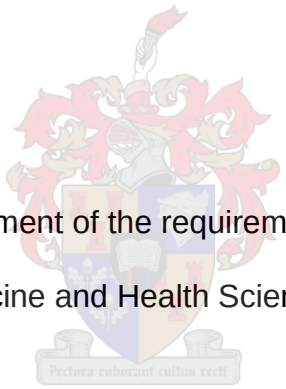
CHRONIC STRESS AND SEMEN PARAMETERS

by

Esmari van der Merwe



Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in the Faculty of Medicine and Health Sciences at Stellenbosch University

Supervisor: Prof. SS du Plessis

Co-supervisor: Dr. E Bosman

March 2020

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2020

Abstract

It has been well documented that stress has adverse effects on the body and can lead to various health issues. Stress has been investigated as a cause for unexplained infertility in both men and women. Semen quality is a key indicator of male reproductive health. Numerous studies have been done on the effect of stress on semen parameters and an association between chronic psychological stress and poor semen parameters have been reported. Managing psychological stress can help to improve the health of an individual. In order to address the problem it is therefore important to determine if an individual experience high levels of stress. This can be established through psychological questionnaires and various biomarkers, such as the screening test for time urgency perfectionism (TUP) and alpha-amylase in saliva.

In general, more or less 84% of couples are estimated to conceive naturally within a year. The remaining 16% of couples are affected by infertility. Within this group, it is estimated that male reproductive factors are the sole cause of one-third of cases and a contributing factor in another 20% of cases. Management of chronic stress in female patients has shown improved IVF rate of 67% or higher. However, as of yet no study has been performed on males to correlate the levels of TUP-stress, alpha-amylase to semen parameters as well as other seminal stress markers such as DNA fragmentation and oxidative stress (ROS).

This study compared TUP-categories (Low, Moderate, High) with respect to semen parameters, alpha-amylase levels, age and BMI and investigated if increased alpha-amylase levels correlate with semen parameters, age and BMI.

The experiments were performed at Medfem Fertility Clinic in Bryanston Johannesburg and the Division of Medical Physiology in the Department of Biomedical Sciences at Stellenbosch University. A total of 62 male patients of Medfem Fertility Clinic adhering to the basic requirements enrolled in the study.

Results showed no significant difference between age, semen parameters and alpha-amylase between TUP categories. Men in the High TUP category had a significant higher BMI compared to those in the Low and Moderate categories. No significant correlation was found between alpha-amylase, age, BMI and semen parameters.

This study was unsuccessful in proving a significant relationship between the TUP categories, age and semen parameters. The High TUP category did show a significantly higher BMI compared to the Low and Moderate TUP groups. This finding confirms that there is a link between psychological stress and elevated BMI. Although there was no significant difference between the TUP categories with regards to sORP values, the Moderate and High categories were both higher than the normal value for sORP in semen. This implies that chronic stress leads to elevated levels of oxidative stress in semen.

No relationship was found between TUP categories and alpha-amylase levels. Although both are used to detect chronic stress, the TUP questionnaire is used to detect personality types who are prone to chronic stress, whilst salivary alpha-amylase is a biomarker for chronic stress and functions in a completely different way. It is possible that whilst both can be used to detect chronic stress it is not advised to attempt to establish a relationship between the two as the mechanisms of both are very different.

Opsomming

Daar is goed gedokumenteer dat stress ‘n nadelige uitwerking het op die liggaam wat tot verskeie gesondheidskwessies aanleiding kan gee. Ondersoek het aangedui dat stress die oorsaak is van ‘n onverklaarbare infertiliteit by beide mans en vrouens. Semen kwaliteit is ‘n sleutel aanwyser vir manlike reproduktiewe gesondheid. Verskeie studies is uitgevoer op die effek van stress op semen parameters, en ‘n assosiasie tussen chroniese sielkundige stress en swak semen parameters is gerapporteer. Die bestuur van sielkundige stress kan help om die gesondheid van ‘n individu te verbeter. Ten einde die probleem aan te spreek is dit derhalwe belangrik om te bepaal of die individu hoë vlakke van stress ervaar. Dit kan vasgestel word deur die voltooiing van sielkundige vraelyste en verskeie biomerkers, soos die siftingstoets vir tydsbeperkende perfektionisme (TUP) en alfa-amilase in speeksel.

In die algemeen, ondervind min of meer 84% van paartjies, na raming, natuurlike konsepsie binne ‘n jaar. Die oorblywende 16% van paartjies word geaffekteer deur onvrugbaarheid. Manlike reproduktiewe faktore is vermoedelik die oorsaak van ongeveer een derde van die gevalle en ‘n bydraende faktor tot ‘n verdere 20% met in die groep. Die bestuur van chroniese stress in vroulike pasiënte het ‘n verbeterde IVB koers van 67% of hoër aangedui. Maar tot nog toe is geen studie op mans onderneem om die vlakke van chroniese stress en alfa-amilase op semen parameters asook ander seminale stress merkers soos DNA fragmentasie en oksidatiewe stress (ROS) te korreleer nie.

Hierdie studie vergelyk TUP-kategorieë (Lae, matige, hoë) met betrekking tot semen parameters, alfa-amilase vlakke, ouderdom en BMI en ondersoek of verhoogde alfa-amilase vlakke korreleer met semen parameters, ouderdom en BMI.

Die eksperimente is uitgevoer by Medfem Fertility Clinic in Bryanston Johannesburg en die Afdeling Mediese Fisiologie en die Departement van Biomediese Wetenskappe van die

Stellenbosch Universiteit. ‘n Totaal van 62 mans, almal pasiënte van Medfem Fertility Clinic, het deelgenaam aan die studie.

Resultate het geen beduidende verskil getoon tussen ouderdom, semen parameters en alfa-amilase tussen TUP kategorieë nie. Mans in die Hoë TUP kategorie het ‘n beduidende hoër BMI in vergelyking met die in die Lae en Matige kategorieë. Geen beduidende korrelasie is gevind tussen alfa-amilase, ouderdom, BMI en semen parameters nie.

Die studie was onsukselvol om ‘n beduidende verwantskap te bewys tussen die TUP kategorieë, ouderdom en semen parameters. Die BMI van die Hoë TUP groep was beduidend hoër as die van die Lae en Matige TUP kategorieë. Dit impliseer ‘n verwantskap tussen chroniese stress en vetsug. Alhoewel daar geen beduidende verskil was tussen die TUP kategorieë in terme van sORP vlakke nie, was die Matige en Hoë kategorieë beide hoër as die normale afsnypunt vir sORP in semen. Dit impliseer dat chroniese stress kan aanleiding gee tot verhoogde vlakke van oksidatiewe stress in semen.

Geen verwantskap was gevind tussen die TUP kategorieë en alfa-amilase vlakke nie. Alhoewel beide gebruik word om chroniese stress te identifiseer, word die TUP vraelys gebruik om persoonlikheidstipes wat geneig is tot chroniese stress te identifiseer, terwyl die speeksel alfa-amilase ‘n biomerker is vir chroniese stress en fungeer op ‘n totaal ander wyse. Terwyl albei gebruik kan word om chroniese stress te identifiseer word ‘n poging om ‘n verwantskap tussen die twee te bepaal nie ondersteun nie omdat die meganisme van albei heeltemal verskil.

Acknowledgements

To:

Prof Stefan du Plessis, thank you for your guidance and insight throughout this process. The ingenuity in which you handled the subject matter was influential. You are someone to look up to for your vast knowledge and leadership.

Edolene, thank you for your support and direction and for granting me the opportunity and time to be able to take on this project.

Alma, thank you for your willingness to teach me new techniques, for your patience and for sharing your knowledge.

My husband Errad, thank you for your endless support and encouragement throughout this project. I would not have been able to do this without you. Thank you for believing in me.

The lab staff at Medfem Fertility Clinic, thank you for your support and for giving me time when I needed it most.

Dr Rodrigues, thank you for allowing me to take on such a project at Medfem Fertility Clinic and for your insights and willingness to assist wherever you could.

My family, thank you for your encouragement and being there when I needed you most.

Contents

LIST OF ABBREVIATIONS

CNS:	Central nervous system
PNS:	Peripheral nervous system
ANS:	Autonomic nervous system
SNS:	Somatic nervous system
SAM:	Sympathetic adrenomedullary
HPA:	Hypothalamus-pituitary-adrenal
ACTH:	Adrenocorticotropic hormone
HPT:	Hypothalamic-pituitary-testicular
GNRH:	Gonadotrophin-releasing hormone
FSH:	Follicle stimulating hormone
LH:	Luteinizing hormone
CRH:	Corticotrophin-releasing hormone
ROS:	Reactive oxygen species
DNA:	Deoxyribonucleic
PUFAS:	Polyunsaturated fatty acids
TUP:	Time urgency perfectionism
WHO:	World health organisation
BMI:	Body mass index
sORP:	Oxidation reduction potential
TUNEL:	Deoxynucleotidyl transferase dUTP nick end labelling
TdT:	Terminal deoxynucleotidyl transferase
FITC:	Fluoroscein isothiocyanate
WC:	Waist circumference
WHtR:	Waist to height ratio
WHR:	Waist to hip ratio
BF%:	Body fat percent

LIST OF FIGURES AND TABLES

FIGURES

TABLES

Chapter 1

Background information and literature overview

1.1 Chronic stress

Stress is defined as “a state of mental or emotional strain or tension resulting from adverse or demanding circumstances”(1). It has been well documented that stress has detrimental effects on the body and can lead to various health issues (1–4). Studies have found anxiety can sometimes be related to stress; it can be provoked by the existence of prolonged stress and multiple stressors. Anxiety is a state of apprehension and disproportionate responses to perceived threats (5). This causes disruption of psychological functioning which in turn leads to physiological symptoms such as increased heartbeat, elevated blood pressure, sweating or dizziness (5). The focus of this study will be on stress. Generally, stress is categorised into two types: Objective/acute (instantaneous) and subjective/chronic or self-induced (perceived) stress (4). Objective/acute stress is a stress response believed to be invariably related to certain events or incidences also referred to as environmental experiences or demands (6,7). Triggers such as death of a family member or a friend, crime, poverty, war and deadlines can all have an effect on even the most relaxed person. These stressors or triggers are induced and originate externally (8). Subjective/chronic or self-induced stress manifests itself due to the way in which a person thinks or perceives that he or she will be able to cope with certain events and demands (6,7). This is also called the psychological stress perspective. When environmental demands are perceived to exceed an individual’s abilities to cope, the individual subsequently feel stressed and experience an accompanying negative emotional response. It is important to note that the perception that one is experiencing stress is a consequence of both the interpretation of the meaning of an event and the assessment of efficacy of coping resources (7).

In reaction to these stressors the body responds by activation of physiological systems that are mainly responsive to physical and psychological demands. These physiological systems and how they are affected by stress will be explored in more details at a later stage. Objective stressors or environmental demands leads to a secretion of adrenalin, the response is acute and short lived (8). In the event that these systems are activated repeatedly or for a prolonged period of time, it can increase the chances of an individual developing a wide range of both physical and psychiatric disorders (7).

A simplified diagram (Figure 1) outlining the integration of environmental demands, subjective or perceived evaluations of the stressfulness of a situation and the biological responses to said stressors or appraisals i.e. stress responses.

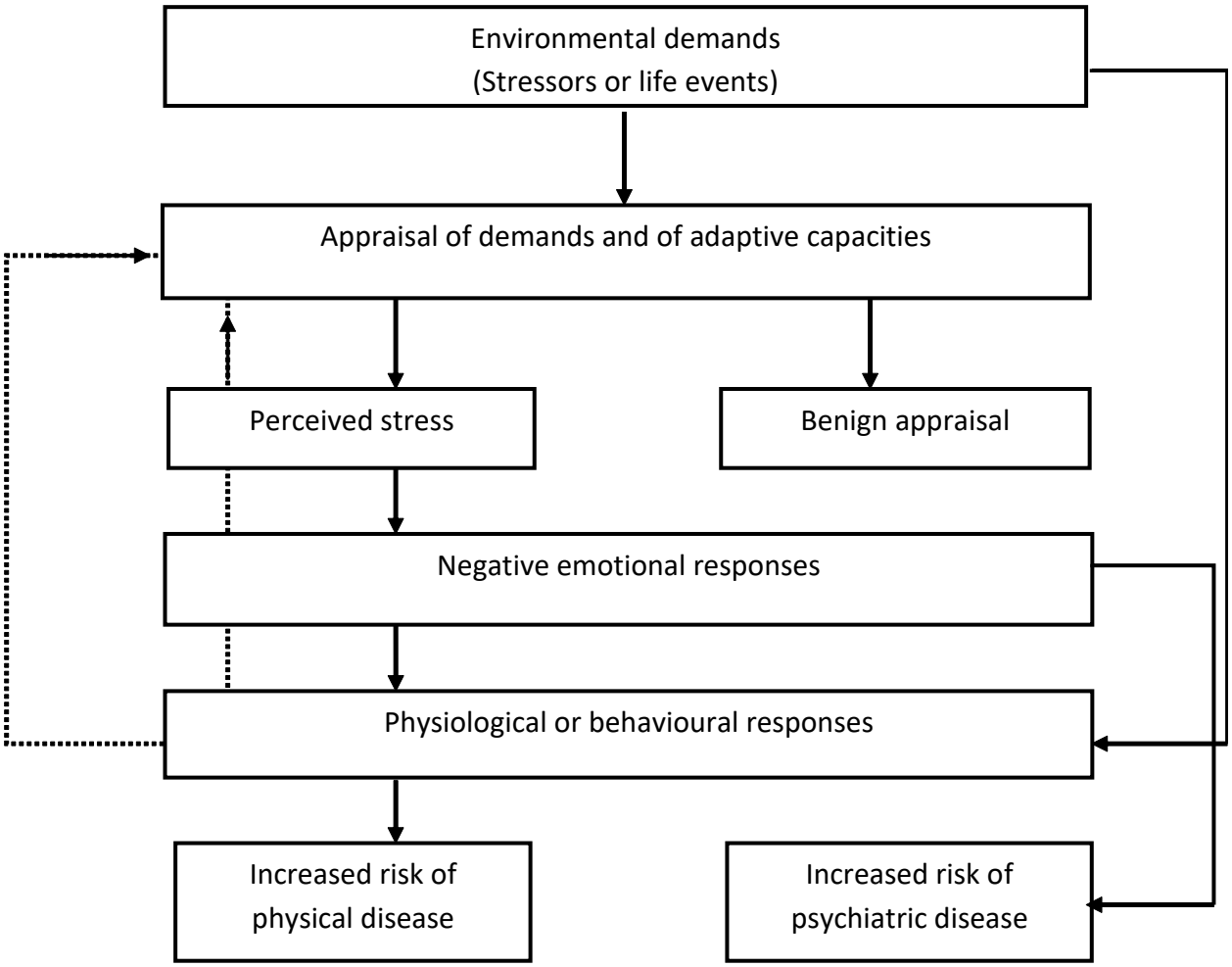


Figure 1: Integration of stressors or life events and biological responses (Adapted from: Measuring stress: a guide for health and social scientists (7))

Personality is “a characteristic way of thinking, feeling, and behaving. Personality embraces moods, attitudes, and opinions and is most clearly expressed in interactions with other people. It includes behavioural characteristics, both inherent and acquired, that distinguish one person from another and that can be observed in people’s relations to the environment and to the social group” (9). There are several theorists that have defined different types of personality. To name a few: personality types A and B, Myers-Brigs 16 personality types, Big Five personality traits and Eysenack’s personality. The consistency of personality types across different studies is a challenge. Researchers use different criteria, study different population groups, sample sizes, language, culture and method of deriving types (10). Type A personality have been by far the most extensive studied personality in health research (11). Personalities that are more competitive, highly organised, ambitious, impatient, and very aware of time management are labelled Type A, while more relaxed, less neurotic and frantic personalities are labelled Type B. This study will focus on Type A personality which was first described by Rosenman and Friedman in 1950 (12). Type A individuals often put themselves at risk of developing subjective or self-induced stress and have an excessive competitive drive. These individuals are easily aggravated and tend to overreact. In addition, they may be hostile, see the worst in others, be envious and lack compassion. A constant sense of urgency is experienced, and they are very impatient. Type A personalities set unrealistic time goals for themselves and when it cannot be met they get frustrated and angry. They are always stressed for time and will often try to do more than one task at a time. These individuals set high standards for themselves and expect others to be equally time-urgent and goal-orientated. If situations feel beyond their control, they tend to get stressed. In addition, Type A personalities worry about things that other people might not deem important and therefore they create extra stress for themselves (8,12,13).

Time urgency perfectionism (TUP) stress describes the constant/chronic/subjective stress these individuals subject themselves to. This is a learned stress and after some time it

becomes a constant stress. Chronic stress persists over an extended period of time. It originates internally and is constantly retained and maintained by the individual (8,14,15). The negative effects of chronic stress are well documented; it has been shown to affect cardiovascular health (16) and suppress immune function, which may lead to increased vulnerability to infections, inflammation, cancers and auto-immune diseases (1,17–19).

Coping responses to stressors manifesting as behavioural changes may influence disease risk, for example people engage in poor health choices: smoking, overeating and excessive drinking of alcohol (20,21). In a study done by Groesz et al. (22) they found that: “greater reported stress, both exposure and perception, was associated with indices of greater drive to eat— including feelings of disinhibited eating, binge eating, hunger, and more ineffective attempts to control eating (rigid restraint)”. They further suggest that stress exposure may bring about a stronger drive to eat and might promote excessive weight gain (22).

1.2 The nervous system

In humans, the nervous system can be divided into the central nervous system (CNS) and the peripheral nervous system (PNS). The PNS is further divided in the autonomic nervous system (ANS) and the somatic nervous system (SNS) (See Figure 2). The SNS receives information from the environment through the sensory organs. Sensory information is then relayed to the CNS which subsequently controls the activity of the voluntary skeletal muscles (23,24). The ANS controls automatic functioning or involuntary muscles. It is often referred to as the “involuntary” nervous system as the control of the organs is not conscious. The ANS basically regulates all organs that contain smooth muscle, such as the heart, blood vessels, visceral organs and exocrine glands (24). Some of the key visceral processes also regulated by the ANS include cardiac output, glandular secretions, reproduction related activities, blood flow to specific organs and waste removal (24). The sympathetic and parasympathetic systems are the two subdivisions of the ANS (1,23,24). These two sub-

systems counteract each other (1). The sympathetic system plays a vital role in the “fight or flight” responses which include: increases in heart rate, blood pressure, ventilation, skeletal muscle perfusion and dilation of the pupils. The parasympathetic system does exactly the opposite of the sympathetic system. Parasympathetic output leads to conservation of energy as well as a decrease of the heart rate, blood pressure and ventilation. It furthermore, leads to increased secretions of saliva and mucous, and constriction of the pupils (23,24).

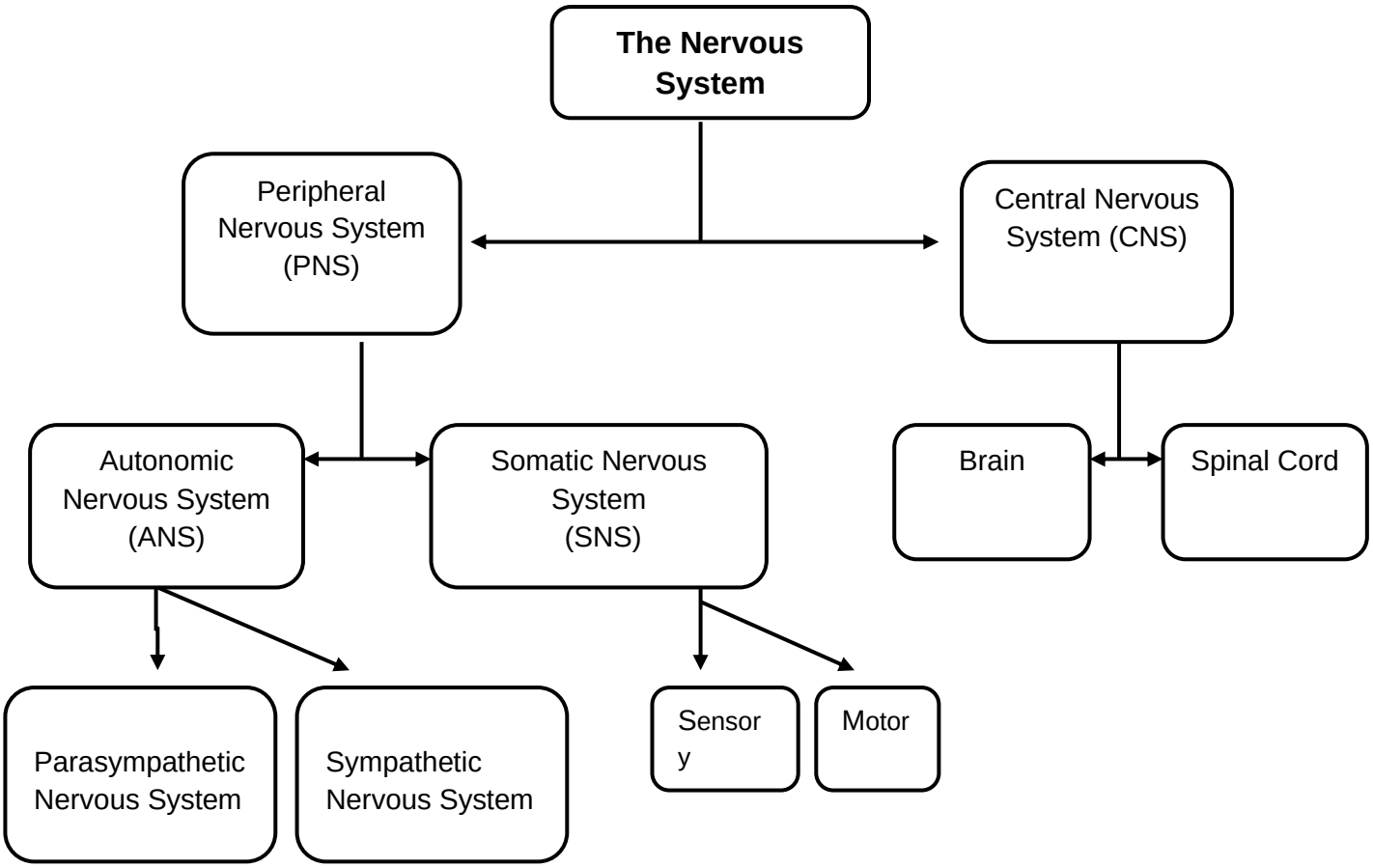


Figure 2: Subdivisions of the nervous system (Adapted from: Introduction to the Autonomic nervous system (23))

In the event that an autonomic imbalance exists, for instance when the sympathetic system is hyperactive and the parasympathetic system is underactive, it can cause various pathological conditions (25,26). Upon perceiving a stressful stimulus, signals are sent to the hypothalamus. The sympathetic adrenomedullary (SAM) pathway is subsequently activated and adrenaline and noradrenaline are secreted by the adrenal medulla (1,27). It has been proposed that activation of the SAM pathway also leads to increased salivary alpha-amylase activity (27–30). The vagal nerve is the primary parasympathetic nerve and is proposed to

regulate allostatic load (31). Physiological set points can change in reaction to chronic stress in a process called allostasis. A ‘load’ is then produced that may have an additive effect to the pathophysiological process, which is involved in a number of chronic illnesses (1,32). The functioning of the SNS, Hypothalamus-pituitary-adrenal (HPA) - axis, immune system and cardiovascular and metabolic processes are all impacted by this dysregulation of physiological systems. In short, allostatic load refers to the total or collective damage caused by a repeated neuroendocrine response resulting from chronic stressors, which over an extended period of time may lead to deteriorations in health (33).

Increased cortisol levels are associated with decreased vagal function (1). When the perceived stressors manifest as chronic stress, the SAM can remain hyperactive, and the HPA-axis is also activated (1,27). The activation of the HPA-axis leads to the release of cortisol in the bloodstream (1,27). Excessive secretion of cortisol over a longer period of time may lead to increased allostatic load and therefore onset of disease (1). Neural structures are damaged by long term cortisol exposure (34). Various conditions or situations can result in a chronically activated HPA-axis; this includes melancholic depression, panic anxiety and obsessive compulsive disorder (35).

A multitude of genetic, environment and developmental factors have an influence on the effects of the HPA-axis, the SNS and their respective hormones, which are considered to be the key components of the “stress system” (36). Dysregulation of the “stress system” will cause individuals to suffer adverse health consequences.

Non-physical events can easily trigger the physiochemical responses of the HPA axis. For example: grief, excitement, fear, anxiety, guilt and embarrassment can all trigger a robust HPA-axis response. Adrenocorticotropic hormone (ACTH) and cortisol will also be increased in most individuals by events such as: public speaking, performance evaluations, and sky diving or clinical appointments. “Research has shown that the magnitude of the response

and recovery to these stressors is based on the individual's perception rather than the stressors themselves" (36).

Every person has a unique stress response. Some qualities, listed hereunder, further individualises each person's stress response (36):

- Age
- Gender
- Hereditary predisposition
- Personality characteristics (for example whether a person is an introvert or has low self-esteem)
- Prenatal and early childhood experiences

There are four key factors that determine the scale to which the HPA-axis will respond to an emotional or mental stressor (37,38):

1. Novelty to the individual
2. Unpredictive nature
3. Threat to the person or ego
4. Sense of loss of control

Some individuals might therefore perceive themselves as being stressed based on their thinking of how well they are able to cope. While another individual under the same "stressful" circumstance might not regard themselves as being stressed.

1.3 Stress and infertility

Stress has been investigated as a cause for unexplained infertility in both men and women (39–41). Semen quality is a key indicator of male reproductive health (6). Numerous studies have been done on the effect of stress on semen parameters (41–45) and an association

between chronic psychological stress and poor semen parameters have been reported (42,46,47). In a study performed by Gollenberg et al. (47) it was found that individuals who experienced two or more stressful life events had a lower sperm concentration and showed a decrease in the percentage motile sperm. Janevic et al. (6) similarly reported decreased sperm concentration, decreased percentage motile sperm and a decrease in percentage of morphologically normal sperm in men suffering from psychological stress. An association between higher perceived stress and decreased sperm concentration and normal morphology was also found by researchers in China (48).

As mentioned previously, psychological stress activates certain systems and pathways within the body, thereby resulting in hormonal and homeostatic changes. When the HPA axis is activated as a result of chronic stress, the activity of the hypothalamic-pituitary-testicular (HPT) axis is reduced (49). The HPT axis involves releasing of gonadotrophin-releasing hormone (GnRH) from the hypothalamus, which stimulates the anterior pituitary to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH binds to the receptors on the Sertoli cells to regulate spermatogenesis, while LH binds to the receptors on the Leydig cells to stimulate testosterone production (50). Corticotrophin-releasing hormone (CRH) released by the hypothalamus with activation of the HPA axis inhibits the release of GnRH, resulting in suppression of reproductive functions (51). The surplus cortisol that is present in the body as a result of stress can influence the reproductive system as the cortisol serves to inhibit GnRH and essentially have a negative impact on FSH, LH and testosterone, all of which are needed for healthy spermatogenesis (50,52). Some studies have also linked psychological stress and reduction in sperm quality to an increase in seminal plasma reactive oxygen species (ROS) generation and a reduction in antioxidant protection (53). ROS are also known as oxidants which are highly reactive and belong to the free radical class (54). There is a common association between compromised sperm quality and oxidative damage/stress. When oxidants outnumber antioxidants

oxidative stress develops (55). ROS has a negative impact on sperm function as it can decrease sperm concentration and motility and also damage sperm nuclear deoxyribonucleic acid (DNA). The sperm plasma membrane contains plentiful polyunsaturated fatty acids (PUFAs) and is therefore very sensitive to the effect of ROS. These PUFAs play a role in sperm motility and the acrosome reaction which is necessary for fertilisation (53). ROS damages the sperm membrane through lipid peroxidation, which reduces the motility of the sperm and its ability to fuse with the oocyte. ROS also directly damages sperm DNA, compromising the paternal genomic contribution to the embryo (50,56). In human spermatozoa the sperm chromatin structure is highly organised to ensure that there is no endogenous and exogenous attacks by toxic elements (57). The sperm DNA bases can be modified by various physical, chemical and biological factors causing lesions, mutations and deletions in the DNA bases. The modification of the DNA base pairs may lead to fragmentation and denaturation of the DNA causing reduced sperm function and fertilisation potential. Sperm DNA integrity is associated with sperm motility, capacitation, the acrosome reaction, normal development of the embryo and birth of healthy offspring. The compact packaging of the sperm chromatin is necessary for maintaining sperm DNA integrity. Understanding the sperm chromatin structure is necessary and valuable in management of infertile men (58,59). From the aforementioned mechanisms it is clear that stress can cause or play a role in infertility.

1.4 Testing for stress

1.4.1 Psychological questionnaires

From the preceding information it is clear that stress can have an impact on male fertility through various pathways and can have a detrimental effect on semen parameters and male reproductive health. Managing psychological stress can help to improve the health of an individual. In order to address the problem, it is therefore important to determine if an

individual experience high levels of stress. This can be established through psychological questionnaires and various biomarkers (29,32,47). Psychological questionnaires have been used to establish stress levels and personality types for a long time. Studies as early as 1974 have used psychological questionnaires in stress related studies (60). From the literature it is clear that quite a large number of questionnaires exist (61–64). While some questionnaires are similar in tone and questions asked, most are compiled with a specific outcome in mind related to the study performed. For example, some studies use psychological questionnaires to determine work related stress (65), stress among students (66), the relationship between psychological stress and testicular function (67), and the link between Type 2 diabetes mellitus and psychological stress (68). One such questionnaire is a screening test specifically designed for diagnosing TUP stress as was discussed earlier. This on-line test will determine if a person falls into any of the three categories of TUP (8). These three categories are defined as follows:

- “Low-TUP refers to a balanced approach in being on time in personal and work activities, and performing work and personal activities to a reasonable degree of correctness”.
- “Moderate-TUP refers to a need to be hurried and perfect in the execution of tasks, in many, but not all areas of life”.
- “High-TUP refers to the tendency to be very hard driving in one’s approach to all activities including an excessive need to hurry and produce perfect results in all areas of activity” (69).

A copy of the questionnaire can be found in Addendum I.

1.4.2 Salivary biomarkers

Salivary biomarkers provide a reliable non-invasive and objective measurement of chronic psychosocial stress (32). Salivary cortisol has been used as a measure for HPA axis activity

whilst salivary alpha amylase is an indicator of the SAM pathway involved in the stress response (6,30). The SAM system is activated when a stressor is perceived; if that stressor becomes chronic the HPA axis is activated whilst the SAM pathway remains active as well (27). An activated HPA axis cause an increase in blood cortisol levels which leads to an increase in salivary cortisol. The SAM system leads to an increase in noradrenaline which results in an increase in salivary alpha-amylase production by the parotid gland (27,28). The response of HPA and SAM activity after repeated stress was investigated by Schommer and co-workers (70). They found that HPA responses adjust quickly, whilst the sympathetic nervous system shows consistent activation patterns with repeated stress. These results suggest that salivary alpha amylase may be used when assessing chronic stress (70). Nater et al. (29) suggest that the two branches of the autonomic nervous system do not act independently, therefore both parasympathetic and sympathetic activation lead to an increase in alpha-amylase levels. Neurotransmitter stimulation activates secretion from salivary glands; these glands are innervated by both the sympathetic and parasympathetic nerves. Therefore salivary alpha-amylase is an ideal indicator of autonomic activity (71). Bosch et al. (72) found a predominant role of the sympathetic nervous system in the secretion process of alpha-amylase, together with vagal withdrawal, under psychosocial stress. In a study conducted by Nater et al. (28) in 2004, they found salivary alpha-amylase to be a variable that is sensitive to psychosocial stress, displaying pronounced increases following induction of stress compared to a rest condition. They concluded that salivary alpha-amylase to be a valid and reliable stress marker. Vineetah et al. (32) also found that salivary alpha amylase activity increases in patients with chronic psychosocial stress and may be used as a biomarker of chronic stress. Cortisol is thought to be the classical biomarker of stress (32); however, several studies have failed to find an association between salivary cortisol and self-reported stress in studies of reproductive outcomes (61,73). Lynch

and co-workers found in two studies that stress as measured by increased salivary alpha-amylase is associated with lower fecundity among affected women (27,61).

Infertility is defined as “a condition in which a couple is unable to conceive, after frequent unprotected sexual intercourse, for twelve months or more” (74). In general, more or less 84% of couples are estimated to conceive naturally within a year. The remaining 16% of couples are affected by infertility. Within this group, it is estimated that male reproductive factors are the sole cause of one-third of cases and a contributing factor in another 20% of cases (53,56). Management of chronic stress in female patients has shown an improved In Vitro Fertilisation (IVF) rate of 67% or higher, whereas chronically stressed female patients had an average success rate that was comparative to IVF results across the world (8).

However, as of yet no study has been performed on males to correlate the levels of the TUP stress categories, alpha-amylase levels and semen parameters as well as other seminal stress markers such as DNA fragmentation and ROS.

1.5 Research Question

Does chronic stress (TUP-stress) have a negative effect on semen parameters, age and Body Mass Index (BMI) and does alpha-amylase levels in saliva correlate with chronic stress (TUP-stress) semen parameters, age and BMI.

1.6 Aims and Objectives

Aim:

To determine the possible relationship, if any, between semen parameters of male individuals, the alpha-amylase saliva test and TUP-stress test.

Objectives:

- Compare TUP-categories (Low, Moderate, and High) with respect to semen parameters, alpha-amylase levels, age and BMI.
- Investigate if alpha-amylase levels correlate with semen parameters, age and BMI.

1.7 Place of Study

The experiments were performed at Medfem Fertility Clinic in Bryanston Johannesburg and the Division of Medical Physiology in the Department of Biomedical Sciences at Stellenbosch University.

Chapter 2

Materials and Methods

2.1 Study method and design

The study aimed to explore the possible correlation, if any, between semen parameters of male individuals, the alpha-amylase saliva test and the TUP- stress test. The outline of the study design is depicted in Figure 3. Male patients attending Medfem Fertility Clinic were asked to produce a semen sample after which a routine semen analysis as well as a DNA fragmentation and Oxidative stress evaluation followed. The routine semen analysis forms part of a standard work-up for infertility diagnosis at Medfem Fertility Clinic. A saliva sample, for evaluation of the Alpha-amylase levels, was collected and the patients also completed the TUP-stress test questionnaire (www.timeurgency.com) (Addendum I). A lifestyle questionnaire was also completed (Addendum II).

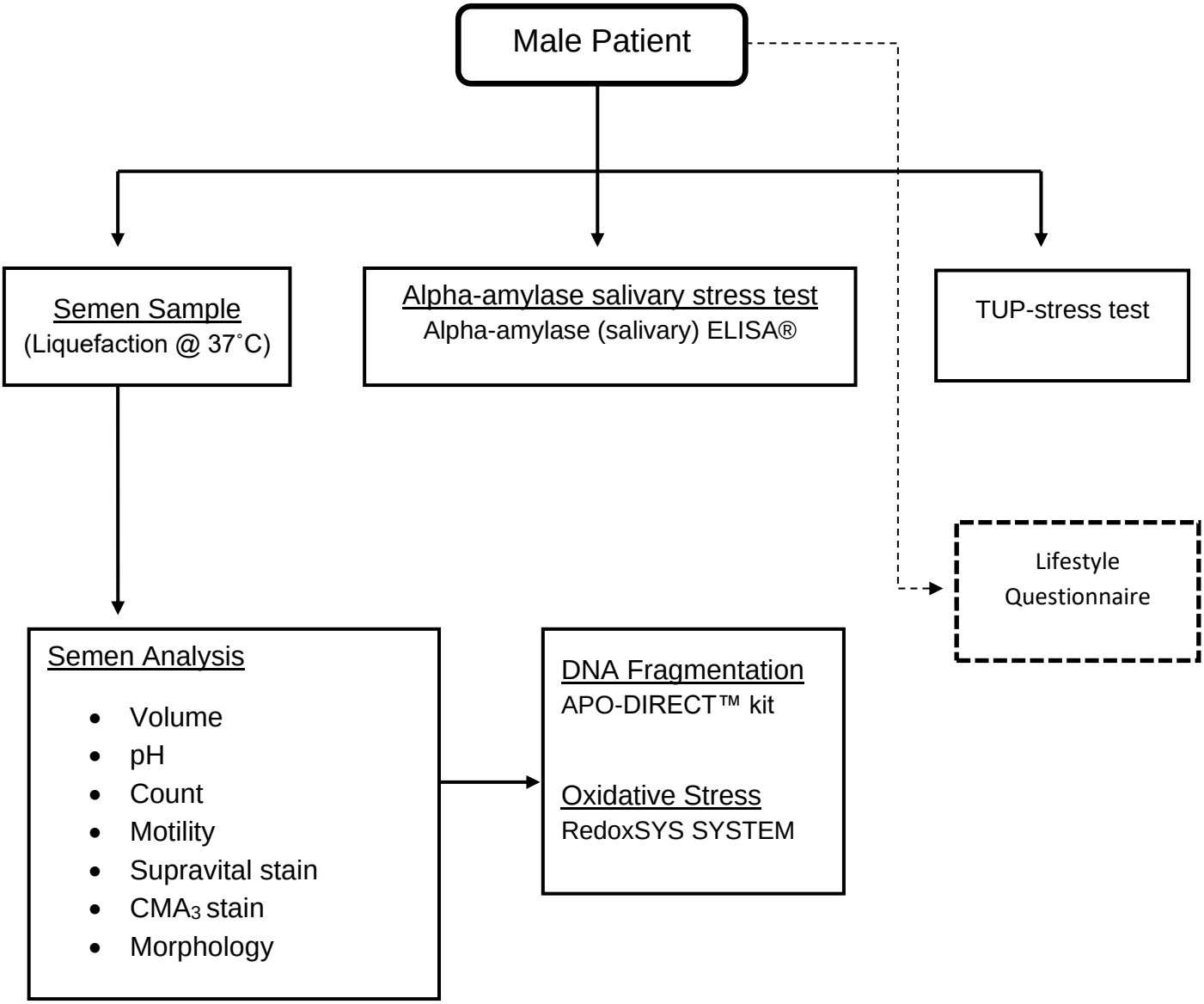


Figure 3: Schematic presentation depicting the study design and procedures

2.2 Participants

Male patients of Medfem Fertility Clinic adhering to the basic requirements were invited to participate in the study.

Inclusion criteria:

Minimum volume: 0.5µl

Age: >18

Exclusion criteria:

Asthma Patients

Trauma to the testes

Varicocele

Diabetics – Type I and Type II

Hyperthyroidism

Hypothyroidism

Hypogonadism

Smokers

2.3 Semen sample collection

Semen samples were collected in private rooms at Medfem Fertility Clinic by means of masturbation, after 2-3 days of sexual abstinence. Sterile wide-mouthed containers were used for collection. Once the sample was produced, it was placed at 37˚C and left to liquefy for 30 minutes. Collection of semen samples were completed according to the World Health organisation (WHO) guidelines (75).

2.4 Semen analysis

Semen samples were evaluated according to standard Medfem Fertility Clinic laboratory protocols and the WHO 2010 semen analysis guidelines after a 30 minute liquefaction time

period. A semen analysis consists of the following evaluations and tests, as described by the WHO (75):

Appearance of the ejaculate

The semen sample was evaluated macroscopically and the colour and transparency of the sample was noted.

Semen volume

The semen sample was transferred from the collection container to a graduated tube (Nunc, Thermo Scientific, Roskilde, Denmark). The volume was read directly from the graduations.

Semen pH

Litmus paper (Merck Millipore®, Massachusetts, United States) was used to measure the pH. A drop of semen was placed on a piece of Litmus paper, and once the colour has changed uniformly it was compared to the calibration strip.

Semen viscosity

The semen sample was drawn up in a 3ml plastic Pasteur pipette (Vitrolife, Göteborg, Sweden) where after the semen was allowed to drop by gravity and the length of any thread was noted. A normal sample leaves the pipette in small drops. A thread of longer than 2cm was regarded as abnormal.

Antisperm antibodies in the semen

Antisperm antibodies were tested by the SpermMAR IgG (Fertipro, Beernem, Belgium) test. On a micro slide (Lasec®, Cape Town, South Africa) 10µl of semen was mixed with 10ul of the latex particles, it was then mixed with 10µl of antiserum. The mixture was covered with a cover slip (Lasec®) and examined microscopically (Nikon Eclipse 50i) using a 400 x

magnification. The result was read after 2-3 minutes. The mixture was then observed for latex particles attached to motile sperm.

Sperm motility

A 5µl aliquot of semen was loaded in each chamber of a 2-Chamber 20µm Leja® slide (Nieuw-Vennep, The Netherlands). The slide was then examined under 200 x magnification, with an eyepiece reticle with a grid. The motility of the spermatozoa was tallied with a laboratory counter in each of the following categories: progressively motile, non-progressively motile and immotile sperm.

Sperm concentration

The sperm concentration was evaluated by using a 2-Chamber 20µm Leja® slide. A 5µl drop of semen is loaded in each of the 20µm chambers. The slide was then examined microscopically under 200 x magnification with an eyepiece reticle with a grid. The grid is composed of 10x10 blocks. The number of sperm inside a row of ten blocks was counted and noted. A count was done for each of the chambers and an average was calculated.

Sperm vitality

The Eosin-Nigrosin dye exclusion staining technique was used to quantify the % of viable sperm. A 50µl aliquot was taken from the semen sample and mixed with equal amounts of Eosin-Nigrosin (Thermo Scientific™, Massachusetts, United States). A smear was made on a glass slide and left to air-dry. The slide was then examined under brightfield optics at 1000 x magnification under oil immersion. Unstained spermatozoa were classified as live, while cells that stained pink were regarded as dead.

Sperm morphology

The Hemacolor® (Merck Millipore®, Massachusetts, United States) staining technique was used to evaluate the morphology of the spermatozoa. A 10µl aliquot was taken to make a smear on a glass slide. The slide was left to air dry. The slide was then placed in the fixing solution for 10 seconds. This was followed by immersing in the Staining Solution-1 for 7 seconds, and subsequently Staining Solution-2 for 7 seconds. Finally, the slide was rinsed in the Buffer Solution. The slide was allowed to air dry. Once the slide was dry, it was evaluated under brightfield optics at 1000 x magnification under oil immersion. Sperm morphology was evaluated according to the 2010 WHO guidelines (56). Samples with normal morphology of ≥4% were reported as “Normal”. Samples with a normal morphology of ≤3% were reported as “Abnormal”.

Chromomycin A_3 staining

CMA_3 is a fluorochrome. Mature DNA in sperm shows a low binding capacity for these fluorochromes. A 10µl aliquot of semen was taken to prepare a smear. The air-dried slide was fixed (3 parts Methanol: 1 part Acetic acid) (Labretoria, Menlo Park, Pretoria) for 20 minutes. The slide was evaluated under 200 x phase-contrast microscopy. An area (approximately 30 spermatozoa per high field magnification) was identified for evaluation; where after a small circle was drawn with a diamond point pen to mark the chosen area. The CMA_3 stain (Labretoria, Menlo Park, Pretoria) (60-100µl) was then applied on the circle and left in the dark in the fridge for 20 min. The slide was taken out and washed in McIlvaine’s buffer (Labretoria) for 20 seconds, then mounted with a coverslip and Dabco® (Aldrich Chemistry, Missouri, United States) mounting solution. It was then stored overnight in the fridge (2°C-8°C) where after it was evaluated using Fluorescence Miscroscopy. At least 100 spermatozoa were evaluated according to the following 4 classes:

1. No Staining (No Fluorescence)
2. Fluorescent band at the equatorial segment

3. Fluorescent staining (faintly yellow)
4. Bright yellow fluorescent staining

Interpretation of the results:

Classes 1 and 2 indicate good quality packaging DNA in the sperm head

Classes 3 and 4 indicate poor packaging DNA in the sperm head

2.5 Oxidative Stress

2.5.1 Materials

Equipment

- RedoxSYS Analyzer (Aytu Bioscience, Colorado, United States)

Disposables

- RedoxSYS Sensors (Aytu Bioscience, Colorado, United States)

2.5.2 Methods

- An individual RedoxSYS sensor was unwrapped and placed into the RedoxSYS Analyzer
- Using a pipette, 30µl of semen was transferred to the sample application port of the inserted sensor
- Once detected, the RedoxSYS Analyzer began processing the sample
- Results was received within 2 minutes
- Samples were evaluated in duplicate

2.5.3 Interpretation of results

- The oxidation reduction potential (sORP) is completed in 2 minutes. Values above the normal range (>1.38/mV/conc) imply a change in the balance between oxidants

and antioxidants that favours the oxidants and signify the presence of oxidative stress in the specimen

- The sORP measurement was 'normalized' using the sperm concentration in order to account for differences in cell count between samples: the sORP ($mV/10^6/ml$) was divided by the sperm concentration (10^6 sperm/ml)
-

$$\frac{mV/10^6/ml}{10^6 \text{ sperm/ml}}$$

- A measurement of 10 $mV/10^6/ml$ or less was regarded as acceptable between duplicate measurements for each sample

2.6 DNA Fragmentation

DNA fragmentation was evaluated using the Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay with the APO-Direct™ kit from BD Pharmingen (Franklin Lakes, United States). An aliquot of the semen sample collected on the day of the semen analysis was used for the DNA fragmentation test.

2.6.1 Materials

Sperm fixation

- Centrifuge (300 x g)
- Serological pipettes
- Pasteur pipettes
- 3.7% paraformaldehyde
- Ice
- 70% (v/v) ethanol
- Centrifuge tubes (12 x 75mm with caps, polystyrene recommended to limit cell loss)

- Phosphate-buffered saline (PBS, pH 7.4)

Staining

- APO-DIRECT Kit components: Reaction buffer, PI/RNase staining buffer, rinsing buffer, wash buffer, Terminal deoxynucleotidyl transferase (TdT) enzyme, Fluoroscein isothiocyanate (FITC)-dUTP. Positive and negative control cells.
- Distilled water
- Aluminium foil

Flow cytrometry

- Flow cytometer equipped with a 488nm Argon laser
- Flow cytometer data acquisition software

Fluorescence microscopy

- Fluorescence microscope (Excitation between 460 and 490 nm and emission >515 nm)
- Microscope slides
- Cover slips

2.6.2 Methods

Semen concentration assessment

- Approximately 2-3 x 10^6 total cells are sufficient to run the assay. To optimize the stains, the sperm concentration should be no more than 5 x 10^6
- For efficiency samples were stored at -20˚C and batched for TUNEL analysis.

Preparation of assay controls

- For the positive sperm control, DNA damage was induced by digestion with DNase I. A sample form a healthy donor was incubated after the cell counting step with 100µl of DNase I (1mg/ml) for 1h at 37˚C
- Similarly, a sample from a healthy donor was used for the negative sperm control, and the TdT enzyme was omitted from the staining step as described below

Fixation of sperm

- The fixation buffer was prepared by adding 10ml of 37% formaldehyde (100% formalin) to 90ml of PBS (pH 7.4) to give a 3.7% (v/v) paraformaldehyde solution
- The sperm sample was centrifuged at 300 x g for 7 minutes to pellet and separate the cells from the seminal plasma. All subsequent centrifugation steps were done at 300 x g for 5 minutes
- The supernatant was discarded by gently aspirating with a Pasteur pipette
- The cells were suspended in the 3.7% paraformaldehyde fixation buffer. The suspension was refrigerated at 4˚C overnight
- The supernatant was discarded, and the pellet suspended in 1 ml of ice cold 70% ethanol at -20˚C

Preparation of samples for staining

- All samples (test and controls) were resuspended by vortexing the tubes since the cells have settled after prolonged storage in ethanol
- For the kit controls, a positive and negative control was provided with the APO-DIRECT kit. 2ml of each of the control suspensions were taken and placed in 12 x 75 mm centrifuge tubes
- Both test and control tubes were centrifuged at 300 x g for 5 minutes and the ethanol supernatant discarded

- The cells were resuspended in 1ml of wash buffer, centrifuged at 300 x g for 5 minutes, and the supernatant removed
- The Wash Buffer treatment was repeated a second time

Staining

- An appropriate volume of the Staining Solution was prepared based on the number of samples to be assayed. For each sample analysed the following was combined: 10µl of Reaction buffer, 0.75µl of TdT Enzyme, 32.25 µl of distilled water, and 8µl FITC-dUTP. The FITC-dUTP reagent was added last as it is light sensitive
- The cell pellets were resuspended in 50µl of the Staining Solution. For negative semen controls, staining solution was added without the TdT enzyme
- To disperse the cells and to allow the staining solution to permeate homogeneously into every cell the tube was vortexed
- The cells were incubated for 60 minutes at 37˚C
- After incubation, 1.0ml of Rinse Buffer was directly added to each sample, centrifuged at 300 x g for 5 minutes, and the supernatant removed by aspiration
- The rinse step was repeated with additional 1.0ml of Rinse Buffer, centrifuged, and the supernatant again removed of each tube
- The cells were resuspended in 0.5ml of the PI/RNase Staining Buffer and incubated at room temperature for 30 minutes
- The cells were analysed in the PI/RNase solution within 3h of completing the staining procedure using flow cytometry

Flow cytometry

- PI stains total DNA, FITC-dUTP stains apoptotic cells. PI is a membrane impermeant dye that is excluded from viable sperm cells with damaged permeable membranes

will be penetrated by the dye. Propidium Iodide (PI) (red) fluoresces at about 623nm and FITC (green) at 520nm. Two dual parameter and two single parameter displays are created with the flow-cytometer data acquisition software

2.7 Alpha-amylase salivary ELISA

The alpha-amylase ELISA assay is an enzyme immunoassay for the quantitative determination of alpha amylase in human saliva. The kit used is from DRG Instruments (Marburg, Germany).

2.7.1 Materials

Equipment

- Thermo Scientific™ Multiskan FC Microplate Photometer
- Serono Diagnostics Vibrax® Lab Shaker

Disposables

All the disposables are provided with the kit

- Coated wells
- Calibrators and controls
- Enzyme conjugate
- Antiserum
- Sample buffer
- Wash buffer
- Chromogen/substrate solution
- Stop solution

2.7.2 Methods

A saliva sample was collected from each patient. The patient was provided with a Salivette® tube for sample collection. Patients were asked to be fasting from 22h00 the night before and abstain from physical exercise on the morning of producing the sample.

Preparation of the patient samples

- Patient samples were diluted 1:201 in sample buffer
- For example, 5µl sample to 1ml sample buffer

Sample incubation

- 20µl of the calibrators, controls and diluted patient samples were transferred into the individual microplate wells according to the pipetting protocol
- 100µl of enzyme conjugate solution was added into each of the microplate wells
- 100µl of antiserum solution was added into each of the microplate wells
- The microplate wells were covered with protective foil provided and incubated for 60 minutes on a microplate shaker (400 U/min) at room temperature (18˚C to 25˚C)

Pipetting protocol

- The pipetting protocol for microtiter strips 1-4 is an example for the quantitative analysis of 24 patient samples (P1-P24) as can be seen in Table 1.

Table 1: Example of the pipetting protocol for microtiter strips for the quantitative analysis of 24 patient samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	C 1	P 1	P 9	P 17								
B	C 2	P 2	P 10	P 18								

Continue...

C	C 3	P 3	P 11	P 19								
D	C 4	P 4	P 12	P 20								
E	C 5	P 5	P 13	P 21								
F	C 6	P 6	P 14	P 22								
G	Co1	P 7	P 15	P 23								
H	Co2	P 8	P 16	P 24								
P = Patient sample, C = Calibrators, Co = Controls												

Washing of the samples

- The wells were emptied and subsequently washed 3 times using 300µl of working strength wash buffer for each wash
- The wash buffer was left in each well for 30 to 60 seconds per washing cycle, and then the wells were emptied
- After washing, all liquid was thoroughly removed from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer

Substrate incubation

- 100µl of chromogen/substrate solution was added into each of the microplate wells
- It was then incubated for 15 minutes at room temperature (18˚C to 25˚C)

Stopping the reaction

- 100µl of the stop solution was added into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced

Measurement

- Photometric measurement of the colour intensity was made at a wavelength of 450nm and a reference wavelength between 620nm and 650nm within 30 minutes of adding the stop solution
- Prior to measuring, the microplate was slightly shaken to ensure a homogeneous distribution of the solution

Calculation of results

The standard curve from which the alpha-amylase concentration of antibodies in the unknown serum samples can be taken was obtained by point-to-point plotting of the extinction values measured for the 6 calibration sera against the corresponding units (linear/log). “4-parameter logistics” plotting was used for calculation of the standard curve by computer. Figure 4 is an example of a typical calibration curve.

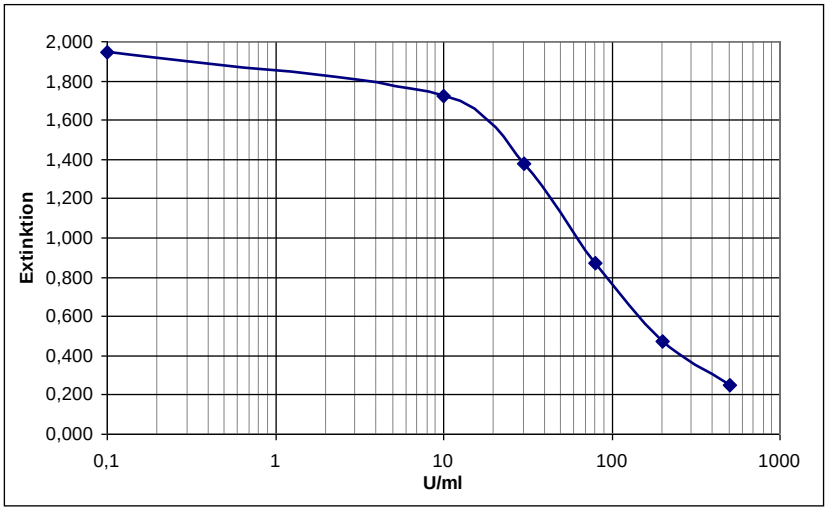


Figure 4: Example of calibration curve

2.8 Time urgency perfectionism stress questionnaire

Patients were pre-registered on www.timeurgency.com. Each patient completed the questionnaire online. The results were available immediately after completion. Each patient was stratified within any of the three categories namely: “Low-TUP”, “Moderate-TUP” and “High-TUP”.

2.9 Body Mass Index

Body Mass Index (BMI) is a value derived from the mass (weight) and height of a person. The BMI is a convenient rule of thumb used to broadly categorize a person as underweight, normal weight, overweight, or obese, based on tissue mass (muscle, fat, and bone) and height. A Lifestyle questionnaire was completed by each patient (Addendum II). The weight (kilograms) and height (meters) were noted on the questionnaire. The BMI of each patient was calculated by dividing the body mass by the square root of the body height.

2.10 Data analysis and statistics

Data was summarised by TUP-category reporting number (N), mean and median for semen parameters, age and BMI. Correlations between the latter and alpha-amylase were assessed using Spearman's rank correlation coefficient (rho).

TUP-categories were compared with respect to semen parameters, alpha-amylase, age and BMI using a one-way analysis of variance (ANOVA). Where data was skewed use was made of one-way ANOVA for ranks (Kruskal-Wallis rank test). When significant, pairwise comparison of TUP-categories was assessed using Bonferroni adjusted criteria, i.e. significant when $p<0.05/3=0.017$.

Stata® Statistics/Data analysis Release 15.1 (Statacorp, College Station, Texas, United States) was used for data analysis and significance was set at $p<0.05$. Data is presented as AVG±SD.

Chapter 3

Results

A total of 62 men were enrolled in the study; however, four men had to be excluded due to diabetes, performance anxiety, smoking and azoospermia respectively. A basic semen

analysis result was available for all 58 remaining participants. Depending on the concentration and motility of the sperm, some tests could not be performed as a minimum number of sperm is required to obtain an accurate test result; these include sperm vitality staining, morphology and CMA_3. A total of 47 men successfully completed the online TUP-test. Of the 47 men, 15 were in the LOW TUP-category, 12 were in the Moderate TUP-category and most of the men (20) were in the HIGH TUP-Category. Three participants who completed the online TUP-test did not have BMI results available. Salivary alpha-amylase results were obtained for 56 of the 58 participants as two participants could not produce a saliva sample.

3.1 Relationship between age, BMI, semen parameter values, and Alpha-Amylase with TUP-categories

3.1.1 Age

From Figure 5 it can be observed that there was no significant difference (p=0.483) detected in the age of the mean age of the men stratified according to TUP-category (Low: 36.13±5.69; Moderate: 38.66±4.16; High: 36.5±6.17).

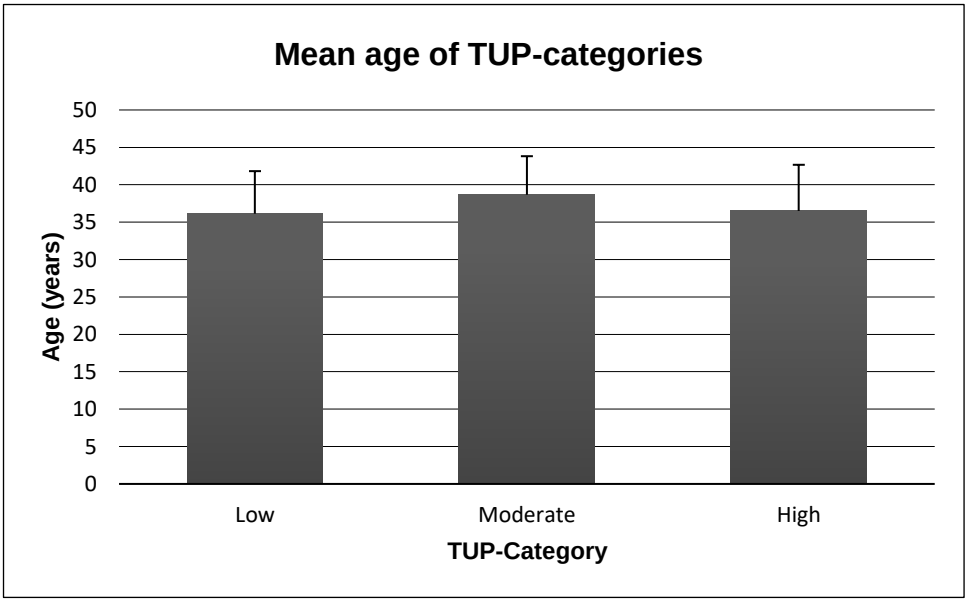


Figure 5: Bar graph depicting the difference between mean ages of TUP-Categories

3.1.2 BMI

Men diagnosed with High TUP had a significantly higher BMI (29.62±4.99; p=0.031) compared to those in the Low (26.22±4.16) and Moderate (25.89±2.59) groups as can be seen in Figure 6.

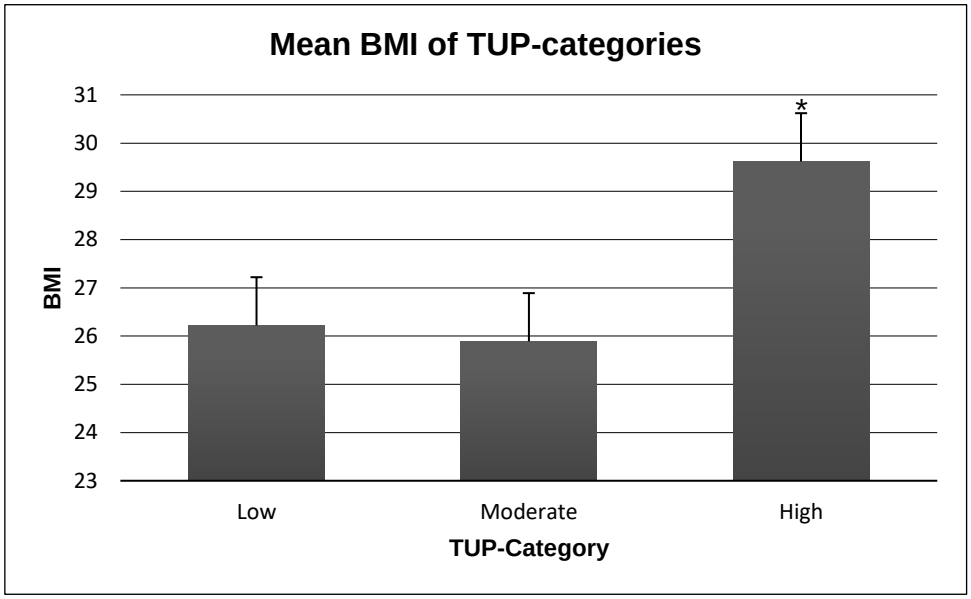


*Figure 6: Bar graph depicting the difference in BMI between TUP-categories (*P<0.05)*

3.1.3 Basic semen parameters

The relationship between basic semen parameter values with TUP-categories is presented in Table 2. There was no statistically significant difference between any of the parameters (volume, viscosity, concentration, motility, morphology, sperm vitality and MAR) between the TUP-categories.

Table 2: Comparison of TUP-categories (Low, Moderate, and High) with respect to basic semen parameters observed means.

	TUP-category: Mean, (SD), (N)			One-way ANOVA p-value
Parameter	Low	Moderate	High	
Volume (mL)	3.30 (1.65) (15)	2.77 (0.83) (12)	3.00 (1.38) (20)	0.596

Continue…

Viscosity (mm)	10.66 (15.34) (15)	5.83 (10.84) (12)	10.00 (14.14) (20)	0.626
Concentration ($x10^6$)	21.67 (14.38) (15)	25.07 (18.99) (12)	23.01 (18.20) (20)	0.879
Motility (%)	52.13 (9.40) (15)	52.50 (16.59) (12)	47.50 (17.51) (20)	0.565
Morphology (%)	4.93 (2.37) (15)	5.33 (2.61) (12)	5.44 (3.0) (18)	0.856
Sperm Vitality (%)	53.66 (9.90) (15)	61.89 (10.04) (9)	57.64 (11.67) (14)	0.195
MAR (%)	6.33 (17.16) (15)	3.75 (12.99) (12)	8.75 (18.84) (16)	0.450†

TUP=time urgency perfectionism; SD= standard deviation; N= number; ANOVA= analysis of variance; MAR= mixed agglutination reaction

† One-way ANOVA for ranks was employed (Kruskal-Wallis rank test)

The Low TUP category had the highest percentage (46.67%) of normal samples compared to the Moderate (33.33%) and the High (25%) TUP categories. Samples were evaluated according to the lower reference limits as set out by the WHO (Addendum III). The High TUP category had an elevated percentage of abnormal motility (25%) compared to the Low (6.67%) and Moderate (8.33%) categories. The remaining number of abnormalities in each TUP category is very similar in outcome as can be seen in Table 3.

Table 3: Summary of percentage (%) normal semen samples and number of abnormalities (%) within each TUP category.

	Low TUP category	Moderate TUP category	High TUP category
Number of normal samples (%)	46,67%	33,33%	25%
Number of abnormalities (%):			
Astenozoospermia (Motility < 32%)	6,67%	8,33%	25%
Oligozoospermia (Concentration <15 x 10^6/mL)	40%	25%	40%
Teratozoospermia (Morphology < 4%)	26,67%	25%	30%
Volume (< 1,5ml)	6,67%	8,33%	5%
Sperm Vitality (< 58%)	33,33%	25%	30%
TUP = Time Urgency Perfectionism; % = Percentage			

3.1.4 Advanced semen parameters

3.1.4.1 Oxidative reduction potential

There was no statistically significant difference (p=0.460) between the Low (1.43±1.48), Moderate (4.23±10.11) and High (12.12±33.50) TUP-categories with regards to Oxidative reduction potential (Figure 7). Both the Moderate and High group means is however above the normal range of (>1.36/mV/conc) as found by literature.

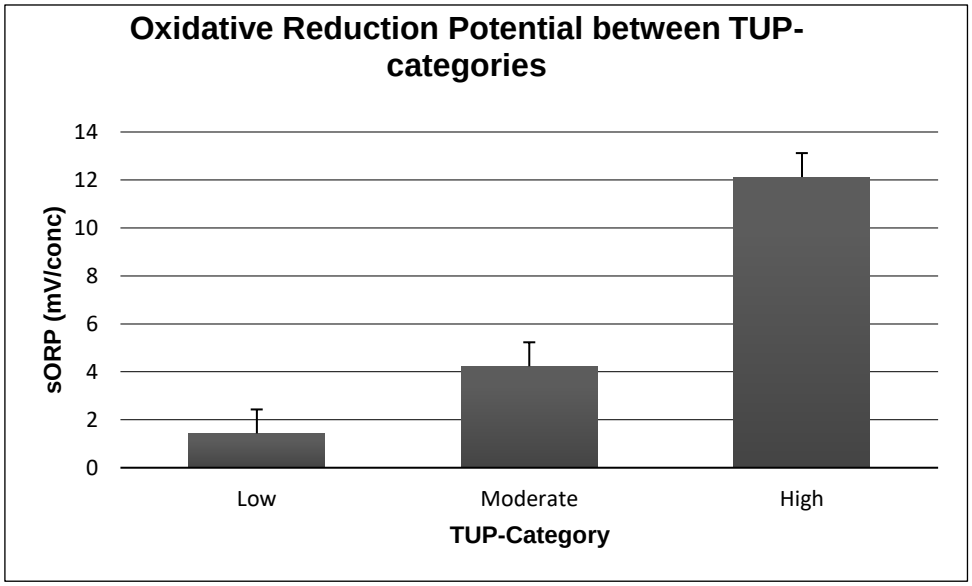


Figure 7: Bar graph of the mean values of Oxidative Reduction Potential of TUP-categories.

3.1.4.2 CMA_3

From Figure 8 it can be observed that there was no significant difference (p=0.713) between the mean CMA_3 values of the TUP-categories (Low: 46.53±15.14; Moderate: 42.16±13.83; High: 42.83±17.82). The mean of each TUP-category is however higher than the 40% cut-off value for the CMA_3 test.

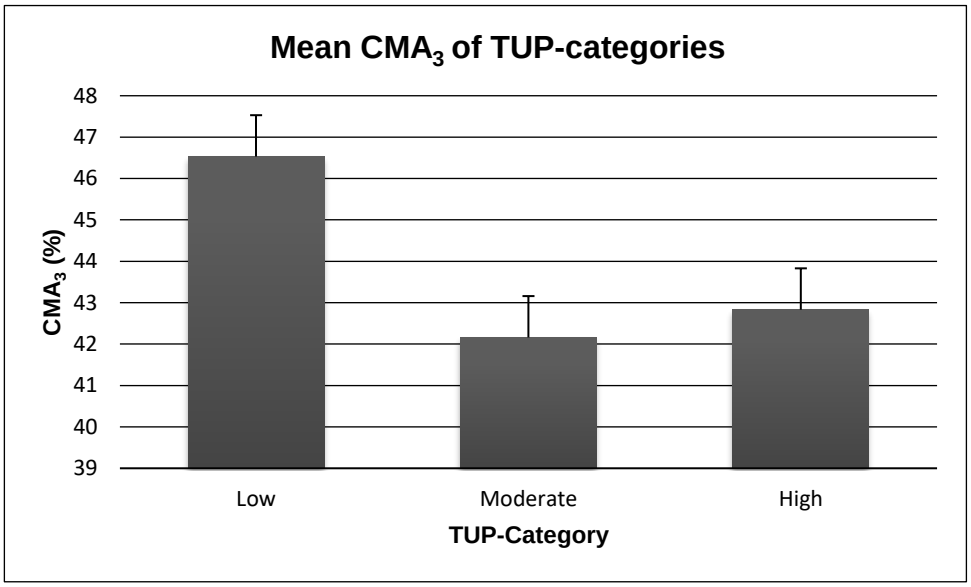


Figure 8: Bar graph of the mean CMA_3 of each TUP-category

3.1.4.3 Sperm viability

There was no significant difference (p=0.384) between the % mean of non-viable sperm of each group (Low: 81±10; Moderate: 72±22; High: 67±26) as can be seen in Figure 9.

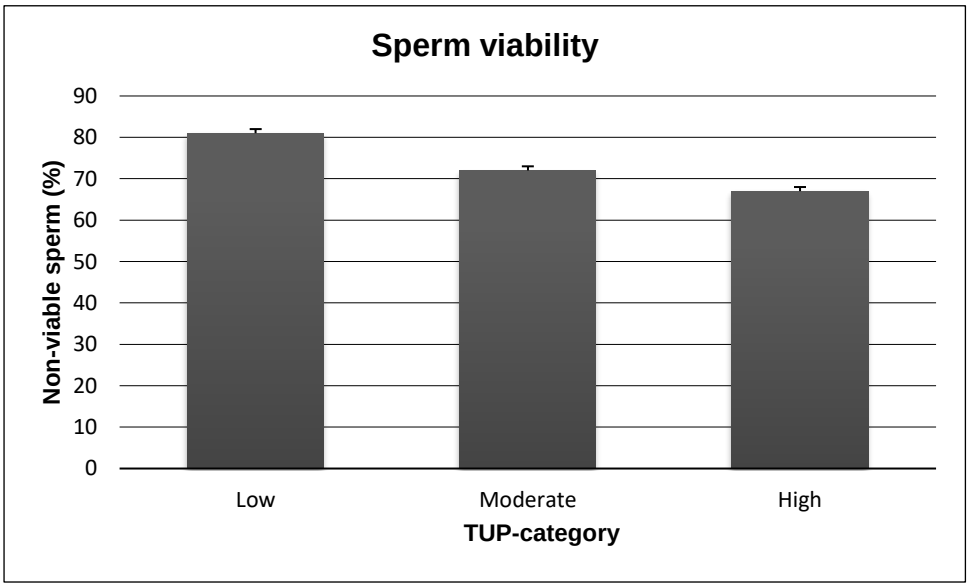


Figure 9: Bar graph depicting the means of non-viable sperm in each TUP-category

3.1.5 Alpha-amylase

As can be seen in Figure 10, no significant difference (p=0.391) was found with regards to Alpha-amylase between the TUP-categories (Low: 172.71±131.80; Moderate: 241.18±139.83; High: 182.19±140.13).

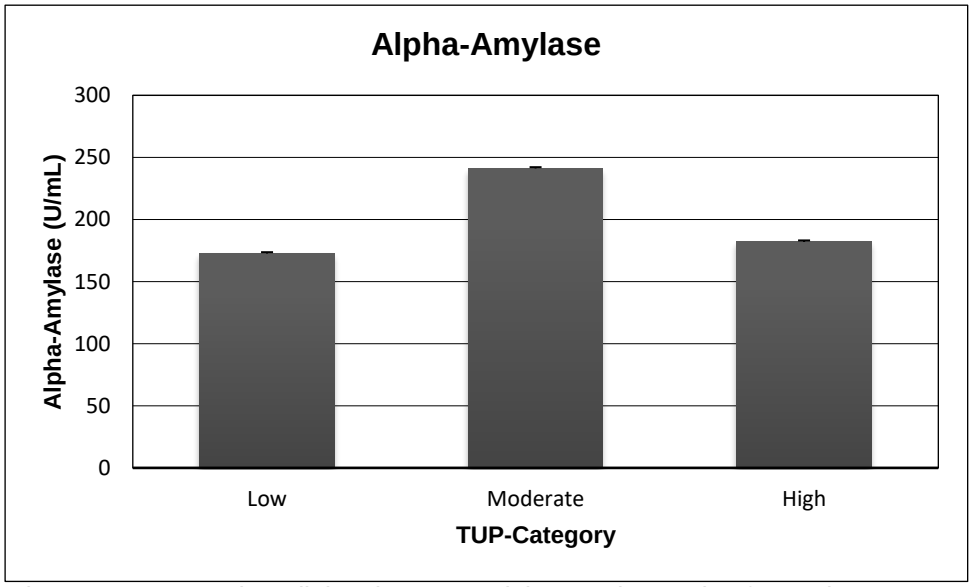


Figure 10: Bar graph outlining the mean Alpha-Amylase value for each TUP-category

3.2 Correlation between semen parameters, age, BMI and alpha amylase

The age, BMI and semen parameters were correlated to alpha-amylase using Spearman's rank correlation coefficient (rho). The results are presented in Table 4. No significant correlation was found between any of the parameters and alpha amylase. However, it was interesting to observe that volume showed a negative correlation with alpha-amylase which was nearing significance. Correlation figures can be found in Addendum IV.

Table 4: Spearman rank correlation of semen parameters, age and BMI with alpha-amylase level

Parameter	Spearman's* rank correlation coefficient	P-value
Age	0.104	0.4502

Continue...

BMI	0.119	0.399
Volume	-0.188	0.170
Viscosity	-0.021	0.877
Concentration	0.217	0.111
Motility	0.097	0.482
Morphology	0.186	0.208
CMA_3	0.112	0.422
Sperm Vitality	0.038	0.806
MAR	0.167	0.243
Oxidative Stress	0.046	0.753
PI	0.047	0.740

BMI= body mass index; CMA_3= chromomycin A_3; MAR= mixed agglutination reaction; PI= propidium iodide

* Spearman's rho

Chapter 4

Discussion and conclusion

Chronic stress is documented to have detrimental effects on the body and can lead to various health issues. It is a constant stress and persists over an extended period of time. It originates internally and is constantly retained and maintained by the individual. TUP stress describes the constant/chronic stress these individuals subject themselves to. There are three categories of TUP-stress in which a person can fall, namely Low, Moderate and High. The exact classification can be determined by the TUP online questionnaire.

Chronic stress has been investigated as a cause for unexplained infertility in numerous studies(39–41). Many studies have found an association between higher stress and poor

semen parameters. It is estimated that male reproductive factors are the cause of one-third of infertility cases (42,46).

Participants of the study were male patients attending Medfem Fertility Clinic seeking assistance with infertility troubles as part of couple infertility. This study attempted to establish if there is a possible relationship between various anthropometric measures (e.g. age, BMI) the stress marker alpha-amylase, semen parameters and the TUP test.

Relationship between age, BMI, semen parameter values, and Alpha-Amylase with TUP-categories

Of the 58 participants a total of 47 men successfully completed the online TUP-test. Fifteen (31.91%) of these men were classified to fall in the Low TUP-category, 12 (25.53%) were in the Moderate TUP-category and 20 (42.55%) were in the High TUP-category. Of interest is the fact that the majority of the men who participated in the study (65%), fell in either the Moderate or High TUP-categories. Therefore most of the participants reported themselves as being stressed. The risk of depression, anxiety and distress is high for infertile patients (76). Most studies relating to stress and infertility report high anxiety, depression and stress rates among the participants (77,78). In a study in Northern California, 352 women and 274 men attending Infertility clinics were assessed. It was determined that 56% of the women and 32% of the men reported significant symptoms of depression. Furthermore 76% of the women and 61% of the men reported significant symptoms of anxiety (77). A review by de Berardis et al. (79) concludes that 25 to 60% of infertile individuals report psychiatric symptoms and their levels of anxiety and depression are significantly higher than fertile controls. Chronic stress is an omnipresent and increasing cause for concern in the modern world (80). As discussed earlier, some personality types are more prone to stress (Type A, Moderate and High TUP) than others while some cope better than others. Some drive an inner stress which is a constant stress. Several studies have found that people that undergo

or seek fertility treatment are more stressed than people who do not suffer from infertility (6,81,82). Infertile couples experience a wide range of physical and emotional stress during their attempts to conceive a child (83). Providing a sufficient semen sample is only one of many concerns of a male patient seeking help for infertility. Sexual infertility stress among men is often linked with feelings of reduced masculinity and a perceived threat to the male identity (84). However, the relationship between stress and infertility may not have a clear cause and effect path. In a 2018 review article by Rooney and Domar, they state that while it is clear that infertility causes stress it is not clear whether stress causes infertility (76). The TUP questionnaire is designed to detect certain personality types, who subject themselves to chronic stress. Therefore, although infertility can cause stress, the questionnaire is able to exclude acute stressors as well as episodes of stress and focus on detecting chronic stress and certain personality types (Moderate and High TUP) who are prone to chronic stress. In this study it is therefore implied that men categorised within the Moderate and High TUP categories are chronically stressed and therefore this might lead to infertility.

Although the relationship between age and chronic stress was not one of the main aims of this study, it was interesting to see there was no significant difference in the mean age between the categories. There are no consistent data for chronic stress and age. Some studies report a decrease in chronic stress with increasing age while others show no difference between younger and older adults (85).

Men in the High TUP category had a significant higher BMI compared to those in the Low and Moderate groups. The World Health Organisation (WHO) categorises the BMI ranges as can be seen in Table 5 (86):

Table 5: Categories of BMI ranges

Category	BMI Range (kg/m^2)
Severe thinness	<16
Moderate thinness	16-17
Mild thinness	17-18.5
Normal	18.5-25
Overweight	25-30
Obese Class I	30-35
Obese Class II	35-40
Obese Class III	>40
BMI = body mass index; kg = kilogram; m^2 = height in meters squared	

All three TUP categories are therefore in the overweight BMI category with the High TUP category borderline Obese Class I. There are numerous studies to suggest that people tend to make poor health choices the more they are stressed (22,87–89). It is however not necessary to overt psychiatric problems to use comfort food for consolation when feeling down and out according to Dallmann et al. (90). In highly developed countries this is a well-recognised and general incidence, with an epidemic of obesity as a consequence. Stress can trigger relapse into obesity. Stress may also be a component of a wide range of obesity risk factors as identified by Keith et al. (91) and therefore could contribute to the increasing incidence of obesity. The high risk for weight gain, and particular accumulation of visceral adipose tissue, during chronic stress is thought to be related to activation of the HPA-axis, sympathoadrenal activity as well as a negative emotional consequence (negative mood causing 'emotional eating') (92). These findings may indicate that men within the High TUP category might be borderline obese Type I due to their high stress levels that may lead to obesity. Tomiyama et al. (93) also found significantly greater BMI in the high stress group

and reported greater eating after stressful events. A very early theory of obesity by Kaplan and Kaplan in 1957 proposed that obese people overeat when anxious and eating reduces this anxiety, thus eating in order to reduce anxiety may lead to compulsive overeating and obesity (94). A problem of using BMI as an indicator of obesity is that the BMI measurement does not differentiate between body lean mass and body fat mass. Gurevich et al. (95) studied the obesity prevalence and accuracy of BMI-defined obesity in Russian firefighters. Their concern was that because of the nature of their duties, firefighters might have greater muscle mass at any given BMI. They also examined the accuracy of BMI-based obesity compared to body fat percent (BF %) and waist circumference (WC). Comparing BMI to analogous BF% and WC categories resulted in low rates of false positives, (3 and 6%), but relatively high rates of false negatives (65 and 36%). They therefore concluded that the use of BMI for determining obesity is realistic but could be improved easily with WC if there are concerns about omitting firefighters with high abdominal adiposity even though they are not obese based on BMI (95). In a review article on BMI by Frank Nuttal (96), he states that BMI is a poor indicator of percent body fat and it does not capture information on the mass of fat in different body sites. However, according to Malina and Katzmarzyk (97), the BMI is a suitable and valid indicator of the risk of overweight and the presence of overweight in adolescents. They further state that the BMI is only a screening tool; adolescents identified as being at risk of overweight or as overweight should be referred for appropriate counselling. It has however been suggested that central obesity measures such as WC, waist to height ratio (WHtR) and waist to hip ratio (WHR) are superior to BMI in predicting visceral adiposity, mortality and cardiometabolic disease (98,99).

There was no significant relationship between any of the basic semen parameters and TUP categories. Although there are numerous studies that found a negative effect of chronic stress on semen analysis outcome, this was not seen in this study. The studies all utilised different questionnaires for determining stress. Questionnaires for the studies were chosen

based on the outcome in mind, accessibility and ease of use of the questionnaire. The TUP-stress questionnaire was developed and used for this study for the same reasons. This could have lead to the difference in outcome between this study and previous studies, due to different stress questionnaires used in each study. In 2010 the WHO revised the normal reference values for semen analysis which have lower cut-off compared to the 1999 WHO which was used for the interpretation of semen analysis up until that point. Consequently several men who, based on their semen analysis were previously diagnosed abnormal, have now become normal using new reference values. In a study by Alshahrani et al. (100) they found that 19% of the population studied changed classification from abnormal to normal when all normal semen parameters were considered. Of the total, 661.44% of the population changed its classification from abnormal to normal when at least one or more abnormal semen parameter was examined. Therefore, this study might have found an effect on semen parameters should the 1999 WHO reference values have been used. However, more recent studies have used the WHO 2010 reference values and did find a negative effect on semen parameters due to chronic stress (49,67). Interestingly in a study by Nouri et al. (101) in 2014 on the decline of semen quality during IVF and subjective male stress, they utilised the WHO 1999 reference values and did not find a decline in semen quality. Our findings are similar to those of Clarke and colleagues (83) in a study done in 1999. They tested the relationship between psychological stress and semen quality among in-vitro fertilization patients and found no correlations between perceived stressfulness and any of the sperm parameters measured pre-IVF. The semen analysis results of the current study were all pre-IVF evaluations and participants were first time patients of the clinic. Only one semen sample was obtained for each man. Subsequent samples produced for IVF purposes were not included in this study. It is possible that this could have lead to misclassification of patients due to within-subject variability that could lead to underestimating the potential

effects of stress. However, one semen sample is considered sufficient to assess semen quality (102).

ORP also known as redox potential, has recently been introduced as a single measure of oxidative stress (103). There was no significant difference between the TUP categories with regards to ORP. Two separate studies have identified an ORP value of > 1.36 mV/10^6/mL and 1.42 mV/10^6/mL as the cut-off value to differentiate infertile from fertile men (104,105). Both the Moderate (4.23±10.11) and the High (12.12±33.50) TUP categories are above the normal range stipulated. This implies a change in the balance between oxidants and antioxidants that favours the oxidants and signifies the presence of oxidative stress in the specimen. It is known that stress increases ROS in the male reproductive tract. Psychological stress can influence oxidative stress and male infertility (106). A main reason for infertility is believed to be excessive production of ROS or decreased antioxidant capacity in semen that causes oxidative stress conditions and ultimately a decrease in sperm motility, increase in sperm death, and fragmentation of DNA (107,108). Low physiological amounts of ROS are however required to trigger essential sperm functions such as capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion (109). Although no significant difference between TUP categories were observed, it is interesting that both the Moderate and High TUP categories are above the cut-off value. This could imply that oxidative stress does increase in semen samples due to an increase in chronic stress. Factors that might have influenced the outcome of this test are semen viscosity and sample size. It was observed that samples that had high viscosity did not have an accurate sORP reading (duplicate sensors with completely different results) or the MiOXSYS analyser simply read error. These results had to be excluded from the statistical analysis. In a clinic trial involving the MiOXSYS analyser, semen samples that have not liquefied within 60 minutes were excluded from the study (110). Furthermore, the clinical trial as well as a study by Homa et al. (111), both excluded semen samples with a concentration lower than 1 x 10^6/mL.

According to the authors ROS and sORP measurement are inaccurate and unreliable when the sperm concentration falls below this value. The current study did not exclude patients with a concentration lower than 1 x 10^6/mL. Two patients had a concentration of 0.1 x 10^6/mL and one had a concentration of 0.2 x 10^6/mL. Interestingly all three these participants were in the High TUP category.

Although there was no significant difference between the mean sperm chromatin packaging (CMA_3 analysis) values of the groups, it is interesting to note that all the mean values were higher than the cut-off value of 40%.

Propidium Iodide is a vital dye that tests for cell viability via dye-exclusion. There was no significant difference between the mean values of PI between the TUP-categories. The Low, Moderate and High TUP categories showed a percentage of 81%, 72% and 67% non-viable cells respectively. During the process of apoptosis, one of the earliest events is the disturbance of the asymmetry of the bilayer of the cell membrane. The most characteristic feature, but a relatively late phenomenon in the apoptosis-cascade, is activation of an endogenous endonuclease, which generates numerous DNA double strand breaks in chromatin (112). The TUNEL assay includes FITC staining for DNA fragmentation but was not reported in the results, as the % of cells affected were nearing 0%. This could imply that some of the cells could have been in the early stages of apoptosis (disturbance of the cell membrane) but DNA damage have not yet occurred.

There was no significant difference with regards to alpha-amylase between the TUP categories. The measurement of salivary alpha-amylase activity has been proposed to reflect stress-related changes in the autonomic nervous system (113,114). In a study performed by Nater et al. (115) in 2006 wherein they studied the determinants of diurnal course of salivary alpha-amylase, they found no association between stress scores and salivary alpha-amylase levels after awakening. Daily salivary alpha-amylase levels were

higher with more chronic stress and stress reactivity. All alpha-amylase samples in the current study were collected between 8am and 12am, yet no significant difference was observed between the TUP categories. Although there is no significant difference in alpha-amylase levels between the TUP categories it is interesting that the mean value of each TUP category is above the normal reference value of the test kit used (DRG Instruments, Marburg, Germany). This can possibly be due to increased stress levels in patients who are due for an infertility evaluation or the idea of taking part in a research study; however in the same study by Nater et al. (115) they found no impact on salivary alpha-amylase levels by perceived stress and momentary daily stress. Furthermore, alpha-amylase have been proven to be a reliable biomarker of chronic stress (30,32,116). The TUP questionnaire is used to identify personality types who subject themselves to chronic stress (Moderate and High types). However, this is but one of many personality and chronic stress questionnaires that can be used (117–122). It is possible that this specific questionnaire is not sensitive enough in identifying chronic stress and that some patients might be incorrectly categorised within a certain TUP group. There is also the possibility that patients do not answer the questions honestly, some might have been in a hurry and did not think the questions through before answering. In a study by Martin et al. (123) they attempted to establish how effective people are at faking on personality questionnaires and concluded that their findings indicates that the participants were able to falsify the normative scale in the desired direction (123).

Correlation between semen parameters, age, BMI and alpha amylase

No correlations between semen parameters, age, BMI and alpha amylase levels were observed. There was however a negative correlation between alpha-amylase and semen volume which did near significance. Giblin et al. (45) reported the same finding in their study entitled: Effects of stress and characteristic adaptability on semen quality in healthy men. In their study they also found a negative correlation with the semen measure of volume, and percent normal morphologic forms. A more recent study among 1215 Danish men also found

an association between stress scores above the reference level and significantly lower semen volume (67). It is not clear what mechanism is involved in the negative correlation between chronic stress and semen volume. The volume of the ejaculate is contributed mainly by the seminal vesicles and prostate gland with a small amount from the bulbourethral glands and epididymides. The ANS is often referred to as the “involuntary” nervous system as the control of the organs is not conscious. Some of the key visceral processes also regulated by the ANS include glandular secretions and reproduction related activities (24). The sympathetic and parasympathetic systems are the two subdivisions of the ANS (1,23,24). These two sub-systems counteract each other (1). The sympathetic system plays a vital role in the “fight or flight” responses which include: increases in heart rate, blood pressure, ventilation, skeletal muscle perfusion and dilation of the pupils. The parasympathetic system does exactly the opposite of the sympathetic system. Is it possible that elevated chronic stress can keep the body in a constant state of ‘fight or flight’? Therefore it is speculated that the parasympathetic system attempts to counteract the sympathetic system and that the constitutive increased parasympathetic output might impact accessory gland secretion and be responsible for the decreased semen volume.

Conclusion

To our knowledge this is the first study to compare Time urgency perfectionism stress with salivary alpha-amylase levels, age, BMI and semen parameters. The study was unsuccessful in proving a significant relationship between the TUP categories, age and semen parameters. The High TUP category did show a significantly higher BMI compared to the Low and Moderate TUP groups. This finding confirms that chronic stress may lead to obesity. Although there was no significant difference between the TUP categories with regards to sORP values, the Moderate and High categories were both greater than the normal value for sORP in semen. This implies that chronic stress can lead to elevated levels

of oxidative stress in semen which has been proven to compromise semen quality and as a result lower male fertility.

No relationship was found between TUP categories and alpha-amylase levels. Although both are used to detect chronic stress, the TUP questionnaire is used to detect personality types who are prone to chronic stress, whilst salivary alpha-amylase is a biomarker for chronic stress and functions in a completely different way. It is possible that whilst both can be used to detect chronic stress it is not advised to attempt to establish a relationship between the two as the mechanisms of both are very different.

The TUP questionnaire is a useful and easy-to-use chronic stress indicator which can be used to identify prone-to-stress personality types. Once identified, these patients can be assisted with stress management by means of a 10 week stress management course available from www.timeurgency.com. This might be helpful to obese patients who overeat due to high levels of chronic stress as well as patients who would like to reduce their chronic stress levels to possibly improve their fertility.

Study limitations

A shortcoming of this study is that only one type of obesity parameter (i.e BMI) was utilised. It has been suggested that central obesity measures such as WC, WHtR and WHR are superior to BMI in predicting visceral adiposity, mortality and cardiometabolic disease.

In 2010 the WHO revised the normal reference values for semen analysis. The current cut-off reference values, which were used for the interpretation of semen analysis in this study, are much lower compared to the 1999 WHO guidelines. Consequently several men, who were previously diagnosed as abnormal, based on their semen analysis, are now classified as normal using new reference values. Therefore, if stricter criteria were employed this study

might have found an effect on semen parameters should the 1999 WHO reference values have been used as more men might have fallen below the reference values.

There are several chronic stress questionnaires which are used in a number of different studies to detect chronic stress. Therefore the TUP-stress questionnaire might not be best suited to correlate with salivary alpha-amylase as there might be another chronic stress questionnaire which will be able to show a correlation between a perceived stress questionnaire and a salivary biomarker. This however requires further investigation.

If the Moderate and High TUP categories were pooled and compared to the Low TUP category it is possible that statistically significant difference might have been observed. However this was not done and can be regarded as a shortcoming of this study.

Another limitation to this study was the small sample size. Various male patients were approached to participate, however several stated that they did not have enough time on the day of visiting the clinic to partake in the study. Others did show interest to participate but did not complete the TUP questionnaires when asked.

Many men who participated in this study suffered from infertility. Therefore, it is possible that even if they were not stressed, their spermatozoa might already be compromised due to other factors, thereby making it difficult to differentiate between stress induced and other factor induced sperm parameter declines.

References

1. Kendall-tackett K. The psychoneuroimmunology of chronic disease. Exploring the links between inflammation, stress, and illness. J Nerv Ment Dis [Internet]. 2010;198(3):235. Available from: http://www.amazon.com/dp/143380476X
2. Kiecolt-Glaser JK, McGuire L, Robles TF, Glaser R. Psychoneuroimmunology: Psychological influences on immune function and health. J Consult Clin Psychol [Internet]. 2002 [cited 2018 Aug 15];70(3):537–47. Available from: http://psycnet.apa.org.ez.sun.ac.za/fulltext/2002-01415-007.pdf
3. Herbert TB, Cohen S. Stress and immunity in humans: A meta-analytic review. Psychosom Med. 1993;
4. Glaser R, Kiecolt-Glaser JK, Malarkey WB. Handbook of human stress and immunity [Internet]. Handbook of human stress and immunity. Elsevier Science; 1994 [cited 2018 Aug 15]. 321–339 p. Available from: https://books.google.co.za/books/handbook+of+human+stress+and+immunity
5. Ratanasiripong P, Park JF, Ratanasiripong N, Kathalae D. Stress and anxiety management in nursing students: Biofeedback and mindfulness meditation. J Nurs Educ. 2015;
6. Janevic T, Kahn LG, Landsbergis P, Cirillo PM, Cohn BA, Liu X, et al. Effects of work and life stress on semen quality. Fertil Steril [Internet]. 2014;102(2):530–8. Available from: http://dx.doi.org/10.1016/j.fertnstert.2014.04.021
7. Deary IJ. Measuring stress: A guide for health and social scientists. J Psychosom Res. 1996;41(2):186.
8. Rodrigues MJ, Rodrigues FA. The Psychological Management of Infertility. In: Handbook of Fertility: Nutrition, Diet, Lifestyle and Reproductive Health [Internet]. Academic Press; 2015 [cited 2019 Jun 5]. p. 293–301. Available from: https://www.sciencedirect.com/science/article/pii/B9780128008720000275
9. personality | Definition, Types, Nature, & Facts | Britannica.com [Internet]. [cited 2019

Nov 5]. Available from: https://www.britannica.com/topic/personality

10. Asendorpf JB. The puzzle of personality types. Eur J Pers. 2002;16(S1):S1–5.
11. Nabi H, Kiviméki M, Zins M, Elovainio M, Consoli SM, Cordier S, et al. Does personality predict mortality: Results from the GAZEL French prospective cohort study. Int J Epidemiol. 2008 Apr;37(2):386–96.
12. Friedman M, Rosenman RH. Type A behavior and your heart [Internet]. 1st ed. New York: Fawcett Crest; 1982 [cited 2018 Aug 15]. Available from: https://books.google.co.za/books/type+A+behaviour+and+your+heart
13. Miller LH, Smith AD. The Stress Solution: An Action Plan to Manage the Stress in Your Life [Internet]. 1st ed. Pocket Books. Pocket Books; 1993 [cited 2018 Aug 15]. 342 p. Available from: https://books.google.co.za/books/the+stress+solution:+an+action+plan+to+manage+stress+in+your+life
14. Rodrigues A, Wolff M, Wolff E. Changing health profiles in women's demographic and behavioral variables. In: Rodrigues A, Wolff E, Wolff M, editors. Changing health profiles in women's demographic and behavioral variables. Grenada Spain: Congress of the European Association for Behavioral and Cognitive Therapies; 2000.
15. Rodrigues A, Wolff E, Wolff M. Stress, health and women: From Type-A to time urgency perfectionism. In: Stress, health and women: From Type-A to time urgency perfectionism. Grenada Spain: Congress of the European Association for Behavioral and Cognitive Therapies; 2000.
16. Stegmann BJ. Other nonstress influences can alter salivary α-amylase activity. Fertil Steril [Internet]. 2011 Jun [cited 2018 Aug 15];95(7):2190–1. Available from: http://linkinghub.elsevier.com/retrieve/pii/S001502821100584X
17. Vitlic A, Lord JM, Phillips AC. Stress, ageing and their influence on functional, cellular and molecular aspects of the immune system. Age (Omaha). 2014;36(3):1169–85.

18. McEwen BS. Seminars in medicine of the Beth Israel Deaconess Medical Center: Protective and damaging effects of stress mediators. Flier JS, Underhill LH, editors. N Engl J Med [Internet]. 1998 Jan 15 [cited 2018 Aug 15];338(3):171–9. Available from: http://www.nejm.org/doi/10.1056/NEJM199801153380307

19. Antoni MH, Lutgendorf SK, Cole SW, Dhabhar FS, Sephton SE, McDonald PG, et al. The influence of bio-behavioural factors on tumour biology: Pathways and mechanisms [Internet]. Vol. 6, Nature Reviews Cancer. 2006 [cited 2018 Aug 15]. p. 240–8. Available from: http://go.galegroup.com.ez.sun.ac.za/ps/i.do?ty=as&v=2.1&u=27uos&it=DIourl&s=RELEVANCE&p=AONE&qt=SP~240~~IU~3~~SN~1474-175X~~VO~6&lm=DA~120060000&sw=w

20. Sinha R, Jastreboff AM. Stress as a common risk factor for obesity and addiction. Biological Psychiatry. 2013.

21. Roberts C, Troop N, Connan F, Treasure J, Campbell IC. The effects of stress on body weight: Biological and psychological predictors of change in BMI. Obesity. 2007;

22. Groesz LM, McCoy S, Carl J, Saslow L, Stewart J, Adler N, et al. What is eating you? Stress and the drive to eat. Appetite. 2012;

23. Jellinger KA. The Central Nervous System -Structure and Function. Eur J Neurol [Internet]. 2004 [cited 2018 Aug 15];11(7):503–503. Available from: https://books.google.co.za/books/the+central+nervous+system:+structure+and+function

24. Crespo L, Wecker L, Dunaway G, Faingold C, Watts S. BRODY'S HUMAN PHARMACOLOGY. Mosby/Elsevier; 2009.

25. Brook RD, Julius S. Autonomic imbalance, hypertension, and cardiovascular risk. Am J Hypertens [Internet]. 2000 [cited 2018 Aug 17];13(6 Pt 2):112S-122S. Available from: https://academic.oup.com/ajh/article-abstract/13/S4/112S/186277

26. Thayer JF, Friedman BH. A neurovisceral integration model of health disparities in aging. Anderson N, Bulatao R, Cohen B, editors. Crit Perspect racial Ethn Differ Heal late life [Internet]. 1st ed. 2004 [cited 2019 Jun 7];567–603. Available from: https://www.ncbi.nlm.nih.gov/books/NBK25521/

27. Lynch CD, Sundaram R, Maisog JM, Sweeney AM, Buck Louis GM. Preconception stress increases the risk of infertility: Results from a couple-based prospective cohort study-the LIFE study. Hum Reprod. 2014;29(5):1067–75.

28. Nater UM, Rohleder N, Gaab J, Berger S, Jud A, Kirschbaum C, et al. Human salivary alpha-amylase reactivity in a psychosocial stress paradigm. Int J Psychophysiol. 2005;

29. Nater UM, La Marca R, Florin L, Moses A, Langhans W, Koller MM, et al. Stress-induced changes in human salivary alpha-amylase activity - Associations with adrenergic activity. Psychoneuroendocrinology. 2006;

30. Rohleder N, Nater UM. Determinants of salivary α-amylase in humans and methodological considerations. Psychoneuroendocrinology. 2009.

31. Thayer JF, Sternberg E. Beyond heart rate variability: Vagal regulation of allostatic systems. Ann N Y Acad Sci. 2006;1088:361–72.

32. Vineetha R, Pai KM, Vengal M, Gopalakrishna K, Narayanakurup D. Usefulness of salivary alpha amylase as a biomarker of chronic stress and stress related oral mucosal changes - a pilot study. J Clin Exp Dent. 2014;6(2).

33. Glei DA, Goldman N, Chuang YL, Weinstein M. Do chronic stressors lead to physiological dysregulation? Testing the theory of allostatic load. Psychosom Med. 2007;

34. Gunnar M, Quevedo K. The Neurobiology of Stress and Development. Annu Rev Psychol. 2007;

35. Tsigos C, Chrousos GP. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. In: Journal of Psychosomatic Research. 2002.

36. Guilliams TG, Edwards L. Chronic Stress and the HPA Axis: Clinical Assessment and Therapeutic Considerations. Stand. 2010;9:1–12.

37. Marin MF, Pilgrim K, Lupien SJ. Modulatory effects of stress on reactivated emotional memories. Psychoneuroendocrinology. 2010;

38. McEwen BS, Wingfield JC. The concept of allostasis in biology and biomedicine. Horm Behav. 2003;

39. Brkovich AM, Fisher WA. Psychological distress and infertility: Forty years of research. J Psychosom Obstet Gynaecol [Internet]. 1998 Jan 7 [cited 2018 Aug 17];19(4):218–28. Available from: http://www.tandfonline.com/doi/full/10.3109/01674829809025700

40. Greil AL. Infertility and psychological distress: A critical review of the literature. Soc Sci Med. 1997;45(11):1679–704.

41. Sheiner EK, Sheiner E, Carel R, Potashnik G, Shoham-Vardi I. Potential association between male infertility and occupational psychological stress. J Occup Environ Med. 2002;

42. Fenster L, Katz DF, Wyrobek AJ, Pieper C, Rempel DM, Oman D, et al. Effects of psychological stress on human semen quality. J Androl [Internet]. 1997 Nov [cited 2018 Aug 17];18(2):194–202. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022534701624601

43. Hjollund NHI, Bonde JPE, Henriksen TB, Giwercman A, Olsen J. Job strain and male fertility. Epidemiology. 2004;15(1):114–7.

44. Hjollund NHI, Bonde JPE, Henriksen TB, Giwercman A, Olsen J. Reproductive effects of male psychologic stress. Epidemiology. 2004;

45. Giblin PT, Poland ML, Moghissi KS, Ager JW, Olson JM. Effects of stress and characteristic adaptability on semen quality in healthy men. Vol. 49, Fertility and Sterility. 1988. p. 127–32.

46. Auger J, Eustache F, Andersen AG, Irvine DS, Jørgensen N, Skakkebæk NE, et al.

Sperm morphological defects related to environment, lifestyle and medical history of 1001 male partners of pregnant women from four European cities. Hum Reprod. 2001;

47. Gollenberg AL, Liu F, Brazil C, Drobnis EZ, Guzick D, Overstreet JW, et al. Semen quality in fertile men in relation to psychosocial stress. Fertil Steril [Internet]. 2010;93(4):1104–11. Available from: http://dx.doi.org/10.1016/j.fertnstert.2008.12.018

48. Li Y, Li Y, Zhou N, Han X, Ma M, Li L, et al. Socio-psycho-behavioural factors associated with male semen quality in China: Results from 1346 healthy men in Chongqing. J Fam Plan Reprod Heal Care. 2013;39(2):102–10.

49. Collodel G, Moretti E, Fontani V, Rinaldi S, Aravagli L, Saragò G, et al. Effect of emotional stress on sperm quality. Indian J Med Res [Internet]. 2008 [cited 2018 Aug 17];128(3):254–61. Available from: https://www.researchgate.net/publication/23567279

50. Agarwal A, Sabanegh Jr. ES. A Complete Guide to Lifestyle and Environmental Factors [Internet]. du Plessis SS, Agarwal A, Sabanegh ESJ, editors. New York: Springer; 2014 [cited 2019 Jul 13]. 19–27 p. Available from: https://books.google.co.za/books/male+infertility:+a+complete+guide+to+lifestyle+and+environmental+factors

51. Rivier C, Rivest S. Effect of Stress on the Activity of the Hypothalamic-Pituitary-Gonadal Axis: Peripheral and Central Mechanisms1. Biol Reprod [Internet]. 1991;45(4):523–32. Available from: https://academic.oup.com/biolreprod/article-lookup/doi/10.1095/biolreprod45.4.523

52. Jóźków P, Mędraś M. Psychological stress and the function of male gonads [Internet]. Vol. 63, Endokrynologia Polska. 2012 [cited 2018 Aug 17]. p. 44–9. Available from: https://journals.viamedica.pl/endokrynologia_polska/article/view/25203

53. Eskiocak S, Gozen AS, Kilic AS, Molla S. Association between mental stress & some

antioxidant enzymes of seminal plasma. Indian J Med Res. 2005;122(6):491–6.

54. Agarwal A, Saleh RA. Role of oxidants in male infertility: Rationale, significance, and treatment. Urologic Clinics of North America. 2002.

55. Sikka SC. Relative impact of oxidative stress on male reproductive function. Curr Med Chem. 2001;

56. Tremellen K. Oxidative stress and male infertility--a clinical perspective. Hum Reprod Update. 2008;

57. Steven Ward W, Coffey DS. DNA Packaging and Organization in Mammalian Spermatozoa: Comparison with Somatic Cell. Biol Reprod [Internet]. 1991 Apr 1 [cited 2018 Aug 17];44(4):569–74. Available from: https://academic.oup.com/biolreprod/article/2762885/DNA

58. Giwercman A, Richthoff J, Hjøllund H, Bonde JP, Jepson K, Frohm B, et al. Correlation between sperm motility and sperm chromatin structure assay parameters. Fertil Steril. 2003;

59. Moskovtsev SI, Willis J, Azad A, Mullen JBM. Sperm DNA integrity: Correlation with sperm plasma membrane integrity in semen evaluated for male infertility. Arch Androl [Internet]. 2005 [cited 2018 Aug 17];51(1):33–40. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15764415

60. Friedman GD, Ury HK, Klatsky AL, Siegelaub AB. A psychological questionnaire predictive of myocardial infarction: results from the Kaiser Permanente epidemiologic study of myocardial infarction. Psychosom Med. 1974;

61. Lynch CD, Sundaram R, Buck Louis GM, Lum KJ, Pyper C. Are increased levels of self-reported psychosocial stress, anxiety, and depression associated with fecundity? Fertil Steril. 2012;

62. Matud MP, Bethencourt JM, Ibáñez I. Gender differences in psychological distress in Spain. Int J Soc Psychiatry. 2015;61(6):560–8.

63. Albert MA, Durazo EM, Slopen N, Zaslavsky AM, Buring JE, Silva T, et al. Cumulative psychological stress and cardiovascular disease risk in middle aged and older women: Rationale, design, and baseline characteristics. Am Heart J. 2017;192:1–12.

64. Hills P, Argyle M. The Oxford Happiness Questionnaire: A compact scale for the measurement of psychological well-being. Pers Individ Dif. 2002;33(7):1073–82.

65. Nübling M, Stößel U, Hasselhorn H-M, Michaelis M, Hofmann F. Measuring psychological stress and strain at work - Evaluation of the COPSOQ Questionnaire in Germany. Psychosoc Med. 2006;

66. Abu-Ghazaleh SB, Sonbol HN, Rajab LD. A longitudinal study of psychological stress among undergraduate dental students at the University of Jordan. BMC Med Educ. 2016;

67. Nordkap L, Jensen TK, Hansen ÅM, Lassen TH, Bang AK, Joensen UN, et al. Psychological stress and testicular function: A cross-sectional study of 1,215 Danish men. Fertil Steril. 2016;

68. Hackett RA, Steptoe A. Type 2 diabetes mellitus and psychological stress-a modifiable risk factor. Nature Reviews Endocrinology. 2017.

69. Rodrigues M. Time urgency perfectionism stress [Internet]. [cited 2016 Mar 28]. Available from: www.timeurgency.com

70. Schommer NC, Hellhammer DH, Kirschbaum C. Dissociation between reactivity of the hypothalamus-pituitary-adrenal axis and the sympathetic-adrenal-medullary system to repeated psychosocial stress. Psychosom Med. 2003;

71. Proctor GB, Carpenter GH. Regulation of salivary gland function by autonomic nerves. Autonomic Neuroscience: Basic and Clinical. 2007.

72. Bosch JA, De Geus EJC, Veerman ECI, Hoogstraten J, Nieuw Amerongen A V. Innate secretory immunity in response to laboratory stressors that evoke distinct patterns of cardiac autonomic activity. Psychosom Med. 2003;

73. Harville EW, Savitz DA, Dole N, Herring AH, Thorp JM. Stress Questionnaires and Stress Biomarkers during Pregnancy. J Women's Heal. 2009;

74. My virtual medical centre [Internet]. 2015 [cited 2016 Sep 7]. Available from: www.myvmc.com/diseases/infertility

75. World Health Organization. WHO laboratory manual for the Examination and processing of human semen. World Health Organization. 2010.

76. Rooney KL, Domar AD. The relationship between stress and infertility. Dialogues Clin Neurosci [Internet]. 2018 [cited 2019 Nov 18];20(1):41–7. Available from: www.dialogues-cns.org

77. Pasch LA, Holley SR, Bleil ME, Shehab D, Katz PP, Adler NE. Addressing the needs of fertility treatment patients and their partners: are they informed of and do they receive mental health services? Fertil Steril. 2016;

78. Lakatos E, Szigeti JF, Ujma PP, Sexty R, Balog P. Anxiety and depression among infertile women: A cross-sectional survey from Hungary. BMC Womens Health. 2017;

79. De Berardis D, Mazza M, Marini S, Del Nibletto L, Serroni N, Pino MC, et al. Psychopathology, emotional aspects and psychological counselling in infertility: A review. Clin Ter. 2014;

80. Ceccato S, Kettner SE, Kudielka BM, Schwieren C, Voss A. Social preferences under chronic stress. PLoS One [Internet]. 2018 [cited 2019 Nov 18];13(7). Available from: https://doi.org/10.1371/journal.pone.0199528

81. Peterson BD, Newton CR, Feingold T. Anxiety and sexual stress in men and women undergoing infertility treatment. Fertil Steril. 2007;88(4):911–4.

82. Negro-Vilar A. Stress and other environmental factors affecting fertility in men and women: Overview. Environ Health Perspect. 1993;101(SUPPL. 2):59–64.

83. Clarke RN, Klock SC, Geoghegan A, Travassos DE. Relationship between psychological stress and semen quality among in-vitro fertilization patients.

1999;14(3):753–8.

84. Elliott S. The relationship between fertility issues and sexual problems in men. Vol. 7, Canadian Journal of Human Sexuality. 1998. p. 295–303.

85. Osmanovic-Thunström A, Mossello E, Åkerstedt T, Fratiglioni L, Wang HX. Do levels of perceived stress increase with increasing age after age 65? A population-based study. Age Ageing [Internet]. 2015 [cited 2019 Nov 19];44(5):828–34. Available from: https://academic.oup.com/ageing/article-abstract/44/5/828/52059

86. WHO | Obesity [Internet]. [cited 2019 Nov 21]. Available from: https://www.who.int/topics/obesity/en/

87. Dallman MF, Pecoraro N, Akana SF, La Fleur SE, Gomez F, Houshyar H, et al. Chronic stress and obesity: A new view of “comfort food.” Proc Natl Acad Sci U S A [Internet]. 2003 [cited 2019 Nov 18];100(20):11696–701. Available from: www.pnas.orgcgidoi10.1073pnas.1934666100

88. Chao A, Grilo CM, White MA, Sinha R. Food cravings mediate the relationship between chronic stress and body mass index. J Health Psychol. 2015;20(6):721–9.

89. Cuevas AG, Chen R, Thurber KA, Slopen N, Williams DR. Psychosocial Stress and Overweight and Obesity: Findings from the Chicago Community Adult Health Study. Ann Behav Med. 2019 Oct 7;53(11):964–74.

90. Dallman MF, Pecoraro N, Akana SF, La Fleur SE, Gomez F, Houshyar H, et al. Chronic stress and obesity: A new view of “comfort food.” Proc Natl Acad Sci U S A. 2003;100(20):11696–701.

91. Keith SW, Redden DT, Katzmarzyk PT, Boggiano MM, Hanlon EC, Benca RM, et al. Putative contributors to the secular increase in obesity: Exploring the roads less traveled [Internet]. Vol. 30, International Journal of Obesity. 2006 [cited 2019 Nov 19]. p. 1585–94. Available from: www.nature.com/ijo

92. Geiker NRW, Astrup A, Hjorth MF, Sjödin A, Pijls L, Markus CR. Does stress influence

sleep patterns, food intake, weight gain, abdominal obesity and weight loss interventions and vice versa? [Internet]. Vol. 19, Obesity Reviews. 2018 [cited 2019 Nov 19]. p. 81–97. Available from: https://www.researchgate.net/publication/319338035

93. Tomiyama AJ, Dallman MF, Epel ES. Comfort food is comforting to those most stressed: Evidence of the chronic stress response network in high stress women. Psychoneuroendocrinology. 2011;

94. KAPLAN HI, KAPLAN HS. The psychosomatic concept of obesity. J Nerv Ment Dis. 1957;

95. Gurevich KG, Poston WSC, Anders B, Ivkina MA, Archangelskaya A, Jitnarin N, et al. Obesity prevalence and accuracy of BMI-defined obesity in Russian firefighters. Occup Med (Chic Ill). 2017;

96. Nuttall FQ. Body mass index: Obesity, BMI, and health: A critical review. Nutrition Today. 2015.

97. Malina RM, Katzmarzyk PT. Validity of the body mass index as an indicator of the risk and presence of overweight in adolescents. Am J Clin Nutr. 1999;

98. Song X, Jousilahti P, Stehouwer CDA, Söderberg S, Onat A, Laatikainen T, et al. Comparison of various surrogate obesity indicators as predictors of cardiovascular mortality in four European populations. Eur J Clin Nutr. 2013;

99. Adab P, Pallan M, Whincup PH. Is BMI the best measure of obesity? BMJ (Online). 2018.

100. Alshahrani S, Aldossari K, Al-Zahrani J, Gabr AH, Henkel R, Ahmad G. Interpretation of semen analysis using WHO 1999 and WHO 2010 reference values: Abnormal becoming normal. Andrologia [Internet]. 2018 Mar [cited 2019 Nov 26];50(2). Available from: http://doi.wiley.com/10.1111/and.12838

101. Nouri K, Litschauer B, Sator M, Tiringer D, Ott J, Walch K, et al. Decline of semen

quality during IVF is not associated with subjective male stress. Asian J Androl. 2014;

102. Stokes-Riner A, Thurston SW, Brazil C, Guzick D, Liu F, Overstreet JW, et al. One Semen Sample or 2? Insights From a Study of Fertile Men. J Androl [Internet]. 2007 May 23 [cited 2019 Nov 19];28(5):638–43. Available from: http://doi.wiley.com/10.2164/jandrol.107.002741

103. Panner Selvam MK, Henkel R, Sharma R, Agarwal A. Calibration of redox potential in sperm wash media and evaluation of oxidation–reduction potential values in various assisted reproductive technology culture media using MiOXSYS system. Andrology [Internet]. 2018 Mar [cited 2019 Nov 19];6(2):293–300. Available from: http://doi.wiley.com/10.1111/andr.12461

104. Agarwal A, Roychoudhury S, Bjugstad KB, Cho CL. Oxidation-reduction potential of semen: What is its role in the treatment of male infertility? [Internet]. Vol. 8, Therapeutic Advances in Urology. 2016 [cited 2019 Nov 20]. p. 302–18. Available from: http://tau.sagepub.com

105. Agarwal A, Wang SM. Clinical Relevance of Oxidation-Reduction Potential in the Evaluation of Male Infertility. Urology [Internet]. 2017 [cited 2019 Nov 19];104:84–9. Available from: http://dx.doi.org/10.1016/j.urology.2017.02.016

106. Bisht S, Faiq M, Tolahunase M, Dada R. Oxidative stress and male infertility [Internet]. Vol. 14, Nature Reviews Urology. 2017 [cited 2019 Nov 20]. p. 470–85. Available from: www.nature.com/nrurol

107. Agarwal A, Sharma R, Roychoudhury S, Du Plessis S, Sabanegh E. MiOXSYS: a novel method of measuring oxidation reduction potential in semen and seminal plasma. Fertil Steril [Internet]. 2016 [cited 2019 Nov 19];106(3):566-573.e10. Available from: http://dx.doi.org/10.1016/j.fertnstert.2016.05.013

108. Agarwal A, Roychoudhury S, Bjugstad KB, Cho CL. Oxidation-reduction potential of semen: What is its role in the treatment of male infertility? Vol. 8, Therapeutic

Advances in Urology. 2016. p. 302–18.

109. Panner Selvam MK, Henkel R, Sharma R, Agarwal A. Calibration of redox potential in sperm wash media and evaluation of oxidation–reduction potential values in various assisted reproductive technology culture media using MiOXSYS system. Andrology. 2018 Mar;6(2):293–300.

110. A Clinical Evaluation of Semen Quality Using the MiOXSYS™ System 2.0 - Full Text View - ClinicalTrials.gov [Internet]. [cited 2019 Nov 21]. Available from: https://clinicaltrials.gov/ct2/show/NCT02839447

111. Homa ST, Vassiliou AM, Stone J, Killeen AP, Dawkins A, Xie J, et al. A comparison between two assays for measuring seminal oxidative stress and their relationship with sperm DNA fragmentation and semen parameters. Genes (Basel). 2019;

112. Oosterhuis GJE, Vermes I. Apoptosis in human ejaculated spermatozoa. J Biol Regul Homeost Agents [Internet]. 2004 [cited 2019 Nov 21];18(2):115–9. Available from: https://www.researchgate.net/publication/8244969

113. Nater UM, Rohleder N, Schlotz W, Ehlert U, Kirschbaum C. Determinants of the diurnal course of salivary alpha-amylase. Psychoneuroendocrinology. 2007;

114. Wolf JM, Nicholls E, Chen E. Chronic stress, salivary cortisol, and α-amylase in children with asthma and healthy children. Biol Psychol. 2008;

115. Nater UM, Rohleder N, Schlotz W, Ehlert U, Kirschbaum C. Determinants of the diurnal course of salivary alpha-amylase. Psychoneuroendocrinology. 2007;32(4):392–401.

116. Nater UM, Rohleder N, Gaab J, Berger S, Jud A, Kirschbaum C, et al. Human salivary alpha-amylase reactivity in a psychosocial stress paradigm. Int J Psychophysiol. 2005;55(3):333–42.

117. McManus IC, Keeling A, Paice E. Stress, burnout and doctors' attitudes to work are determined by personality and learning style: A twelve year longitudinal study of UK

medical graduates. BMC Med. 2004;

118. McCrae RR, John OP. An Introduction to the Five-Factor Model and Its Applications. J Pers. 1992;

119. Ebstrup JF, Eplov LF, Pisinger C, Jørgensen T. Association between the five factor personality traits and perceived stress: Is the effect mediated by general self-efficacy? Anxiety, Stress Coping. 2011;

120. Cohen S, Tyrrell DAJ, Smith AP. Negative Life Events, Perceived Stress, Negative Affect, and Susceptibility to the Common Cold. J Pers Soc Psychol. 1993;

121. McDonald JD. Measuring personality constructs: The advantages and disadvantages of self-reports, informant reports and behavioural assessments. Enquire. 2008;

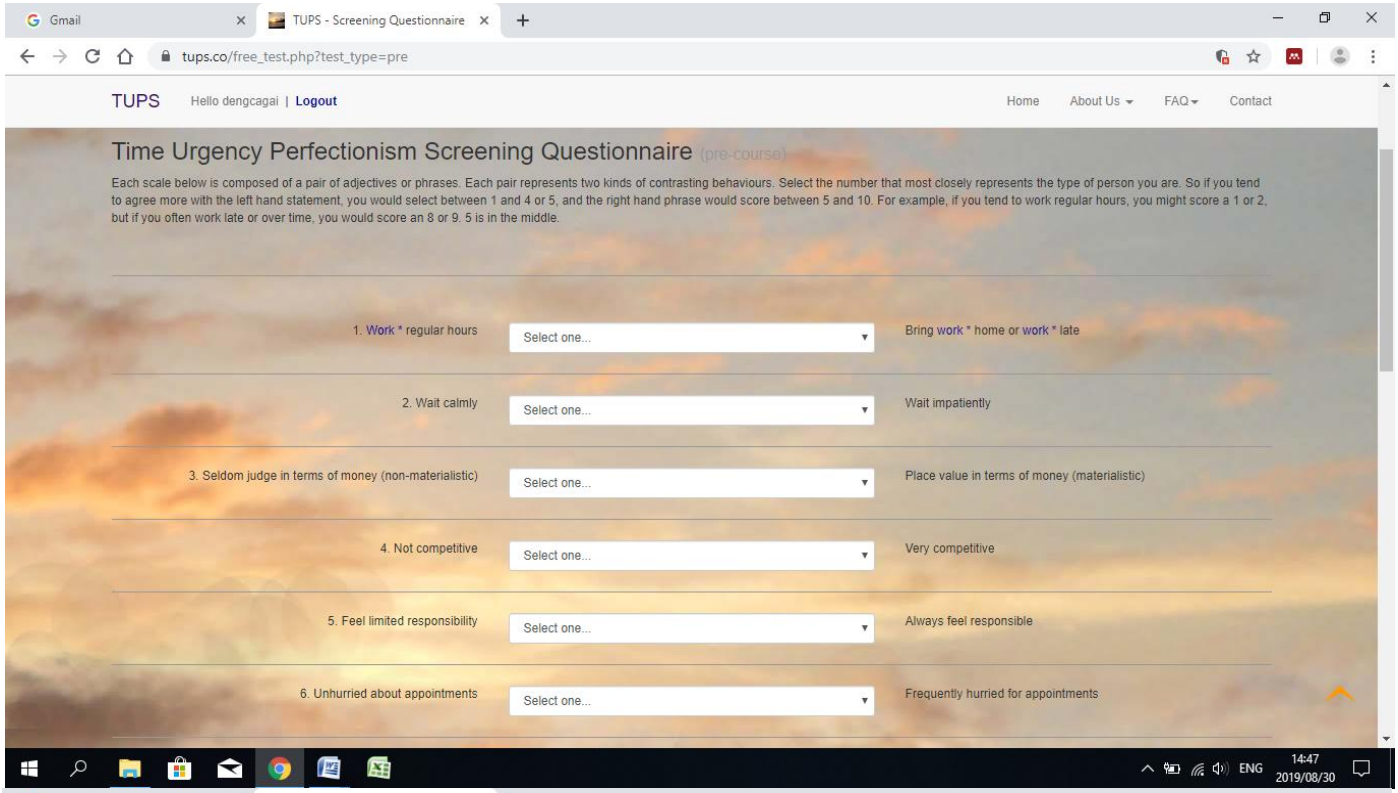
122. Lawrence JW, Fauerbach JA. Personality, coping, chronic stress, social support and PTSD symptoms among adult burn survivors: A path analysis. J Burn Care Rehabil. 2003;

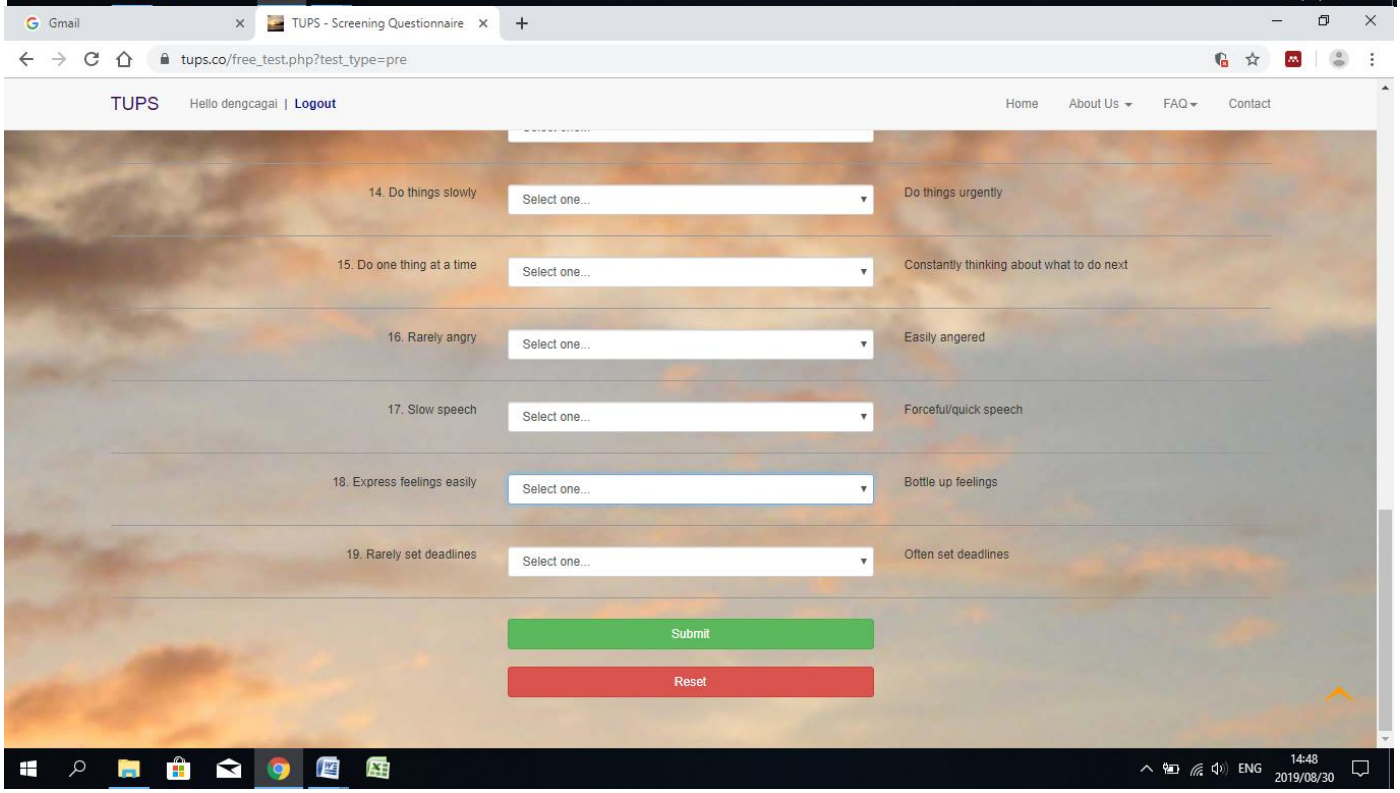
123. Martin BA, Bowen CC, Hunt ST. How effective are people at faking on personality questionnaires? Pers Individ Dif. 2002;

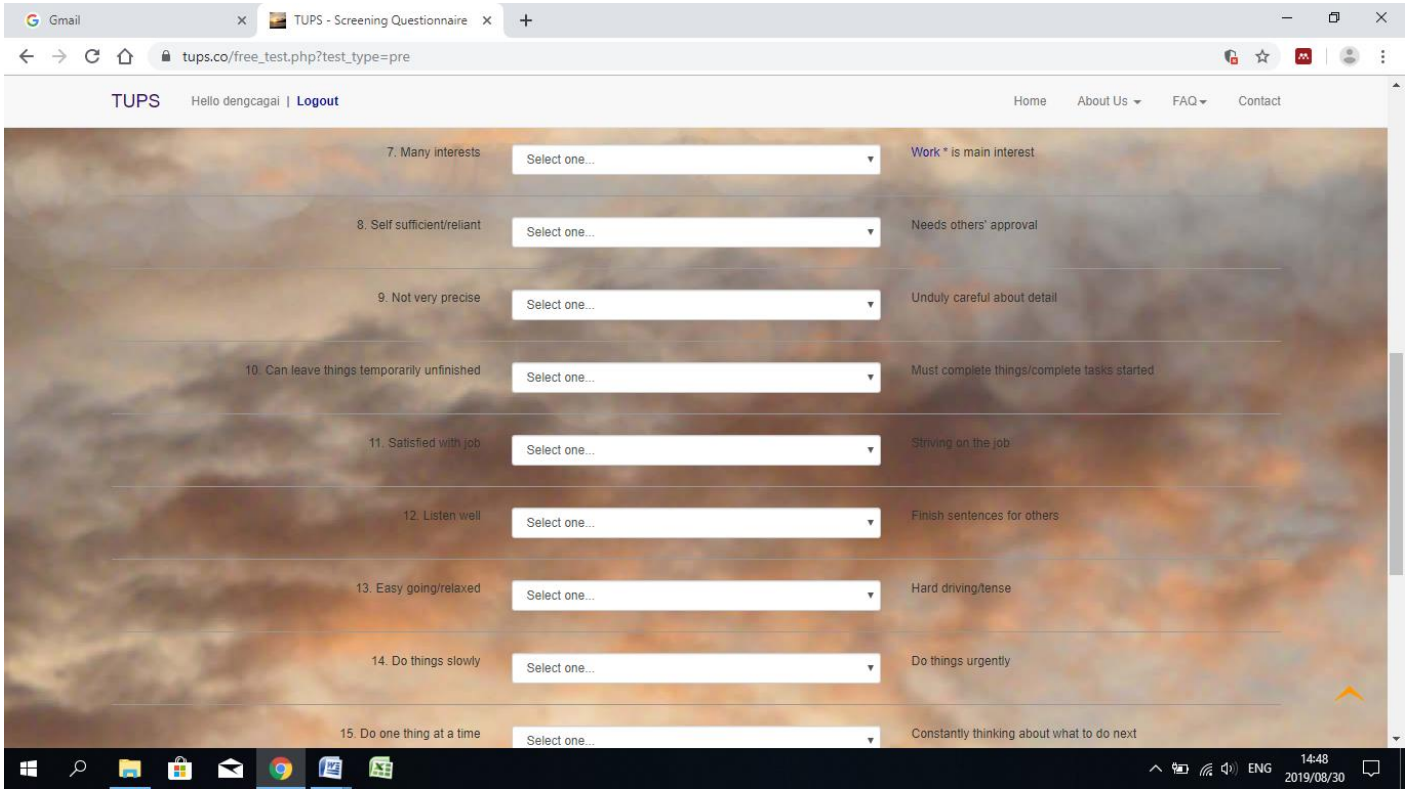
Chapter 5

Addenda

Addendum I: Time Urgency Perfectionism Questionnaire







Addendum II: Lifestyle Questionnaire

Lifestyle Questionnaire

Personal Information

Name and surname: _____

Medfem file no: _____

Cellphone number: _____

E-mail address: _____

Medical History

Abstinence period: _____ (preferably 3-4 days)

Medication: _____

Age: _____

Weight: _____

Length: _____

Previous accidents or operations on genital parts:

_____

_____

_____

Questionnaire

1. Do you smoke? If so, please specify brand and how many daily?

Yes		No		Per day	

Brand: _____

2. Do you consume any alcoholic beverages? (e.g. 1 glass of wine/ 1 beer/ 1 tot or ale/ 25ml spirit) If so, how many units a day/week?

Yes		No		Per day/week	

3. Have you consumed any alcoholic beverages in the past 72 hours? If so, how much?

Yes		No		Quantity	

4. Do you follow a regular exercise programme? If yes, please specify the type of exercise and how many times weekly?

Yes		No		Per week	

Type: _____

5. Have you exercised in the last 72 hours? If so, please specify the type of exercise.

Yes		No	

Type: _____

6. Do you suffer from any chronic diseases? If so, what kind of disease and when were you first diagnosed?

Yes		No	

Disease: _____

Date first diagnosed: _____

7. Are you using any prescription medication? If yes, what is it called and since when have you been using it?

Yes		No	

Name of medication: _____

Month and year: _____

8. Are you taking any alternative medication, vitamins or mineral supplements, herbal or traditional remedies? If yes, specify the name of medication and how often you use it.

Yes		No		Per week	

Name: _____

9. Have you gained or lost more than 5kg in the past 12 months? If so, how much?

Yes		No		kg	

10. Do you suffer from stress related disorders, such as tension headaches / panic attacks / anxiety? If yes, what symptoms and how often per week?

Yes		No		Per week	

Symptoms: _____

11. Are you exposed to hazardous chemicals on a daily basis (at work or at home)? If yes, where do you work (type of industry) or live (suburb)?

Yes		No	

Details:

_____

_____

_____

12. How many meals a day do you eat?

	Meals per day

13. Do you follow a healthy diet?

Yes		No	

14. Do you eat breakfast in the mornings?

Always		Most days		Occasionally		Never	

15. Do you wear tight fitting clothing around your genitals, either by choice or otherwise?

Always		Most days		Occasionally		Never	

Addendum III: Lower reference values (5th centiles and their 95% confidence intervals) for semen characteristics

Parameter	Lower reference limit
Semen volume (ml)	1.5 (1.4–1.7)
Total sperm number (10^6 per ejaculate)	39 (33–46)
Sperm concentration (10^6 per ml)	15 (12–16)
Total motility (PR + NP, %)	40 (38–42)
Progressive motility (PR, %)	32 (31–34)
Vitality (live spermatozoa, %)	58 (55–63)
Sperm morphology (normal forms, %)	4 (3.0–4.0
Other consensus threshold values	
pH	≥7.2
Peroxidase-positive leukocytes (106 per ml)	<1.0
MAR test (motile spermatozoa with bound particles, %)	<50

Addendum IV: Correlation Figures

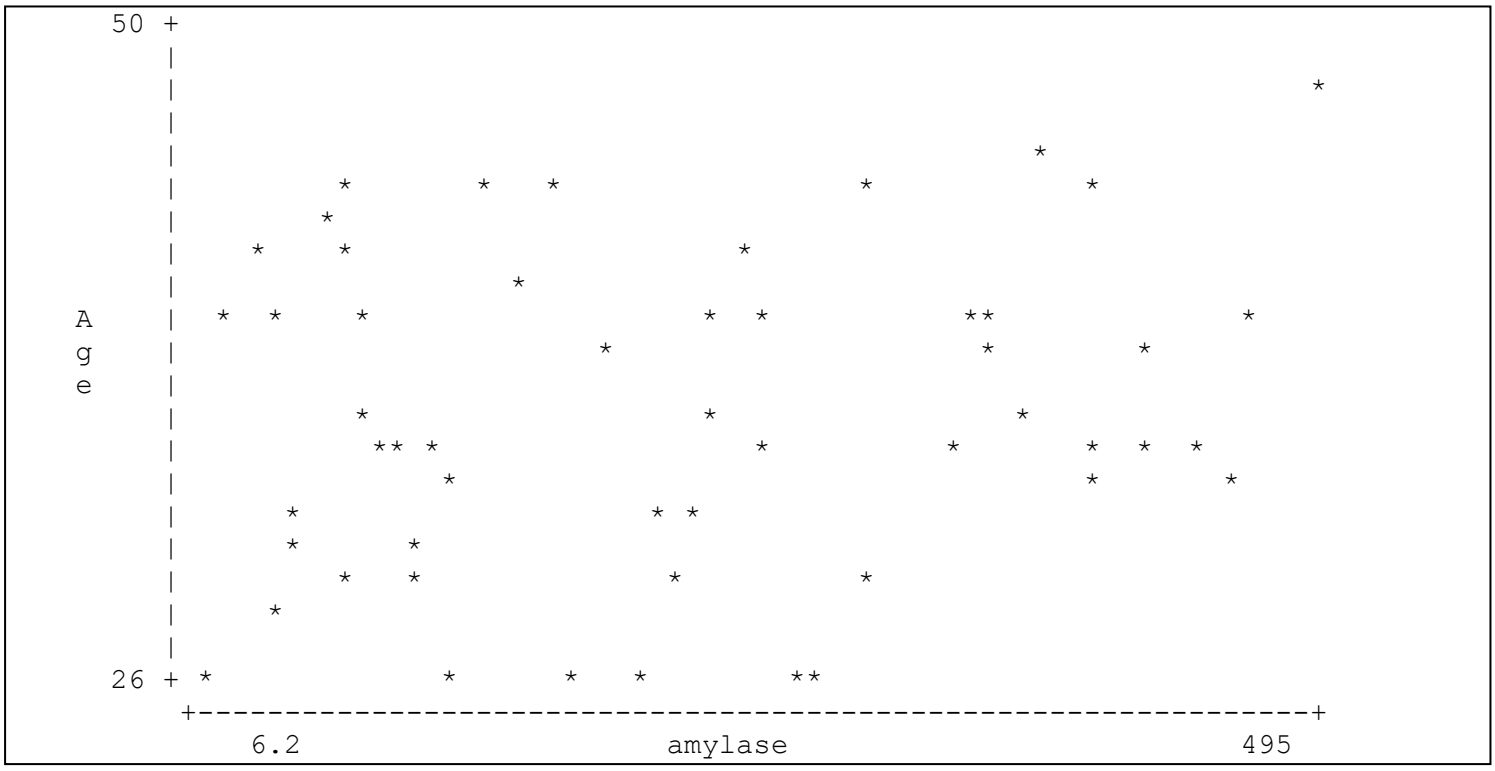


Figure a: Correlation between age and alpha-amylase (Spearman's rho=0.104; p=0.4502)

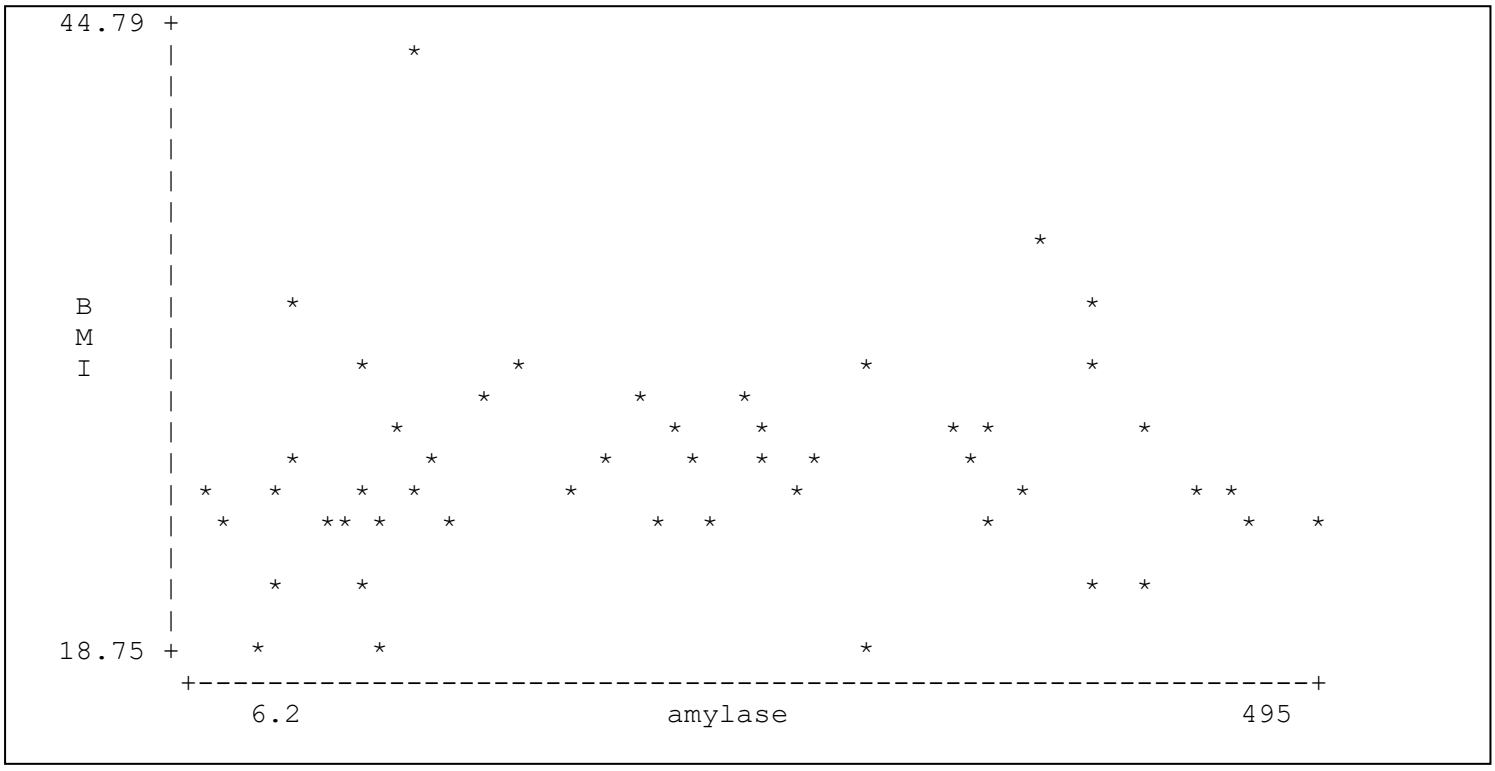


Figure b: Correlation between BMI and alpha-amylase (Spearman's rho=0.119; p=0.399)

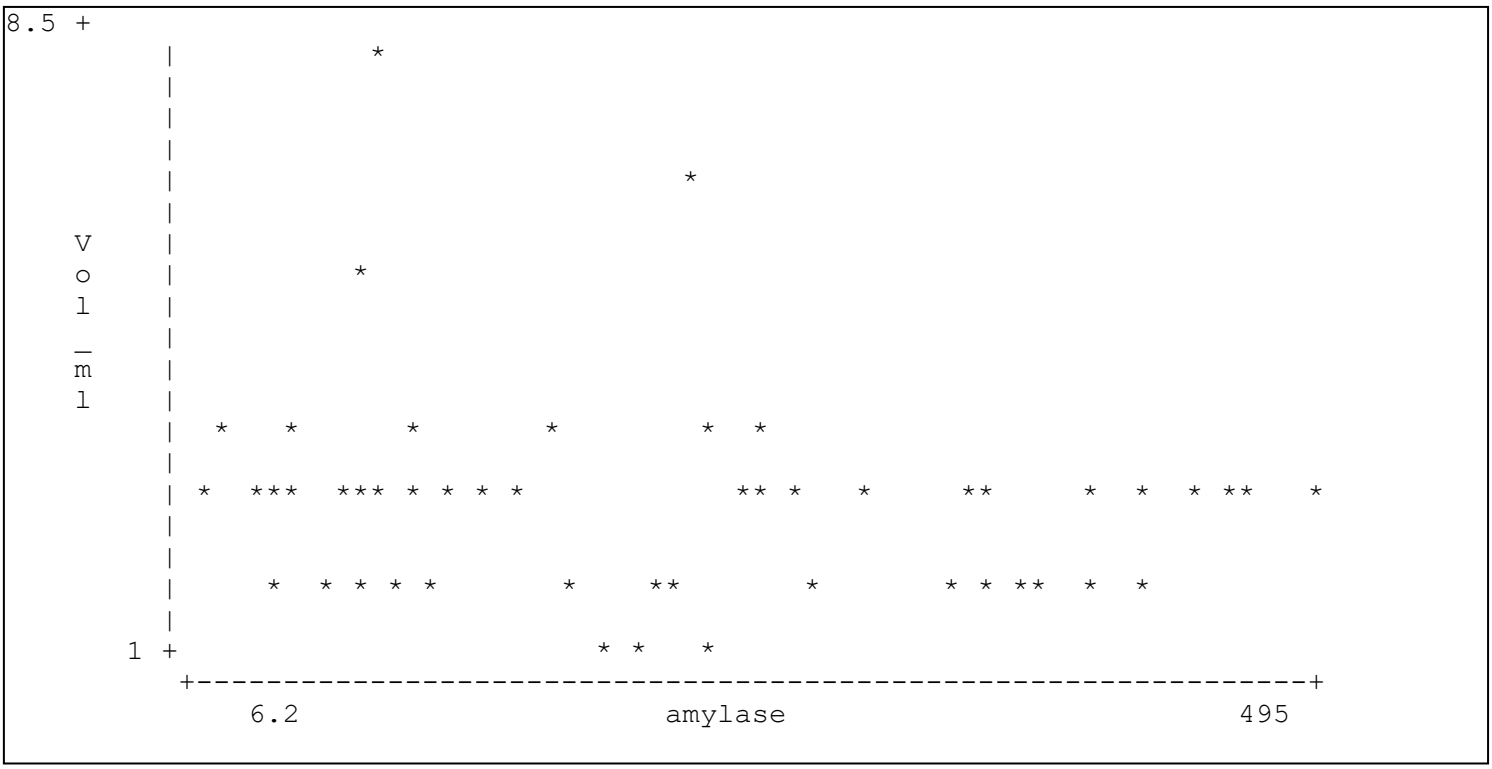


Figure c: Correlation between semen volume and alpha-amylase (Spearman's rho= -0.188; p=0.170)

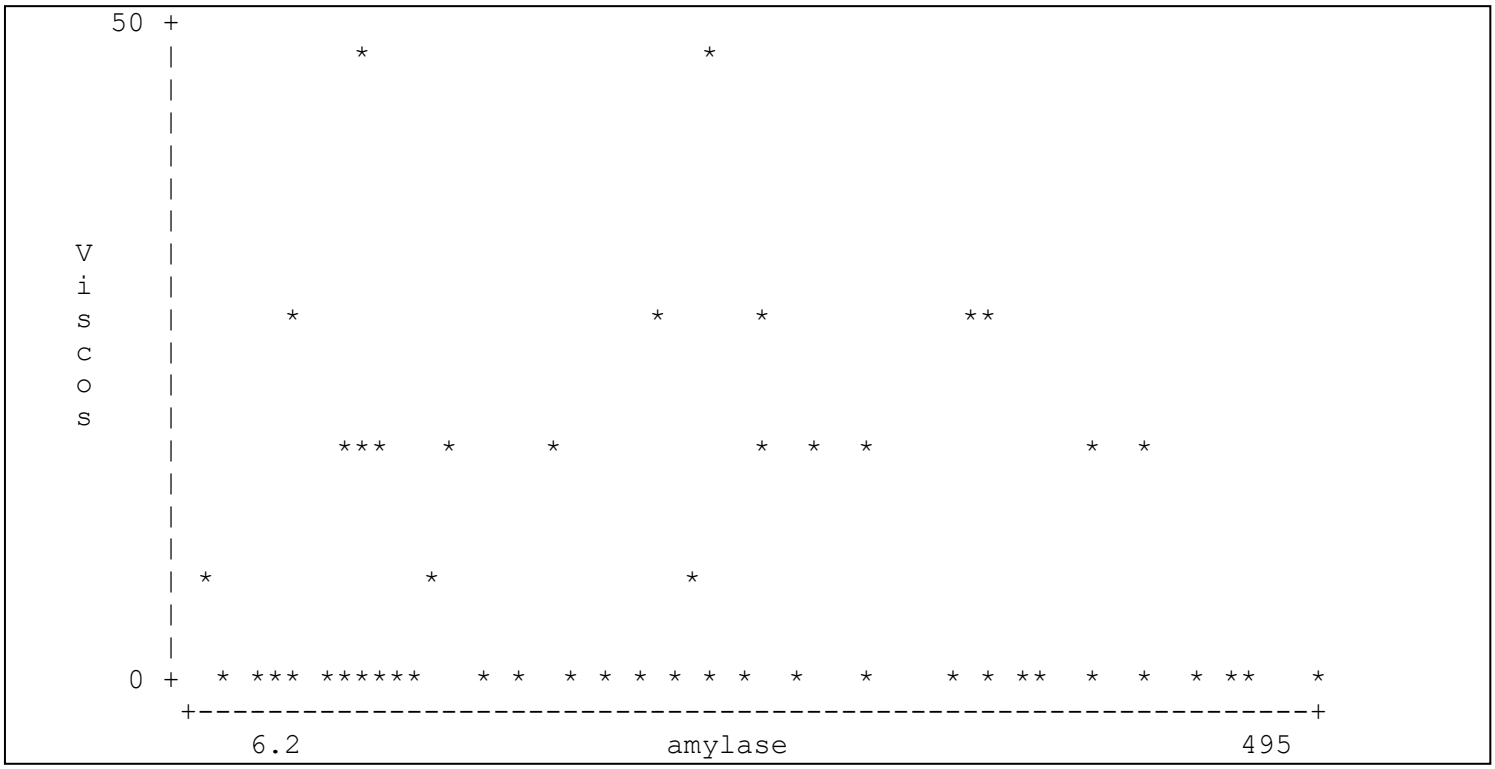


Figure d: Correlation between semen viscosity and alpha-amylase (Spearman's rho= -0.021; p=0.877)

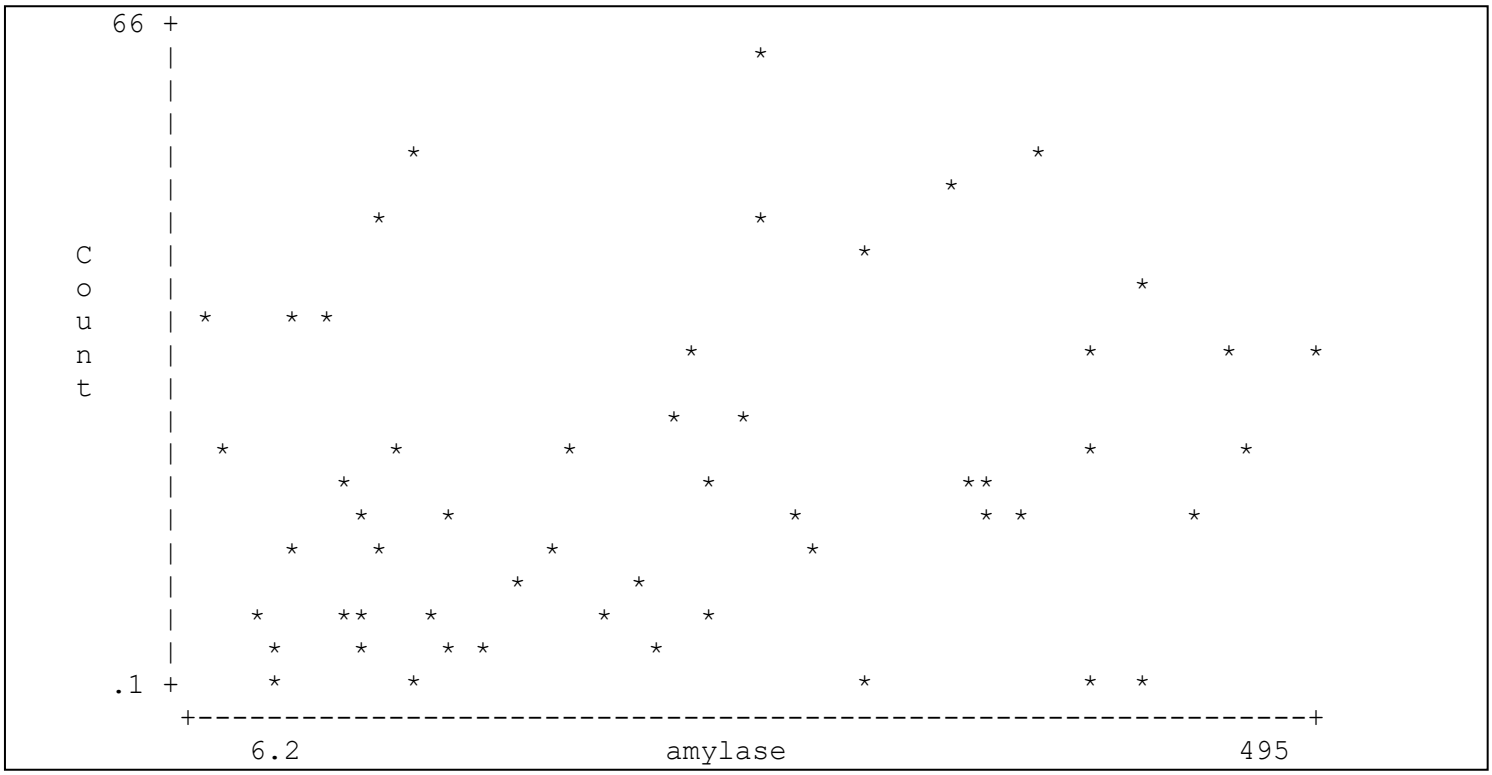


Figure e: Correlation between concentration and alpha-amylase (Spearman's rho= 0.217; p=0.111)

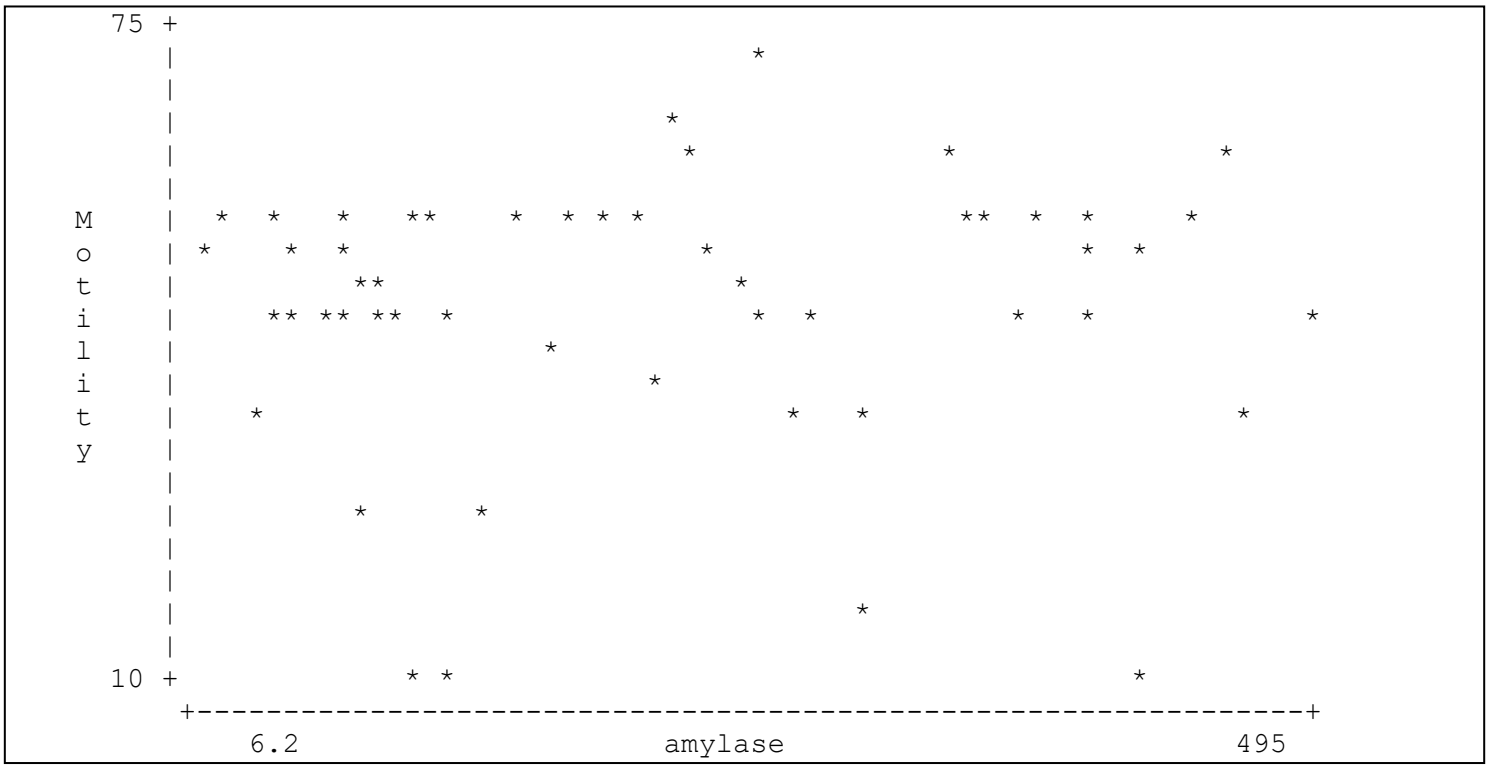


Figure f: Correlation between Motility and alpha-amylase (Spearman's rho=0.097; p=0.482)

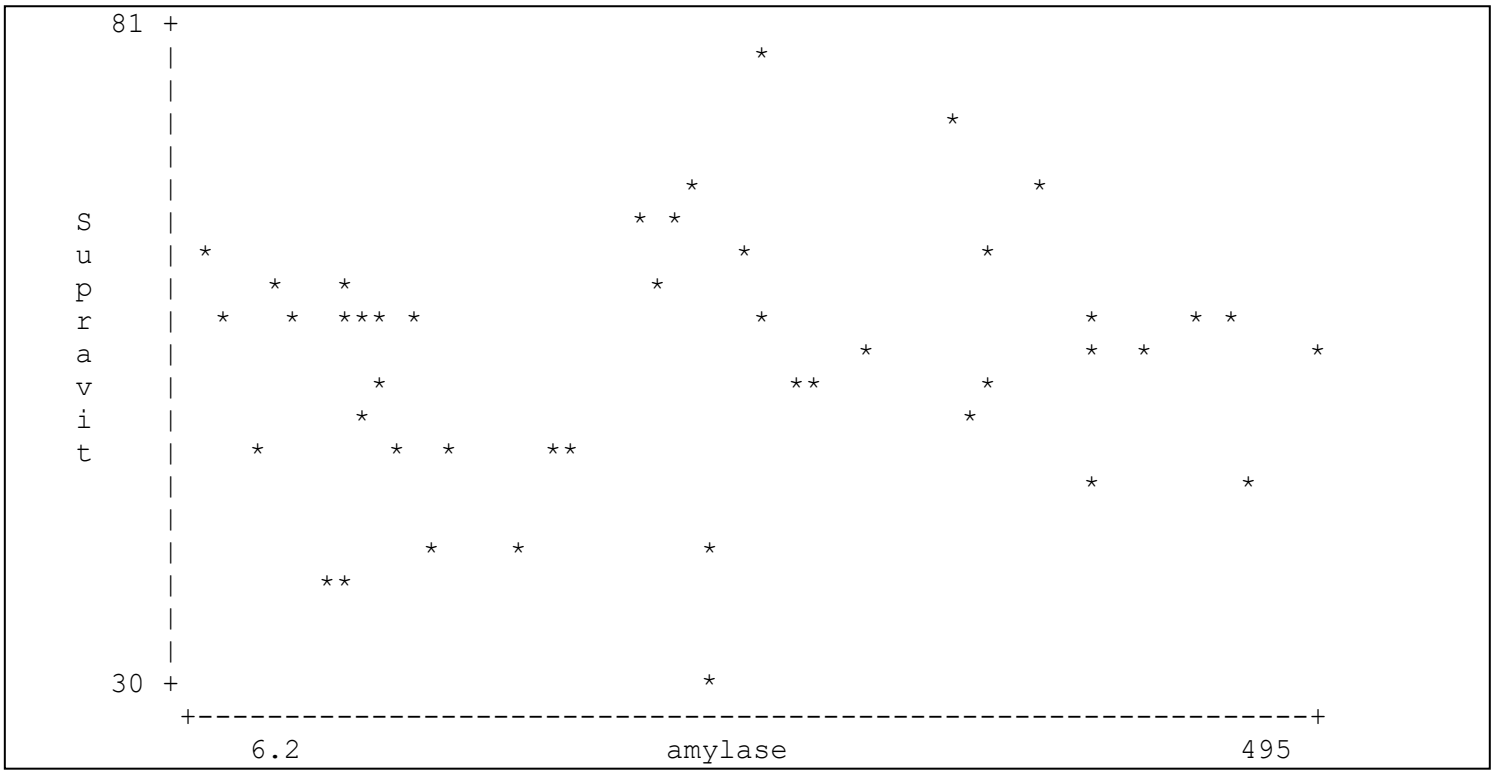


Figure g: Correlation between sperm vitality and alpha-amylase (Spearman's rho= 0.038; p=0.806)

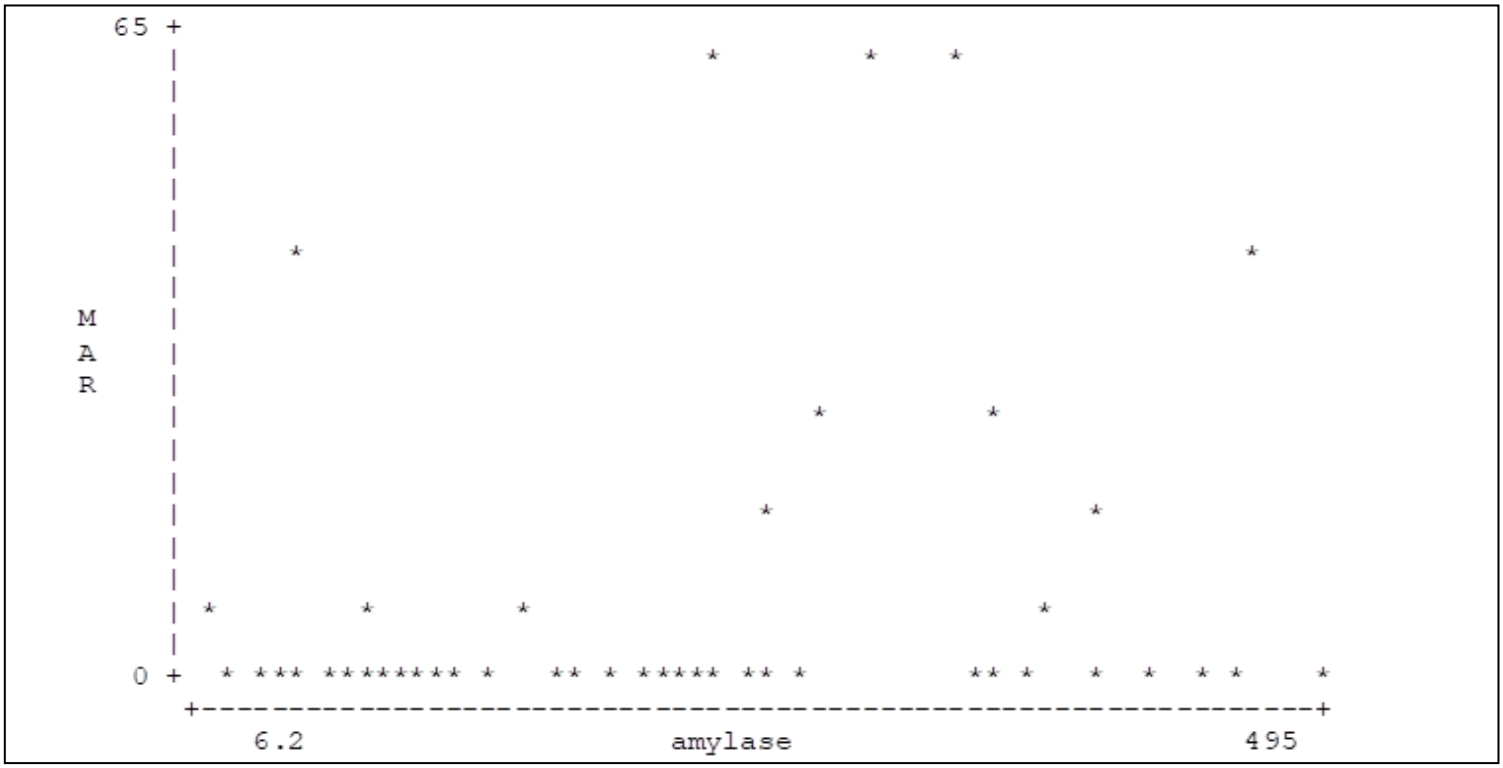


Figure h: Correlation between MAR and alpha-amylase (Spearman's rho= 0.167; p=0.243)

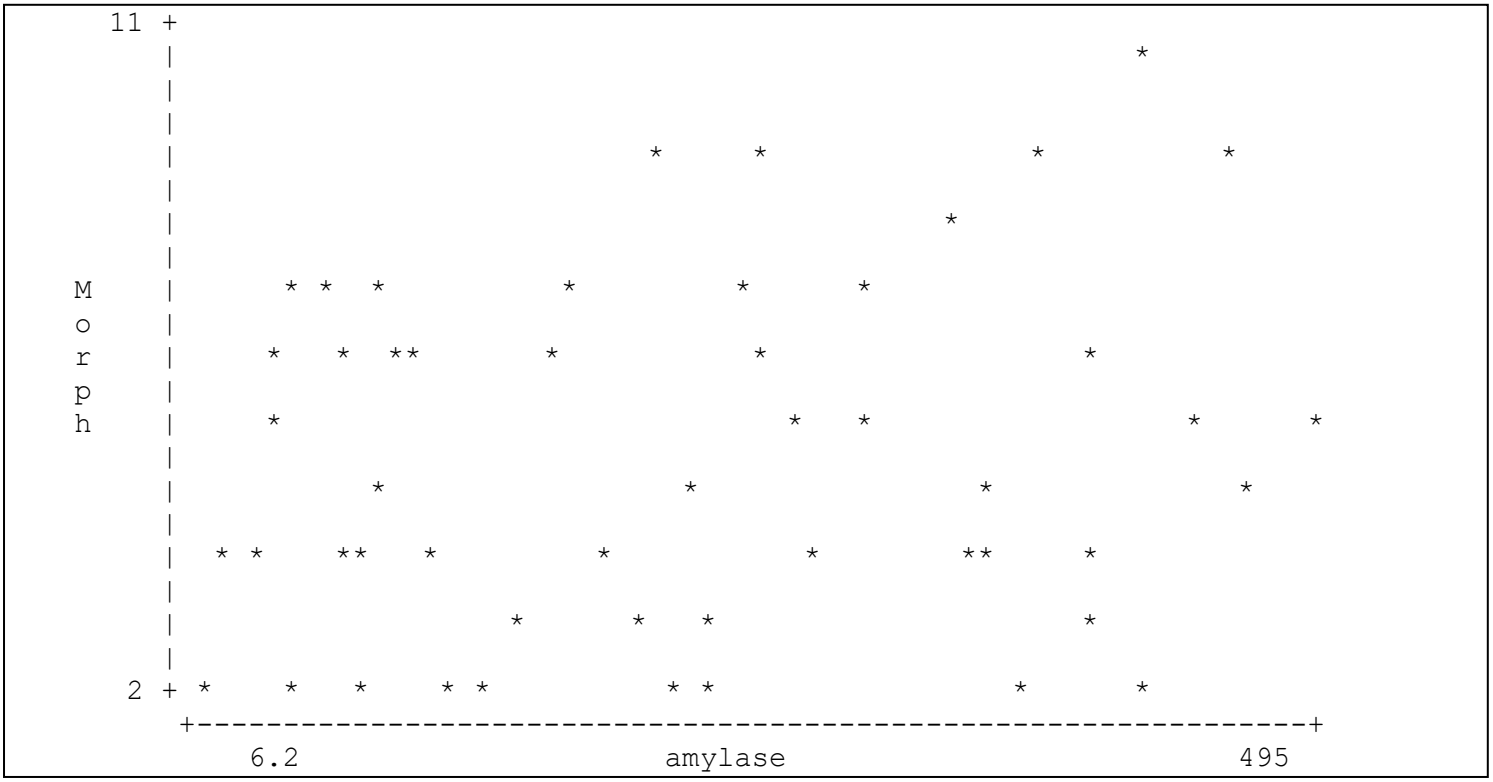


Figure i: Correlation between morphology and alpha-amylase (Spearman's rho= 0.186; p=0.208)

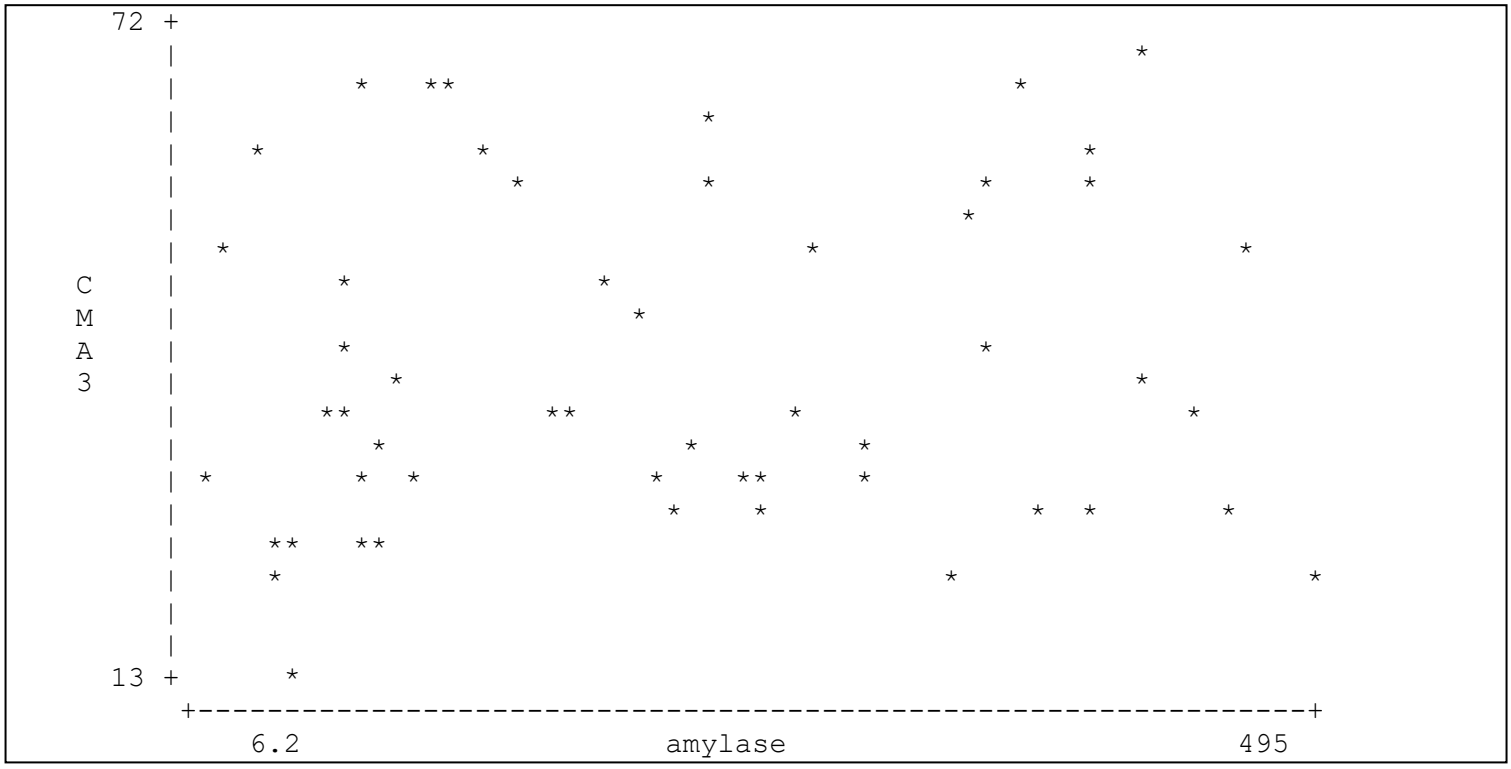


Figure j: Correlation between CMA_3 and alpha-amylase (Spearman's rho= 0.112; p=0.422)

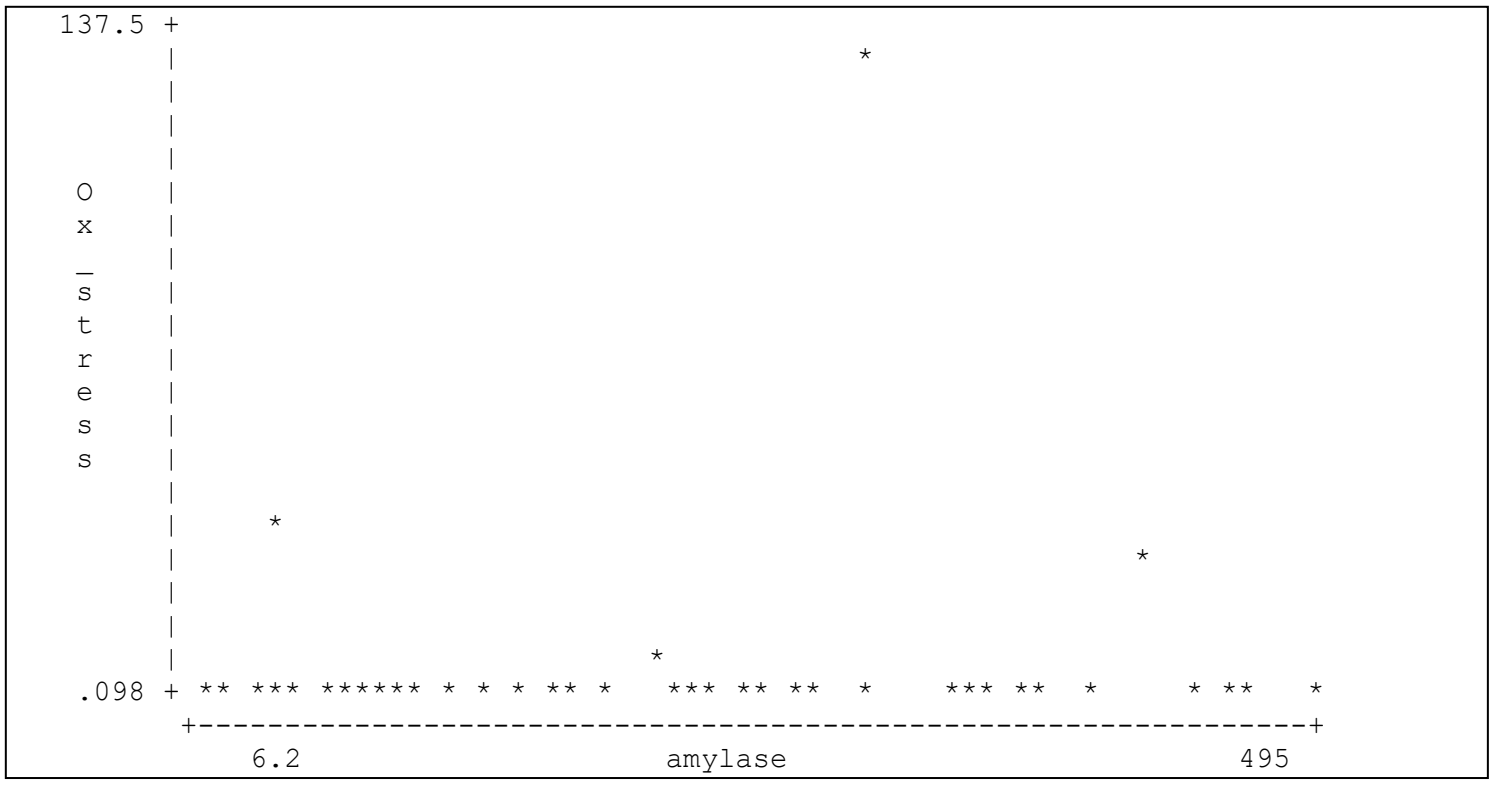


Figure k: Correlation between oxidative stress and alpha-amylase (Spearman's rho=0.046; p=0.753)

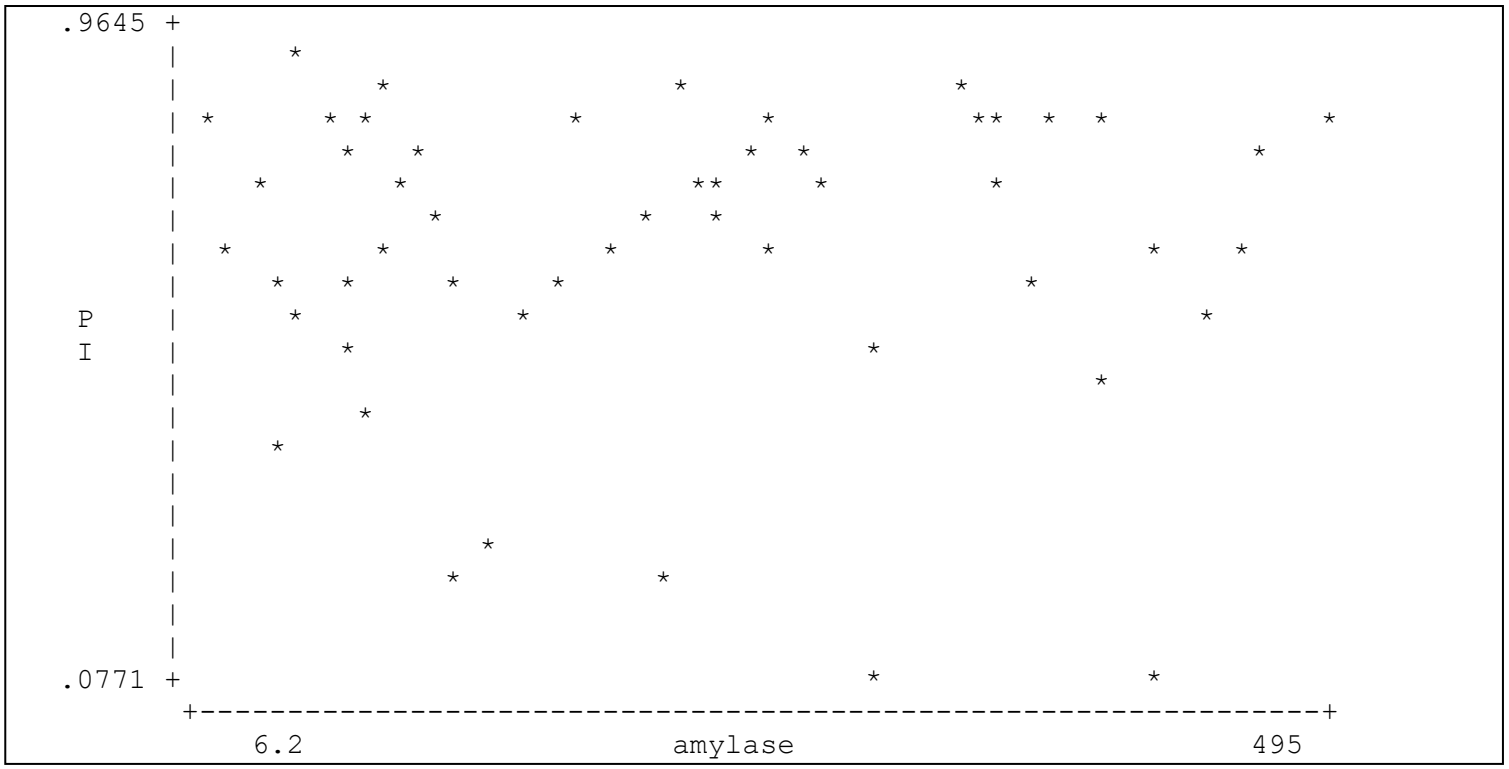


Figure l: Correlation between sperm non-viability and alpha-amylase (Spearman's rho= 0.047; p=0.740)