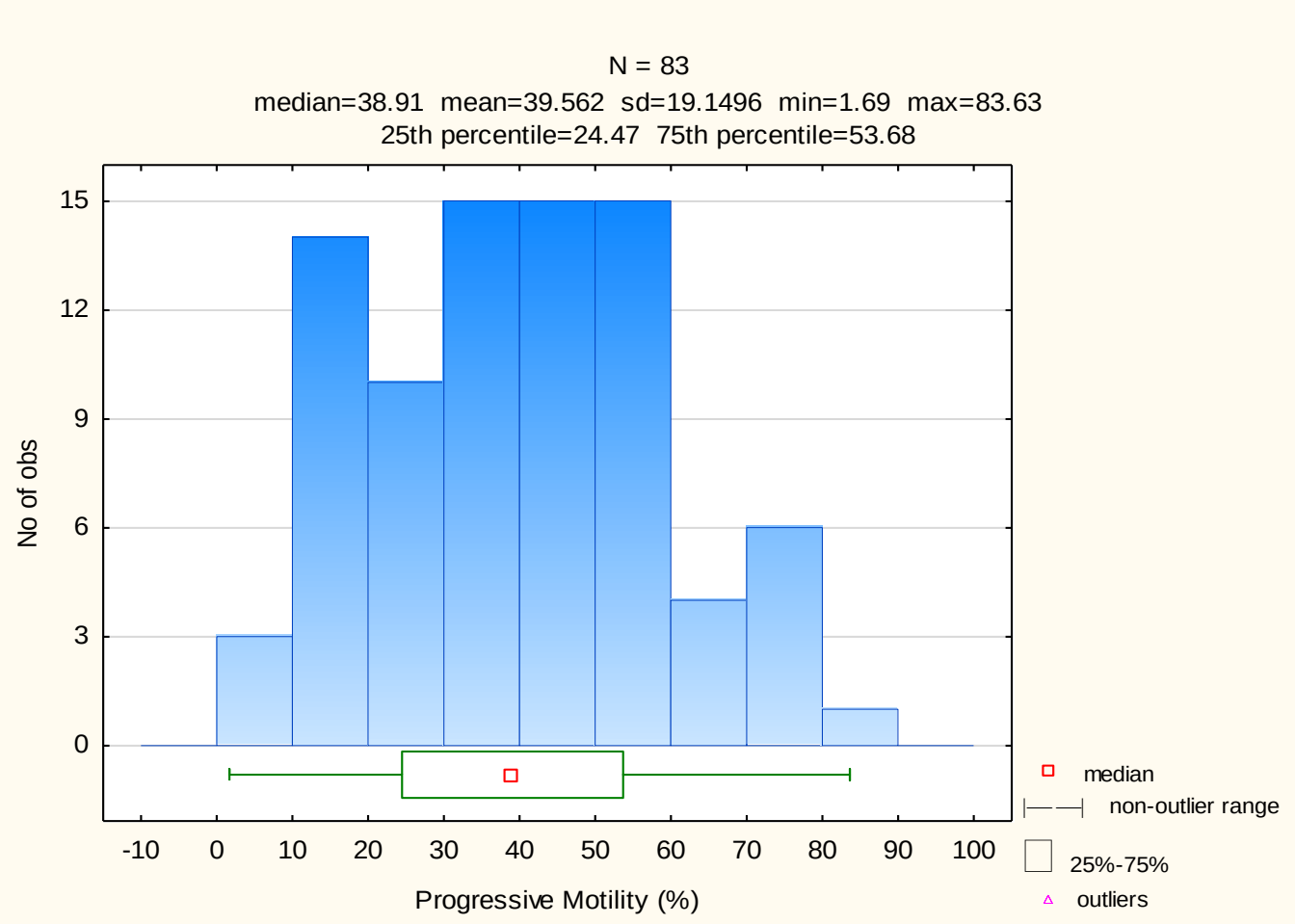



Figure 32 Histogram depicting the number of observations and distribution of progressive motility (%) of spermatozoa of the participant population

### 4.4.8. Round Cells

CASA is able to measure round cells. Round cells consist of both immature spermatozoa and leukocytes. However, CASA is unable to differentiate between immature spermatozoa and leukocytes in the semen sample. The WHO reference limit for round cells is <1x10$^6$/ml (WHO, 2010). Of the 83 samples obtained in the study, the average round cells values observed is 0.2302 x10$^6$/ml (SD=0.3376). The minimum round cell value observed is 0.00 while the highest round cell value observed is 2.46 x10$^6$/ml as depicted in *Figure 33*.

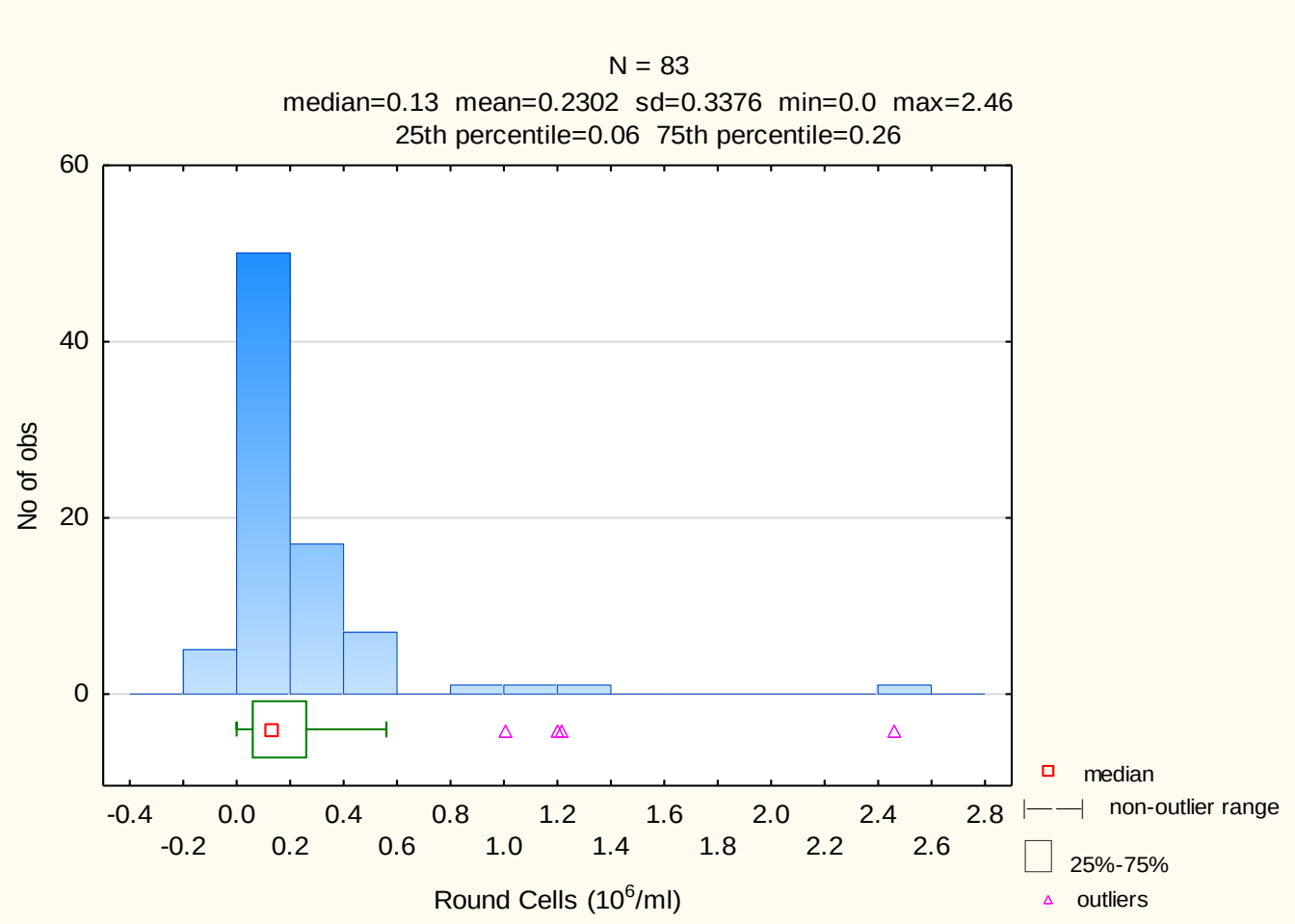



Figure 33 Histogram depicting the number of observations and distribution of round cells (10$^6$/ml) of the participant population

### 4.4.9. Kinematics

The kinematic parameters provide more information about the motility and trajectory of the spermatozoa. These parameters consist of the velocity parameters: curvilinear velocity/VCL (µm/s), straight-line velocity/VSL (µm/s) and average path velocity/VAP (µm/s). The trajectory shape is able to influence the velocity values. Therefore, the velocity values are compared. The linearity index/LIN (%) compares the VSL and VCL parameters as described in *Equation 3*. The straight-line index/STR (%) compares the VS.L and VAP parameters as described in *Equation 4*. The oscillation index/WOB (%) compares the VAP and the VCL parameters as described in *Equation 5*.

$$LIN = \frac{VSL}{VCL} \times 100$$

Equation 3 The Equations used to calculate LIN kinematic parameter

$$STR = \frac{VSL}{VAP} \times 100$$

Equation 4 The Equation used to calculate STR kinematic parameter

$$WOB = \frac{VAP}{VCL} \times 100$$

Equation 5 The Equation used to calculate the WOB kinematic parameter

The amplitude of lateral health displacement/ALH (µm) is a kinematic parameter that describes the width of the lateral movement of the sperm head and the beat cross frequency/BCF (Hz) which describes the number of times the sperm head crosses the direction of movement. The last kinematic parameter to be reported in hyperactive motile sperm cells.

#### 4.4.9.1. VCL

The average VCL observed during this study was 51.284 µm/s (SD=11.724). With a minimum value of 26.03 and a maximum value of 81.71 µm/s observed for the VCL parameter as displayed in *Figure 34*.

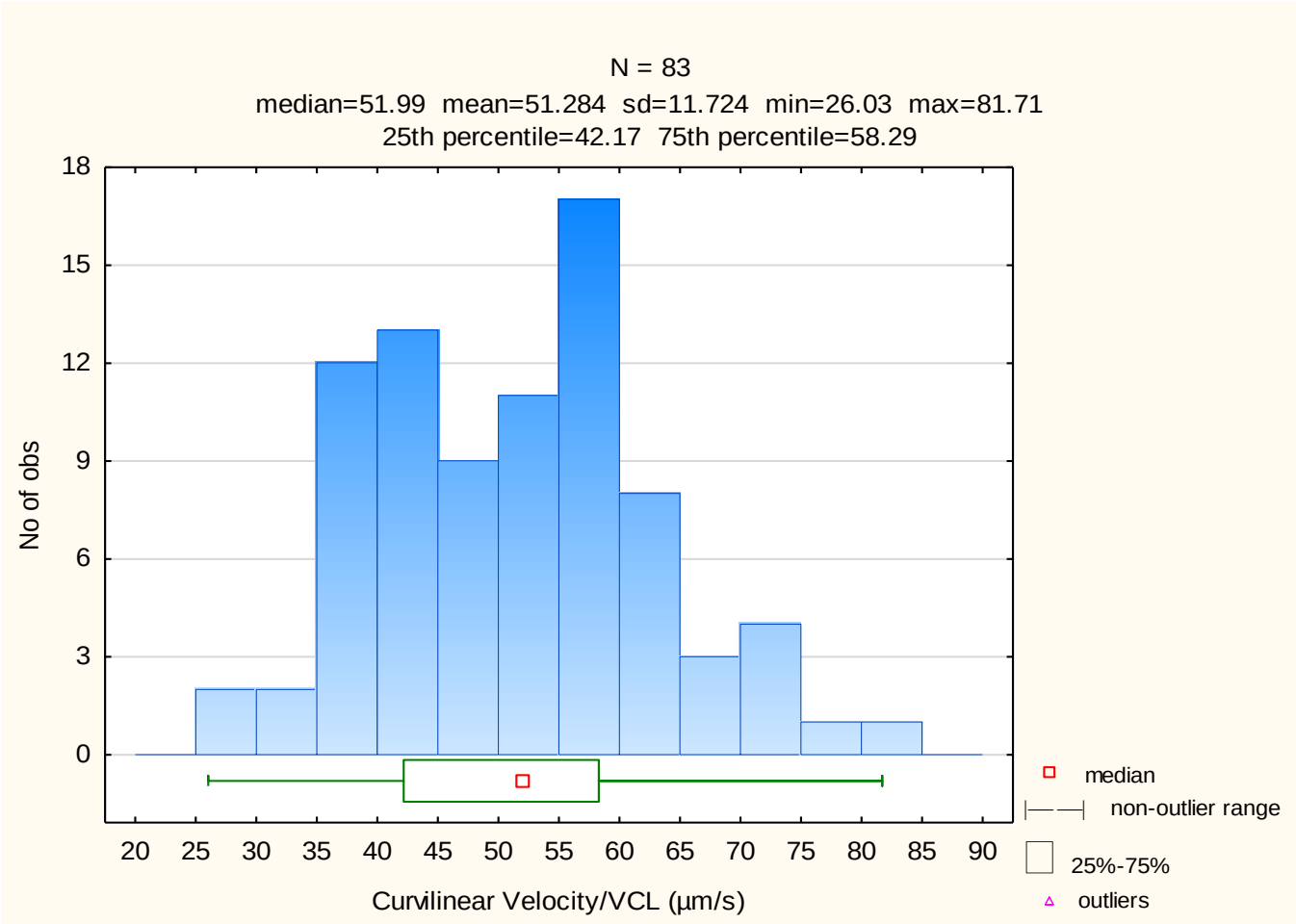



Figure 34 Histogram depicting the number of observations and distribution of spermatozoa Curvilinear Velocity/VCL of the participant population

#### 4.4.9.2. VAP

For the participant population, the average VAP measured in this study is 26.3555 µm/s (SD=9.2609) as depicted in *Figure 35*. A maximum VAP value of 47.3 µm/s was observed, while a minimum value of 9.28 µm/s was observed.

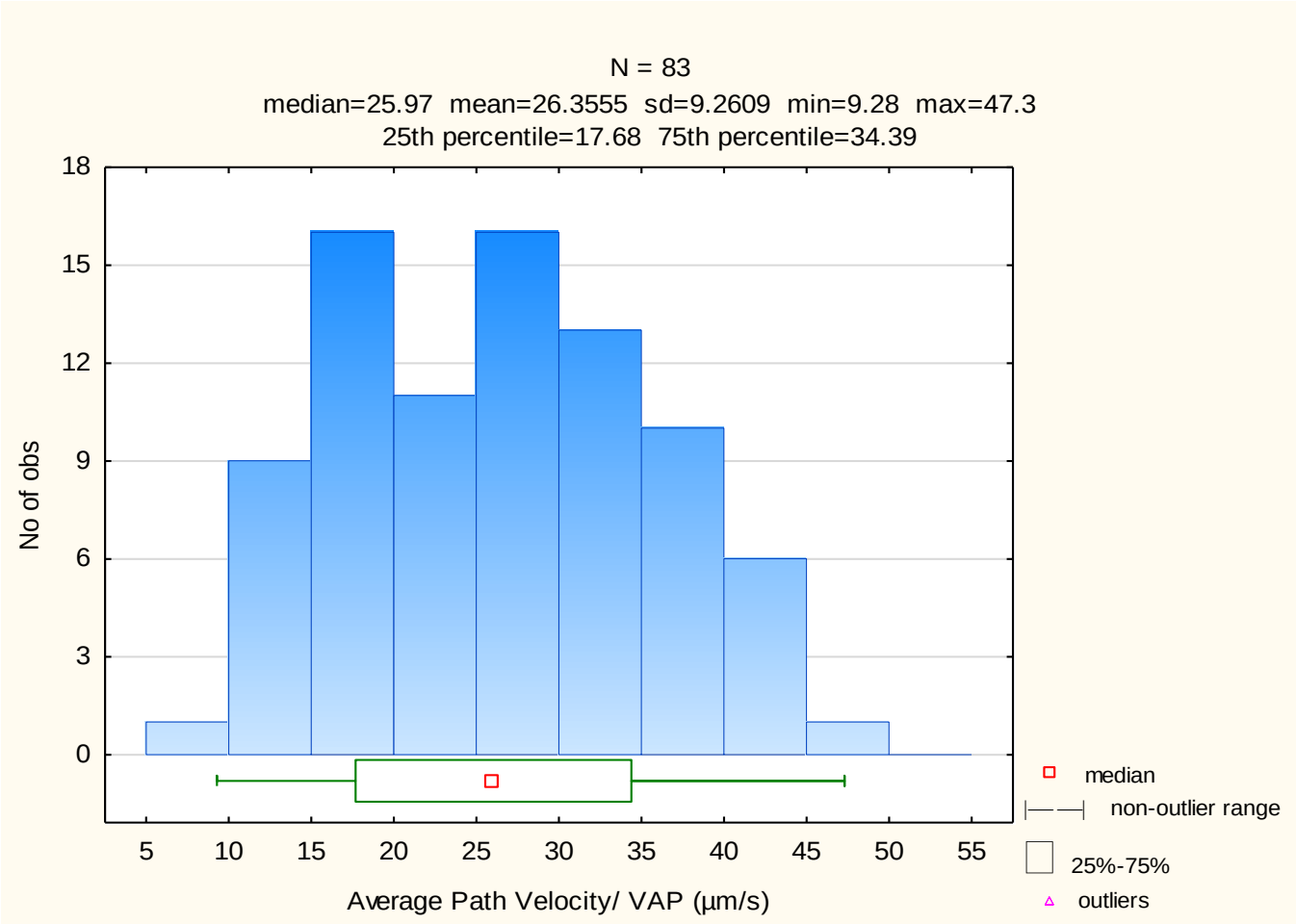



Figure 35 Histogram depicting the number of observations and distribution of spermatozoa average path velocity/VAP of the participant population

#### 4.4.9.3. VSL

The average VSL observed in this study is 23.1849 µm/s (SD=8.1595). The lowest observed VSL value was 4.65 µm/s and the highest observed VSL value was 44.6 µm/s as depicted in *Figure 36*.

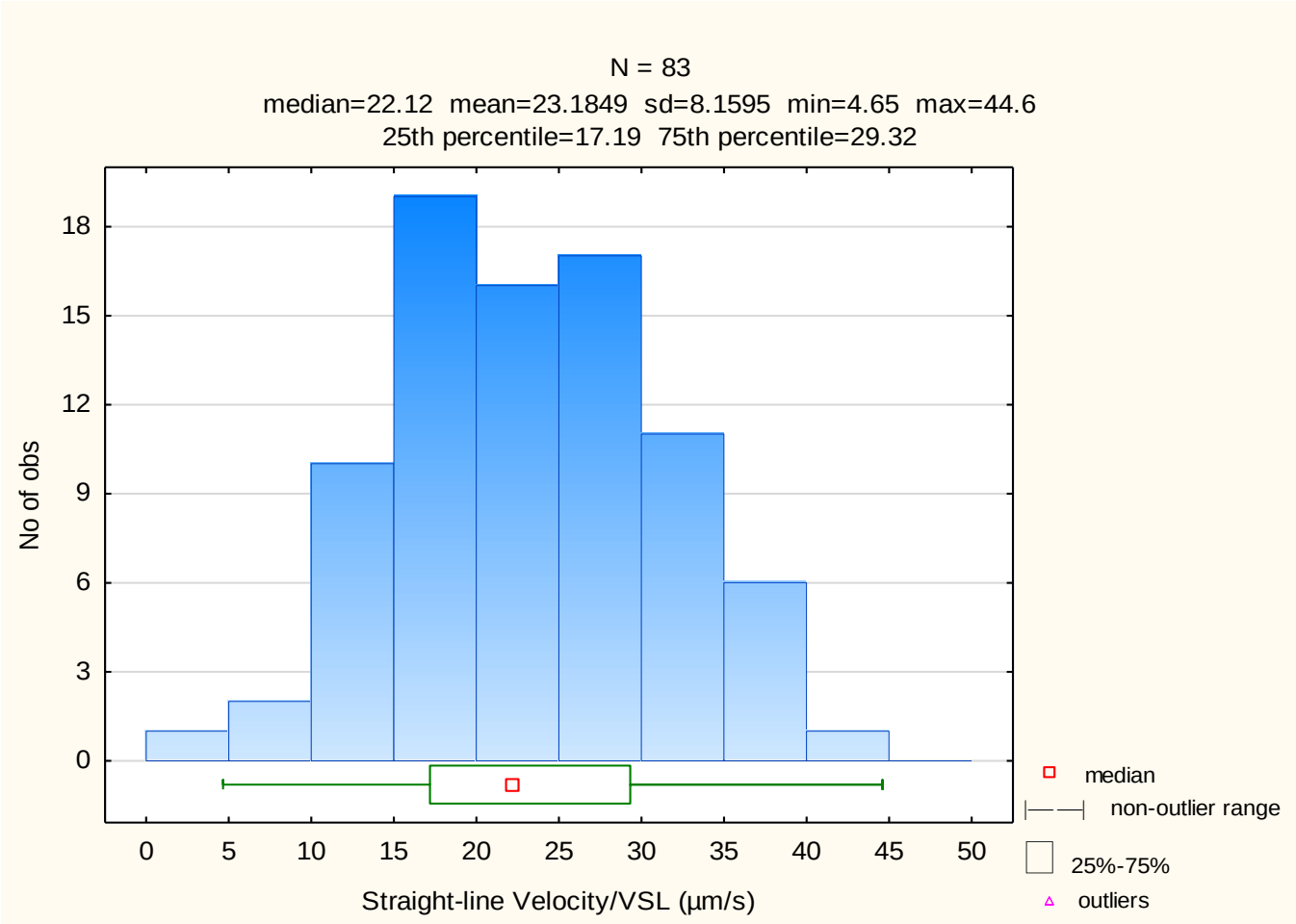



Figure 36 Histogram depicting the number of observations and distribution of spermatozoa straight-line velocity/VS.L of the participant population

#### 4.4.9.4. STR

The mean STR value observed in this study was 47.981% (SD=11.3436), with a minimum STR value of 25.37% and a maximum STR value of 65.96% as depicted in *Figure 37*.

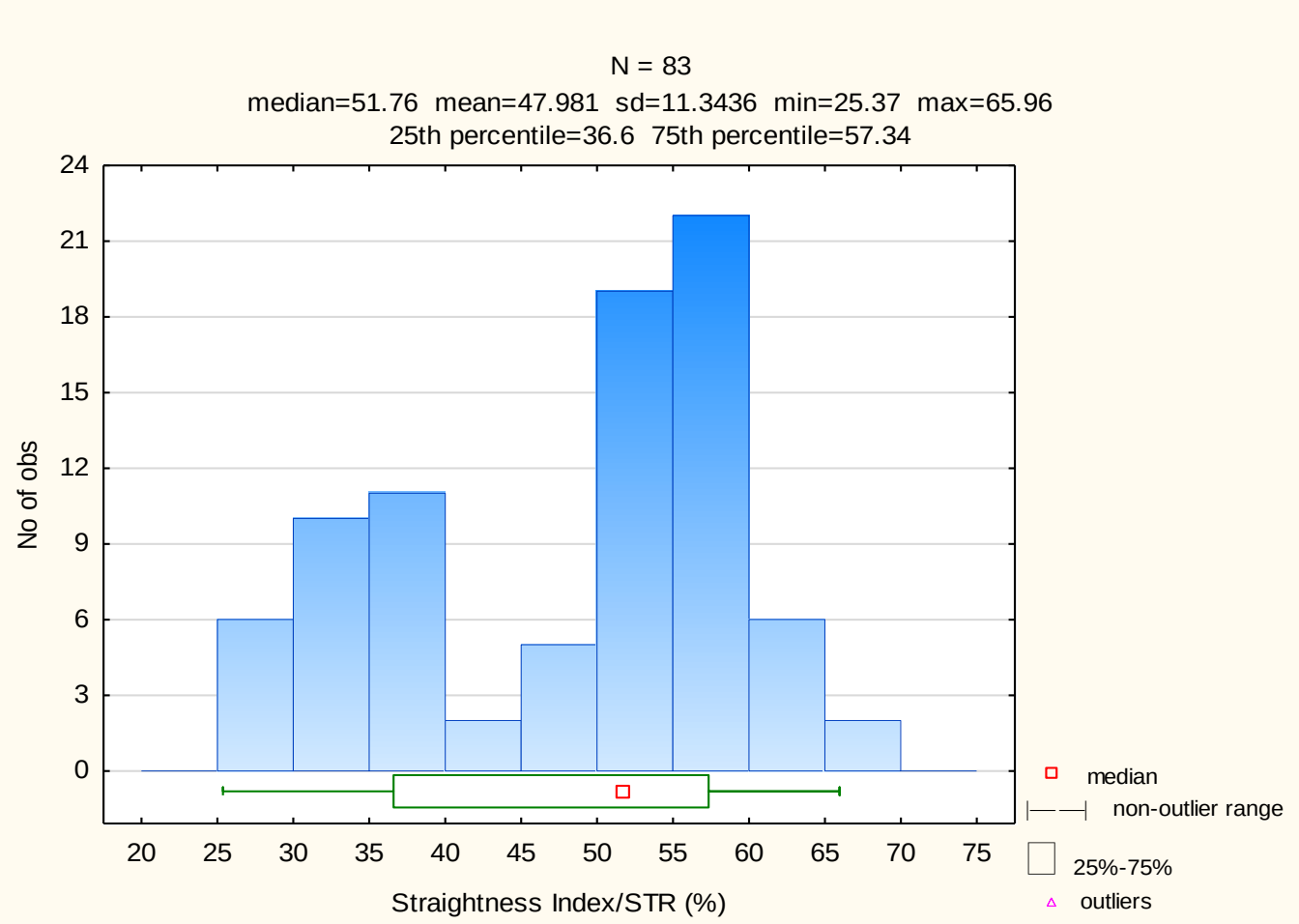



Figure 37 Histogram depicting the number of observations and distribution of the spermatozoa straightness index/ STR of the participant population

#### 4.4.9.5. LIN

Of the 83 samples collected, an average LIN value of 41.2146% (SD=10.5702) was observed in this study. The highest LIN value observed was 67.34% and the lowest LIN value observed was 14.97% as depicted in *Figure 38*.

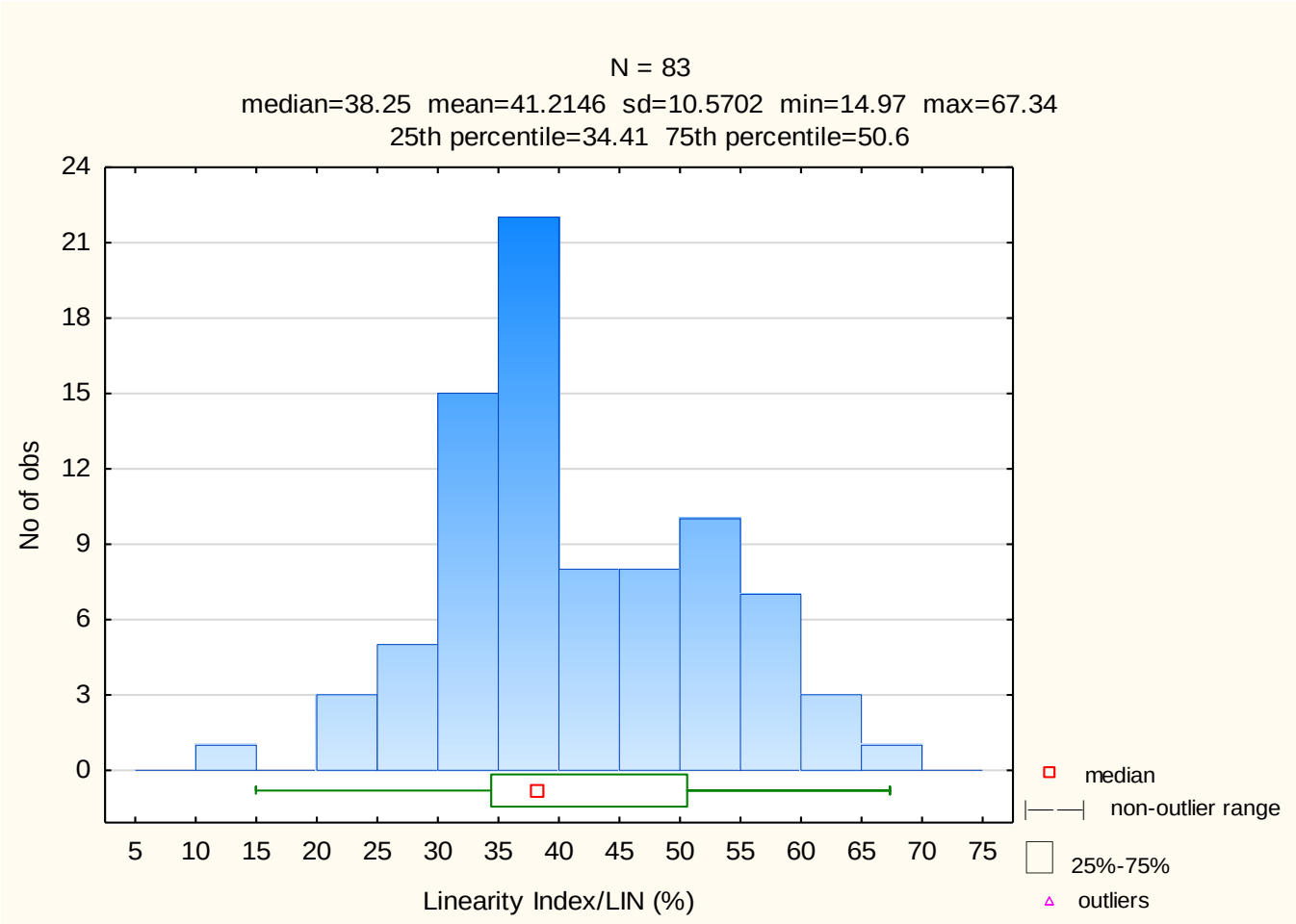



Figure 38 Histogram depicting the number of observations and distribution of spermatozoa linearity index/ LIN of the participant population

#### 4.4.9.6. WOB

The highest WOB value observed was 79.79% and the lowest WOB value observed was 29.42% as depicted in *Figure 39*. The average WOB value observed was 59.3399% (SD=8.1606).

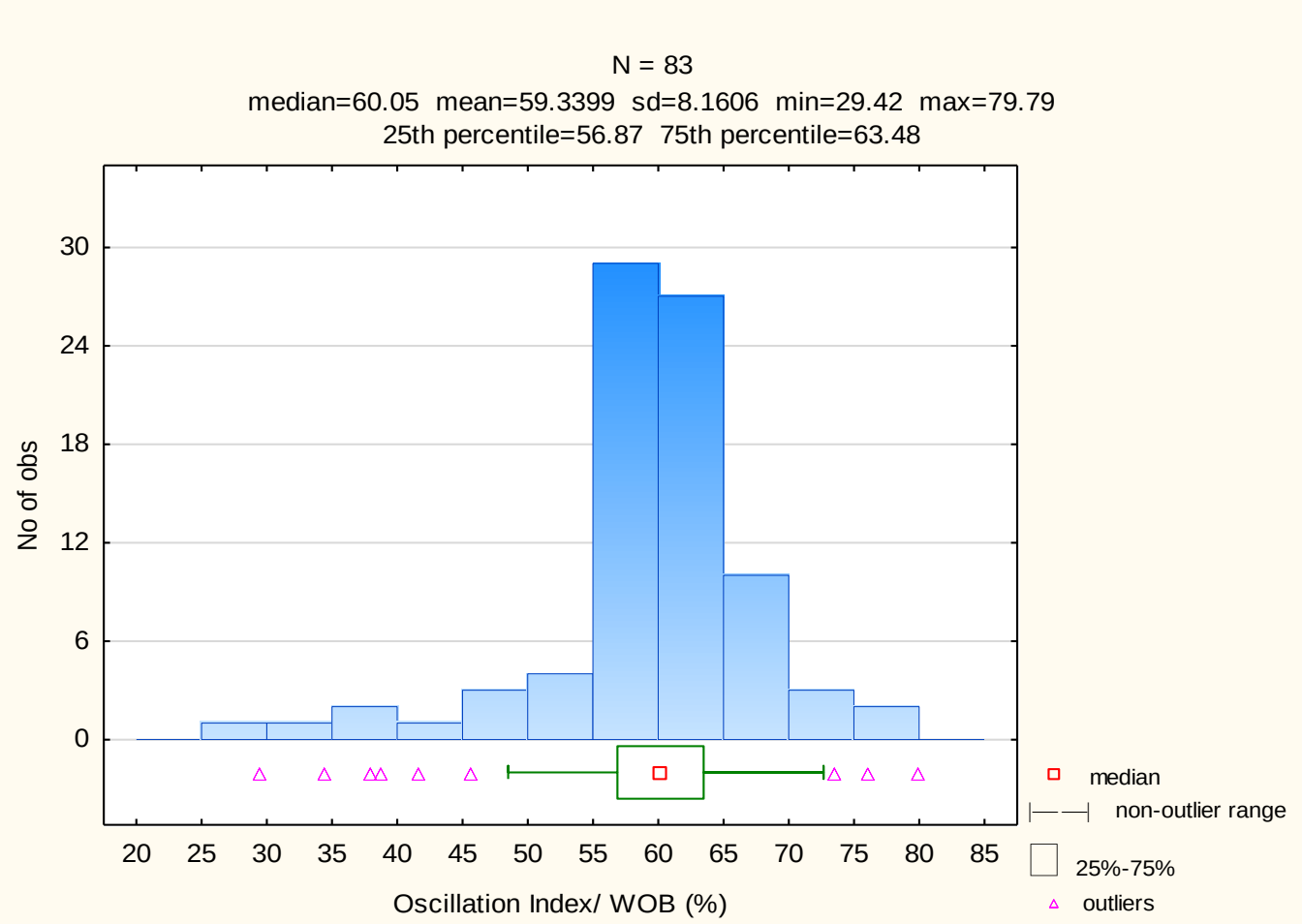



Figure 39 Histogram depicting the number of observations and distribution of spermatozoa oscillation index/WOB of the participant population

#### 4.4.9.7. ALH

The sample size for ALH and is 79, as a result of missing data sets. The average ALH value observed in the participant population is 1.8048 µm (SD=0.3283), the highest ALH value observed was 2.57 µm and the lowest ALH value observed was 1.08 µm as depicted in *Figure 40*.

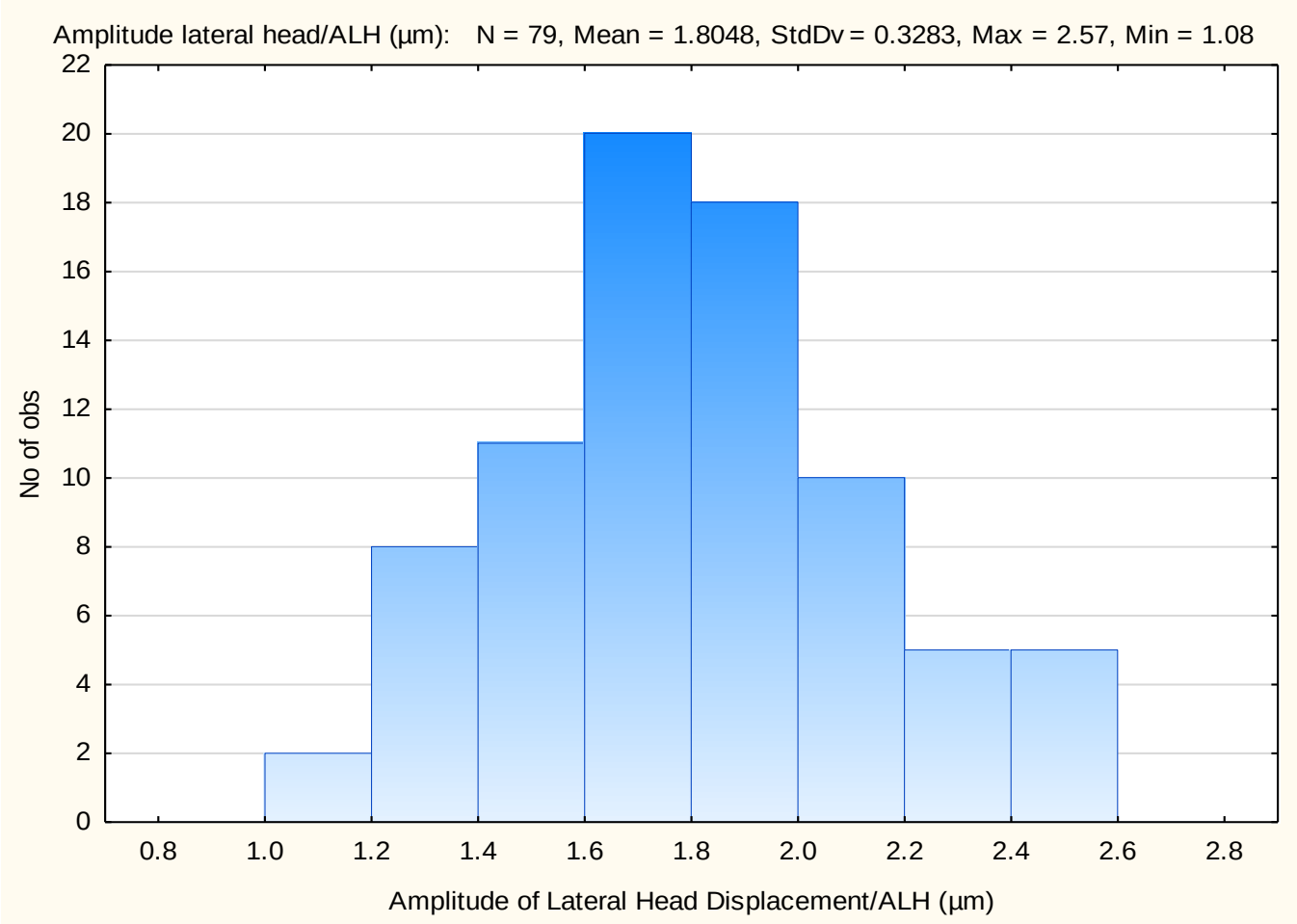



Figure 40 Histogram depicting the number of observations and distribution of spermatozoa amplitude of lateral head displacement/ALH of the participant population

#### 4.4.9.8. BCF

The sample size for BCF is 79, as a result of missing data. The mean BCF observed was 12.3919 Hz (SD=2.8459), with a minimum value of 2.73 Hz and a maximum value of 18.1 Hz observed as depicted in *Figure 41*.

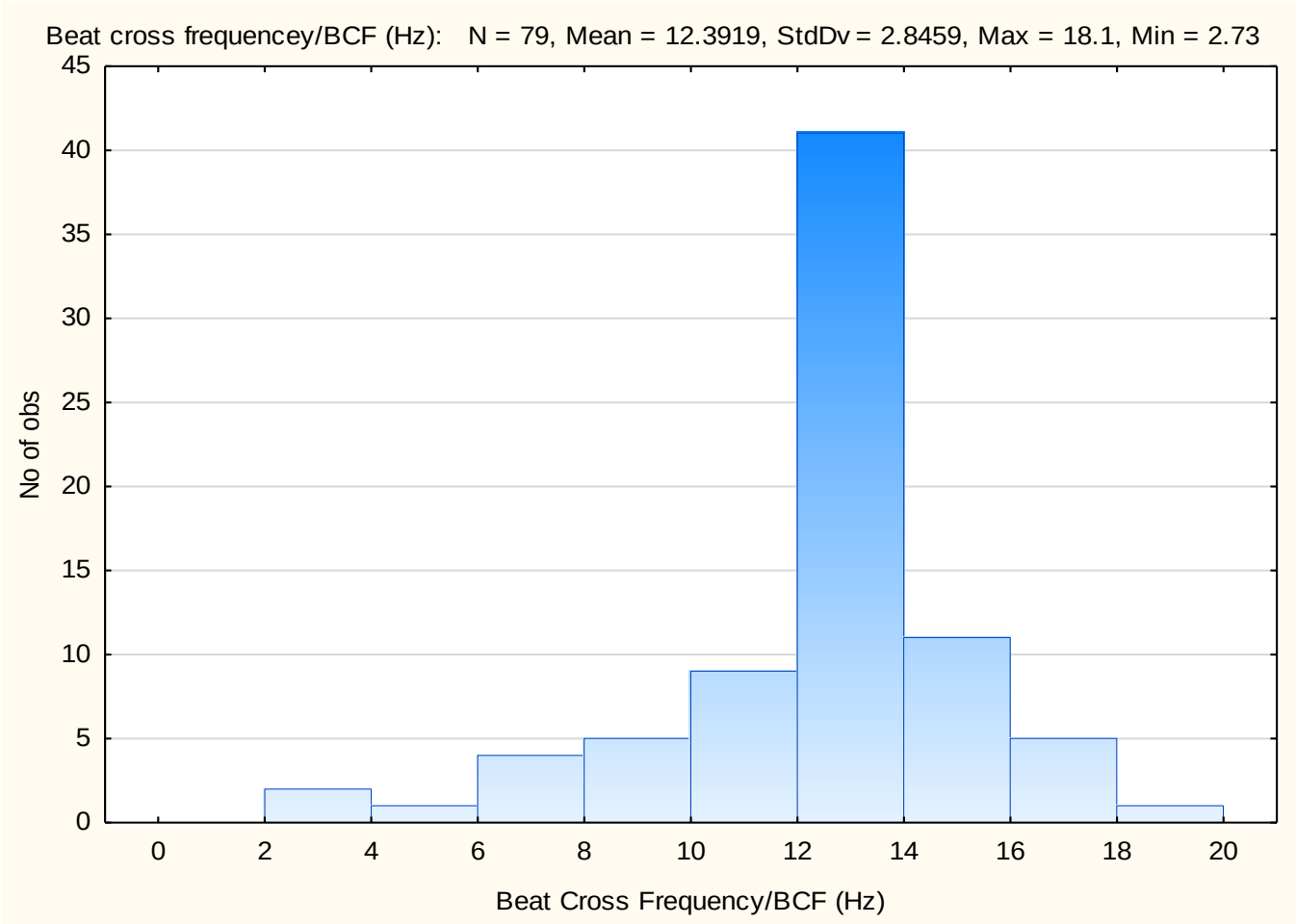



Figure 41 Histogram depicting the number of observations and distribution of spermatozoa beat cross frequency/BCF of the participant population

#### 4.4.9.9. Hyperactive Motile

Of the 83 samples collected for the hyperactive motile parameter, the average value observed was 1.1882 % (SD=1.163). The lowest observed hyperactive motile value is 0.00 while the highest observed value is 4.91%. as displayed in *Figure 42*.

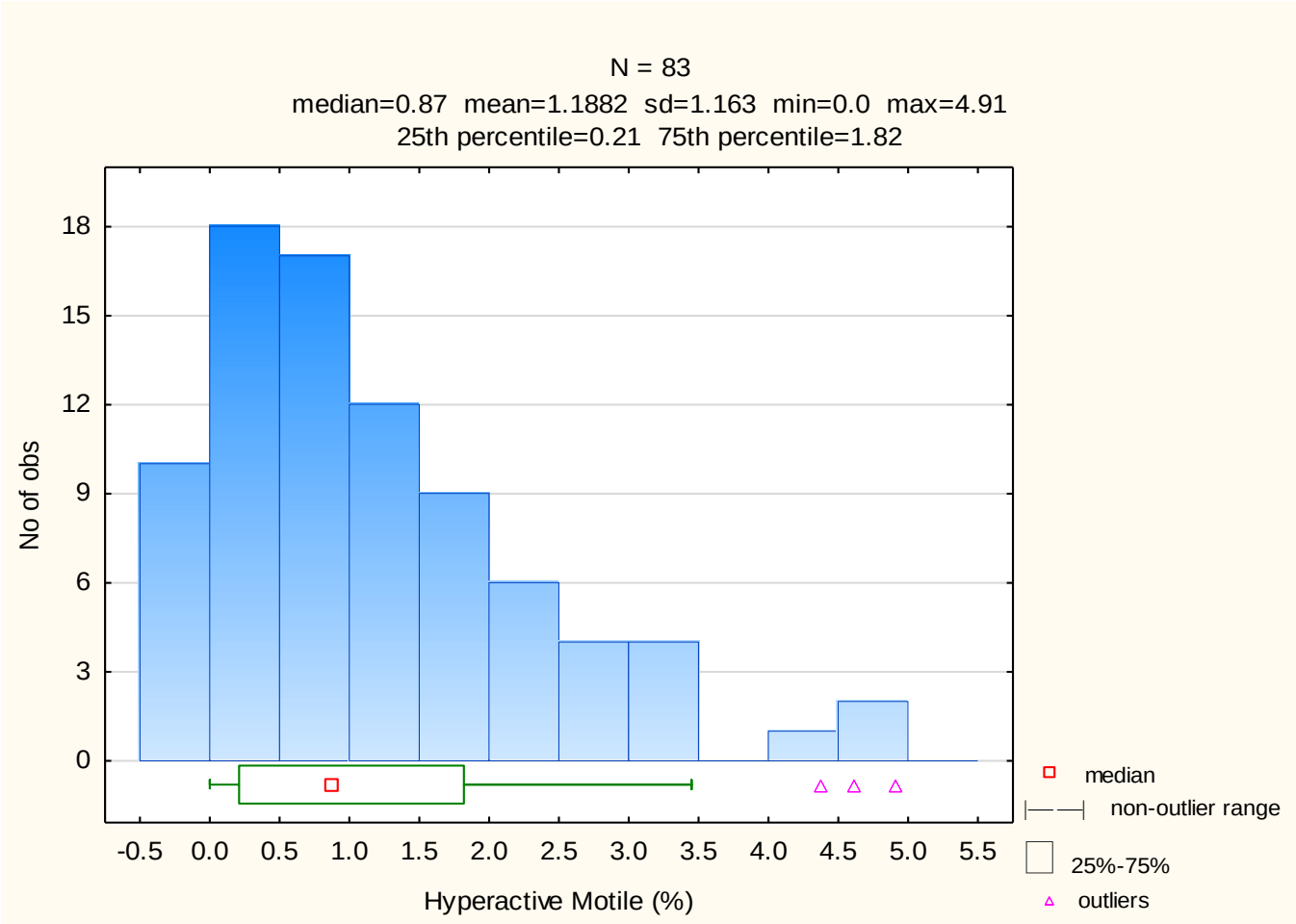



Figure 42 Histogram depicting the number of observations and distribution of hyperactive motile spermatozoa (%) of the participant population

### 4.4.10. Viable Spermatozoa

The WHO lower reference limit for viability is 58% (WHO, 2010). The average viable spermatozoa observed in this participant population is 57.5181% (SD=16.8578). The lowest observed viability values for spermatozoa is 3.5% while the highest observed viability values are 85.0% as depicted in *Figure 43*

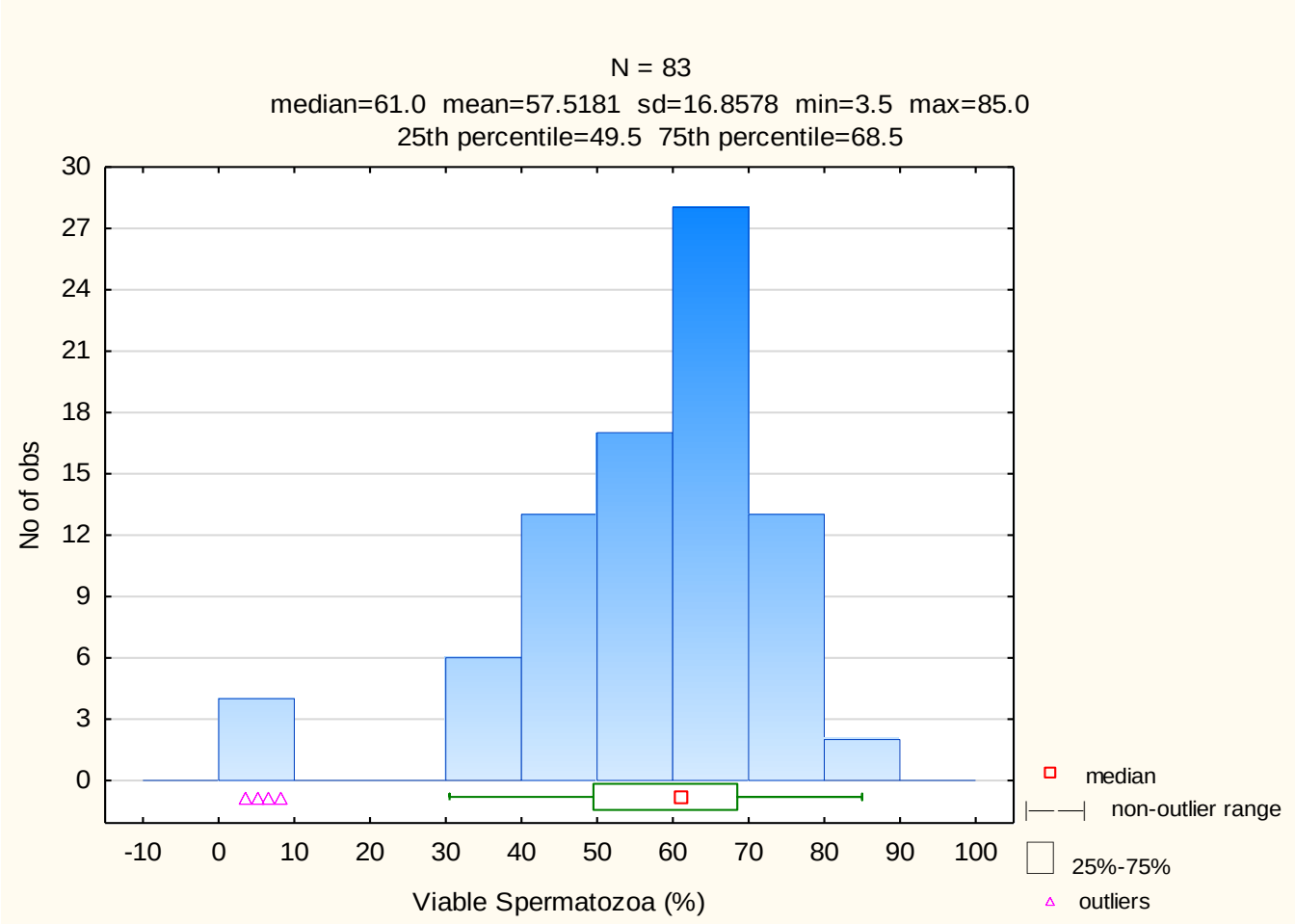



Figure 43 Histogram depicting the number of observations and distribution of viable spermatozoa (%) of the participant population

### 4.4.11. Reactive Oxygen Species

Some datasets have been misplaced, therefore the sample size for ROS is 81. The average ROS value observed was 392.7009 RLU/sec/$10^6$ (SD=2294.88). The data observed was massively spread out and the median ROS value observed was 2.2291 RLU/sec/$10^6$ as displayed in *Figure 44*.

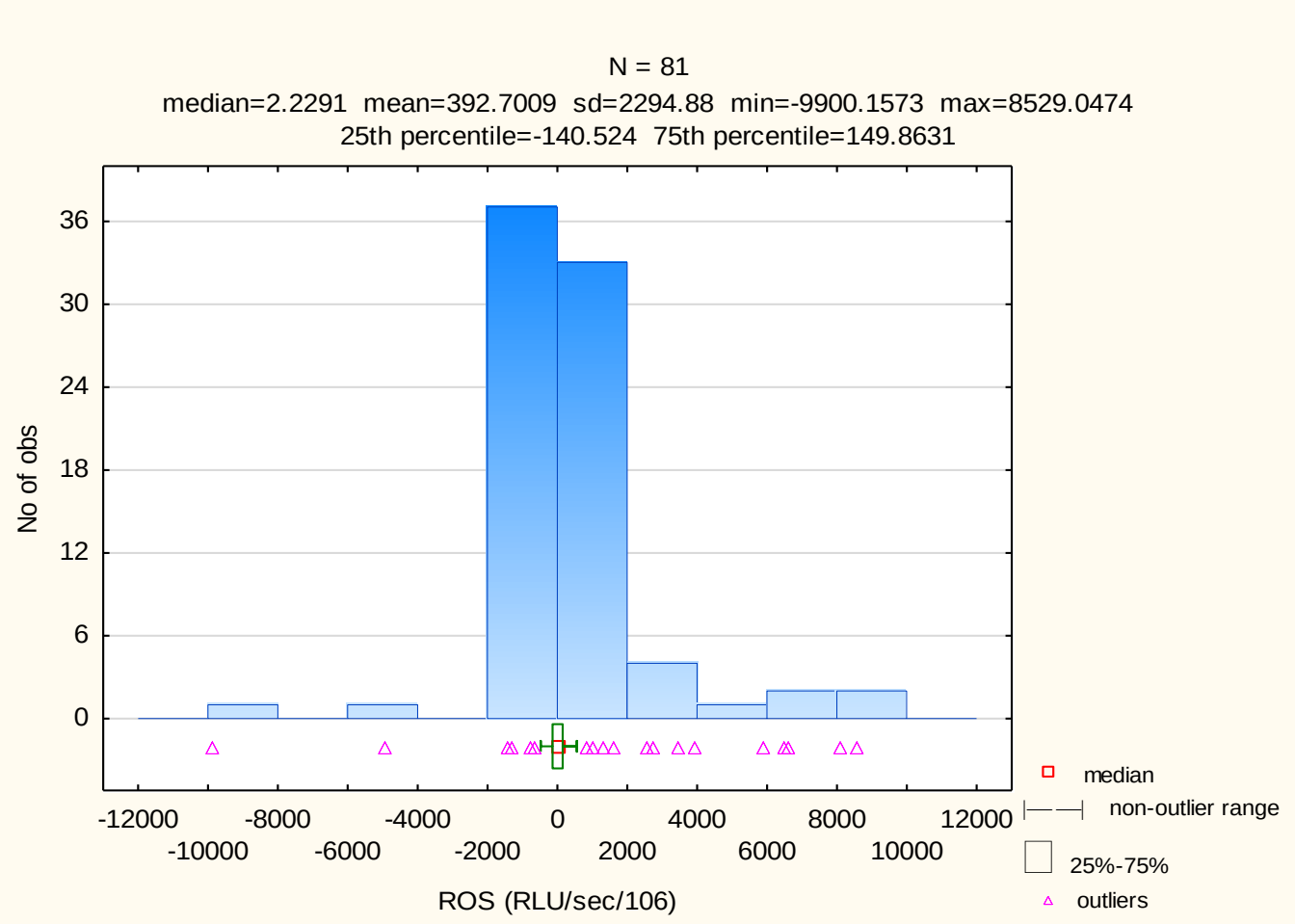



Figure 44 Histogram depicting the number of observations and distribution of semen ROS level (MFI) of the participant population

### 4.4.12. DNA Fragmentation

Two datasets have been misplaced for DNA fragmentation. Therefore, the sample size is 81 instead 83. The average DNA fragmentation observed during this study is 4.7557 % (SD=4.412). The lowest DNA fragmentation value observed was 0.11% and the highest DNA fragmentation value observed was 19.8% as depicted in *Figure 45*

.

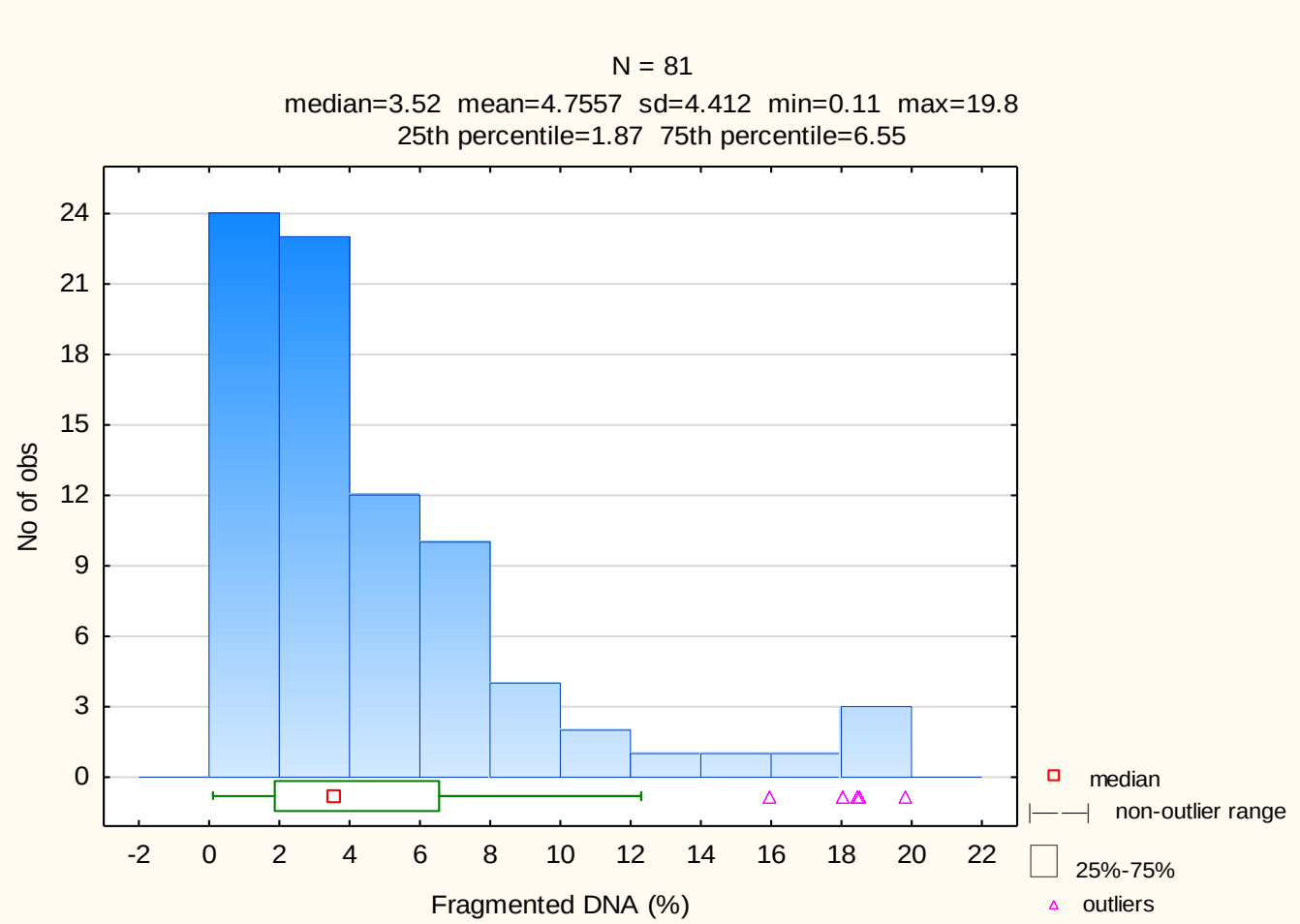



Figure 45 Histogram depicting the number of observations and distribution of DNA fragmentation (%) of spermatozoa in the participant population

### 4.4.13. Nitric Oxide

Missing datasets have resulted in the sample size for Nitric Oxide being 65 instead of 83. The mean NO MFI observed was 4818.7077 (SD=2303.7596), while the minimum observed NO MFI was 288.0 and the highest observed NO MFI was 12899.0 as depicted in *Figure 46*.

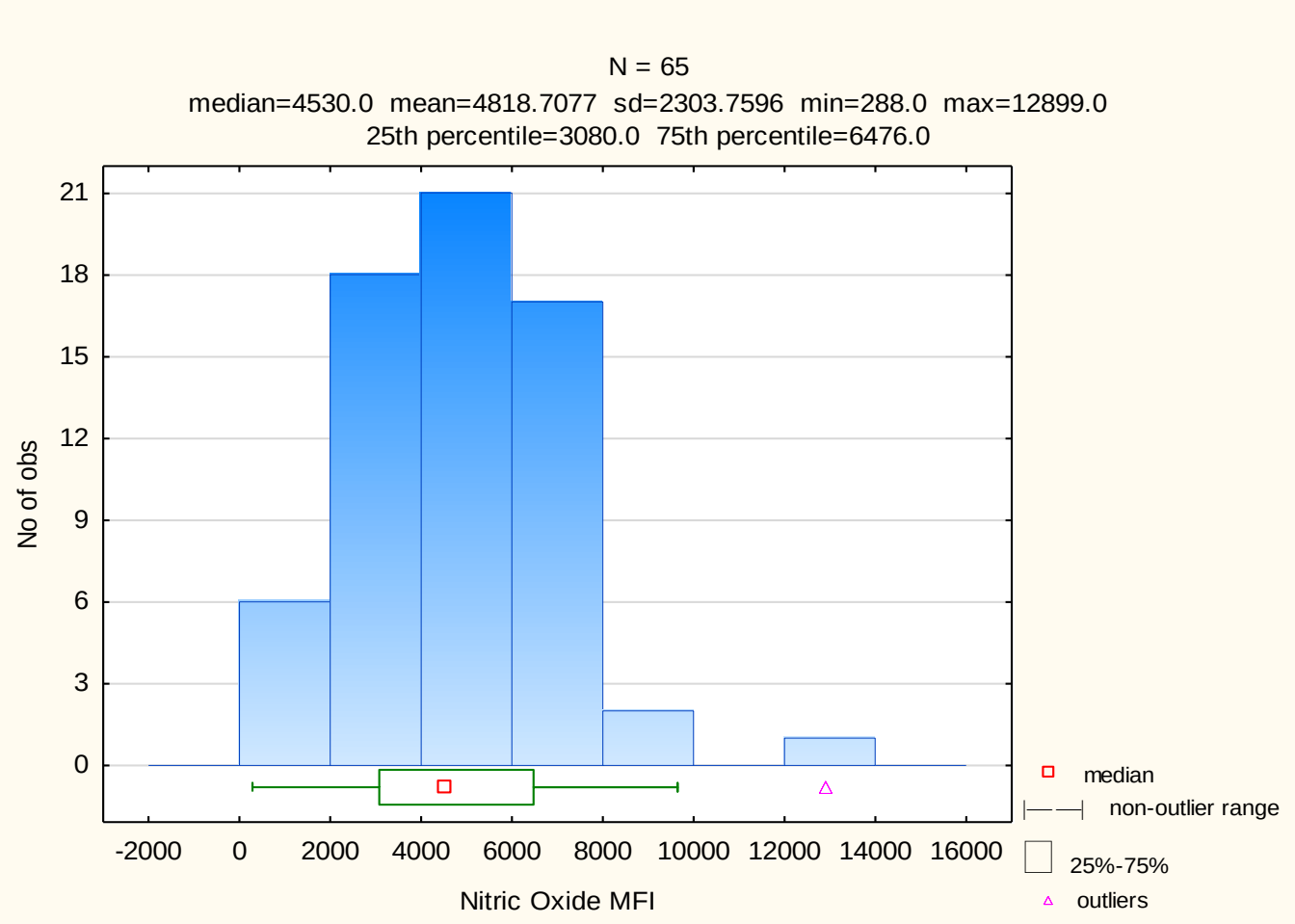



Figure 46 Histogram depicting the number of observations and distribution of nitric oxide median fluorescence intensity (NO MFI) of spermatozoa in the participant population

### 4.4.14. Cytokine Profile: Seminal Plasma

When considering the seminal plasma cytokine levels of IFN-γ, IL-6, TNF-α and IL-1β the sample size was considerably lower (n=20). Observing the cytokine profile was a pilot project to investigate immunological changes observed in the population. Samples used were chosen at random.

#### 4.4.14.1. Seminal Plasma IFN- γ

It was found that the average level of IFN-γ was 29.0575 pg/ml (SD=8.4427). The lowest recorded IFN-γ value was 15.79 pg/ml and the highest recorded IFN-γ value was 52.98 pg/ml as depicted in *Figure 47*.

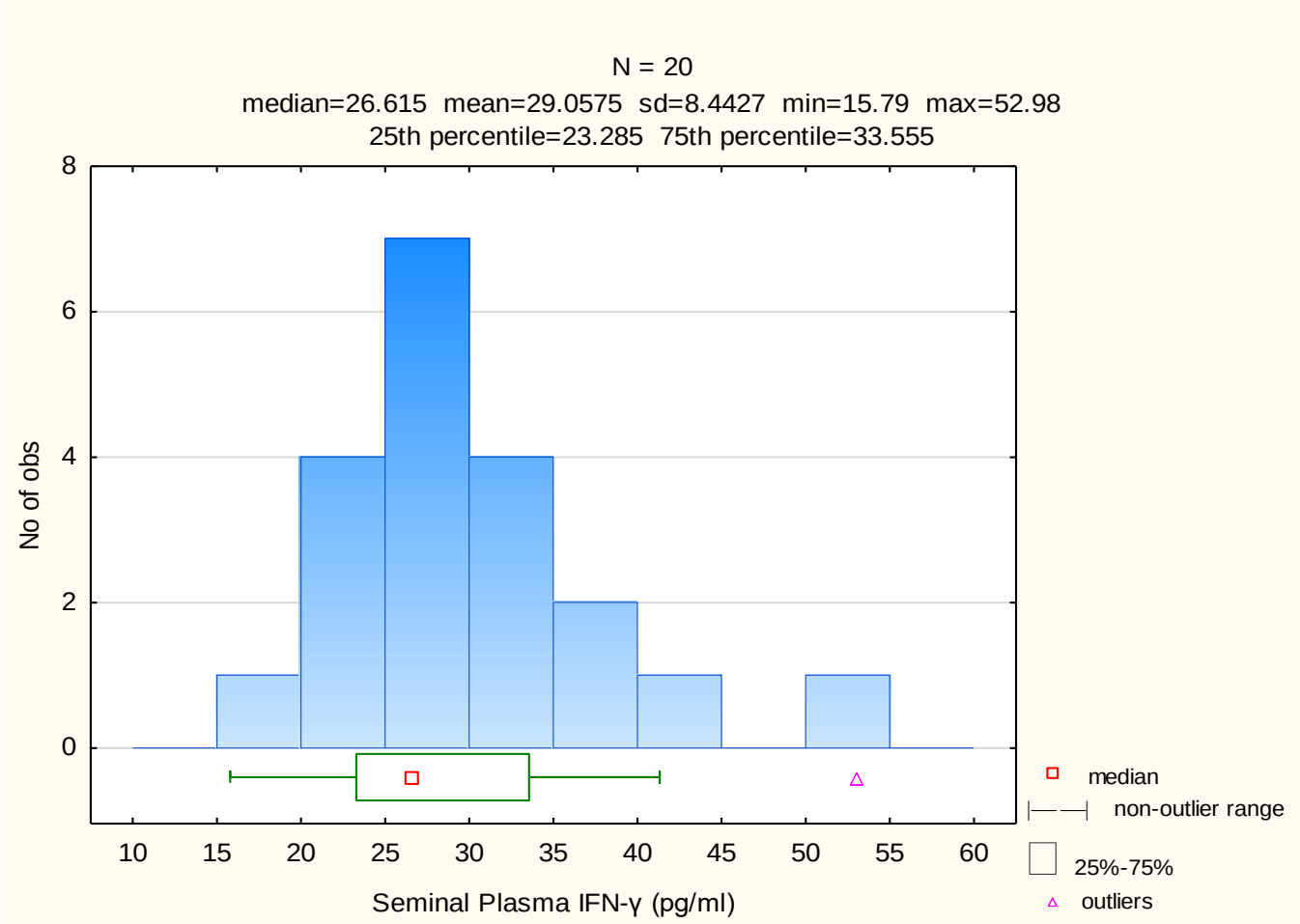



Figure 47 Histogram depicting the number of observations and distribution of seminal plasma IFN-γ levels of a portion the participant population

#### 4.4.14.2. Seminal Plasma IL-6

The average value of seminal plasma IL-6 was 16.485 pg/ml (SD=20.8781) as depicted in *Figure 48*. The lowest observed value of IL-6 was 1.93 pg/ml while the highest observed value of IL-6 was 77.74 pg/ml.

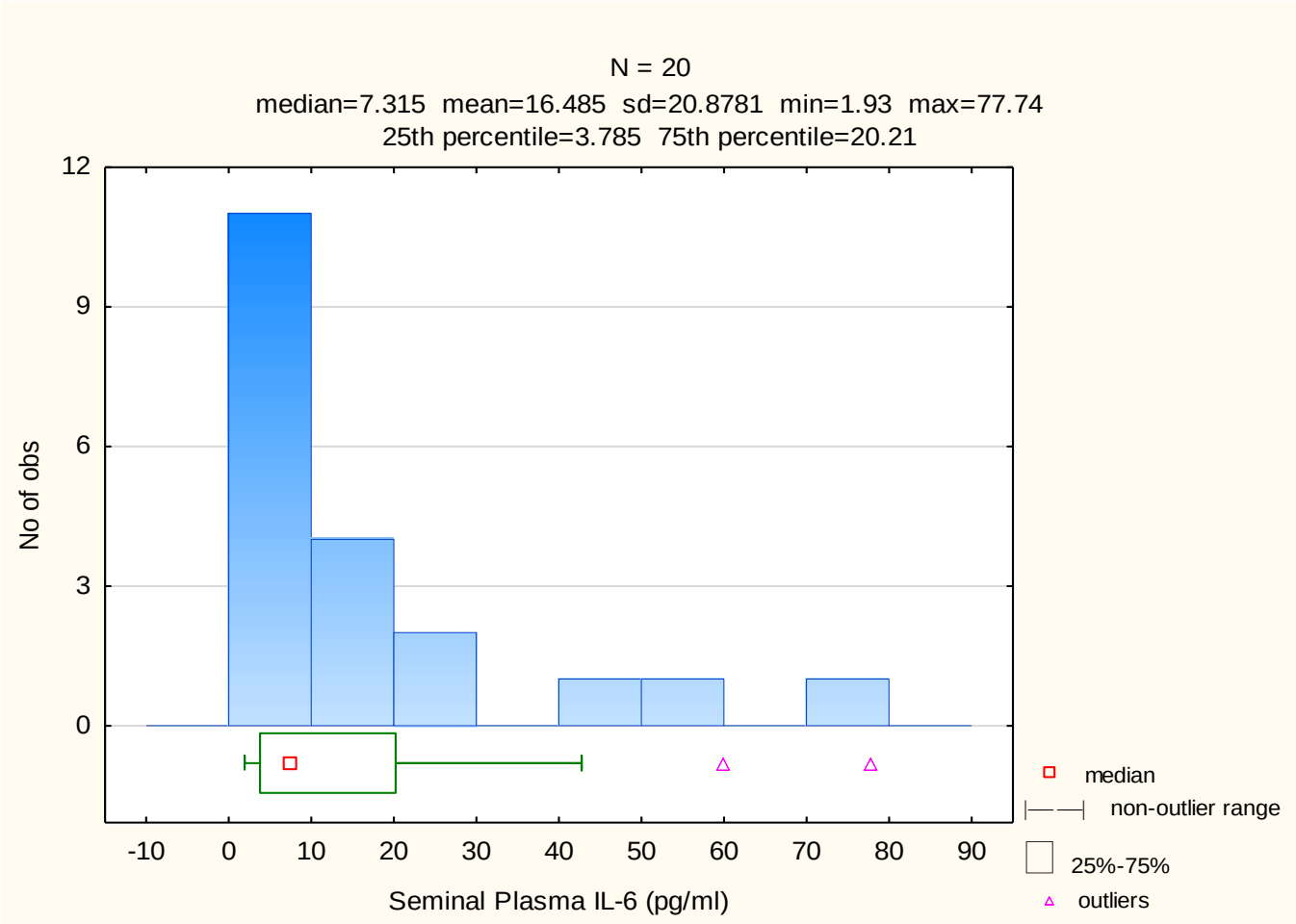



Figure 48 Histogram depicting the number of observations and distribution of seminal plasma IL-6 levels of a portion the participant population

### 4.4.14.3. Seminal Plasma TNF- α

The lowest observed value of TNF-α in seminal plasma was 0.8 pg/ml while the highest observed value was 22.66 pg/ml. The mean TNF-α value observed was 3.959 pg/ml (SD=5.0428) and is depicted in *Figure 49*.

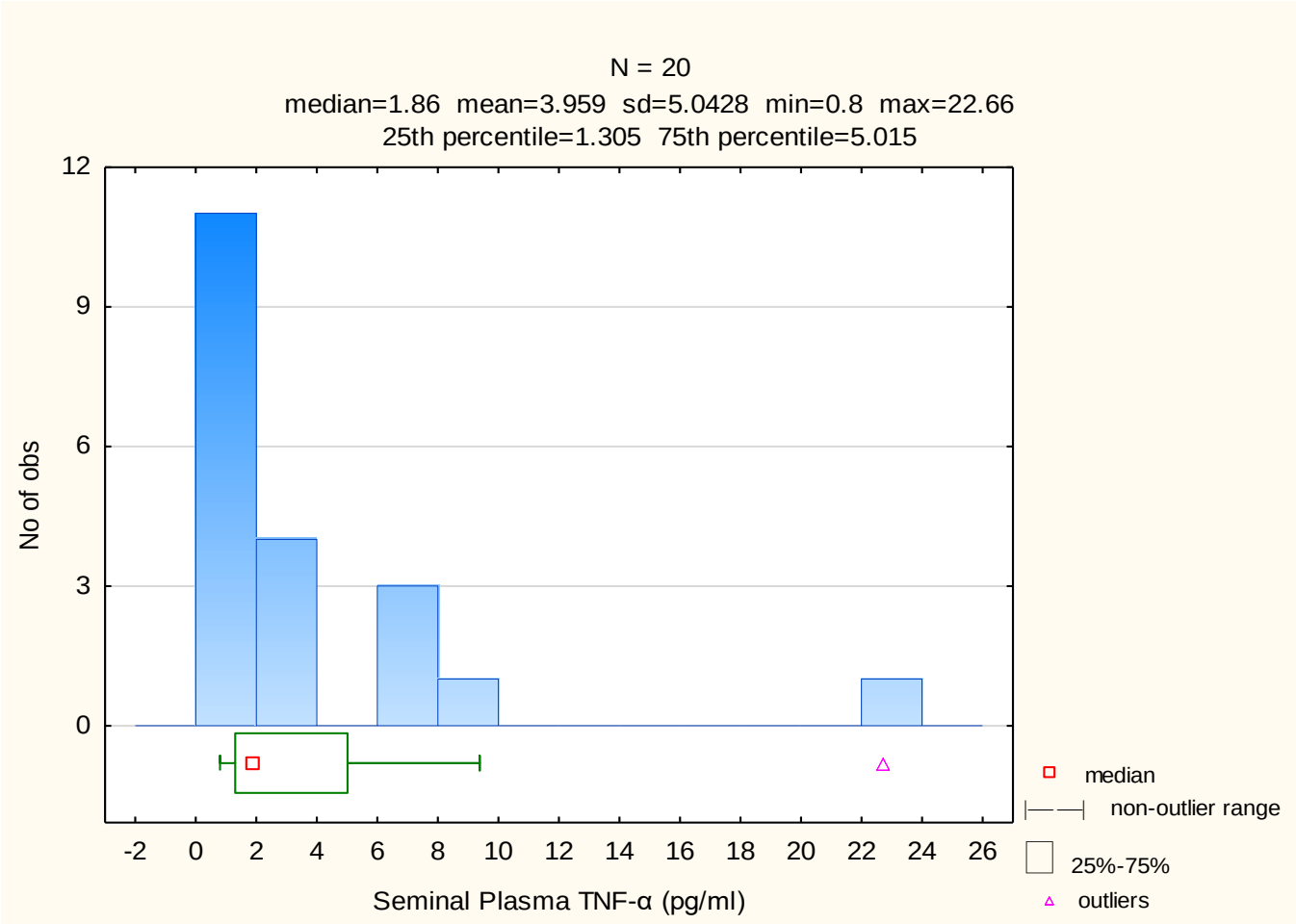



Figure 49 Histogram depicting the number of observations and distribution of seminal plasma TNF-α levels of a portion the participant population

#### 4.4.14.4. Seminal Plasma IL-1β

The mean observed IL-1β value in the participant population was 15.161 pg/ml (SD=32.3765). The lowest value observed was 1.05 pg/ml and the highest value observed was 130.35 pg/ml as depicted in *Figure 50*.

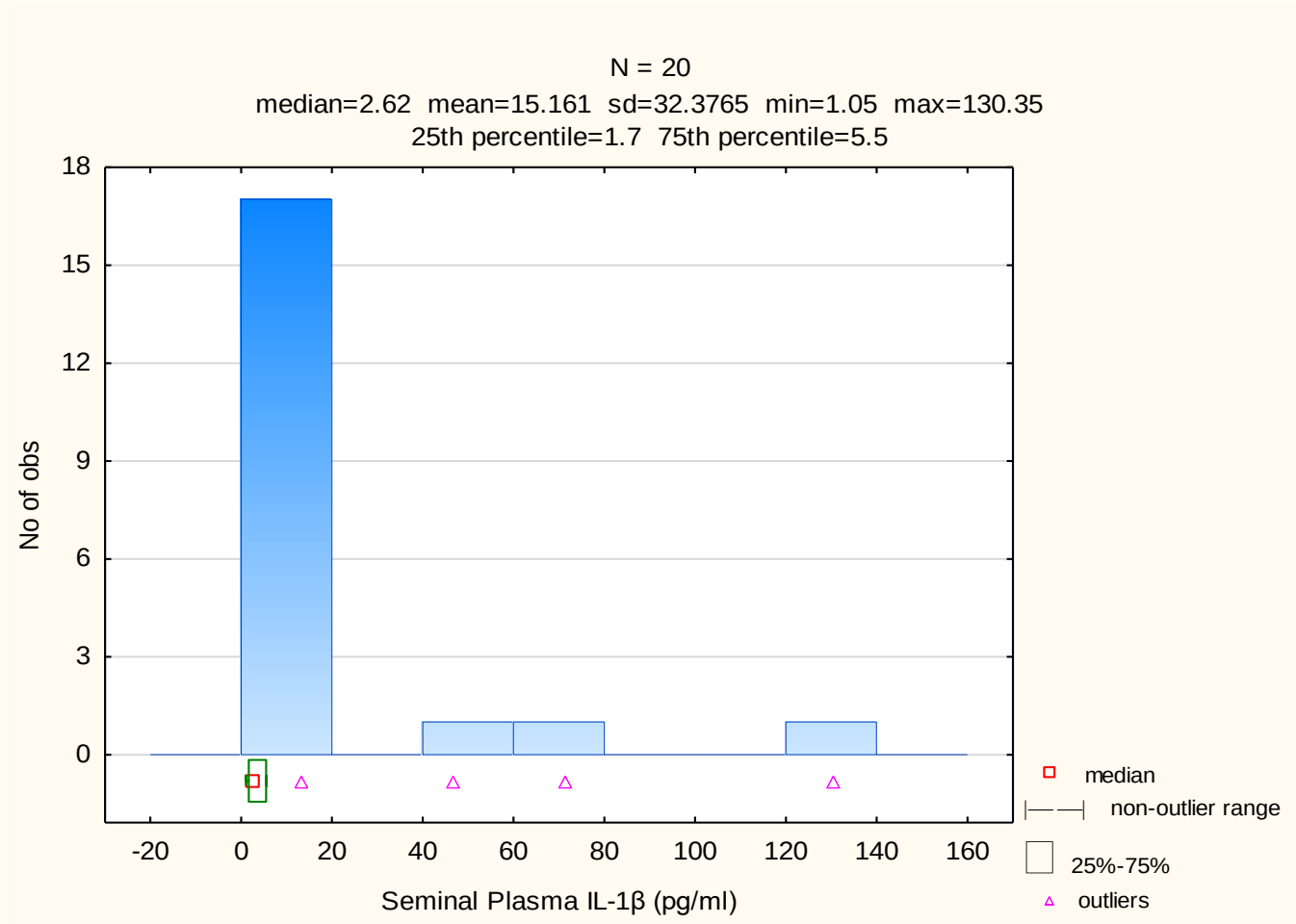



Figure 50 Histogram depicting the number of observations and distribution of seminal plasma IL-1β levels of a portion the participant population

### 4.4.15. Cytokine Profile: Blood Plasma

#### 4.4.15.1. Blood Plasma IFN- γ

The same cytokines were measured in the blood plasma. The sample size for IFN-γ was 12 as a result of some of the data being out of range, as the values were too low to observe. The average IFN-γ level observed in the participant population was 16.5242 pg/ml (SD=17.8269) with a minimum value of 0.54 pg/ml and a maximum value of 64.88 pg/ml being observed and depicted in *Figure 51*.

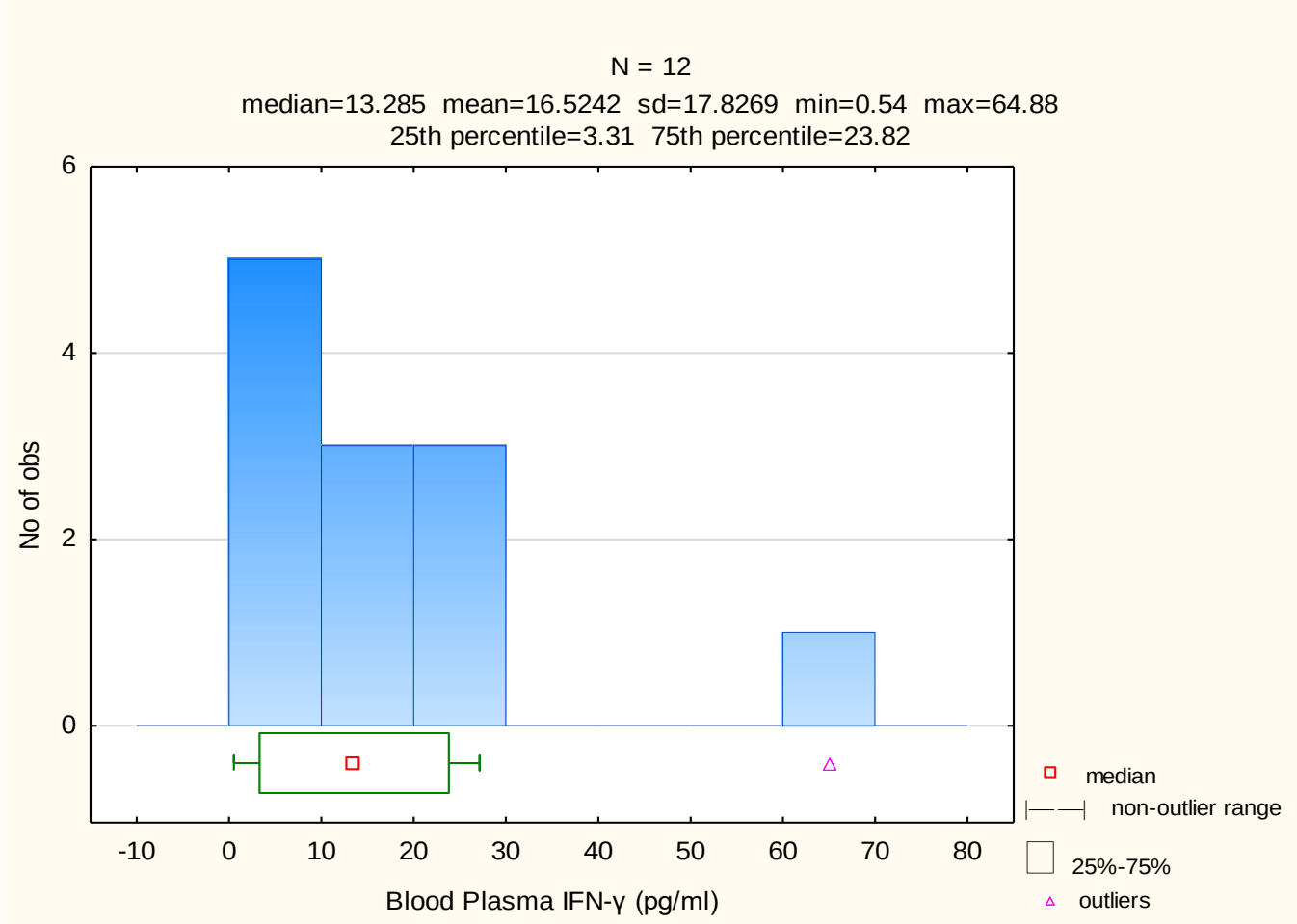



Figure 51 Histogram depicting the number of observations and distribution of blood plasma IFN-γ levels of a portion the participant population

### 4.4.15.2. Blood Plasma IL-6

On average the amount of blood plasma IL-6 observed in the 20 samples was 2.1455 pg/ml which is lower than the values observed in the seminal plasma. The lowest value observed in this study is 1.67 pg/ml, the median value observed 2.09 pg/ml and the highest value observed was 4.17 pg/ml as depicted in *Figure 52*.

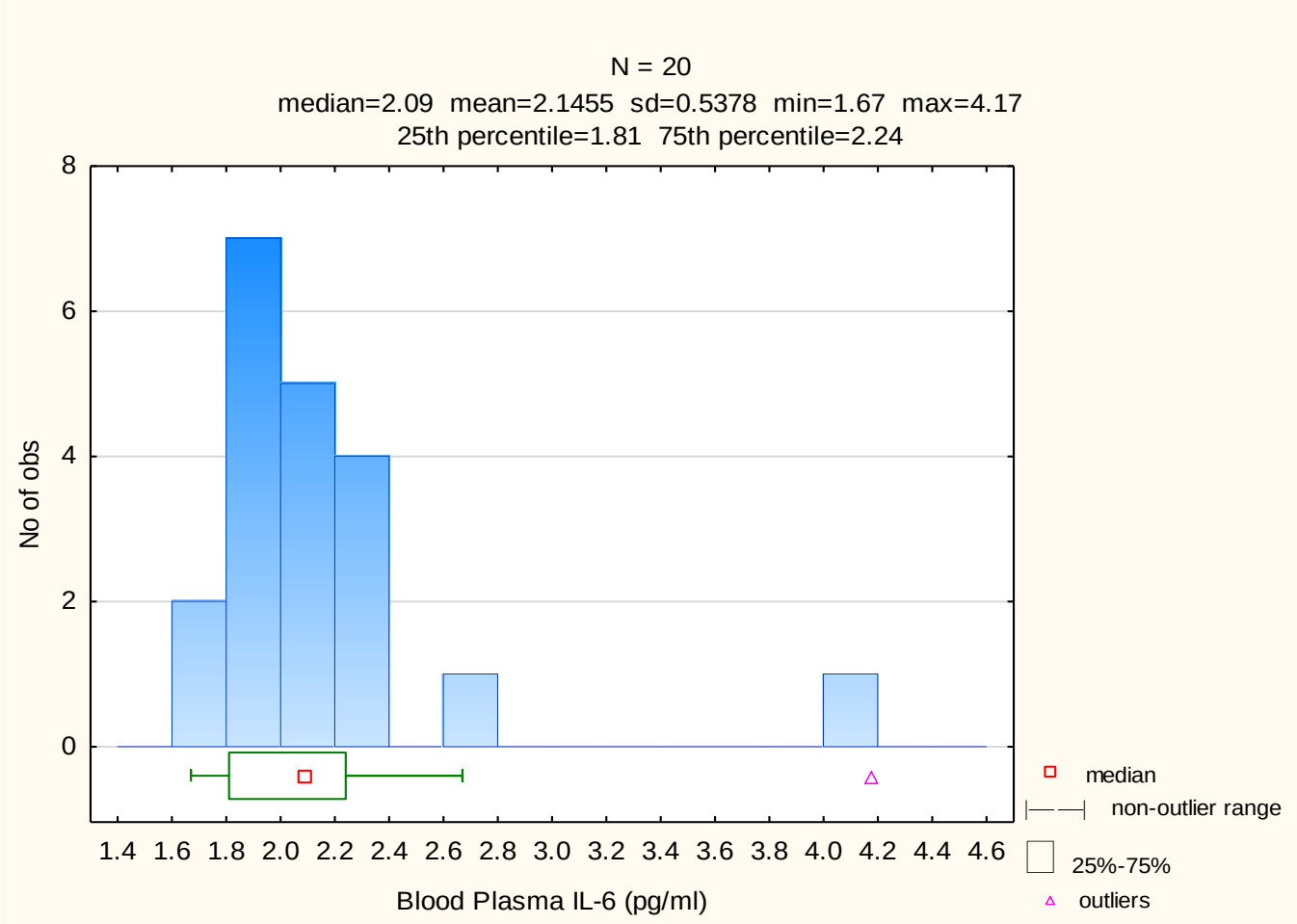



Figure 52 Histogram depicting the number of observations and distribution of blood plasma IL-6 levels of a portion the participant population

#### 4.4.15.3. Blood Plasma TNF-α

The mean value of TNF-α measured in the blood plasma was 2.728 pg/ml (SD=0.9695) as depicted in *Figure 53*. The maximum observed TNF-α value for the 20 samples analysed was 3.8 pg/ml while the minimum value observed was 0.63 pg/ml.

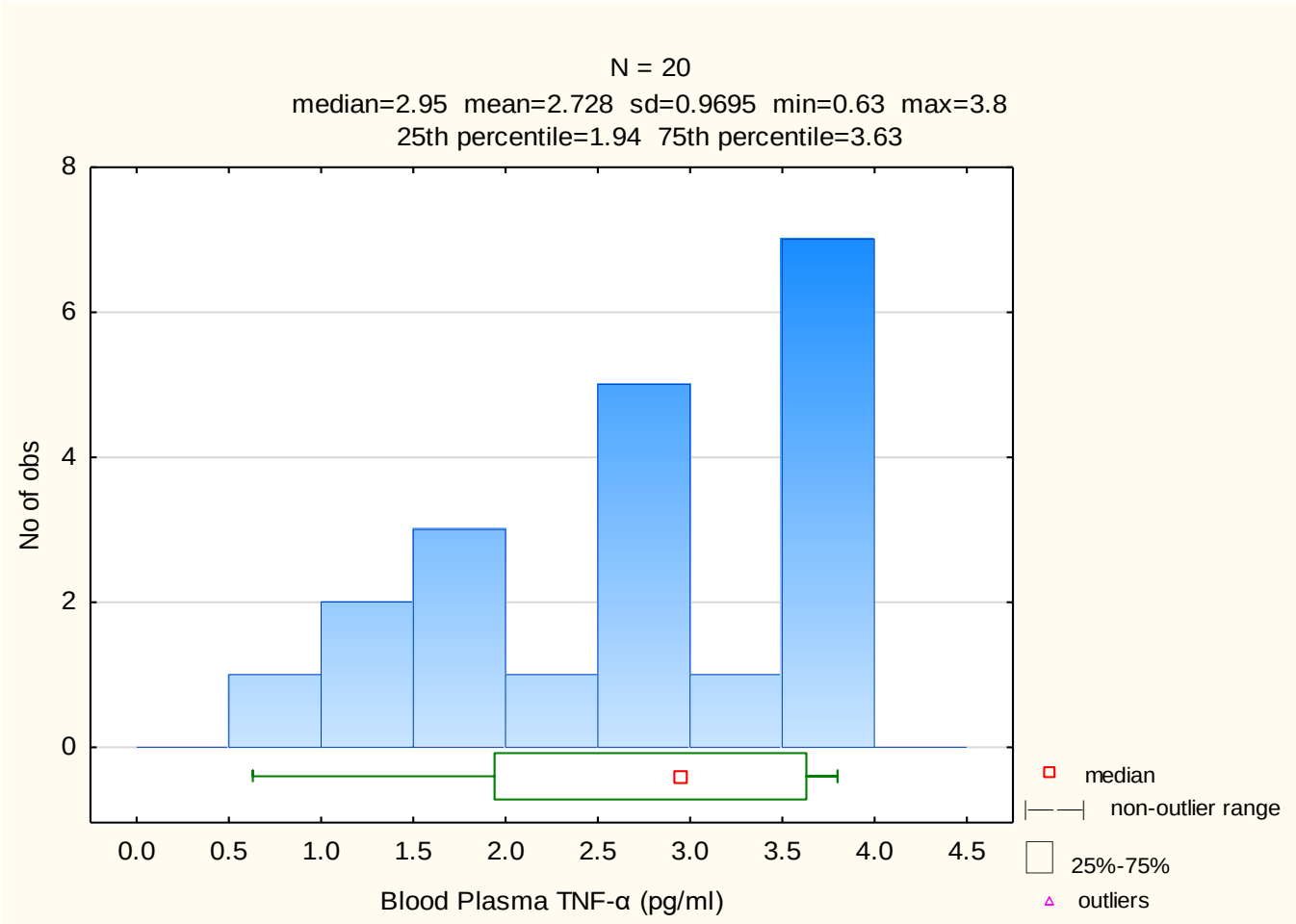



Figure 53 Histogram depicting the number of observations and distribution of blood plasma TNF-α levels of a portion the participant population

### 4.4.15.4. Blood Plasma IL-1β

Blood plasma IL-1β was found to have an average value of 1.852 pg/ml (SD=0.9247). The lowest detected IL-1β value was 0.05 pg/ml, with a median value of 1.852 pg/ml and a highest value of 4.45 pg/ml detected in the analyzed samples as depicted in *Figure 54*.

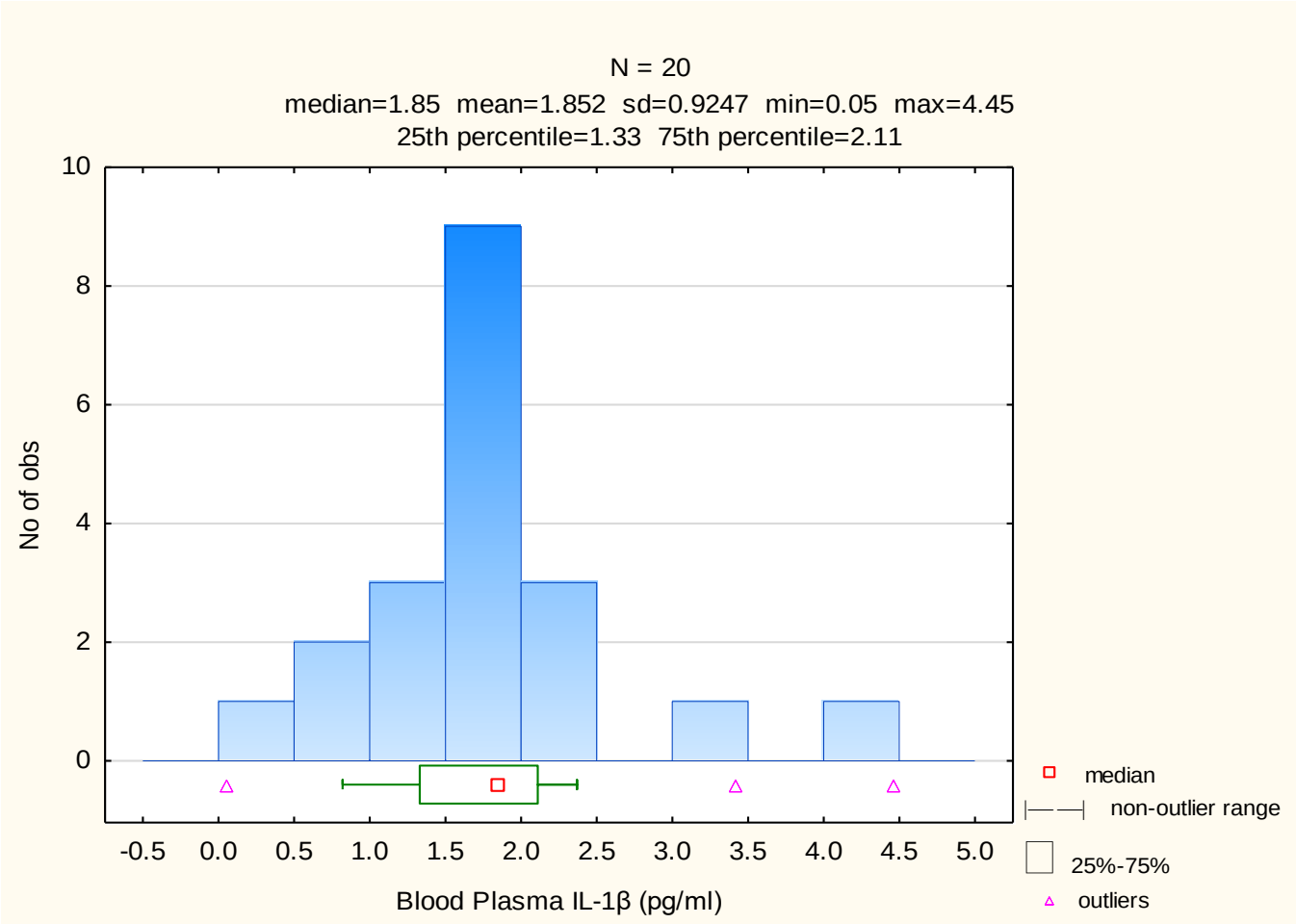



Figure 54 Histogram depicting the number of observations and distribution of blood plasma IL-1β levels of a portion the participant population

### 4.4.16. Cortisol Level: Seminal Plasma and Blood Plasma

As a result of budget constraints, it was only possible to observe the cortisol levels in 59 blood plasma samples and 60 seminal plasma samples. Donors were randomly selected for cortisol level analysis and their samples were used for this analysis. The kit allocated reference values for morning collection (08:00-10:00) is between 60-230 ng/ml, while afternoon collections (16:00) reference values vary between 30 ng/ml and 150 ng/ml. Most of the samples were collected at morning collection session.

#### 4.4.16.1. Blood Plasma Cortisol

The average blood plasma cortisol value observed was 86.562 ng/ml (SD=76.1821) as depicted in *Figure 55*. The lowest observed blood plasma cortisol level was 22.425 ng/ml and the highest value observed was 429.104 ng/ml which is much higher than the reference values.

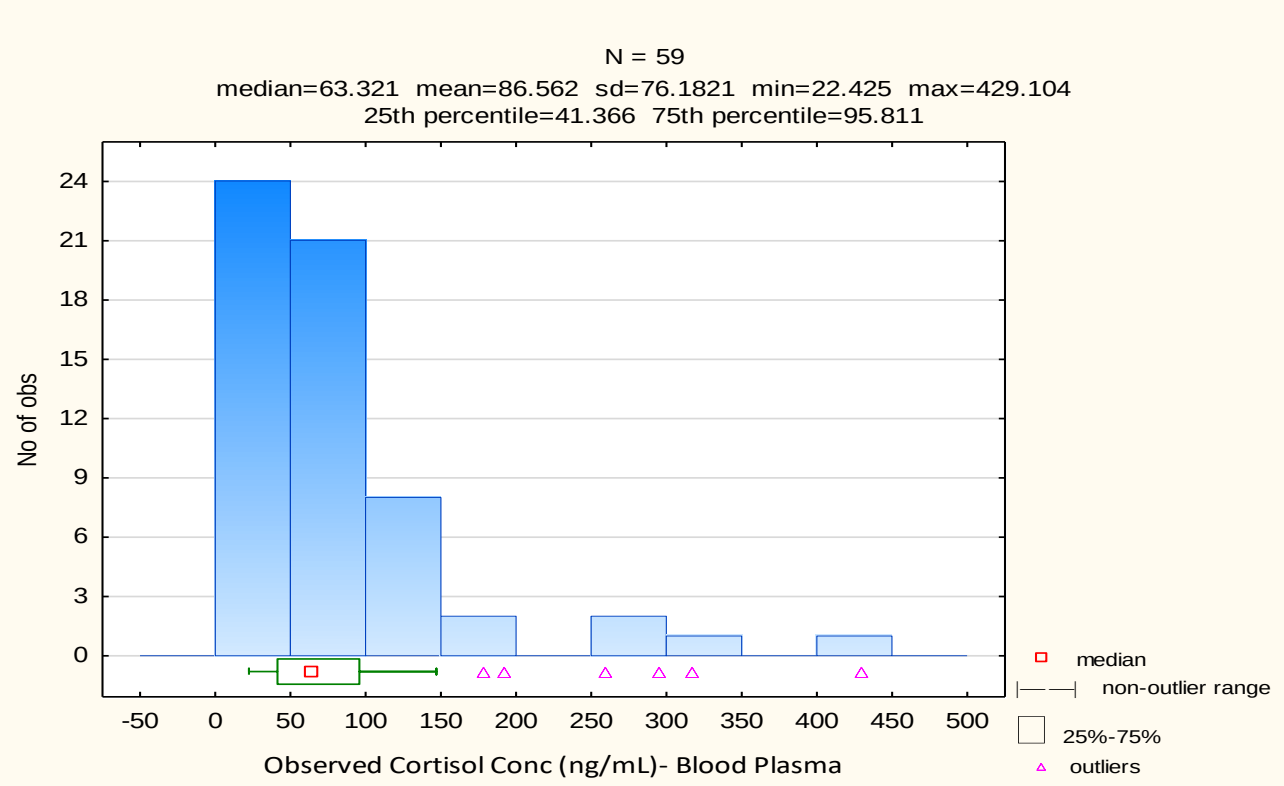



Figure 55 Histogram depicting the number of observations and distribution of the observed cortisol concentration in blood plasma of the participant population

### 4.4.16.2. Seminal Plasma Cortisol

Seminal plasma cortisol levels were lower than those observed in the blood plasma. The 60 samples analyzed to detect seminal plasma cortisol level generated an average of 19.4335 ng/ml (SD=13.378). A maximum value of 54.954 ng/ml was detected while a minimum value of 3.378 ng/ml as depicted in *Figure 56*.

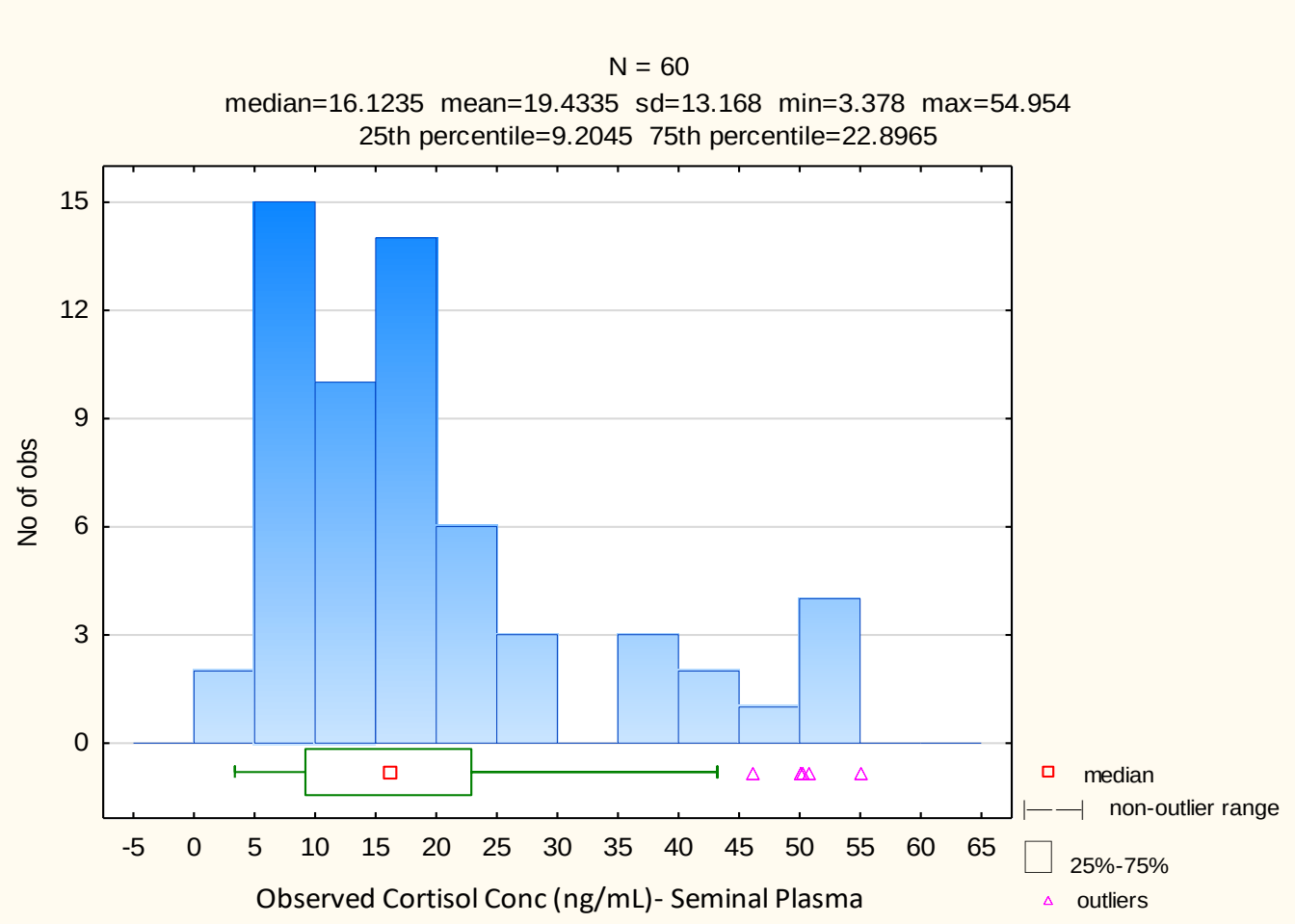



Figure 56 Histogram depicting the number of observations and distribution of the observed cortisol concentration in seminal plasma of the participant population

## 4.5. Correlations

First, the relationship between state and trait anxiety was investigated. It was found that state and trait share a significantly strong positive relationship (0.6202, p = <0.0001) as depicted in *Figure 57*.

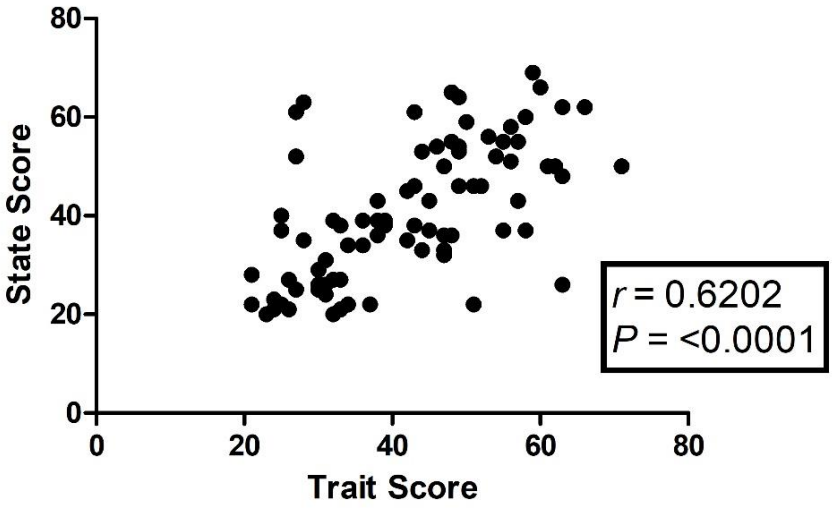



Figure 57 Plot depicting the positive relationship between state score and trait score

Next, the correlations of the single dependent variables were compared to state anxiety. Pearson or Spearman correlations were reported where appropriate, taking outliers and sample sizes into consideration. Only strong trends and statistically significant results will be reported in *Table 5*.

Table 5 Table displaying the strong trends and statistically significant correlations between state anxiety scores and single dependent variables.

| **Independent variable** | **Dependent variable** | ***P* value** | **Correlation Coefficient (R)** |
|---|---|---|---|
| **State Anxiety Score** | VAP | 0.02 | -0.26 |
| | STR | 0.05 | -0.22 |
| | LIN | 0.05 | 0.22 |
| | Blood Plasma Cortisol | 0.02 | 0.30 |
| | Seminal Plasma TNF-α | 0.07 | -0.42 |
| | Seminal Plasma IL-1β* | <0.01 | -0.57 |

For state anxiety vs. the single dependent variables, most of the data sets Pearson correlation coefficients and P values were reported, except for seminal plasma IL-1β. Here the Spearman correlation results were reported due to the small sample size and number of outliers present in this parameter.

Next, the correlations of the single dependent variables were compared to trait anxiety. Only strong trends and statistically significant results will be reported in *Table 6*. Similarly, either Pearson or Spearman correlations were reported where appropriate, taking outliers and sample sizes into consideration. Most of the data sets Pearson correlation coefficients and P values were reported, with the exception of semen volume, round cells and seminal plasma IL-1β. Here the Spearman correlation results were reported due to the small sample size or number of outliers present in this parameter.

Table 6 Table displaying the strong trends and statistically significant correlations between trait anxiety scores and single dependent variables.

| **Independent variable** | **Dependent variable** | ***P* Value** | **Correlation Coefficient (R)** |
|---|---|---|---|
| **Trait anxiety score** | Semen Volume* | 0.07 | 0.20 |
| | Round Cells* | <0.01 | 0.30 |
| | Total Motility | 0.10 | -0.18 |
| | VAP | <0.01 | -0.29 |
| | STR | 0.05 | -0.22 |
| | LIN | 0.05 | 0.21 |
| | Blood Plasma Cortisol | 0.03 | 0.29 |
| | Blood Plasma TNF-α | 0.06 | -0.42 |
| | Seminal Plasma IL-1β* | 0.02 | -0.53 |

Additionally, biologically significant relationships between dependent variables was investigated and summarized in *Table 7.* Spearman correlation coefficients were reported with the exception of the results describing the relationship between seminal plasma TNF-α and IL-1β due to the small sample size and sensitivity to outliers.

Table 7 Summary of biologically significant relationships between dependent variables.

| **Independent variable** | **Dependent variable** | ***P* Value** | **Correlation Coefficient (R)** |
|---|---|---|---|
| Abstinence period | Concentration | 0.05 | 0.22 |
| | TSC | 0.03 | 0.24 |
| | Sperm DNA Fragmentation | 0.05 | 0.22 |
| Volume | TSC | <0.01 | 0.51 |
| Concentration | TSC | <0.01 | 0.79 |
| | Round cells | 0.02 | 0.27 |
| Total Motility | Progressive Motility | <0.01 | 0.93 |
| | VCL | <0.01 | 0.74 |
| | VAP | <0.01 | 0.53 |
| | VS.L | <0.01 | 0.56 |
| | LIN | <0.01 | 0.32 |
| | Hyperactive Motile | <0.01 | 0.54 |
| | ALH | <0.01 | 0.78 |
| | BCF | <0.01 | 0.73 |
| Progressive Motility | VCL | <0.01 | 0.82 |
| | VAP | <0.01 | 0.59 |
| | VS.L | <0.01 | 0.56 |
| | LIN | 0.03 | 0.24 |
| | Hyperactive Motile | <0.01 | 0.64 |
| | ALH | <0.01 | 0.87 |
| | BCF | <0.01 | 0.74 |
| | Blood plasma TNF-α | 0.04 | 0.46 |
| | Blood plasma IFN-γ* | 0.03 | 0.62 |
| Round Cells | Seminal plasma IL-1β | 0.02 | 0.53 |
| | Viable spermatozoa | 0.07 | -0.20 |
| Spermatozoa DNA Fragmentation | Seminal plasma TNF-α | <0.01 | 0.72 |
| NO | Blood Plasma IFN-γ | 0.06 | 0.64 |
| Seminal Plasma TNF-α * | Seminal Plasma IL-1β | <0.01 | 0.59 |

## 4.6. Mixed Model ANOVA Results and T-tests

A mixed model analysis of variance is used to test for differences between two or more independent groups while still subjecting participants to repeated measures design (repeated measures with a between subject factor). The purpose of the ANOVA was to compare the means of the stratified dependent variables which will allow us to verify the relationships observed throughout the correlations. T-tests were used to achieve the same purpose for the cytokine profile data due to the small sample size. The state and trait scores were stratified into low (<40) and high anxiety (≥40) groups.

### 4.6.1. Descriptive Statistics: 'High anxiety' vs. 'Low anxiety'

For the stratified state anxiety score 54% of the participant population samples formed the 'low anxiety' group, while the remaining 38 samples formed the 'high anxiety' group as depicted in *Figure 57*. The average low state anxiety score is 29.9111 (SD=6.5177), while the average high state anxiety score is 53.3947 (SD=7.4394) as tabulated in *Table 8*. Conversely, 39 samples formed the low trait anxiety group and the remaining 53% formed the high trait anxiety group as depicted in *Figure 58*. The average low trait anxiety score is 30.6410 (SD=5.1415) which is higher than low state anxiety score. While the average high trait anxiety score is 52.1818 (SD=7.2057) which is lower than the average high state anxiety score as tabulated in *Table 8*. A summary of the descriptive statistics of the stratified single dependent data is included in *Tables 9-14*.

Table 8 Descriptive statistics of the stratification of state and trait anxiety into 'low' and 'high' anxiety scores

| | Mean | SD | Min | Max | N |
|---|---|---|---|---|---|
| Low State | 29.9111 | 6.5177 | 20 | 39 | 45 |
| High State | 53.3947 | 7.4394 | 40 | 69 | 38 |
| Low Trait | 30.6410 | 5.1415 | 21 | 39 | 39 |
| High Trait | 52.1818 | 7.2057 | 42 | 71 | 44 |

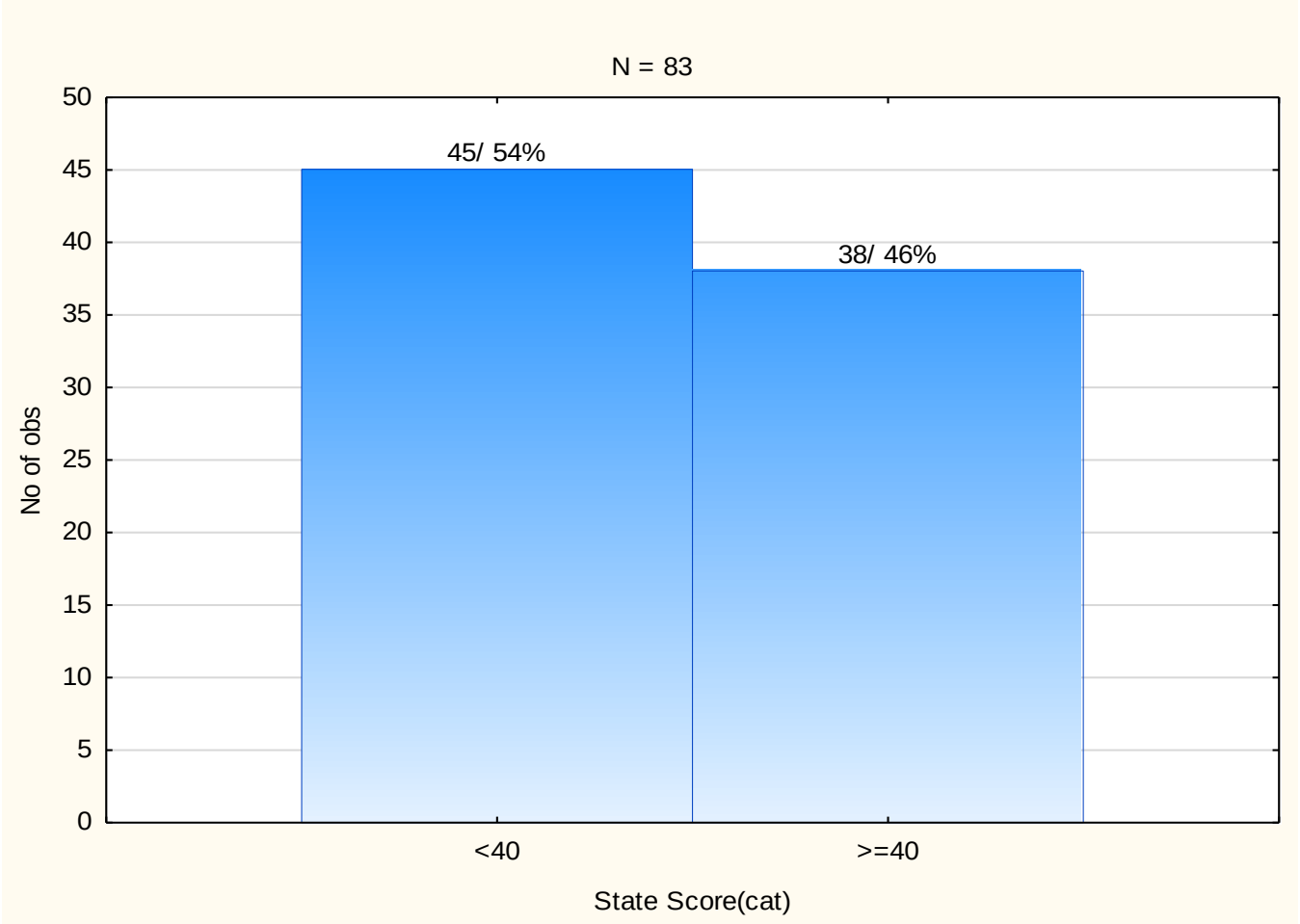



Figure 58 Histogram displaying the distribution of the stratified state anxiety scores.

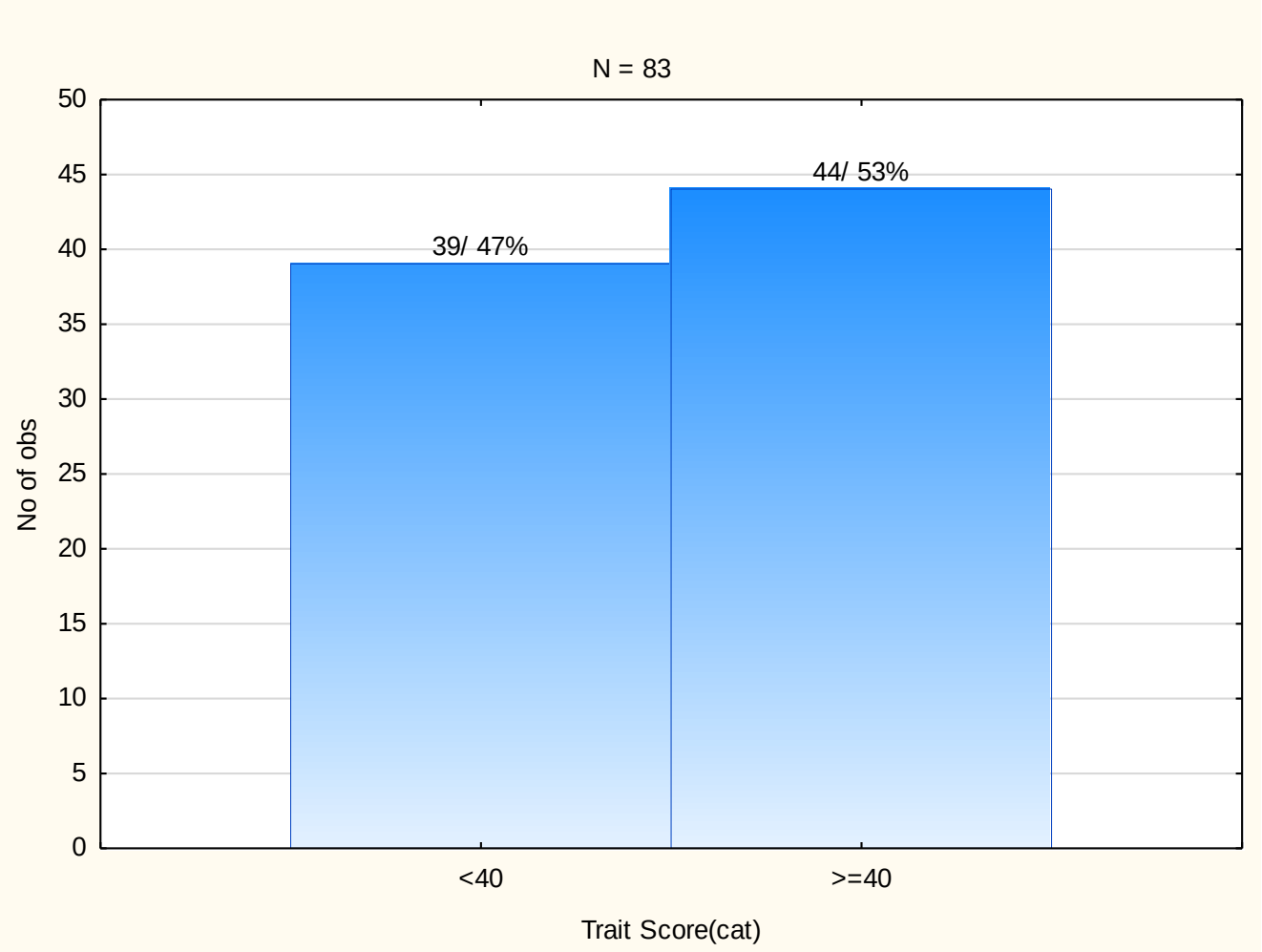



Figure 59 Histogram displaying the distribution of the stratified trait anxiety scores

Table 9 . A summary of the descriptive statistics of the stratified single dependent data

| Independent Variable | Dependent Variable | Distribution | N | Mean | SD |
|---|---|---|---|---|---|
| Abstinence Period (n=82) | State Score | <40 | 44 | 3.7045 | 1.1119 |
| | State Score | >=40 | 38 | 3.9211 | 2.1357 |
| | Trait Score | <40 | 38 | 3.8158 | 1.0096 |
| | Trait Score | >=40 | 44 | 3.7955 | 2.0751 |
| Volume (n=83) | State Score | <40 | 45 | 3.6329 | 1.5108 |
| | State Score | >=40 | 38 | 3.8753 | 1.4691 |
| | Trait Score | <40 | 39 | 3.5741 | 1.6116 |
| | Trait Score | >=40 | 44 | 3.8943 | 1.3698 |
| Concentration (n=83) | State Score | <40 | 45 | 58.8720 | 38.3322 |
| | State Score | >=40 | 38 | 55.2753 | 32.6084 |
| | Trait Score | <40 | 39 | 60.6397 | 40.7227 |
| | Trait Score | >=40 | 44 | 54.1989 | 30.6466 |
| Total Sperm Count (n=83) | State Score | <40 | 45 | 229.1929 | 227.5334 |
| | State Score | >=40 | 38 | 195.6118 | 117.8750 |
| | Trait Score | <40 | 39 | 227.7626 | 239.0037 |
| | Trait Score | >=40 | 44 | 201.4589 | 121.0712 |
| Total Motility (n=83) | State Score | <40 | 45 | 58.2636 | 19.3209 |
| | State Score | >=40 | 38 | 52.2337 | 21.8285 |
| | Trait Score | <40 | 39 | 61.0436 | 18.1097 |
| | Trait Score | >=40 | 44 | 50.5918 | 21.6113 |

Table 10 A summary of the descriptive statistics of the stratified single dependent data (continued)

| Independent Variable | Dependent Variable | Distribution | N | Mean | SD |
|---|---|---|---|---|---|
| Progressive Motility (n=83) | State Score | <40 | 45 | 42.3584 | 19.1416 |
| | State Score | >=40 | 38 | 36.2505 | 18.8735 |
| | Trait Score | <40 | 39 | 44.2856 | 18.6508 |
| | Trait Score | >=40 | 44 | 35.3752 | 18.8075 |
| Round Cells (n=83) | State Score | <40 | 45 | 0.1729 | 0.2046 |
| | State Score | >=40 | 38 | 0.2982 | 0.4406 |
| | Trait Score | <40 | 39 | 0.1287 | 0.1192 |
| | Trait Score | >=40 | 44 | 0.3202 | 0.4327 |
| VCL (n=83) | State Score | <40 | 45 | 53.4660 | 12.3721 |
| | State Score | >=40 | 38 | 48.7000 | 10.4856 |
| | Trait Score | <40 | 39 | 53.6531 | 12.7417 |
| | Trait Score | >=40 | 44 | 49.1841 | 10.4423 |
| VAP (n=83) | State Score | <40 | 45 | 28.9953 | 9.7910 |
| | State Score | >=40 | 38 | 23.2295 | 7.5869 |
| | Trait Score | <40 | 39 | 28.9172 | 10.2461 |
| | Trait Score | >=40 | 44 | 24.0850 | 7.7168 |
| VSL (n=83) | State Score | <40 | 45 | 22.5860 | 6.9444 |
| | State Score | >=40 | 38 | 23.8942 | 9.4471 |
| | Trait Score | <40 | 39 | 23.0036 | 6.6831 |
| | Trait Score | >=40 | 44 | 23.3457 | 9.3506 |

Table 11 A summary of the descriptive statistics of the stratified single dependent data (continued)

| Independent Variable | Dependent Variable | Distribution | N | Mean | SD |
|---|---|---|---|---|---|
| STR (n=83) | State Score | <40 | 45 | 50.2784 | 10.8373 |
| | State Score | >=40 | 38 | 45.2603 | 11.4669 |
| | Trait Score | <40 | 39 | 49.4777 | 11.3414 |
| | Trait Score | >=40 | 44 | 46.6543 | 11.3085 |
| LIN (n=83) | State Score | <40 | 45 | 39.1467 | 8.4655 |
| | State Score | >=40 | 38 | 43.6634 | 12.2896 |
| | Trait Score | <40 | 39 | 40.1787 | 9.1627 |
| | Trait Score | >=40 | 44 | 42.1327 | 11.7063 |
| WOB (n=83) | State Score | <40 | 45 | 59.4896 | 7.0583 |
| | State Score | >=40 | 38 | 59.1626 | 9.3961 |
| | Trait Score | <40 | 39 | 60.4103 | 7.4719 |
| | Trait Score | >=40 | 44 | 58.3911 | 8.7005 |
| ALH (n=79) | State Score | <40 | 42 | 1.8671 | 0.3390 |
| | State Score | >=40 | 37 | 1.7341 | 0.3050 |
| | Trait Score | <40 | 37 | 1.8603 | 0.3586 |
| | Trait Score | >=40 | 42 | 1.7560 | 0.2949 |
| BCF (n=79) | State Score | <40 | 42 | 12.6840 | 2.5261 |
| | State Score | >=40 | 37 | 12.0603 | 3.1730 |
| | Trait Score | <40 | 37 | 13.0416 | 2.3270 |
| | Trait Score | >=40 | 42 | 11.8195 | 3.1523 |

Table 12 A summary of the descriptive statistics of the stratified single dependent data (continued)

| Independent Variable | Dependent Variable | Distribution | N | Mean | SD |
|---|---|---|---|---|---|
| Hyperactive Motile Spermatozoa (n=83) | State Score | <40 | 45 | 1.3973 | 1.2998 |
| | State Score | >=40 | 38 | 0.9405 | 0.9339 |
| | Trait Score | <40 | 39 | 1.3882 | 1.3288 |
| | Trait Score | >=40 | 44 | 1.0109 | 0.9749 |
| Viable Spermatozoa (n=83) | State Score | <40 | 45 | 59.4000 | 14.9980 |
| | State Score | >=40 | 38 | 55.2895 | 18.7860 |
| | Trait Score | <40 | 39 | 59.7821 | 15.4544 |
| | Trait Score | >=40 | 44 | 55.5114 | 17.9471 |
| Blood Plasma Cortisol (n=59) | State Score | <40 | 32 | 63.5100 | 35.2942 |
| | State Score | >=40 | 27 | 113.8828 | 100.1617 |
| | Trait Score | <40 | 33 | 63.4325 | 35.2194 |
| | Trait Score | >=40 | 26 | 115.9187 | 101.3630 |
| Seminal Plasma Cortisol (n=60) | State Score | <40 | 32 | 19.6381 | 10.9589 |
| | State Score | >=40 | 28 | 19.1997 | 15.5211 |
| | Trait Score | <40 | 30 | 19.9808 | 13.6850 |
| | Trait Score | >=40 | 30 | 18.8862 | 12.8402 |
| Semen ROS level (n=81) | State Score | <40 | 44 | 326.3893 | 2590.6228 |
| | State Score | >=40 | 37 | 471.5580 | 1917.0821 |
| | Trait Score | <40 | 38 | 141.0241 | 2483.8385 |
| | Trait Score | >=40 | 43 | 615.1129 | 2118.5917 |

Table 13 A summary of the descriptive statistics of the stratified single dependent data (continued)

| Independent Variable | Dependent Variable | Distribution | N | Mean | SD |
|---|---|---|---|---|---|
| Spermatozoa DNA Fragmentation (n=81) | State Score | <40 | 44 | 4.7116 | 4.1405 |
| | State Score | >=40 | 37 | 4.8081 | 4.7723 |
| | Trait Score | <40 | 38 | 4.3100 | 3.9690 |
| | Trait Score | >=40 | 43 | 5.1495 | 4.7814 |
| NO MFI (n=65) | State Score | <40 | 37 | 4867.8108 | 2234.7350 |
| | State Score | >=40 | 28 | 4753.8214 | 2431.8502 |
| | Trait Score | <40 | 32 | 4714.9688 | 2600.3504 |
| | Trait Score | >=40 | 33 | 4919.3030 | 2010.6973 |
| Seminal Plasma INF-γ (n=20) | State Score | <40 | 9 | 30.59 | 9.761 |
| | State Score | >=40 | 11 | 27.80 | 7.439 |
| | Trait Score | <40 | 8 | 27.59 | 5.323 |
| | Trait Score | >=40 | 8 | 27.52 | 8.626 |
| Seminal Plasma IL-6 (n=20) | State Score | <40 | 9 | 22.84 | 27.39 |
| | State Score | >=40 | 11 | 11.28 | 12.73 |
| | Trait Score | <40 | 8 | 8.789 | 8.255 |
| | Trait Score | >=40 | 8 | 6.430 | 3.517 |
| Seminal Plasma TNF-α (n=20) | State Score | <40 | 9 | 5.782 | 6.812 |
| | State Score | >=40 | 11 | 2.467 | 2.398 |
| | Trait Score | <40 | 8 | 6.008 | 7.259 |
| | Trait Score | >=40 | 8 | 2.658 | 2.824 |

Table 14 A summary of the descriptive statistics of the stratified single dependent data (continued)

| Independent Variable | Dependent Variable | Distribution | N | Mean | SD |
|---|---|---|---|---|---|
| Seminal Plasma IL-1β | State Score | <40 | 9 | 30.83 | 44.56 |
| | State Score | >=40 | 11 | 2.345 | 1.408 |
| | Trait Score | <40 | 8 | 32.91 | 47.20 |
| | Trait Score | >=40 | 8 | 1.931 | 1.026 |
| Blood Plasma IFN-γ (n=12) | State Score | <40 | 6 | 12.36 | 8.607 |
| | State Score | >=40 | 6 | 20.69 | 24.16 |
| | Trait Score | <40 | 5 | 13.84 | 8.730 |
| | Trait Score | >=40 | 4 | 28.54 | 26.80 |
| Blood Plasma IL-6 (n=20) | State Score | <40 | 9 | 2.207 | 0.7604 |
| | State Score | >=40 | 11 | 2.095 | 0.2843 |
| | Trait Score | <40 | 8 | 2.203 | 0.8128 |
| | Trait Score | >=40 | 8 | 2.078 | 0.2982 |
| Blood Plasma TNF-α (n=20) | State Score | <40 | 9 | 3.048 | 0.7849 |
| | State Score | >=40 | 11 | 2.466 | 1.061 |
| | Trait Score | <40 | 8 | 3.354 | 0.4446 |
| | Trait Score | >=40 | 8 | 2.566 | 1.065 |
| Blood Plasma IL-1β (n=20) | State Score | <40 | 9 | 1.967 | 0.7231 |
| | State Score | >=40 | 11 | 1.758 | 1.088 |
| | Trait Score | <40 | 8 | 2.014 | 0.7453 |
| | Trait Score | >=40 | 8 | 2.110 | 1.002 |

The repeated measures design is utilized when numerous independent variables exist in a data set, however, all participants have each variable measured (Field, 2009). It takes into account the possible dependency of the data that may arise as a result of each donor having contributed 4 samples. A 2 (state low/state high) x 2 (trait low/trait high) mixed model ANOVA was conducted to evaluate the effect of the anxiety scores on the single dependent variables. The mixed model design allows that the individual effect of each independent variable is tested, as well as the possible interaction effect of both independent variables: state and trait scores. This is useful as trait anxiety describes the inclination to an anxious state, which naturally will influence the state anxiety scores to some measure. Only strong trends or statistical significance in the data set will be reported.

### 4.6.2. Volume

Anxiety might have an influence on the semen volume observed in the study, but this influence might vary depending on whether the anxiety is more short term (state anxiety) or more long term (trait anxiety) as well as if the anxiety is considered low (<40) or high (≥40).

A trend was observed between state and trait anxiety scores on the volume parameter was noted ($F$ =3.17, $p$ = 0.08). When considering the effect of low trait anxiety on the volume observed in the low state and high state anxiety, the difference between the means is not as different (3.9237±0.3544 vs. 3.5769±0.5305). The difference between the means of low state and high state anxiety when considering the effect of high trait anxiety, the means are different (3.1301±0.4290 vs. 3.8255±0.3497). Any relationship observed between state anxiety and the volume parameter was affected by trait anxiety and vice versa and the interaction graphs of the means is displayed in *Figure 60*.

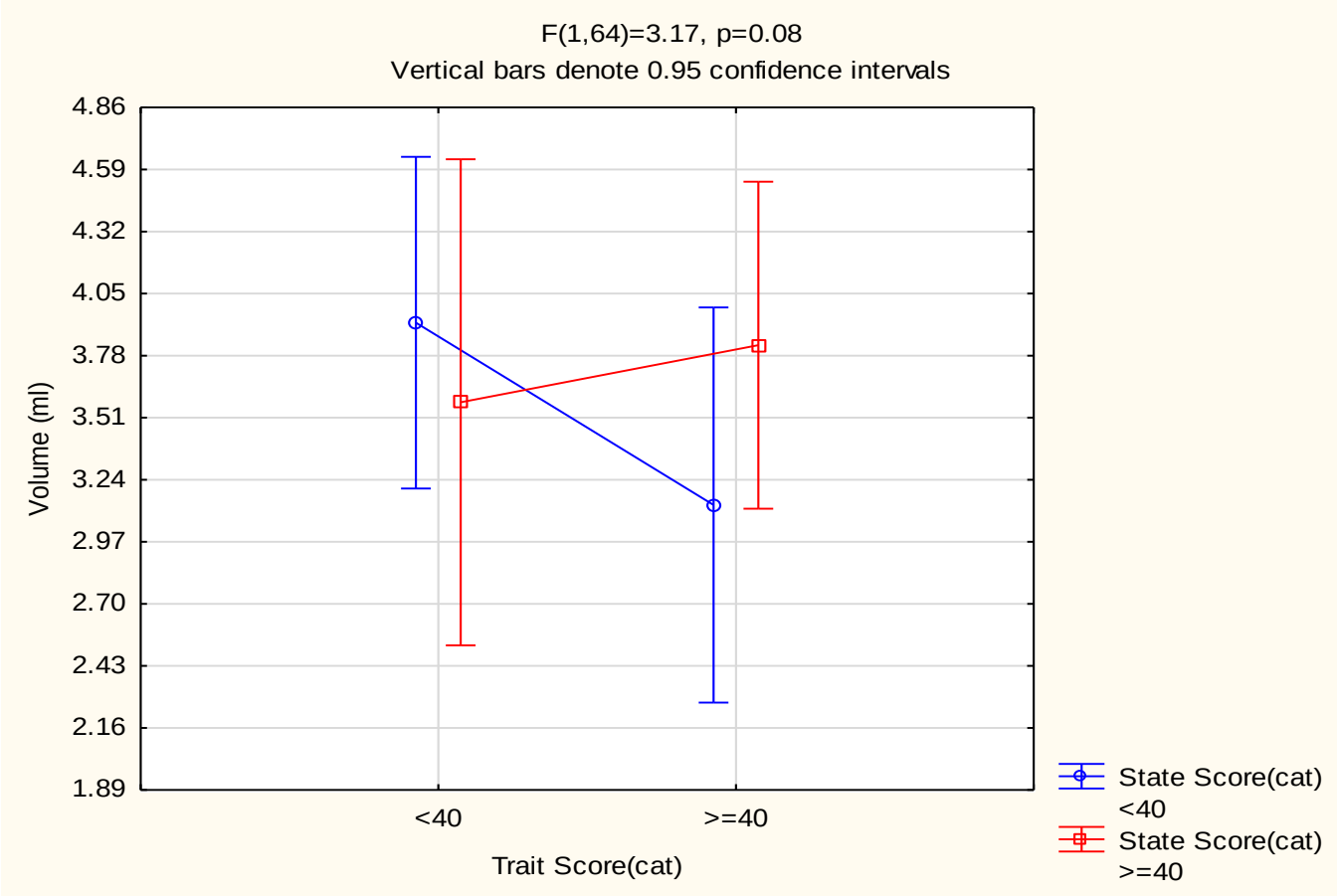



Figure 60 Mean volume and confidence intervals for stratified trait and state anxiety scores

### 4.6.3. Round Cells

Anxiety might have an influence on the number of round cells observed in the study, but this influence might vary depending on whether the anxiety is more short term (state anxiety) or more long term (trait anxiety) as well as if the anxiety is considered low (<40) or high (≥40).

The main effect of trait anxiety on round cells was approaching statistical significance ($F$ = 2.41, $p$ = 0.13). The interaction effect of trait anxiety and state anxiety is negligible. The round cells increased in the high trait anxiety group compared to the low trait anxiety group (0.2974±0.0699 vs. 0.1331±0.0845) as displayed in *Figure 61*.

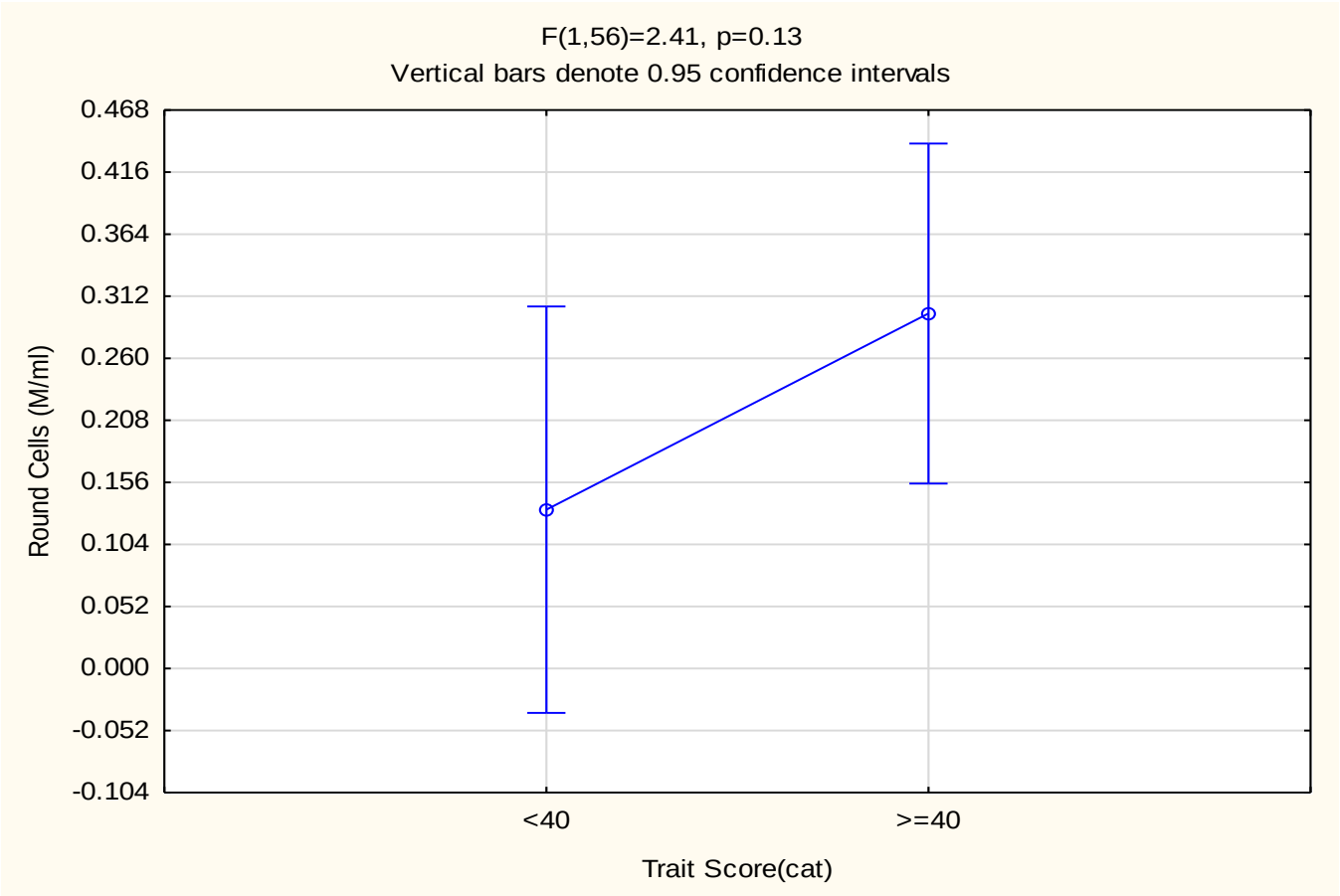



Figure 61 Mean number of round cells and confidence intervals for stratified trait and state anxiety scores

### 4.6.4. Kinematic Parameter: VCL

The VCL kinematic parameter might be influenced by anxiety, but this influence might vary depending on whether the anxiety is more short term (state anxiety) or more long term (trait anxiety) as well as if the anxiety is considered low (<40) or high (≥40).

The main effect of state anxiety on the VCL parameter was approaching statistical significance ($F = 2.21$, $p = 0.14$). The interaction effect of trait anxiety and state anxiety is negligible. The VCL decreased in the high trait anxiety group compared to the low anxiety group ($48.4803 \pm 2.6931$ vs. $52.4765 \pm 2.3394$) as displayed in *Figure 62*.

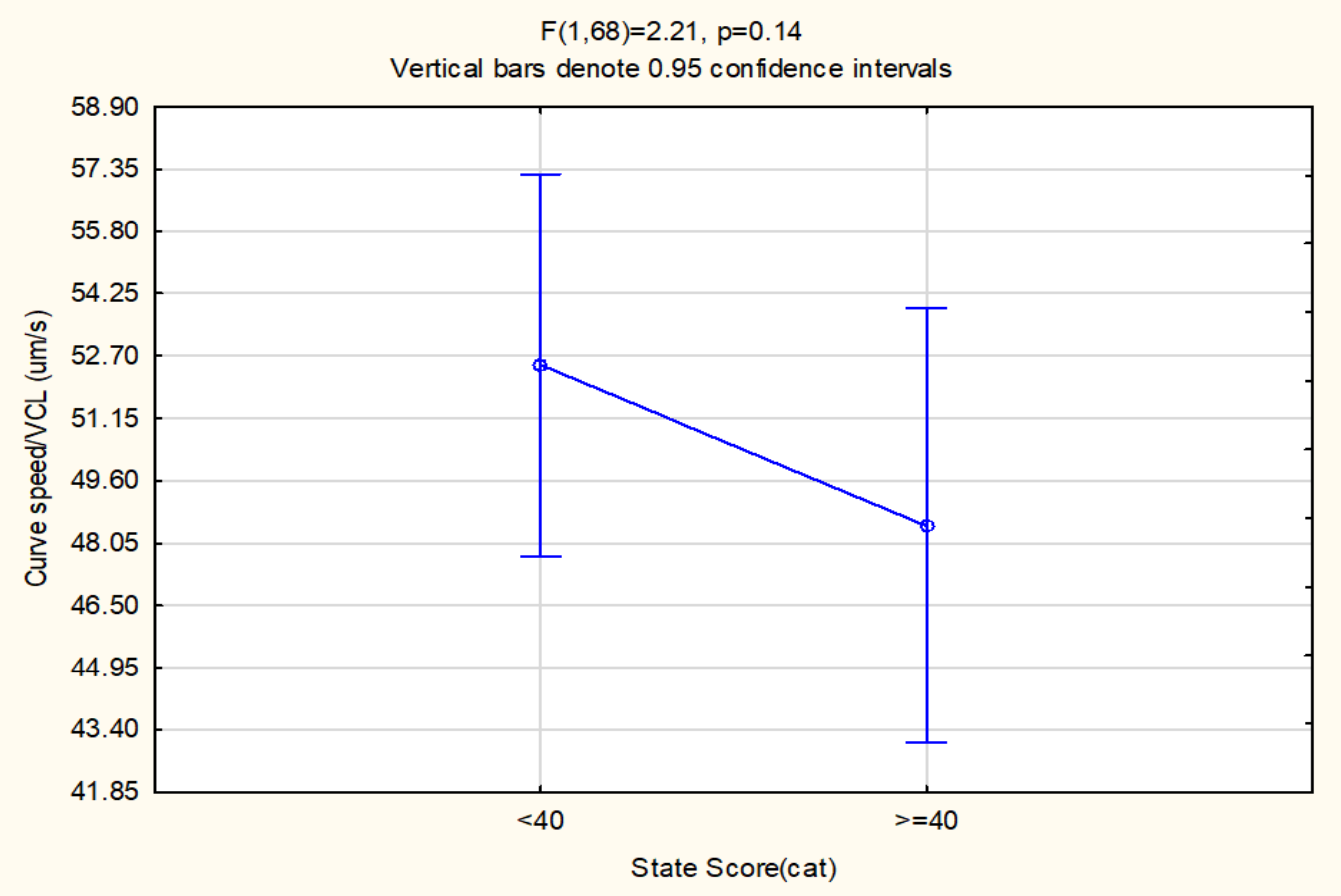



Figure 62 Mean VCL and confidence intervals for stratified state anxiety scores

### 4.6.5. Kinematic Parameter: LIN

The LIN kinematic parameter might be influenced by anxiety, but this influence might vary depending on whether the anxiety is more short term (state anxiety) or more long term (trait anxiety) as well as if the anxiety is considered low (<40) or high (≥40).

The main effect of state anxiety on the LIN parameter was approaching statistical significance ($F = 4.11$, $p = 0.05$). The interaction effect of trait anxiety and state anxiety is negligible. The LIN increased in the high trait anxiety group compared to the low anxiety group (43.0939± 2.3786 vs. 39.8471 ± 2.2430) as displayed in *Figure 63*.

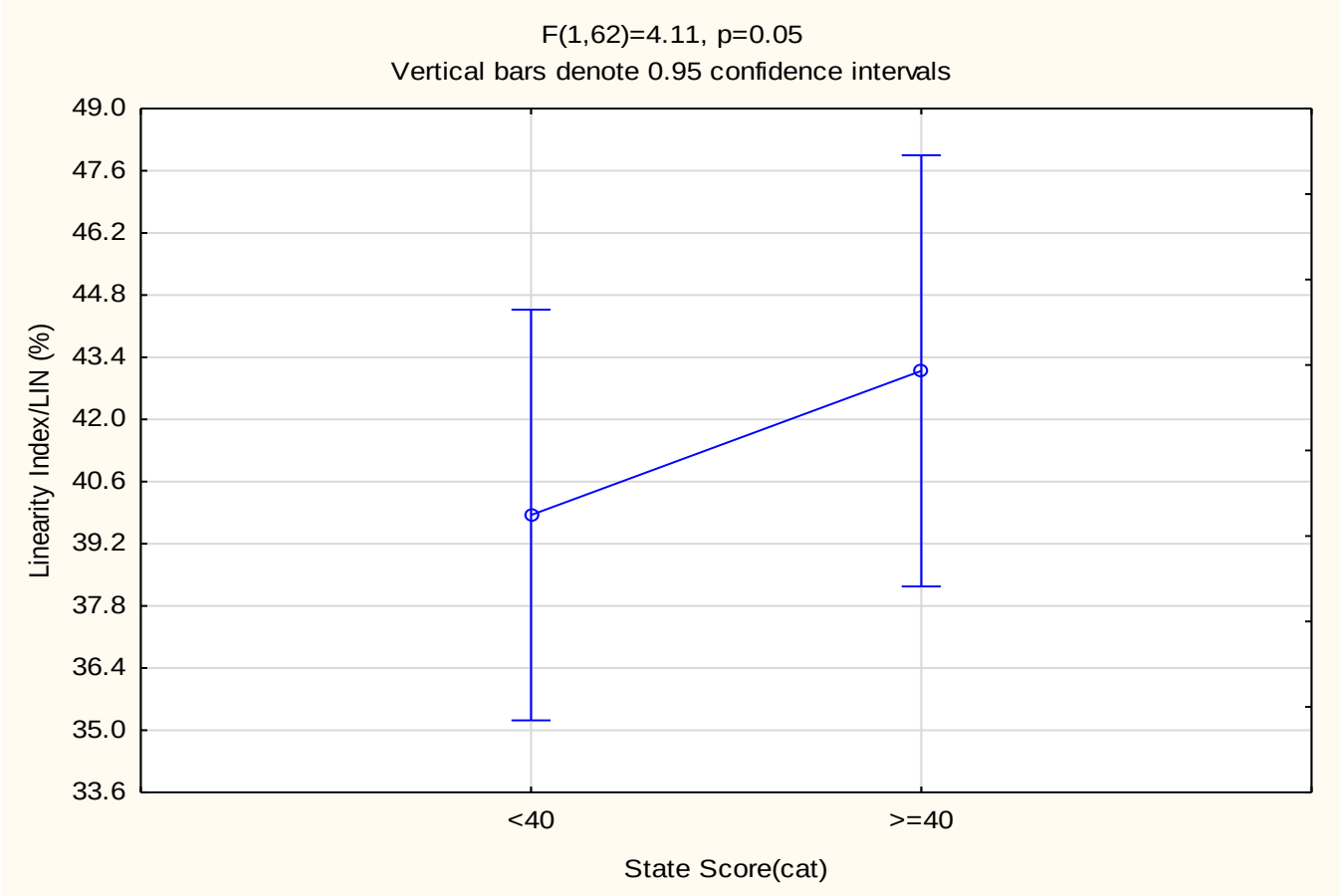



Figure 63 Mean LIN and confidence intervals for stratified state anxiety scores

### 4.6.6. Kinematic Parameter: WOB

Anxiety might have an influence on the WOB kinematic parameter observed in the study, but this influence might vary depending on whether the anxiety is more short term (state anxiety) or more long term (trait anxiety) as well as if the anxiety is considered low (<40) or high (≥40).

The main effect of state anxiety on the WOB parameter was statistically significant ($F = 7.37$, $p = 0.01$). The interaction effect of trait anxiety and state anxiety is negligible. The WOB increased in the high trait anxiety group compared to the low anxiety group (61.7673± 1.9461 vs. 57.3377 ± 1.7709) as displayed in *Figure 64*.

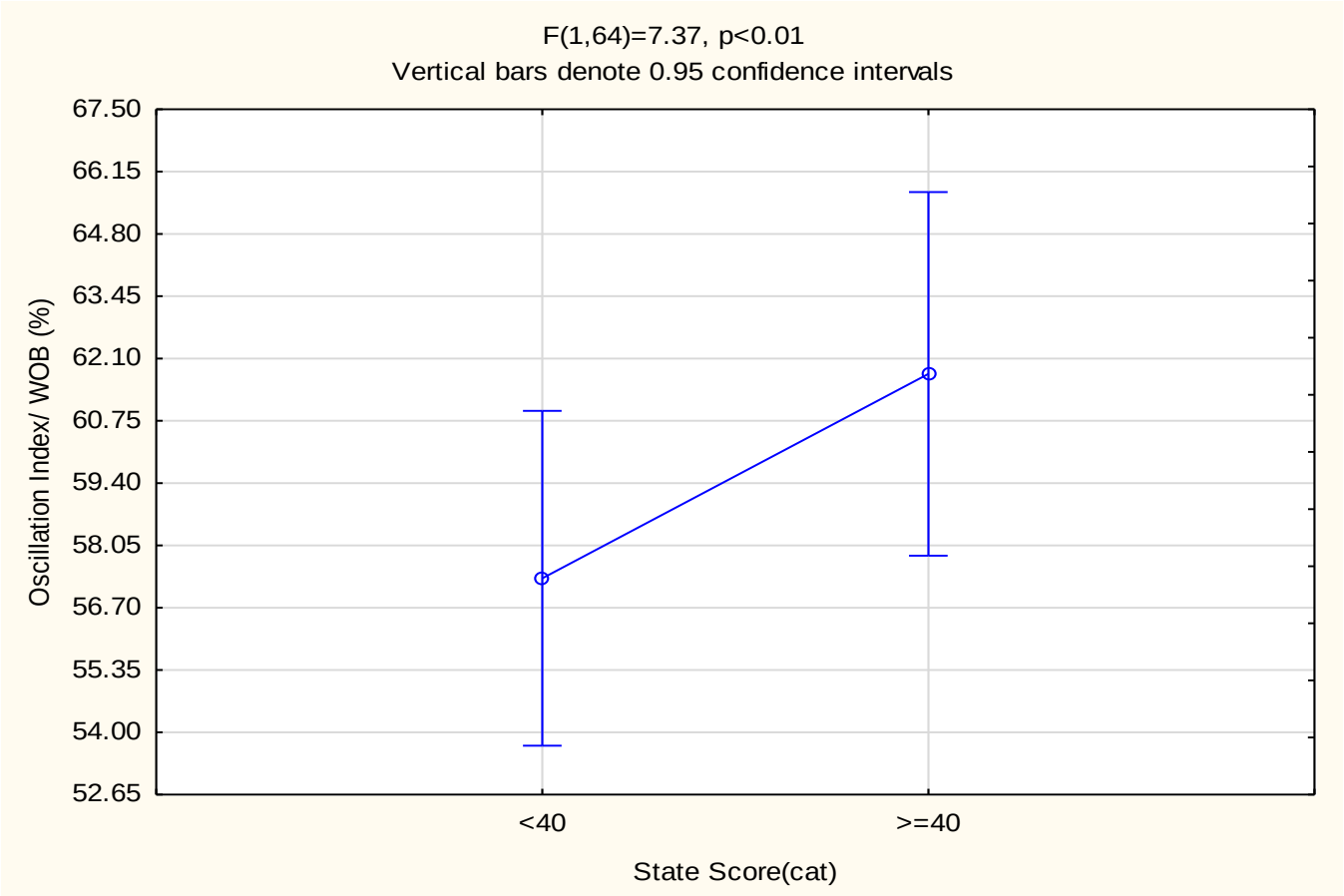



Figure 64 Mean WOB and confidence intervals for stratified state anxiety scores

### 4.6.7. Blood Plasma Cortisol

Anxiety might have an influence on the blood plasma cortisol level observed in the study, but this influence might vary depending on whether the anxiety is more short term (state anxiety) or more long term (trait anxiety) as well as if the anxiety is considered low (<40) or high (≥40).

A trend towards a significant interaction effect between state and trait anxiety scores on the volume parameter was noted ($F$ =3.70, $p$ = 0.06). When considering the effect of low trait anxiety on the volume observed in the low state and high state anxiety, the difference between the means is not as different (72.4070±18.5880 vs. 56.3593±27.1873). The difference between the means of low state and high state anxiety when considering the effect of high trait anxiety, the means are different (60.8365± 32.1125 vs. 121.9255± 20.9587). Any relationship observed between state anxiety and the blood plasma cortisol parameter was affected by trait anxiety and vice versa and the interaction graphs of the means is displayed in *Figure 65*.

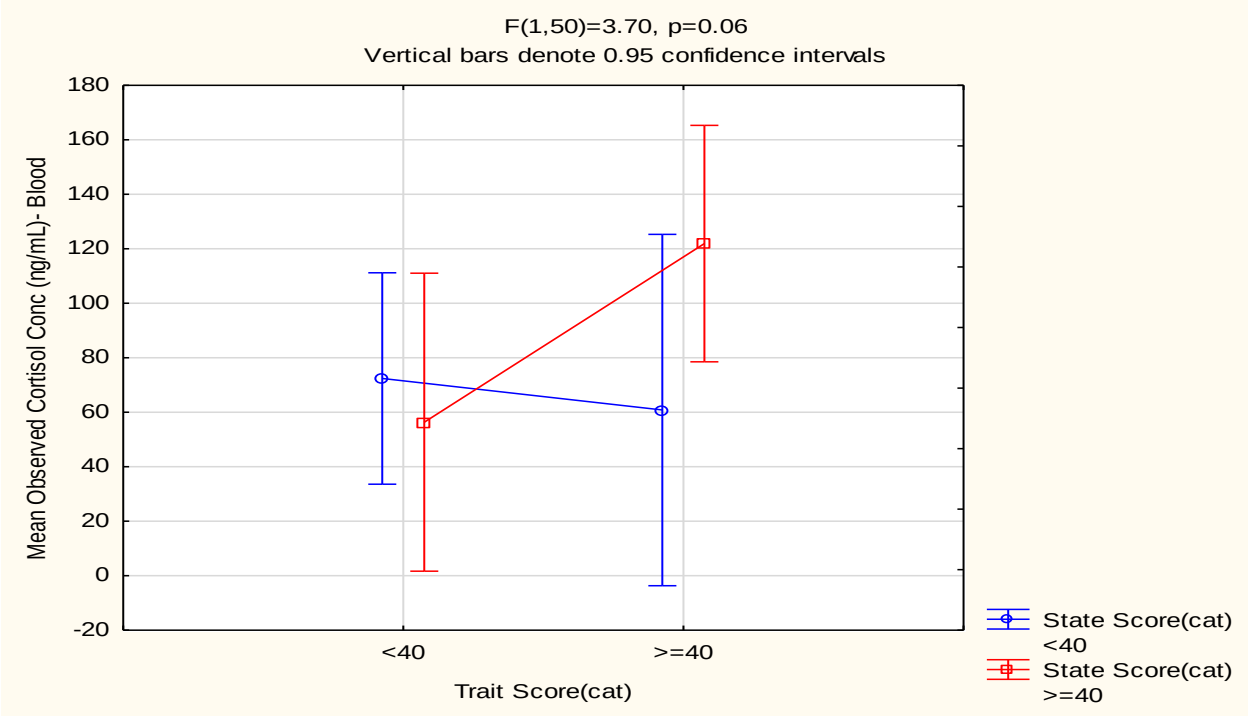



Figure 65 Mean blood plasma cortisol level and confidence intervals for stratified trait and state anxiety scores

### 4.6.8. ROS

Anxiety might have an influence on the ROS level observed in the study, but this influence might vary depending on whether the anxiety is more short term (state anxiety) or more long term (trait anxiety) as well as if the anxiety is considered low (<40) or high (≥40).

A trend towards a significant interaction effect between state and trait anxiety scores on the volume parameter was noted ($F$ =3.36, $p$ = 0.07). When considering the effect of low trait anxiety on the ROS level observed in the low state and high state anxiety, the difference between the means is very different (-154.2725± 547.9029 vs. 1337.0524± 866.9290). The difference between the means of low state and high state anxiety when considering the effect of high trait anxiety, the means are not as different (1015.7835±686.9728 vs. 671.0464±541.2252). Any relationship observed between state anxiety and the volume parameter was affected by trait anxiety and vice versa and the interaction graphs of the means is displayed in *Figure 66.*

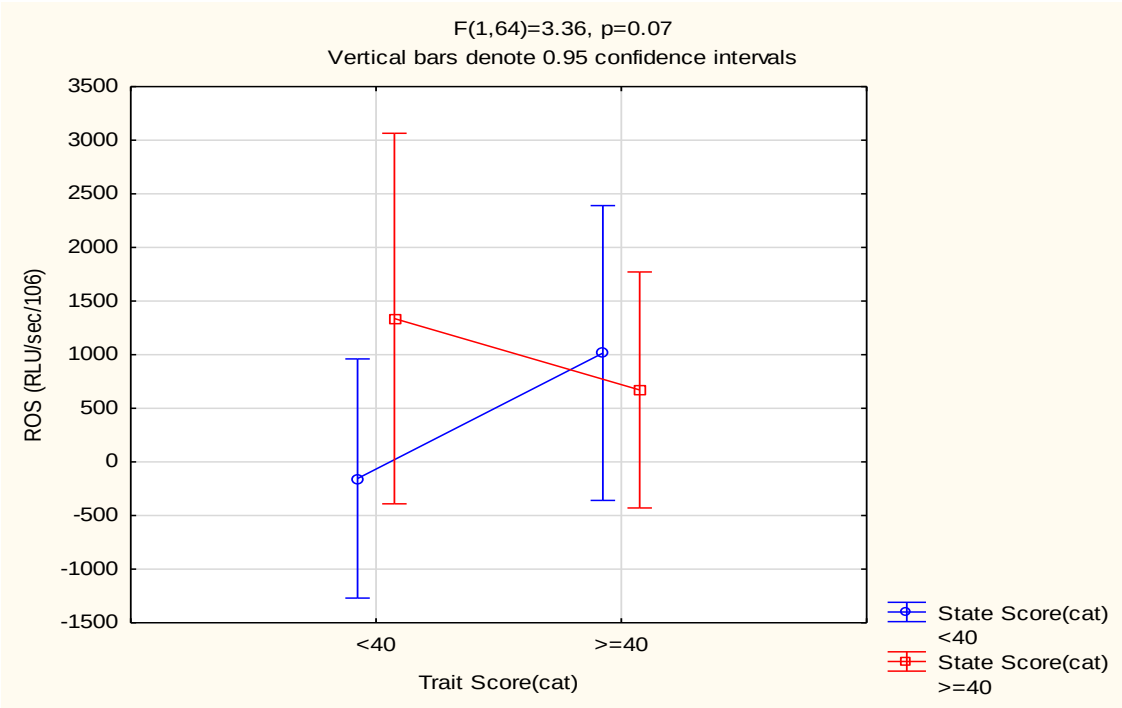



Figure 66 Mean ROS level and confidence intervals for stratified trait and state anxiety scores

No significant or strong trends were observed using the t-tests for the cytokine profile when considering state anxiety. Similarly, no significant or strong trends were observed using the t-tests for the cytokine profile when considering state anxiety

## 4.7. Cortisol: Blood /Seminal Plasma Cortisol vs. Single Dependent Variables

Cortisol levels were measured as a method of quantifying the biological level of anxiety. Cortisol was measured in the blood plasma as well as in the seminal plasma. Blood cortisol is depicted in *Figure 55*, the average blood cortisol observed is 86.562 ng/ml (SD=76.1821). The observed range is between 22.425 ng/ml and 429.104 ng/ml.

Seminal plasma cortisol level was measured too (*Figure 56*). The average seminal plasma cortisol level measured was 19.4335 ng/ml (SD= 13.168). Seminal plasma cortisol was measured within the range of 3.378 ng/ml and 54.954 ng/ml. The descriptive statistics for the single dependent variables have previously been described in *Figure 27- Figure 54*.

### 4.7.1. Correlations

First the correlations of the single dependent variables were compared to blood plasma cortisol levels. Pearson or Spearman correlations were reported where appropriate, taking outliers and sample sizes into consideration. Only strong trends and statistically significant results will be reported in *Table 15*.

Table 15 Table displaying the strong trends and statistically significant correlations between blood plasma cortisol level and the single dependent variables.

| **Independent variable** | **Dependent variable** | ***P* value** | **Correlation Coefficient (R)** |
|---|---|---|---|
| Blood Plasma Cortisol | Concentration | <0.01 | 0.43 |
| | TSC | 0.08 | 0.23 |
| | Round Cells | <0.01 | 0.55 |
| | VAP | 0.02 | -0.30 |
| | VSL | 0.03 | 0.29 |
| | STR | <0.01 | -0.43 |
| | WOB* | 0.05 | -0.26 |
| | Sperm Viability | <0.01 | -0.38 |
| | NO | 0.03 | 0.32 |
| | Blood Plasma IL-6 | 0.01 | 0.54 |

For blood plasma cortisol level vs. the single dependent variables, most of the data sets Pearson correlation coefficients and *P* values were reported, with the exception of the oscillation index (WOB). Here the Spearman correlation results were reported due to the small sample size and number of outliers present in this parameter.

Next, the correlations of the single dependent variables were compared to seminal plasma cortisol levels. Only strong trends and statistically significant results will be reported in *Table 16*. Similarly, either Pearson or Spearman correlations were reported where appropriate, taking outliers and sample sizes into consideration.

Table 16 Table displaying the strong trends and statistically significant correlations between seminal plasma cortisol level and single dependent variables. Only Pearson correlations were reported.

| **Independent variable** | **Dependent variable** | ***P* value** | **Correlation Coefficient (R)** |
|---|---|---|---|
| Seminal Plasma Cortisol | TSC | 0.12 | 0.20 |
| | Blood IL-6 | 0.03 | 0.48 |

## 4.8. History of Stress, Anxiety and/or Depression: Yes, vs. No

Students were asked whether they have a history of stress, anxiety and/ or depression in the donor profile questionnaire and could answer the question as either ‘yes’ or ‘no’. Using the answers to this question, a ‘yes’ and a ‘no’ group were created. A total of 18 participants answered the donor profile questionnaires, of which 39% of this population answered the question as ‘no’ and 61% answered ‘yes’, as shown in *Figure 24.* Pearson or Spearman correlations were reported where appropriate, taking outliers and sample sizes into consideration.

### 4.8.1. 'Yes' Group

For those who answered yes (11 individuals) to having a history of stress, anxiety, or depression the descriptive and correlation results were summarized into *Table 17* and *Table 18.*

Table 17 Descriptive statistics of the 'yes' group variables that shared a statistically significant relation with state and trait anxiety

| Independent Variable | Dependent Variable | Distribution | N | Mean | SD |
|---|---|---|---|---|---|
| VAP | State Score | <40 | 18 | 27.77 | 11.07 |
| | State Score | >=40 | 26 | 22.03 | 8.007 |
| | Trait Score | <40 | 12 | 29.01 | 12.99 |
| | Trait Score | >=40 | 32 | 22.64 | 7.674 |
| VSL | State Score | <40 | 18 | 22.31 | 7.910 |
| | State Score | >=40 | 26 | 26.27 | 9.698 |
| | Trait Score | <40 | 12 | 21.88 | 7.426 |
| | Trait Score | >=40 | 32 | 25.69 | 9.586 |
| LIN | State Score | <40 | 18 | 39.05 | 9.883 |
| | State Score | >=40 | 26 | 46.15 | 12.44 |
| | Trait Score | <40 | 12 | 37.00 | 10.39 |
| | Trait Score | >=40 | 32 | 45.59 | 11.69 |
| STR | State Score | <40 | 18 | 49.06 | 10.07 |
| | State Score | >=40 | 26 | 42.37 | 11.47 |
| | Trait Score | <40 | 12 | 48.91 | 9.368 |
| | Trait Score | >=40 | 32 | 43.68 | 11.76 |
| Seminal Plasma IL-6 | State Score | <40 | 2 | 68.71 | 12.77 |
| | State Score | >=40 | 6 | 15.84 | 16.19 |
| | Trait Score | <40 | 3 | 60.06 | 17.49 |
| | Trait Score | >=40 | 5 | 10.45 | 10.50 |
| Blood plasma IFN-γ | State Score | <40 | 1 | 4.970 | 0.00 |
| | State Score | >=40 | 5 | 24.71 | 24.65 |
| | Trait Score | <40 | 2 | 6.630 | 2.348 |
| | Trait Score | >=40 | 4 | 28.82 | 26.42 |
| Round cells | State Score | <40 | 18 | 0.200 | 0.2872 |
| | State Score | >=40 | 26 | 0.2872 | 0.5071 |
| | Trait Score | <40 | 12 | 0.1033 | 0.0804 |
| | Trait Score | >=40 | 32 | 0.3881 | 0.4893 |
| Blood plasma cortisol | State Score | <40 | 13 | 46.05 | 15.05 |
| | State Score | >=40 | 18 | 143.7 | 110.1 |

| | | | | | |
|---|---|---|---|---|---|
| | Trait Score | <40 | 10 | 46.48 | 17.39 |
| | Trait Score | >=40 | 21 | 129.6 | 107.5 |

Table 18 Summary of 'yes' group relationship between state anxiety scores and single dependent variables

| **Independent variable** | **Dependent variable** | ***P* Value** | **Correlation Coefficient (R)** |
|---|---|---|---|
| **State Anxiety** | Seminal plasma IL-6 | 0.012 | -0.83 |
| | Blood plasma INF-γ | 0.05 | 0.82 |
| | Round cells | 0.04 | 0.32 |
| | Blood plasma cortisol | 0.0012 | 0.54 |

Independent-samples t-tests were conducted to compare the single dependent variables stratified by high and low state and trait anxiety scores. For round cells, values stratified by low state anxiety scores are significantly lower than high state anxiety scores (0.200±0.287 vs. 0.387±0.507, p=0.0248). Blood plasma cortisol low state anxiety data is significantly lower than high state anxiety blood plasma cortisol (46.05±15.05 vs. 143.7±110.1, p=0.0007). For semen IL-6, values stratified by low state anxiety scores are significantly higher than high state anxiety scores (68.71±12.77 vs. 15.84±16.19, p=0.0061). Graphs are displayed on *Figure 67.*

For those who answered yes to having a history of stress, anxiety, or depression the correlation results were summarized into *Table 19.*

Table 19 Summary of 'yes' group relationship between trait anxiety scores and single dependent variables

| **Independent variable** | **Dependent variable** | ***P* Value** | **Correlation Coefficient (R)** |
|---|---|---|---|
| **Trait Anxiety** | VAP | 0.019 | -0.35 |
| | VSL | 0.04 | 0.31 |
| | LIN | 0.002 | 0.44 |
| | STR | 0.04 | -0.31 |
| | Seminal plasma IL-6 | 0.0069 | -0.86 |
| | Blood plasma INF-γ | 0.01 | 0.90 |
| | Round cells | 0.006 | 0.41 |
| | Blood plasma cortisol | 0.0014 | 0.55 |

For round cells, values stratified by low trait anxiety scores are significantly lower than high trait anxiety scores (0.1033±0.0804 vs. 0.3881±0.489, p=0.0064). 'Low trait' viability is significantly higher than 'high trait' viability (64.71 ±18.43 vs. 54.40±20.40, p=0.0213). Blood plasma cortisol levels (low trait anxiety) are significantly lower than 'high trait anxiety' blood plasma cortisol levels (46.48±17.39 vs. 129.6±107.5, p=0.0033). Seminal plasma IL-6 levels (low trait anxiety) are significantly higher than seminal plasma IL-6 levels (high trait anxiety) (60.06 ±17.49 vs..10.45±10.50, p=0.0469). Graphs are displayed in *Figure 68*.

### 4.8.2. ‘No’ Group

The population size was relatively small for the ‘no’ group (7 individuals) However, LIN was still positively correlated to state score, r=0.52, p=0.005. Abstinence period was also found to be negatively correlated to trait score, r= -0.28, p=0.005.

Independent t-tests were performed to compare the single dependent variables stratified by high and low state and trait anxiety scores. Volume (low state anxiety) was found to be higher compared to high state anxiety volume (3.531 $\pm$0.6652 vs.2.682$\pm$0.7401, p=0.0358) as shown in *Figure 69*. There were too few data sets in the low trait anxiety column for analysis to be performed.

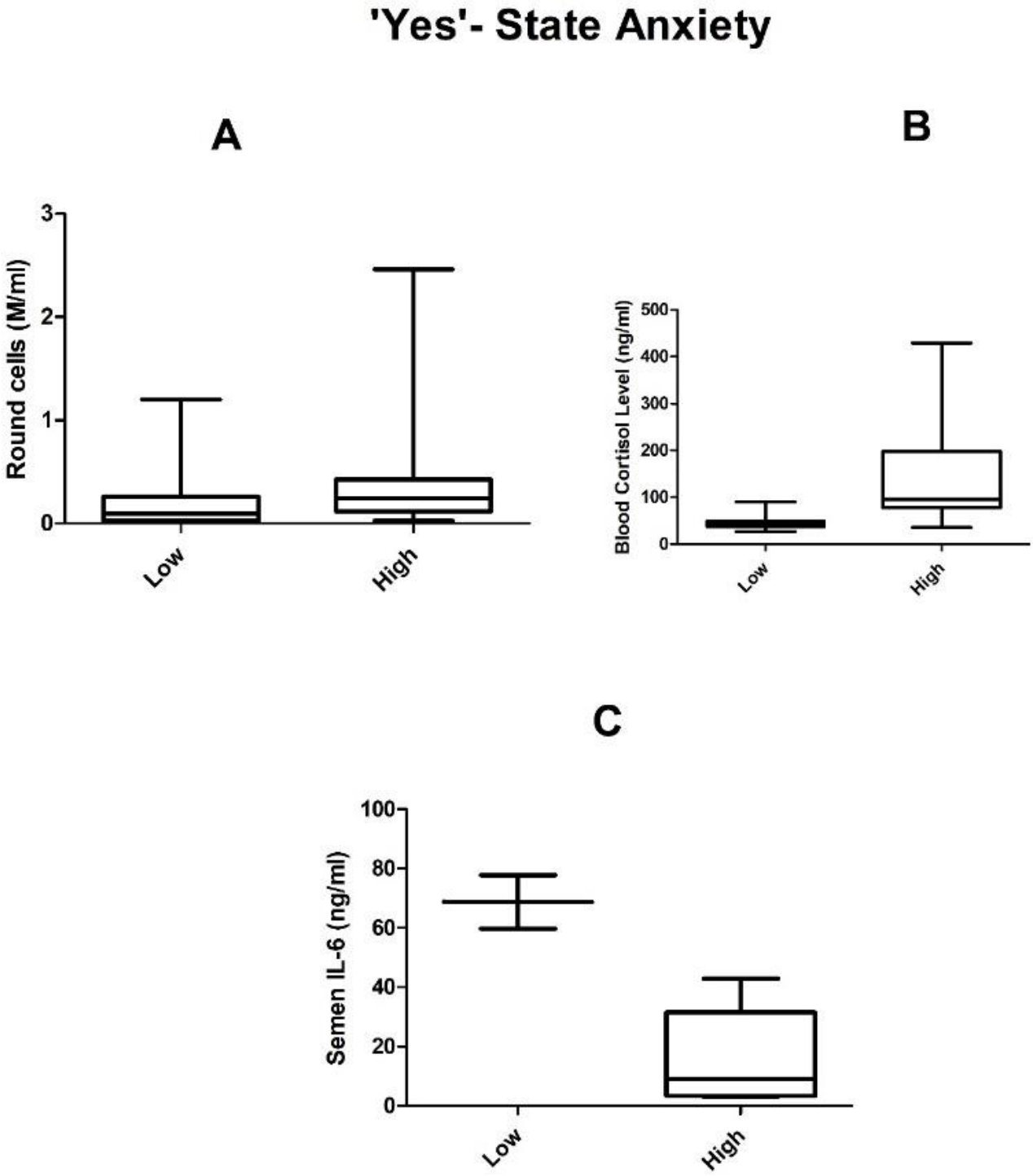



Figure 67 Box and whisker plot displaying the statistically significant findings for those who answered ‘yes’, with the state scores stratified into high and low. A) Round cells, B) Cortisol- Blood, C) Semen IL-6.

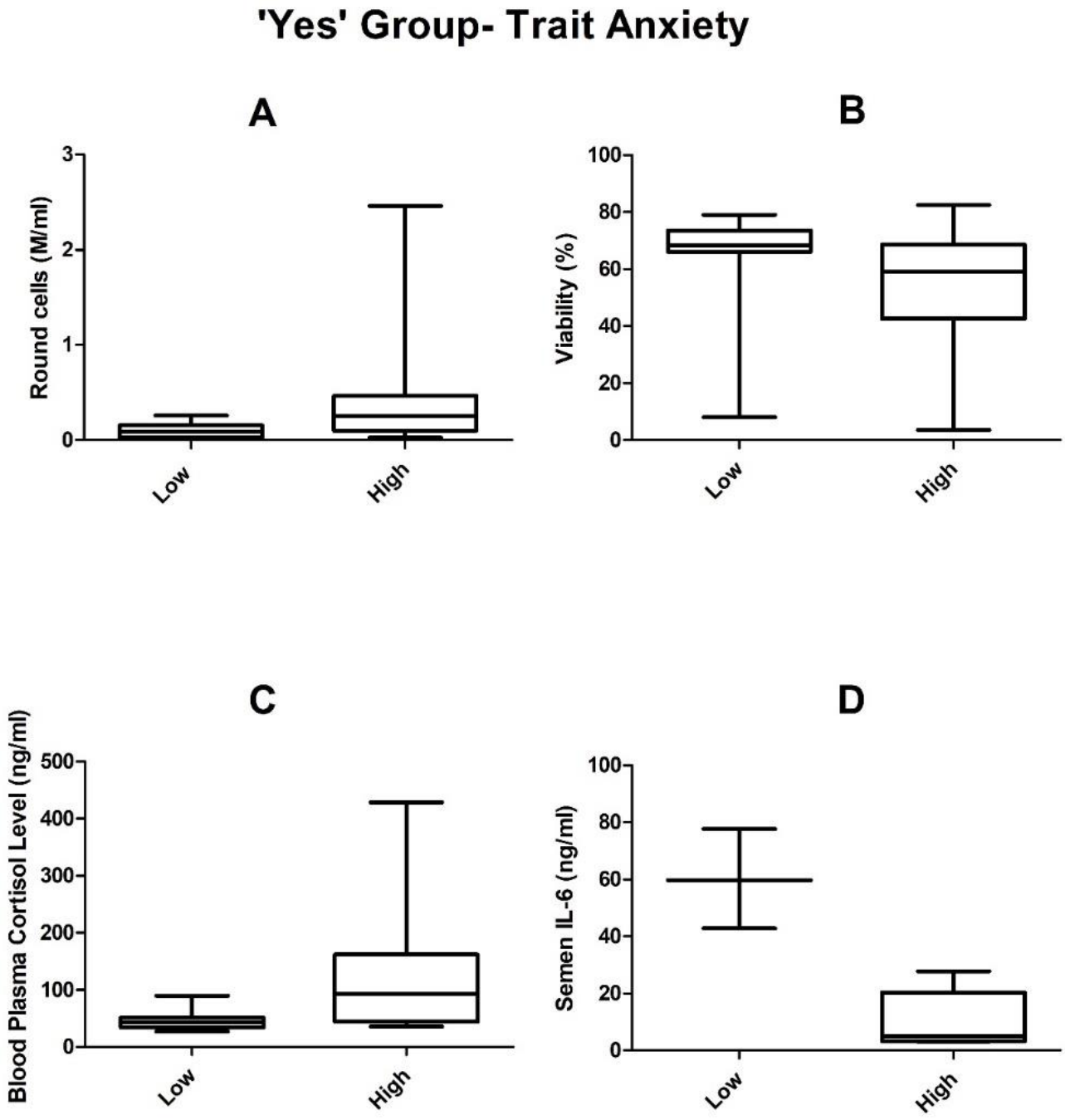



Figure 68 Box and whisker plot displaying the statistically significant findings for those who answered ‘yes’, with the trait scores stratified into high and low. A) Round cells, B) Viability, C) Blood Plasma Cortisol Level, D) Semen IL-6.

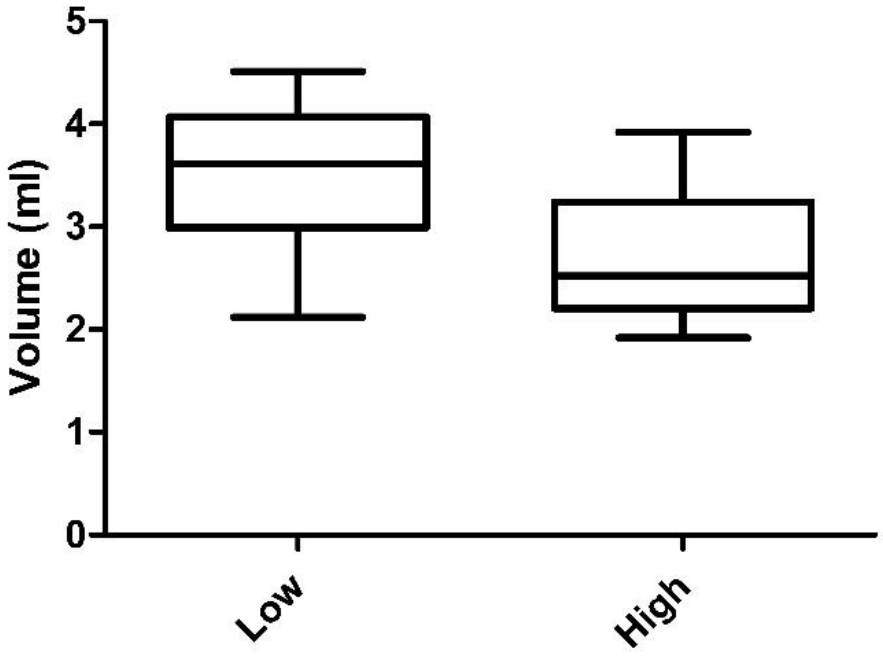



Figure 69 Box and whisker plot displaying the statistically significant findings for those who answered ‘No’, with the state scores stratified into high and low for volume

# Chapter 5

# Discussion

## 5.1. Introduction

As previously mentioned, literature presents a complex and often contradictory picture of the relationship between psychological stress and male fertility. Very limited information is available on the specific relationship between anxiety level and male fecundity. The aim of the study was to determine if there is a relationship between anxiety and reproductive parameters in a male student model and what that relationship is. Various objectives were defined to achieve the aim of this study, and the findings will be discussed in this chapter.

## 5.2. Self-reported Anxiety: STAI Scores

In a recent study it has been observed that South African first year students have a life-time prevalence rate of 22.6% (20.4-24.9) and a 12- month prevalence rate of 20.8% (18.7-23.0) of having a generalized anxiety disorder (Bantjes, et al., 2019). One of the objectives of this study was to determine the reported level of anxiety of the male students by means of the State Trait Anxiety Index (STAI) questionnaire.

The STAI questionnaire utilized in this study provided information about both state and trait anxiety which describes both the transient anxiety level as well as the proclivity to an anxious response, respectively. The average state anxiety score measured during this study (39,0, SD=13.69, n=83) is higher than the Spielberger et al. normative state anxiety scores for the male student population (36.47, SD=10.02, n=324) (Spielberger, et al., 1983). It is however, similar to the state anxiety scores of both the first- attempt *in vitro* fertilization (IVF) group

(39.84, SD= 10.10,n=94) and the control group (38.15, SD=8.59, n=85) collected in the article investigating the association of state and trait anxiety to semen quality of in IVF patients (Vellani, et al., 2013). This overall increase state anxiety scores indicate that the participants in this study were anxious. The majority of the participants of this study were enrolled for programs within the Faculty of Medicine and Health Sciences which are known for being academically very demanding programs and students in these programs have been shown to exhibit decreased mental health (Aktekin, et al., 2001). More than just examination stress can affect the students' mental health, often the importance of the task determines the associated level of anxiety experienced (de Pablo, et al., 1990). The state scores observed can be explained by the constant stressors experienced by the students. The university experience can be exceedingly stressful. Students often have to leave home, integrate into a new social environment, face financial pressures and maintain academic success while being exposed to increased opportunities for substance abuse. These pressures and stressors can often negatively impact the mental health of said student and cause elevated levels of anxiety.

Average trait scores collected in this study (43.0, SD=12.55, n=83) are higher than the Spielberger et al. normative data available for the trait anxiety level of male students (38.30, SD=9.22, n=324) (Spielberger, et al., 1983). It is also higher than the mean trait scores of both the first time IVF patients (37.63, SD=7.95, n=94) and the control group (36.84, SD=7.16, n=85) observed in a study by Vellani et al. (Vellani, et al., 2013). Trait anxiety is reflected in most models of personality and refers to the relatively stable disposition within the individual to assess events as potentially threatening (Mascarenhas & Smith, 2011). It has been proposed that trait anxiety should be considered as a measure of overall susceptibility to emotional disorder and distress (Nordahl, et al., 2019). The observed trait scores are likely linked to the large number of participants in this study who indicated that they have a history of stress, anxiety and/or depression. The increased prevalence of

anxiety observed in this study differs from the epidemiological study (22.6% life-time prevalence rate) for two reasons. The first reason is that the small sample size used in this study might not be representative for the entire population of students. The second reason is that the epidemiological study only sampled first year students. This study selected from a broader range of academic level and thus older students could have increased workload and therefore display increased anxiety.

The state and trait scores were stratified into high (≥40) and low (<40) anxiety scores. For state anxiety, more observations were noted in the low state anxiety group than in the high state anxiety group. Trait anxiety saw more observations of high trait anxiety were observed for the low trait anxiety group. Overall the state and trait scores observed in this study indicates that the student population observed displays overall increased anxiety.

The increased trait anxiety scores observed, to an extent, explains the state anxiety scores, as trait anxiety which measures the proclivity to an anxious state is known to affect the state anxiety scores. Increased trait scores often result in increased state scores, this is confirmed by the statistically significant positive correlation between trait anxiety scores and state anxiety scores observed in this study as well as in literature (Spielberger, et al., 1983; Leal, et al., 2017).

One of the objectives of this study was to investigate the relationship between reported anxiety (STAI questionnaire) and the single dependent variables such as: basic sperm parameters, reactive oxygen species levels, nitric oxide levels, percentage DNA fragmentation, blood and seminal plasma cytokine profile and blood and seminal plasma cortisol levels. These relationships will be reported next.

## 5.3. Macroscopic Analysis of Semen Sample

### 5.3.1. Semen Volume

Concentration of spermatozoa and non-sperm cells, as well as the total sperm count parameter are calculated using the initial volume of the semen sample. For this reason, it is very important that volume is measured accurately. Semen volume is also considered to be a dependable gauge of the secretory function of accessory sex glands, such as the seminal vesicles (WHO, 2010).

A trend towards a positive correlation was observed between semen volume and trait anxiety. Therefore, as trait anxiety scores increased, the observed semen volume increases. The interaction effect between state and trait anxiety on semen volume means that both state and trait scores influence the results that we see with the semen volume parameter. Therefore, we cannot make any concrete assumptions about the relationship between trait anxiety and semen volume as it is influenced by both trait anxiety scores and state anxiety scores to an extent.

However, previous studies have demonstrated an overall decrease in volume with an increase in trait and state anxiety levels in the IVF patient group (Vellani, et al., 2013; Nordkap, et al., 2016). It is well accepted that increased stress/anxiety results in increased HPA-axis activity and increased cortisol levels which can result in inhibition of the HPG-axis which is associated with lower average value of semen volume (Klimek, et al., 2005). Literature explains this decrease in semen volume as an increase in ACTH and cortisol causing an increase in androstenedione levels and subsequent decreased testosterone production. Testosterone level was not investigated in this study.

It was also found that the average semen volume remained unchanged during periods of stress and non-stress (Clarke, et al., 1999; Eskiocak, et al., 2006). This difference in results observed could be explained by difference in project design: the within-subject design of this

study vs. the independent design of the comparative study. The statistical tools differ across the various sources. The ages and purpose of the investigative groups (IVF patients vs. students) differ in the populations of the two respective studies. Abstinence period was investigated as a possible reason for the observed increased semen volume. No significant statistical relationship between semen volume and abstinence period was noted in this study. Convenience sampling is a possible reason for the higher semen volumes, the participants are young and of reproductive age with good overall health. Whether this increase in semen volume with increased trait anxiety is simply a result of sample size, convenience sampling or as a result of more complex neuroendocrinological changes warrants further investigation.

## 5.4. Microscopic Analysis of Semen Sample

### 5.4.1. **Concentration, TSC, R**ound cells

Increased state anxiety scores appear to have no statistically significant relationship with sperm concentration, total sperm count or round cells. Similarly, concentration and total sperm count were not affected by trait anxiety scores. This is in disagreement with the findings of Vellani et al., but is in agreement with Hjollund et al. who found that psychological stress had no effect on sperm concentration and other parameters (Hjollund, et al., 2004; Vellani, et al., 2013). However, increased round cells were associated with increased trait anxiety scores. As mentioned before, round cells consist of both leukocytes and immature germ cells present in the semen (WHO, 2010).

The technique used in this study is unable to differentiate between leukocytes and immature spermatozoa. Therefore, the increased round cells could be a result of inflammation due to increased leukocytes or could indicate testicular damage that resulted in germ cells being released prematurely or both. It is accepted that the dysregulation of the HPA-axis leads to

inhibition of the HPG-axis and eventual decrease in testosterone levels (Nargund, 2015). Decreased levels of testosterone have been described, in literature, to lead to a malfunctioning blood-testis barrier (Smith & Walker, 2014). The malfunctioning blood-testis barrier is a possible reason of increased germ cells being released prematurely by the Sertoli cells. In literature, 80-90% of the observed round cells were immature germ cells and the remaining 10-20% of the round cells were found to be leukocytes (Patil, et al., 2013). It is therefore speculated that the proportion of germ cells to leukocytes are similar for this study.

### 5.4.2. **Total Motility, Progressive Motility and Kinematic Parameters**

State anxiety scores do not share a statistically significant relationship with the motility and kinematic parameters. Trait anxiety shares a negative relationship, which is approaching significance, with the total motility parameter which is a parameter that includes progressive and non-progressive motility. This agrees with Vellani et al. who noted decreased motility in both IVF patients and controls for the state and trait anxiety parameters (Vellani, et al., 2013). No significant relationship was found between state anxiety and progressive motility nor between trait anxiety and progressive motility which disagrees with the findings of Eskiocak et al. and Janevic et al. (Eskiocak, et al., 2006; Janevic, et al., 2014). However, kinematic values were strongly correlated to both total and progressive motility for both state and trait anxiety groups and changes observed in kinematics could be a function of total and progressive motility.

Kinematic values were captured for individual spermatozoa. The kinematics can be categorized into describing: velocity of movement, width of the sperm head trajectory and the frequency with which there is a change in direction of the sperm head (Mortimer, 2000). Curvilinear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP) describe the velocity values that are measured. The VAP is described as the distance that the spermatozoon travels in the average direction of movement throughout the observation period- time is corrected for. The lowest of the velocity values calculated for each

spermatozoon is always the VSL. It uses the first and last points of the trajectory to calculate the straight-line distance between these points. While the VCL can be defined as the total distance that the head of the spermatozoon covers in the observation period. This value is always the highest of all velocity values (Mortimer, 2000). The VAP values will usually be very similar to VSL values in the case of spermatozoa with following linear paths. However, in the case of spermatozoa that follow irregular trajectories and display increased lateral movement, the VAP is then usually much higher than the VSL. The velocity values are influenced by trajectory shape. Therefore, the ratios of the velocity values are also compared using LIN, STR and WOB as described in Equation 1.

Both state and trait anxiety were negatively correlated to the VAP and STR kinematic parameters. VAP influences the STR ratio, so that could be the reason that the STR parameter decreased. The VAP value was only slightly higher than the VSL value, indicating that the spermatozoa, on average, swam in a relatively linear path. The LIN parameter was positively correlated to both state and trait anxiety, which describes the inverse relationship between VSL and VCL. Using mixed model ANOVA, it was found that VCL decreases in the high trait anxiety group. Both LIN and WOB increased in the high trait anxiety group and is influenced by VCL values. To our knowledge, this is the first study that demonstrates the kinematic changes observed that occur during anxious states in a student population. Clarke et al. who observed the effect of psychological stress on IVF patients, noted reduced ALH values during a stressful event. This was not observed for the student population observed in this study.

Various pathways, such as the calcium pathway, the protein kinase A pathway and the cyclic adenosine monophosphate- dependent protein kinase pathway are involved in the control of sperm motility (Darszon, et al., 2001; Darszon, et al., 2006; Pereira, et al., 2017). Superoxide, hydrogen peroxide and nitric oxide are also possibly involved in sperm motility mechanisms. At physiological concentrations ROS is crucial for hyperactivation and

capacitation (Ford, 2004; Pereira, et al., 2017). However, increased ROS could lead to impaired motility by decreasing axoneme protein phosphorylation and causing sperm immobilization (De Lamirande & Gagnon, 1995). Although ROS and NO do not display any statistically significant relationship with trait or state anxiety, the physiological effects of the free radicals could possibly be responsible for the changes observed in the motility and kinematics.

### 5.5. ROS and NO

Modest antioxidant defenses, abundance of lipids and high oxygen consumption of the brain make it particularly vulnerable to increased ROS levels or oxidative stress (Bouayed, et al., 2009). Similarly, the spermatozoon also has limited antioxidant defenses and a high content of polyunsaturated fatty acids in the plasma membranes rendering the spermatozoon vulnerable to redox imbalances. A link between oxidative stress, and obsessive-compulsive disorder and panic disorder was established by Kulogla et al. in two different papers (Kuloglu, et al., 2002a; Kuloglu, et al., 2002b). There is also a growing body of literature that supports the argument that oxidative stress negatively affects sperm function (Bisht & Dada, 2017). Only semen ROS levels were measured in this study and no significant relationships were found between ROS levels and state and trait anxiety scores. Nitric oxide is synthesized from NO synthases (NOS). NOS have been detected in the male reproductive system (Zini, et al., 2001). Depending on the concentration of NO present it can have either a physiological or pathophysiological effect on sperm parameters, particularly on motility (Rosselli, et al., 1995). The variation in technique used to measure NO levels in semen make it difficult to compare results between studies and thus it is not possible to conclude if the results observed in this study have any biological relevance on parameters such as motility. However, blood plasma cortisol level was found to be positively correlated to NO level and not to state and trait scores as observed in a separate study (Eskiocak, et al., 2006).

## 5.6. Spermatozoa Viability and DNA Fragmentation

Increased DNA fragmentation is associated with reduced sperm viability, as DNA fragmentation is one of the final steps of spermatozoa death (Irvine, et al., 1994; Samplaski, et al., 2015). The state and trait anxiety scores share no statically significant relationship with the viability and DNA fragmentation parameters. The average DNA fragmentation observed in this study is about 4.76%. No normative data exists about spermatozoa DNA fragmentation levels using flow cytometry analysis, so it is difficult to compare it to values in literature. However, it was found that DNA fragmentation is positively associated with increased levels of both state and trait anxiety (Vellani, et al., 2013). The average percent of viable spermatozoa observed in this participant population is 57.52% (SD=16.86), which is lower than WHO lower reference limit, yet it still falls within the 95% confidence interval. When the state and trait scores are stratified, for both high state and high trait anxiety the viability parameters were below the WHO lower reference limit. This low percentage of live cells can be defined as necrozoospermia (WHO, 2010).

## 5.7. Cytokine Profile

The immune system communicates with the nervous and endocrine system by cytokines and chemokines, and vice versa. The soluble bioactive mediators known as cytokines e.g. interleukins and tumor necrosis factors are generally associated with inflammation and possible cell death (Hou & Baldwin, 2012). Cytokines are thought to possibly have a detrimental impact on spermatozoa function (Hill, et al., 1987). This study investigated the levels of IFN-γ, IL-6, IL-1β and TNF-α in both the blood and seminal plasma to observe the relationship between these cytokines and state and trait anxiety. The mean observed cytokine levels in this study are 29.0575 pg/ml for IFN-γ, 16.485 pg/ml for IL-6, 15.161 pg/ml for IL-1β and 3.959 pg/ml for TNF-α. Overall, the mean values observed throughout this research project are lower compared to the literature (Eggert-Kruse, et al., 2007; Seshadri, et al., 2011). Mean IFN-γ was observed at 79 pg/ml, IL-6 at 36 pg/ml and 9pg/ml for TNF- α

in a study observing the cytokine expression in the seminal plasma of men participating in an IVF cycle (Seshadri, et al., 2011). The mean IL-1β observed in the literature varied between 6.9- 7.8 pg/ml (Eggert-Kruse, et al., 2007; Politch, et al., 2007). Glucocorticoids, such as cortisol, are known to exert an inhibitory effect on leukocytes and immune accessory cells. Both IL-6 and IL-1 production have been found to be suppressed by glucocorticoids decreasing transcription rates of these interleukins (Chrousos, 2000). This provides a possible explanation for the decreased cytokine expression observed in this study. Alternatively, the decreased cytokine expression observed could be as a result of the comparative study observing men with existing issues to a relatively healthy student population or the small sample size of this study.

IFN-γ *in vitro* is thought to exert an inhibitory effect on testosterone production by affecting cholesterol transport into the mitochondria (Orava, et al., 1986; Seshadri, et al., 2011). No relation was found between IFN-γ and fertilization rates, but IFN-γ was found to negatively affect sperm motion parameters (Hill, et al., 1987; Seshadri, et al., 2011). At the concentrations that blood plasma IFN- γ was observed in this study, it was found to have a significant positive correlation with progressive motility. Therefore, it is very unlikely that the concentrations at which IFN-γ is observed at this study bear a negative effect on sperm motility parameters. IFN-γ was also not found to be correlated to state or trait anxiety and the value detected was lower than previously observed in literature.

The relationship between IL-6 and sperm parameters is unclear. An inverse correlation was noted between increased seminal IL-6 levels and motility parameters (Naz & Evans, 1994). No statistically significant relationship was noted between IL-6, and state and trait anxiety levels so it is not possible to determine if IL-6 was involved in the motility changes observed throughout this study.

State anxiety was negatively correlated to semen TNF-α levels only, while trait anxiety was negatively correlated to blood semen TNF-α levels. As previously mentioned, the seminal

plasma TNF-α level is lower than observed in literature which could be attributed to the sample size of this study or to decreased immune activity in the genital tract (Eggert-Kruse, et al., 2007; Seshadri, et al., 2011). Spermatozoa motility parameters were found to be decreased in the presence of elevated TNF-α (Hill, et al., 1987). TNF- α was also found to have no effect on sperm numbers or motility (Eggert-Kruse, et al., 2007). However, exposure to TNF-α and IFN-γ in combination have demonstrated decreased cellular integrity and viability (Estrada, et al., 1994). It is only possible to speculate that the physiological level of TNF-α observed in this study may have played a role in the motility differences noted in this study.

Basal TNF-α in the blood was found to be significantly associated with anxiety symptom severity (Vogelzangs, et al., 2016). The mean blood plasma TNF-α observed in this study is 2.73 pg/ml. These values are higher compared to basal TNF-α observed in participants with no depressive or anxiety score (0.70 pg/ml), remitted depressive or anxiety disorder (0.80 pg/ml) and participants with a current depressive or anxiety disorder (0.80 pg/ml) (Vogelzangs, et al., 2016). The same study found cytokine production capacity to be increased when anxiety symptoms increase which is not what was observed in this study. The age difference and overall health differences of study participants could be the reason for the observed differences in TNF- α levels. The average TNF-α level observed in this study was similar to levels in a study observing TNF-α levels during psychological stress in students that were either freshly admitted to university, mid-term students, or examination taking students (Chandrashekara, et al., 2007). In this study however, increased trait anxiety negatively correlates to blood plasma TNF-α levels which could be a result of glucocorticoid immunosuppression (Chrousos, 2000). Chandrashekara et al. (2007) also found that in their study the examination taking students with high anxiety scores had significantly lower TNF-α levels. It is important to note that psychological stress can affect the immune system in a variety of ways and could be individual- or situation- specific. A decline in natural killer cell

activity was also observed in students with high anxiety (Borella, et al., 1999). It was suggested that stress may be associated with elevated cortisol level. In situation specific anxiety states this elevated cortisol level could lead to reduced TNF-α levels by negative feedback system between the immune system and neuroendocrine functions (Chandrashekara, et al., 2007).

The IL-1β level observed in this study was 15.16 pg/ml. The mean IL-1β observed in literature ranged from 37.8 pg/ml in sub-fertile patients to 3 pg/ml in control participants in two respective studies (Eggert-Kruse, et al., 2007; Papadimas, et al., 2009). Increased IL-1β levels are noted in infertile patients. The interleukin level observed in the participant population of this study suggests that IL-1β had a negligible effect on the sperm parameters. Similarly, IL-1β was not found to have a significant effect on sperm count or progressive motility when investigating the semen samples of sub-fertile men (Eggert-Kruse, et al., 2007). Both state and trait anxiety were significantly negatively correlated to IL-1β levels.

Seminal plasma TNF-α and IL-1β were found to be strongly positively correlated in this study. This is supported by literature, and both were found to be decreased in state and trait anxiety (Eggert-Kruse, et al., 2007). Similarly, it was noted that TNF-α and IFN-γ in combination can affect spermatozoa cell integrity. Cytokines are known to act in a network and can antagonize each other's interactions, this makes immunological marker results difficult to interpret.

### 5.8. Cortisol

Cortisol is a steroid hormone that is released, in response to ACTH, by the adrenal cortex. Enzyme-mediated reactions convert cholesterol to cortisol (Spiga & Lightman, 2015; Spencer & Deak , 2017). Cortisol is a lipid-soluble steroid molecule that passively diffuses out of vesicles as it is formed. Cortisol is known to exert its effects through specific intracellular receptors. Only free/unbound cortisol is available to most receptors while most

of the cortisol is bound to albumin or corticosteroid-binding globulin (McKay & Cidlowski, 2003). This hormone was investigated as a biological correlate for anxiety levels.

Cortisol is secreted in the range of 15 to 20 mg/day in men (McKay & Cidlowski, 2003). Blood plasma cortisol levels are highest in the morning because of diurnal variation (Krieger, et al., 1971). Reference values, provided by the kit used for cortisol level determination, for morning cortisol levels (08:00-10:00) are between 60-230 ng/ml. While afternoon cortisol levels (16:00) are much lower between 30-150 ng/ml. Patients treated with ACTH typically produce cortisol in the range of 280-600 ng/ml. At normal hormone levels the plasma clearance half-life of cortisol is 66 minutes.

The average blood plasma cortisol value observed was 86.562 ng/ml. Seminal plasma cortisol levels were lower than those observed in the blood plasma. Considering the diurnal rhythm of cortisol plasma levels most samples were donated between 8am and 10am and the observed cortisol levels were within the range for that time period. However, considering that patients treated with ACTH to stimulate the HPA-axis produce cortisol in the range of 280-600 ng/ml, the mean value observed in this study is quite low. Still both state and trait anxiety were positively correlated to blood plasma cortisol levels. It was observed that the mean blood plasma cortisol level was elevated for both high state and high trait anxiety. The ANOVA results confirm this observation as an interaction effect of both state and trait anxiety on the blood plasma cortisol levels.

Blood plasma cortisol levels was found to be positively correlated to concentration, total sperm count. This is unexpected, as elevated glucocorticoid levels are thought to disrupt the conversion of androstenedione into testosterone with deleterious downstream effects (Klimek, et al., 2005). Blood plasma cortisol levels were also positively correlated to round cells which is a possible downstream effect of reduced testosterone levels caused by HPA-axis activation. This elevation in round cells could point to increased immune action or raised levels of immature germ cell release.

Like increased state and trait anxiety, elevated blood plasma cortisol levels were shown to have a negative relationship with VAP and STR kinematic parameters. Additionally, a positive correlation was observed between VSL and blood plasma cortisol level as well as negative correlation was observed between blood plasma cortisol and the WOB kinematic parameter.

Increased blood plasma cortisol levels were negatively correlated to spermatozoa viability. Viable spermatozoa levels were found to decrease when round cells increase, as observed for elevated blood plasma cortisol levels.

Semen IL-1β and blood IL-6 levels were both positively correlated to blood cortisol levels. While seminal plasma IL-1β increases with increased blood cortisol levels and blood cortisol increases with both state and trait anxiety and yet this increase in state and trait anxiety lead to decreased seminal plasma IL-1β provides quite the conundrum. IL-6 has been found to elevate nitric oxide production in a dose-dependent manner (Lampiao & du Plessis, 2008). NO was found to be positively correlated to blood plasma cortisol levels. Additionally, NO was found to share a positive relationship with blood plasma IFN-γ and could possibly be investigated in future as a mediator of NO production in combination with TNF-α and IL-6.

The 60 samples analyzed to detect seminal plasma cortisol level generated an average of 19.4335 ng/ml. This is significantly lower than the levels observed in the blood plasma and could possibly be attributed to the blood-testis barrier. Total sperm count and blood plasma IL-6 were found to share a positive relationship with seminal plasma cortisol levels.

### 5.9. History of Stress, Anxiety and/or Depression

The question ‘do you have history of stress, anxiety and/or depression?’ was asked in the donor profile questionnaire. Participants were stratified into ‘yes’ or ‘no’ groups based on the answer to this question. The goal of this question was to investigate if and how having a

history of stress, anxiety and/or depression would affect the results observed in the single dependent variables and their relationship with state and trait anxiety scores.

More than half of the population, who were compliant in answering the donor profile questionnaire questions, answered ‘yes’ to the question.

Seminal plasma IL-6 previously yielded no statistically significant relationship with state anxiety. However, it was found to significantly decline as state anxiety scores increased in the ‘yes’ group. An inverse correlation was noted between increased seminal IL-6 levels and motility parameters (Naz & Evans, 1994). No changes in motility were observed within the ‘yes’ group for state anxiety.

Similarly, blood plasma INF-γ previously yielded no statistically significant relationship with state anxiety scores. For the ‘yes’ group blood plasma IFN-γ increased as state anxiety scores increased. Previously it was discussed that IFN-γ *in vitro* is thought to exert an inhibitory effect on testosterone production by affecting cholesterol transport into the mitochondria (Orava, et al., 1986; Seshadri, et al., 2011). Increased testosterone levels have been found to lead to increased immature germ cell release, and an increase in round cells were noted with increased state anxiety scores. IFN-γ was found to negatively affect sperm motion parameters (Hill, et al., 1987; Seshadri, et al., 2011), however no changes to motility parameters were observed for the ‘yes’ group. Once again blood plasma cortisol levels were found to increase with increased state anxiety scores.

VAP and STR kinematic parameters were found to be negatively correlated to trait anxiety scores within the ‘yes’ group while LIN and VSL were positively correlated. The same relationship between state anxiety and seminal plasma IL-6, blood plasma IFN-γ, round cells and blood plasma cortisol was observed between trait anxiety and these parameters.

The observations in the ‘no’ group will not be discussed, as the sample size was too small to make any deductions.

## 5.10. Summary

In summary the student population observed in this study display increased anxiety levels when considering their state and trait anxiety scores, respectively. During the stress response the HPA-axis is activated. The body releases CRF in response to the stress leading to the release of ACTH and the eventual release of the body's most abundant glucocorticoid, cortisol. Cortisol is released even once the stressor is passed, and cortisol secretion only ceases once it reaches circulatory concentrations that allow it to have a protective effect in the body. Thereafter, cortisol will activate a negative feedback loop and decrease CRF and ACTH levels to achieve homeostasis (Shulkin, et al., 1998; Steimer, 2002). It is well accepted that the activation of the HPA-axis during the stress response leads to the dysregulation of the HPG-axis. The HPG-axis is inhibited by inhibition of GnRH by glucocorticoids such as cortisol (Nargund, 2015). Glucocorticoid receptors are present in the testes, pituitary and hypothalamus. It has been described that GnRH can be downregulated as a result of elevated glucocorticoids. At a hypothalamic level, it can lead to impaired pulsatile release of the gonadotropic hormones LH and FSH eventually resulting in a decline in the testosterone level. At a testicular level, glucocorticoids have also been found to reduce the testicular response to LH resulting in reduced testosterone secretion (Hu & et al., 2008; Whirledge & Cidlowski, 2010). Blood plasma cortisol levels were noted to increase as state and trait scores increased. This relationship between measured cortisol levels and reported anxiety allows us to speculate that reported anxiety levels are linked to the biological changes associated with the anxious state such as HPA-axis activation and HPG-axis inhibition.

HPA-axis activation in both acute and prolonged psychological stress scenarios can play a role in the immune response of the individual (Petrovsky, et al., 1998). In fact, cortisol has a role as a powerful immuno-suppressant. Overall, the function of the immune system is to prevent or limit infection in the body. An immune response in the body is either innate or

adaptive. Simply put, the innate immunity is an immediate response while the adaptive immunity is dependent on the coordination of specific adaptive immune cells (NIH: National Institute of Allergy and Infectious Diseases, 2014). Innate immune cells consist of eosinophils, basophils, neutrophils, monocytes and macrophages. Adaptive immune cells recognize specific signals and are either B- or T- cells, natural killer cells have both innate and adaptive function (NIH: National Institute of Allergy and Infectious Diseases, 2014). Both innate and adaptive immune responses can occur in the male reproductive tract and immune cells play an important role in the regulation of spermatogenic functions and the maintaining testicular immune privilege (Nguyen, et al., 2014). Cytokines such as IL-1β, IL-6 and TNF-α are primarily expressed by macrophages and IFN-γ by natural killer cells. Additionally, both the Leydig and Sertoli cells have been shown to secret IL-1 and IL-6 under the control of the gonadotrophic hormones (Cudicini, et al., 1997). An overall decrease in TNF-α levels in seminal plasma was observed with an increase in state anxiety scores. TNF-α is produced by activated macrophages. Therefore, we can speculate that the effect of the acute anxiety, measured by state anxiety scores had an immuno-suppressant effect in the male reproductive tract. Similarly, blood plasma TNF-α levels were seen to decrease as trait anxiety scores increased. This systemic response once again suggests that anxiety could produce an immuno-suppressant effect. The trait anxiety scores provide reliability in the long term, as trait anxiety is often described as a part of the individuals personality. Therefore, it is interesting to note that the change in TNF-α levels is seen as a localized effect in ‘acute anxiety’ and as a systemic effect in ‘prolonged anxiety’. As mentioned, the measured cortisol level does increase with an increase in both state and anxiety scores in this study. Increased plasma cortisol was found to reduce pro-inflammatory cytokine production (Petrovsky, et al., 1998). This supports the relationship between cortisol levels and pro-inflammatory cytokines that are observed in this study.

IL-1β levels in the seminal plasma have been found to be closely related to male reproductive function. Decreased levels of seminal plasma IL-1β were previously observed in patients with idiopathic infertility compared to healthy controls in a study comparing cytokines in the blood and semen of infertile patients (Havrylyuk, et al., 2015). Therefore, the inverse relationship between seminal plasma IL-β levels and both state and trait anxiety scores in this study is interesting. Especially because for the 'yes' group blood plasma IFN-γ increased as state anxiety scores increased. In the same study by Havrykyuk et al. an increase in IFN-γ was observed in the blood plasma of patients presenting with idiopathic infertility. Acute stress has been found to increase the number of circulating natural killer cells or by increasing their cell activity, and IFN-γ is predominantly secreted by the natural killer cells (Naliboff, et al., 1991; Segerstrom & Miller, 2004). These findings suggest a possible role for increased anxiety or psychological stress to elucidate idiopathic infertility by means of affecting the cytokine profile.

Given the overall decrease in cytokine concentrations observed in this study, it is speculated that the increase in round cells observed consists primarily of immature spermatozoa. In a study investigating men with possible infertility, 80-90% of the observed round cells were immature germ cells and the remaining 10-20% of the round cells were found to be leukocytes (Patil, et al., 2013). As mentioned before it is accepted that the activation of the stress response leads to inhibition of the HPG-axis and eventual decrease in testosterone levels (Nargund, 2015). Decreased levels of testosterone or malfunctioning androgen receptors have been described, in the literature, to lead to a malfunctioning blood-testis barrier (Smith & Walker, 2014). The malfunctioning blood-testis barrier is a possible reason of increased germ cells being released prematurely by the Sertoli cells. We can speculate that the proportion of germ cells to leukocytes are similar for this study. It was suggested that the presence of larger numbers of immature germ cells were associated with poor IVF success rates (Tomlinson, et al., 1992).

An increase in immature germ cells will in turn lend a possible explanation for the changes in motility observed in this study. Negative correlations between spermatozoa motility and germ cells were observed in the literature (Patil, et al., 2013). This suggests that the presence of immature spermatozoa and altered motility parameters could lead to male-subfertility in this population. Throughout the study it was observed that VAP and STR kinematic parameters decreased as state anxiety scores, trait anxiety scores and blood plasma cortisol levels increased. To state it simply, it appears as though the overall distance travelled by spermatozoa in its average direction of movement decreases with an increase in anxiety in this study. Kinematics alone tell no convincing scientific tale, mostly because of the lack of normative/ comparative data available (Mortimer, 2000). Although it is interesting to note that the average VAP and STR values observed for this study are much lower than the average VAP and STR values in a comparable age group of healthy men enrolled in a study investigating the effects of age on sperm (Sloter, et al., 2006). To date, this is the first study that has described the relationship between spermatozoon kinematics and anxiety. Given the strong statistical relationship between kinematic parameters such as VAP and STR with more conventional parameters such as total- and progressive motility, kinematic values should be investigated more regularly. An inverse relationship between total motility and trait anxiety was observed. Sperm motility is an important parameter to consider when assessing the fertilizing ability of a spermatozoon, as optimum motility is necessary to navigate the vaginal tract and result in fertilization (Amelar, et al., 1980). Although ROS and NO do not display any statistically significant relationship with trait or state anxiety, the physiological effects of the free radicals could also possibly elucidate the changes observed in the motility and kinematics. The inverse relationship observed between elevated blood plasma cortisol and decreased spermatozoa viability is possibly a function of the increased number of round cells observed during this study. Similarly, the lifestyle factors were not

controlled for and it is possible that factors such as medication taken by the participants, nicotine and alcohol consumption influenced the results observed throughout this study.

## 5.11. Limitations and future research

A major limitation of this study is that testosterone level of the participants was not measured. Future studies could investigate the mechanisms of how a dysregulated HPG-axis could influence testosterone, androgen receptors and the integrity of the blood-testis barrier in an anxious model. Similarly, the medication used by certain participants could have influenced the results obtained in this study. The sample size of this study is a possible limitation as this study was designed in a longitudinal fashion where subjects served as their own control to reduce variability. This possibly resulted in non-significant findings due to lack of statistical power. Additionally, this study was unable to differentiate the round cell population identified as having a relationship with anxiety levels in this study. Time was also a limiting factor in this study, and future studies could investigate the effect of anxiety on male parameters in a more longitudinal fashion, taking the spermatogenic cycle into consideration. Future studies could further investigate the possible mechanisms by which cortisol affects premature germ cell release in an anxious model. Future studies could also investigate if the kinematic changes observed in an anxious model influences sperm fertilizing ability. Additionally, models investigating long term-anxiety or anxiety disorders could investigate any possible morphological changes anxiety may lead to in spermatozoa.

# Chapter 6

# Conclusion

In conclusion, increased levels of anxiety were observed in the student population observed for the purposes of this study. The reported anxiety level (STAI scores) was positively correlated to the measured anxiety level (blood plasma cortisol level). Cortisol was thought to have an overall immuno-suppressant effect both locally, in the reproductive tract, as well as systemically. Inhibition of the HPG-axis by the glucocorticoid, cortisol, is speculated to decrease testosterone levels. This suggests a compromised blood-testis barrier in increased anxiety, particularly in individuals more prone to an anxious response. The increase in number of round cells in this study could therefore be a result of increased release of immature germ cells from a compromised blood-testis barrier. The decreases in motility parameters observed in this study also support the presence of increased immature germ cells. The changes in the cytokine profile, round cells and motility often correspond to values observed in men presenting with idiopathic infertility. Overall an increase in anxiety level was found to negatively affect male reproductive parameters as observed in this study.

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# Appendices

## 6.1. Appendix A: Ethics Approval Notice

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

**Health Research Ethics Committee (HREC)**

**Approval Notice**

**New Application**

**Ethics Reference #:0920**

**Title: Observing the effects of anxiety on male reproductive parameters**

**HREC Reference # S17/08/137**

Dear Ms T De Jager

The **New Application** received on 24/01/2018 16:21 was reviewed by members of **Health Research Ethics Committee** via **expedited** review procedures on 25/01/2018 and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Period: **25-Jan-2018 – 24-Jan-2019**

Please remember to use your protocol number Project Id on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

**After Ethical Review**

Please note you can submit your progress report through the online ethics application process, available at: https://applyethics.sun.ac.za/Project/Index/995 and the application should be submitted to the Committee before the year has expired. Please see Forms and Instructions on our HREC website for guidance on how to submit a progress report.

The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Translation of the consent document(s) to the language(s) applicable to your study participants should now be submitted to the HREC.

**Provincial and City of Cape Town Approval**

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Departement of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-research-approval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

Figure 70 Excerpt of the Ethics Approval Notice

## 6.2. Appendix B: Institutional Permission Agreement

UNIVERSITEIT • STELLENBOSCH • UNIVERSITY
jou kennisvennoot • your knowledge partner

**INSTITUTIONAL PERMISSION:**

**AGREEMENT ON USE OF PERSONAL INFORMATION IN RESEARCH**

**Name of Researcher:** Terri-Ann de Jager

**Name of Research Project:** Obersiving the effects of anxiety on male reproductive parameters

**Service Desk ID:** IRPSD 593

**Date of Issue:** 6 September 2017

You have received institutional permission to proceed with this project as stipulated in the institutional permission application and within the conditions set out in this agreement.

| **1 WHAT THIS AGREEMENT IS ABOUT** | |
|---|---|
| What is POPI? | 1.1 POPI is the Protection of Personal Information Act 4 of 2013.<br>1.2 POPI regulates the entire information life cycle from collection, through use and storage and even the destruction of personal information. |
| Why is this important to us? | 1.3 Even though POPI is important, it is not the primary motivation for this agreement. The privacy of our students and employees are important to us. We want to ensure that no research project poses any risks to their privacy.<br>1.4 However, you are required to familiarise yourself with, and comply with POPI in its entirety. |
| What is considered to be personal information? | 1.5 'Personal information' means information relating to an identifiable, living, individual or company, including, but not limited to:<br>1.5.1 information relating to the race, gender, sex, pregnancy, marital status, national, ethnic or social origin, colour, sexual orientation, age, physical or mental health, well-being, disability, religion, conscience, belief, culture, language and birth of the person;<br>1.5.2 information relating to the education or the medical, financial, criminal or employment history of the person; |

Figure 71 Excerpt from the Institutional Permission Agreement