

**The Effect of Insulin, Leptin and Inflammatory Cytokines
on Reproductive Health and Hypogonadism in Males
Diagnosed with the Metabolic Syndrome.**

by

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**A thesis submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy in the Department of Medical Biosciences, University of
the Western Cape.**

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Co-Supervisor: Professor Patrick JD Bouic

DECLARATION

Hereby I, the undersigned, declare that the thesis '*The effect of insulin, leptin and inflammatory cytokines on reproductive health and hypogonadism in males diagnosed with the metabolic syndrome*' is my own work, that it has not been submitted previously for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Kristian Leisegang

Signed: _____

Date: _____



DEDICATION

This thesis is dedicated to my wife, Alta, and children Daniel and Kyle.

Not just this work is dedicated to you, but my whole being.



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ABSTRACT

The metabolic syndrome (MetS) is a collection of various metabolic, hormonal and immunological risk factors that cluster together, closely related to poorly understood phenomena such as hyperinsulinaemia (insulin resistance), hyperleptinaemia (leptin resistance), a low grade, systemic and chronic inflammation and, in males, hypogonadism. Infertility is increasing globally, and male factor infertility accounts for a large percentage of couples who are not able to conceive. The relationship between components of MetS and male reproductive health is not clear, and requires further investigation, as does the impact of MetS on male reproductive health in a case controlled study. The impact of hyperinsulinaemia, hyperleptinaemia and inflammatory cytokines on the male reproductive tract also requires investigation. Furthermore, it is hypothesised that these phenomena negatively impact steroidogenesis cascades. In order to investigate this, a case controlled study and TM3 Leydig cell culture experiments were designed.

Participants were recruited from public advertisements, and screened for strict exclusion criteria, including acute or chronic inflammation, hormonal treatments, vasectomy and leukocytospermia ($> 10^6/\text{ml}$). Following clinical diagnostics, 78 males were either placed into a control group (CG) or the MetS group, with numerous parameters compared between them. Serum was assayed for routine risk markers including HDL cholesterol, triglycerides, glucose and C-reactive protein (CRP). Saliva was assayed for free testosterone and progesterone. Semen samples underwent semen analysis for ejaculation volume, sperm concentration and motility, vitality, morphology and leukocyte concentration, in addition to mitochondrial membrane potential (MMP) and DNA fragmentation (DF). Both serum and seminal fluid were further assayed for insulin, leptin, tumour necrosis factor- α (TNF α) and interleukins 1- β (IL1 β), 6 (IL6) and 8 (IL8). Glucose was also assayed in seminal fluid. Separately, hCG stimulated TM3 Leydig cells were exposed to varying concentrations of insulin (0.01, 0.1, 1 & 10 pg/ml), TNF α , IL1 β , IL6 and IL8 (0.1, 1, 10 & 100 pg/ml) for 48 hours at optimal cell culture conditions. TM3 cell viability, protein concentration and testosterone and progesterone concentrations were assessed.

Results indicated that males in the MetS group (n=34) had significantly increased body mass index, waist circumference, blood pressure, triglycerides, glucose, and C-reactive protein (CRP) with decreased HDL cholesterol, as compared to the CG. Furthermore, ejaculation volume, sperm concentration, total sperm count, progressive and total motility were significantly decreased in the MetS group, and sperm with abnormal MMP and DF were increased in this group. No difference was found for morphology. Serum and seminal insulin, leptin, TNF α , IL1 β , IL6 and IL8 were all significantly increased in the MetS group. Both testosterone and progesterone were also significantly decreased in the MetS group. Insulin increased testosterone and decreased progesterone in the TM3 cells. TNF α , IL1 β and IL6 all decreased testosterone and progesterone concentrations and TM3 cell viability. IL8 increased TM3 cell viability and decreased progesterone, will no effect on testosterone.

These results suggest MetS is associated with decreased fertility potential in males. Furthermore, a significant increase in seminal insulin, leptin, TNF α , IL1 β , IL6 and IL8 suggests local reproductive tract inflammation in the absence of leukocytospermia. Strong correlations between serum and seminal insulin, leptin, TNF α , IL1 β , IL6 and IL8, as well as serum CRP, imply that these systemic phenomena are related to the reproductive tract changes observed. Therefore, the underlying pathophysiology of MetS negatively affects male reproduction, in addition to general health and well-being. A decrease in progesterone and testosterone suggests a collapse in steroidogenesis cascades. Additionally, inflammation, increased leptin and insulin resistance likely contribute to this collapse in steroidogenesis based on TM3 cell culture experiments. These results provide novel avenues for further investigations.

KEYWORDS

Metabolic syndrome

Obesity

Insulin

Leptin

Inflammation

Testosterone

Progesterone

Infertility

TM3 Leydig cells

Steroidogenesis



CHAPTER 1: INTRODUCTION

1.1. Metabolic syndrome

The metabolic syndrome (MetS) is considered to be a collection of various metabolic risk factors that tend to cluster together, resulting in an increased risk for cardiovascular disease (CVD), type-2 diabetes mellitus (T2DM) and various cancers (Grundy et al., 2004; Huang, 2009). Although the exact aetiology and pathophysiology of MetS is still a matter of contention (Taslim & Tai, 2009), the syndrome comprises of various poorly understood pathophysiological phenomena, associated with complex metabolic, hormonal and immune dysfunctions. The common features that cluster together include central (abdominal) obesity, hypertension, dyslipidaemia (particularly low HDL-cholesterol) and glucose intolerance, as illustrated in Figure 1 (Eckel et al., 2005; Kasturi et al., 2008; Huang, 2009). These appear to be linked together by insulin resistance (IR) and a low grade proinflammatory state (Kasturi et al., 2008; Monteiro & Azevedo, 2010). In males, a state of hypogonadism is also well defined (Kasturi et al., 2008; Saad & Gooren, 2011). These are all well documented risks for CVD and T2DM (Eckel et al., 2005; Kasturi et al., 2008; Huang, 2009). It is not clear in the scientific literature which components are considered as aetiological phenomena or as a consequence of the syndrome.

Complications of obesity have been known for centuries, with Hippocrates observing that 'sudden death is more common in those that are naturally fat than lean' (Chadwick & Mann, 1950). Obesity was linked to lipid abnormalities and hypertension in the 17th century by Nicholas Tulp (Tulp syndrome) (Erkelens et al., 1993). In the 18th century, Giovanni Battista Morgagni associated visceral adiposity with hypertension, dyslipidaemia, hyperuricaemia, CVD and sleep apnoea (Enzi et al., 2003). The Swedish physician Kylin reported the clustering of hypertension, hyperglycaemia, dyslipidaemia and hyperuricaemia (gout) within certain patients in the 1920's (Nilsson, 2001) followed by the association of the 'android' obesity phenotype with CVD and T2DM in the 1940's (Vague, 1947). The term 'metabolic syndrome' appears to be first coined in 1977 by Haller, in which he described a collection of obesity, diabetes, hyperlipidaemia, hyperuricaemia and hepatic

steatosis in the German population that predisposed them to developing ischaemic heart disease (Haller, 1977). The concept was later described by Reaven in 1988, with hyperinsulinaemia and IR identified as a common underlying thread linking these associations together (Reaven, 1988). MetS has been described using various terminologies in the past, such as 'syndrome X', 'insulin resistance syndrome' and even the 'deadly quartet' (Reaven, 1988; Kaplan, 1989; DeFronzo & Ferrannini, 1991). The more modern terminology and descriptions was formalised in 1998 by the World Health Organisation (WHO) (Alberti & Zimmit, 1998).

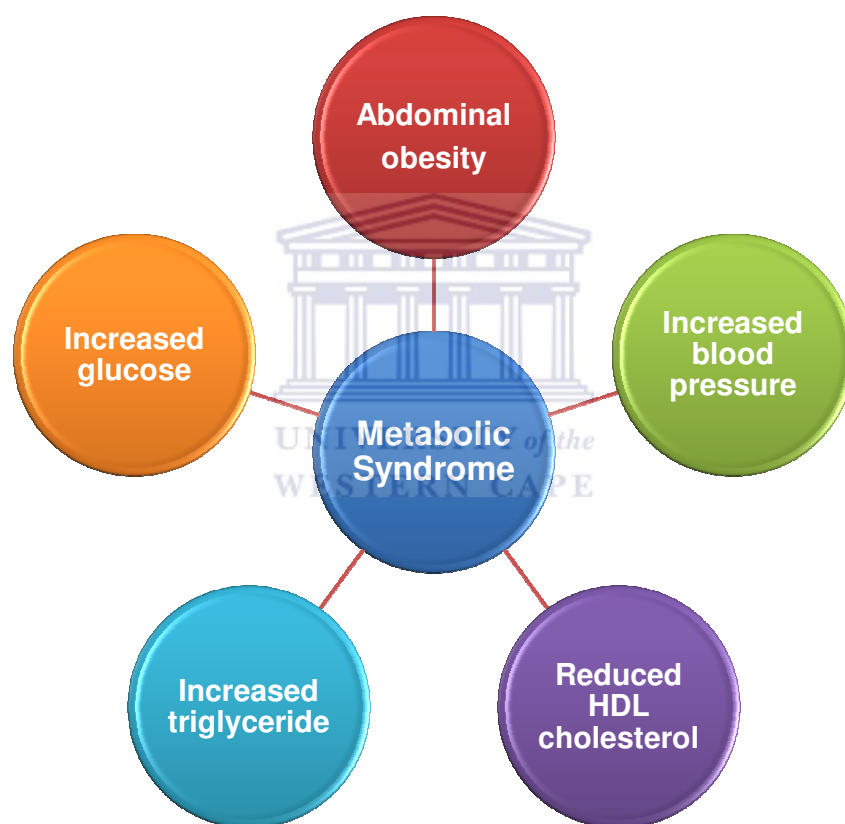


Figure 1: The five central features in the definition and current diagnostic criteria for metabolic syndrome as described by Alberti et al. (2009). This definition is based on the syndrome being a clustering of various risk factors for cardiovascular disease and type 2 diabetes mellitus.

cHDL = High Density Lipoprotein Cholesterol

Significant controversies continue to be associated with MetS. Critics argue that this collection of risk factors should not be considered a syndrome at all, as it lacks a clear aetiology and the pathophysiology is not well defined, in addition to continuing arguments over the evidence that has led to the currently accepted cut off values for diagnostics (Kahn, 2006; Gale, 2008; Alberti et al., 2009; Kuk & Ardern, 2009). The clinical relevance of the syndrome is also questioned, with arguments that MetS adds little to no additional predictive value for CVD over the traditional Framingham risk factors, and that the risks associated with MetS are not greater than the some of its parts (Gale, 2008; Schweiger et al., 2008). It is further argued by some authors that there is no real change in clinical management of patients as compared to the individual components of MetS (Kahn, 2006; Gale, 2008; Kuk & Ardern, 2009). However, studies indicating that up to 40% of obese individuals may be metabolically normal, and therefore have reduced risk of complications associated with MetS and obesity in general. Conversely, there are also lean patients who are diagnosed with MetS, and this can provide important clinical information (Brochu et al., 2001; Grundy, 2006; Stephan et al., 2008; Kuk & Ardern, 2009).

1.1.1. Prevalence and epidemiology

There has been a dramatic global increase in the incidence of MetS over the past 20 years, closely associated with the global epidemic of obesity and T2DM, making the syndrome a global epidemic in its own right (Zimmet et al., 2001; Potenza & Meckanick, 2009). In fact, the global epidemics of obesity, CVD and T2DM have drawn attention to this cluster of metabolic and fat derangements as common and interrelated underlying pathophysiologies (Potenza & Meckanick, 2009). Although numerous studies have assessed prevalence and epidemiological factors, they generally differ widely in design and diagnostic criteria that are used (Cameron et al., 2004; Potenza & Meckanick, 2009).

Epidemiologic studies have demonstrated wide variations and differences in prevalence by age, gender, and ethnicity. The prevalence in adults varies from as low as 8% in India to as high as 24% in America for men, and as low as 7% in France to as high as 43% in Iran for Woman. Overall, prevalence in America is approximately 34.4%, approximately 24.6% - 30.9% in Europe, with significantly

lower rates in Japan of approximately 8.1% - 9.9% (Cameron et al., 2004; Pais et al., 2009; Potenza & Meckanick, 2009; Razzouk & Muntner, 2009). The association with age is a generally constant variable, indicating MetS is highly age dependent. However, there is an increase in incidence of MetS in younger people, too (Sinha et al., 2002; Wei et al., 2003; Weiss et al., 2004; Ervin, 2009; Potenza & Meckanick, 2009). This is again parallel with an increase in obesity, T2DM and CVD in younger adults and even children (Sinha et al., 2002; Wei et al., 2003; Weiss et al., 2004).

1.1.1.1. Prevalence in South Africa

The prevalence of MetS in the South African population is not well defined. Ker and colleagues (2007) reported that 31% of a group of 1410 corporate executives that belonged to a specialist health and fitness company in South Africa had MetS. An additional 33% had two criteria of MetS, thus considered borderline. Similar findings were reported in a smaller subset of black executives assessed. In a study of 500 black and 254 white diabetic patients in South Africa, 46.5% and 74.1% of the cohort were found to have MetS, respectively (Kalk & Joffe, 2008). Similar prevalence was found in a corresponding female cohort. This was slightly higher in women (25%) than in men (10.5%). A more recent publication by George and colleagues (2013) indicates a 29% and 46% prevalence of MetS in 374 African and 350 Asian Indian adults, respectively. A study assessing children aged 10 – 16 years old in South African schools identified MetS prevalence rates of 8.9% (n=158), 6.4% (n=281) and 14% (n=57) in black, coloured and Caucasians males respectively, and 5.2% (n=288), 5.7% (n=415) and 6.8% (n=73) in black, coloured and Caucasian females. The overall rate of MetS in this study was 6.5% (Matsha et al., 2009). Generally, based on scanty data available, it appears that MetS is highly prevalent in the urban setting across numerous socio-economic and racial backgrounds.

1.1.2. Definitions, diagnosis and classification

MetS can be described as a master in disguise, as it can present in various ways according to different components that are being expressed in each individual (Eckel et al., 2005). It has proven difficult, and even controversial, to define and diagnose (Alberti et al., 2009). Since the formulation of criteria by the World Health

Organisation (WHO) in 1998 (Alberti & Zimmit, 1998), numerous criteria have been published (Alberti et al., 2009). Although the diagnostic criteria proposed by the organisations had various similarities, key differences are found. These differences were particularly evident in the inclusion of obesity and the role of insulin resistance as diagnostic criteria (Alberti et al., 2009).

The WHO criteria were the first to tie together the key components of insulin resistance, obesity, dyslipidaemia and hypertension (Alberti & Zimmit, 1998). However, the definition mandates that insulin resistance be present in order to achieve the diagnosis (Alberti & Zimmit, 1998). Furthermore, the use of the euglycaemic clamp for assessment of insulin resistance is not easily applied in the clinical setting. The WHO criteria also included the presence of microalbuminaemia as a possible diagnostic criterion, absent in all other definitions (Alberti & Zimmit, 1998; Grundy et al., 2004; Alberti et al., 2009; Taslim & Tai, 2009).

In 1999, the European Group for the Study of Insulin Resistance (EGIR) published a set of criteria (Balkau & Charles, 1999). Similar to the WHO, this also requires insulin resistance to be mandatory in order to achieve a diagnosis of MetS (Alberti et al., 2009). The definition of insulin resistance is based on a fasting serum insulin measurement. This is easier to achieve in a clinical setting, but risks the exclusion of patients with T2DM in the diagnosis (Huang, 2009; Taslim & Tai, 2009).

The Adult Treatment Panel III (ATPIII) guidelines (National Cholesterol Education Programme, 2002), although criticised for not including insulin resistance (Cheal et al., 2004; Liao et al., 2004), tended to be the most widely used in medical research for the definition of MetS for both clinical and research purposes as it is considered to be the most easily applied in the clinical setting (Grundy et al., 2004; Alberti et al., 2009; Huang 2009; Taslim & Tai, 2009). The ATPIII was developed in 2001 by the National Cholesterol Education Programme, and requires that any three of five set criteria must be met for a diagnosis of MetS. These include increased waist circumference, hypertension, hypertriglyceridaemia, reduced high density lipoprotein (HDL) cholesterol and increased fasting glucose (National Cholesterol Education Programme, 2002).

The criteria set by the American Association of Clinical Endocrinology (AACE) in 2003 are essentially a combination of the WHO and ATP III definitions except that no specific defined number of risk factors are specified. This leaves the diagnosis to clinical judgement, and thus more subjective (Grundy et al., 2004; Taslim & Tai, 2009).

In 2005, the International Diabetes Foundation (IDF) published criteria for MetS that required obesity, and not insulin resistance, as a central and mandatory criterion (Zimmet et al., 2005). This was an important development, recognising the relationship between different abdominal fat risk associations with different populations. The criticism with these criteria is the fact that obesity is the emphasis, and not insulin resistance (Alberti et al., 2009; Huang, 2009; Taslim & Tai, 2009).

In 2009, a joint scientific statement was published following meetings between the *International Diabetes Federation Task Force on Epidemiology and Prevention*, the *National Heart, Lung and Blood Institute*, the *American Heart Association*, the *World Heart Federation*, the *International Atherosclerosis Society*, and the *International Association for the Study of Obesity* (Alberti et al., 2009). This was an attempt to unify the various criteria, to 'harmonise' the variations of definitions and diagnostic criteria for MetS. It was agreed that there should not be an obligatory component, and that waist measurement would be a very useful screening tool (and not other assessments of obesity such as the body mass index). Five central criteria are set, and any three would qualify for the diagnosis of MetS. A single set of cut off points are suggested for all variables except waist circumference, which has variations based on ethnic backgrounds. There are, however, differences for male and female cut off values in waist circumference and HDL-cholesterol measurements (Alberti et al., 2009). The key components of this criterion are outlined in Table 1. Based on the purposes for this thesis, these cut off values are components for males only and are used to define MetS in this study.

Table 1: A summary of the current criteria for the clinical diagnosis of metabolic syndrome in Asian, Caucasian and Sub-Saharan African males. A minimum of three criteria need to be fulfilled to obtain a clinical diagnosis. Categorical cut off values for waist circumference vary based on ethnic background (Alberti et al., 2009).

Criteria	Categorical Cut Off Points
Waist Circumference	Sub-Saharan African ≥ 94 cm; Caucasian ≥ 94 cm; Asian ≥ 90 cm;
Blood pressure (or relevant medication)	Systolic ≥ 130 mmHg and/or diastolic ≥ 85 mmHg
Fasting triglycerides (or relevant medication)	> 1.70 mmol/L
HDL Cholesterol (or relevant medication)	< 1.00 mmol/L
Fasting glucose (or relevant medication)	> 5.5 mmol/L

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1.1.3. Complications and clinical consequences

Almost by definition, MetS is closely associated with a risk for CVD and T2DM. The syndrome is further associated with a general risk of morbidity and mortality, with numerous other co-morbidities being associated with the syndrome. These include, but are not limited to, various cancers (particularly colorectal, breast, endometrial, prostate, hepatic, renal and pancreatic) (Calle et al., 2003; Pais et al., 2009; Gallagher et al., 2010; Braun et al., 2011; Esposito et al., 2013a; Esposito et al., 2013b), polycystic ovarian syndrome, non-alcoholic hepatic steatosis and/or steatitis, cholelithiasis, obstructive sleep apnoea, gout and hyperuricaemia (Eckel et al., 2005; Huang, 2009) and Alzheimer's disease (De Felice, 2013). Many of these phenomena are also closely related to the development of IR in numerous studies, again highlighting the important central role of IR in MetS (Cheal et al., 2004).

A meta-analysis of prospective studies indicates that MetS is associated with an increased relative risk (RR) of developing T2DM of 2.99, alongside a RR of developing CVD of 1.65 and a RR of all causes of mortality of 1.27 (Ford, 2005). Furthermore, mortality from any cause in those diagnosed with MetS has been suggested to increase 2.26 fold in males and 2.78 fold in females after adjustments for age, BMI, cholesterol levels, exercise, alcohol consumption and smoking (Potenza & Meckanick, 2009).

1.2. Pathophysiology of metabolic syndrome

As with an adequate and generally well accepted definition and set of diagnostic criteria for MetS, the exact aetiology and pathophysiology is also a matter of contention. A simplification of the pathophysiology as discussed below is represented in Figure 2 below.

1.2.1. Aetiology and risk factors

Although there is a clear genetic predisposition, and an unfavourable genotype is indeed an important risk, environmental factors are considered more influential in the development of the syndrome. Well defined risk factors that contribute to the MetS epidemic include a sedentary lifestyle, increased caloric intake and poor dietary choices (Eckel et al., 2005; Huang 2009).

Genetic predisposition is highly complex, and involves multiple genes and numerous single nucleotide polymorphisms. Support is offered in numerous studies that associate heritability with components of MetS, including obesity, insulin resistance, hypertension, HDL-cholesterol levels and triglycerides, as well as the consequences, such as CVD and T2DM. Genes encoding leptin and the leptin receptor have also been found to be important in the predisposition of obesity and MetS, as are genes encoding lipolysis and β 2- and β 3-adrenoreceptors, peroxisome-proliferator activated receptor- γ (PPAR γ) (Groop, 2000; Hegele, 2003; Dallongeville et al., 2003; Fumeron et al., 2004).

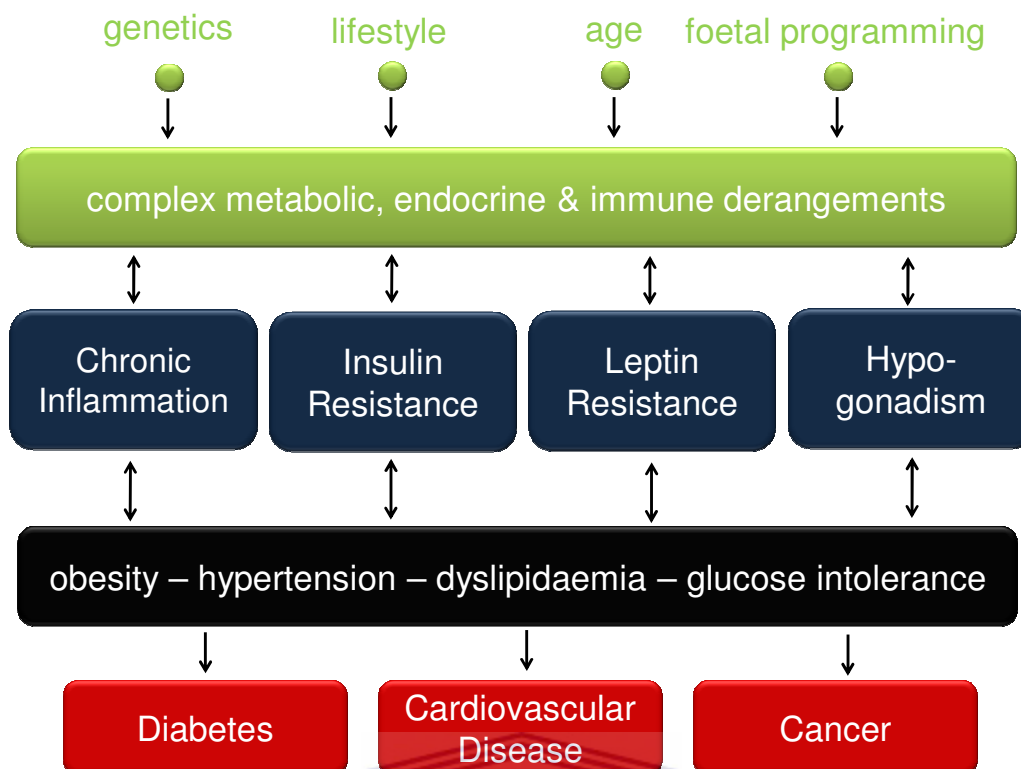


Figure 2: The simplified schematic summarising the complex interactions associated with the pathophysiology of metabolic syndrome in males. This includes phenomena such as insulin resistance, leptin resistance, chronic inflammation and hypogonadism.

In a modern 'Westernised' society, the abundance of an excess supply of calories is associated with an obesity epidemic, MetS and the various clinical associations. An increase in the prevalence of obesity is associated with rising levels of hypertension, dyslipidaemia, insulin resistance, CVD and T2DM, which are interrelated, culminating in the MetS (Huang, 2009; Zeelie et al., 2010). In addition to abundance of calories, the food quality is of importance (Eckel et al., 2005). Lack of dietary fibre, fruit and vegetables and even a lack of moderate alcohol consumption, has been associated with MetS (Potenza & Meckanick, 2009). A lack of adequate exercise, the so called 'sedentary lifestyle' is also strongly associated with an increased risk for obesity and MetS (Eckel et al., 2005).

Associations between total fat intake, insulin resistance, MetS and coronary heart disease are primarily mediated through the saturated and unsaturated fatty acid

components (Hodson et al., 2001). Saturated fatty acids have long been associated with obesity, and have been related to a corresponding risk of the metabolic syndrome (insulin resistance), T2DM and CVD. Unsaturated fatty acids are widely considered to have a more beneficial biological effect in humans, with many arguing in favour of replacing saturated fats with unsaturated fats, rather than carbohydrates, to cause a favourable change in serum lipid profiles. This is associated with the so-called Mediterranean diet, considered protective against MetS (Hodson et al., 2001; Riccardi et al., 2004; Potenza & Meckanick, 2009).

Chronic psychosocial and work stress is also considered an important factor in the development of MetS. The mechanisms and pathways for this association is complex, mediating specific imbalances with the hypothalamic-pituitary-adrenal system and an activation of the sympathetic nervous system (Björntorp, 2001; Tsigos & Chrousos, 2002; Vitaliano et al., 2002; Chandola et al., 2006; Tentolouris et al., 2006).

1.2.2. Obesity and the role of adipose tissue

In adults, predominant white adipose tissue (WAT) can be classified into two broad types, namely subcutaneous and visceral, with distinct gene expressions (phenotypes) associated with obesity. Subcutaneous adipose tissue is generally considered to be protective against features associated with obesity and MetS (Kwon & Pessin, 2013). The inability to convert excess carbohydrates to lipids for storage in subcutaneous tissue is thought to be a prominent mechanism in the development of MetS and associated complications (Bastard et al., 2006; Kwon & Pessin, 2013).

The global obesity pandemic is a prominent driving force behind the increased incidence of MetS. There is indeed a close relationship between obesity, particularly visceral obesity assessed by a waist circumference measurement, and MetS. However, the manner of inclusion and the definition of obesity has generated much discussion and controversy (Alberti et al., 2009; Huang, 2009; Taslim & Tai, 2009). To illustrate the importance of BMI as a predictor of MetS, being overweight, with

high triglycerides, low HDL or hypertension, more often resulted in a diagnosis of MetS the identification of IR (Cheal et al., 2004).

Waist circumferences (WC) or the waist-to-hip ratio (WHR) is included and favoured above BMI for several definitions of MetS (Grundy et al., 2004; Alberti et al., 2009; Huang, 2009; Taslim & Tai, 2009). WC correlates with IR and has a stronger association with the development of CVD and T2DM than BMI alone (Alberti et al., 2009; Gallagher et al., 2010). It is important to be certain that an increased WC is due to intra-abdominal (visceral) fat deposits and not subcutaneous fat, which is only possible with MRI technology (Eckel et al., 2005). A further area of controversy is the observed variation of the role of visceral obesity in different sexes, and especially in those with different ethnic backgrounds (Taslim & Tai, 2009). South Asian populations have greater amounts of visceral adiposity for given WC measurements compared to European populations (Alberti et al., 2005; Lear et al., 2007; Alberti et al., 2009; Gallagher et al., 2010). As an example, those in Singapore have a greater amount of adiposity compared to Caucasians with the same BMI (Deurenberg et al., 2002), indicating that those with different ethnic backgrounds carry different amounts of intra-abdominal fat and therefore risk of MetS (Araneta & Barrett-Connor, 2005; Kadowaki et al., 2006; Lear et al., 2007).

Normal weight individuals can also be diagnosed with MetS (Ruderman, 1998; Eckel et al., 2009), as obesity is just one of five components according to the criteria suggested by Alberti and colleagues (2009). Conversely, there are patients with increased WC and BMI that do not exhibit other features of MetS, and are also associated with a lower risk for CVD and T2DM compared to others in similar weight categories with MetS features (Huang, 2009). However, with literature indicating that weight loss can lead to improvements of multiple features of MetS simultaneously, visceral obesity appears to be a core central feature involved in the pathophysiology (Huang, 2009).

Adipose tissue comprises of adipocytes (containing a single, large fat droplet), preadipocytes, macrophages and lymphocytes. Traditionally, this tissue was thought to act predominantly as a major storage site for energy and insulator. However, adipose tissue further secretes various proteins termed adipokines (general term

used to denote any protein synthesised and secreted by adipocytes) amongst numerous other metabolically active molecules (Trayhurn & Wood, 2004; Wozniak et al., 2009). These proteins circulate in the body acting predominantly as hormones, communicating with tissues such as the brain, liver, muscle, the immune system and adipose tissue itself (Kwon & Pessin, 2013). Examples of these molecules include leptin, resistin, retinol binding protein-4, chemerin, CC-chemokine ligand 2, CC-chemokine receptor type 5, angiopoitin-like protein, adiponectin, omentin-1 apelin and traditional cytokines such as tumour necrosis factor-alpha (TNF α), interleukin 1 β (IL1 β) and interleukin 6 (IL6) (Bastard et al., 2006; Kwon & Pessin, 2013). Adipose tissue is therefore considered to be an important hormonally active organ and a prominent controller of energy homeostasis, metabolic function, immune activity and reproduction (Trayhurn & Beattie, 2001; Nawrocki & Scherer, 2004; Juge-Aubry et al., 2005; Wang et al., 2008; Wozniak et al., 2009). Via the activity of adipokines, adipose tissue has been shown to actively modulate and participate in various metabolic and inflammatory processes. (Trayhurn & Beattie, 2001; Nawrocki & Scherer, 2004; Juge-Aubry et al., 2005; Wang et al., 2008).

1.2.3. Insulin and insulin resistance

Insulin is a polypeptide based hormone produced predominantly in the Islets of Langerhans of the endocrine pancreas by β -cells and is well described as the key hormone involved in the regulation of glucose and free fatty acid uptake by tissue cells uptake, with roles in the promotion and regulation of growth, differentiation and metabolism. This action is mediated mostly through action on liver, adipose and skeletal muscle tissue, although many other tissues have receptors for the action of insulin. Insulin is essentially a 51 amino acid dimer of two chains (A and B), each containing three α -helices linked together by disulfide bonds (Brange & Langkjoer, 1993; Menting et al., 2013).

Insulin stimulates the uptake and use of glucose differently in various tissues, mediated predominantly via hepatocytes, adipocytes and skeletal muscle. In an insulin sensitive individual, increased blood glucose stimulates the β -cells of the pancreas to synthesis and release insulin. Acting via the insulin responsive glucose transporter 4 (GLUT4) receptor, glucose is taken up by these tissues (Kim et al.,

2006a). Via this mechanism, insulin stimulates glycogen synthesis from glucose for storage, and suppresses hepatic gluconeogenesis (Kim et al., 2006a; Karnieli & Armoni, 2008). Insulin sensitivity correlates positively with cellular expression of GLUT4 (Karnieli & Armoni, 2008). The net effect of these predominant functions of insulin is to reduce glucose concentration in the blood stream via an increase in GLUT4 mediated cellular uptake. Glucose is then stored primarily as glycogen, with excess glucose being stored as fat (Kim et al., 2006a; Huang, 2009).

Insulin mediates metabolic and mitogenic effects through binding to cell surface insulin receptors, leading to activation of two pathways: the phosphoinositide 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways (Huang, 2009; Gallagher et al., 2010). PI3K results in activation of 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt kinase (Huang, 2009). This PI3K-Akt pathway is responsible for most downstream metabolic effects of insulin function, which includes activation of endothelial nitric oxide synthase (eNOS) and translocation of GLUT4 in skeletal muscle and adipocytes (via increased PPAR γ gene expression and actin mediated mobilisation) (Taniguchi et al., 2006).

The activation of the MAPK pathway generally mediates transcription of factors involved with cell growth (Gallagher et al., 2010). Effects of metabolites in this pathway result in vasoconstriction, increased expression of vascular cell adhesion molecules and growth and mitogenesis of vascular smooth muscle cells (Kim et al., 2006a; Huang, 2009; Gallagher et al., 2010). In brief, under normal function, insulin promotes cellular glucose uptake, inhibits gluconeogenesis, decreases adipose tissue lipolysis and hepatic very low density lipoprotein synthesis, in addition to decreasing appetite in the brain (Gallagher et al., 2010). It further mediates endothelial and vascular smooth muscle function (Huang, 2009).

1.2.3.1. Insulin resistance

Impaired insulin signalling is central to development of the metabolic syndrome and can promote cardiovascular disease indirectly through development of abnormal glucose and lipid metabolism, hypertension, and a proinflammatory state (Rask-Madsen & Kahn, 2012). Changes in insulin function are illustrated in Figure 3.

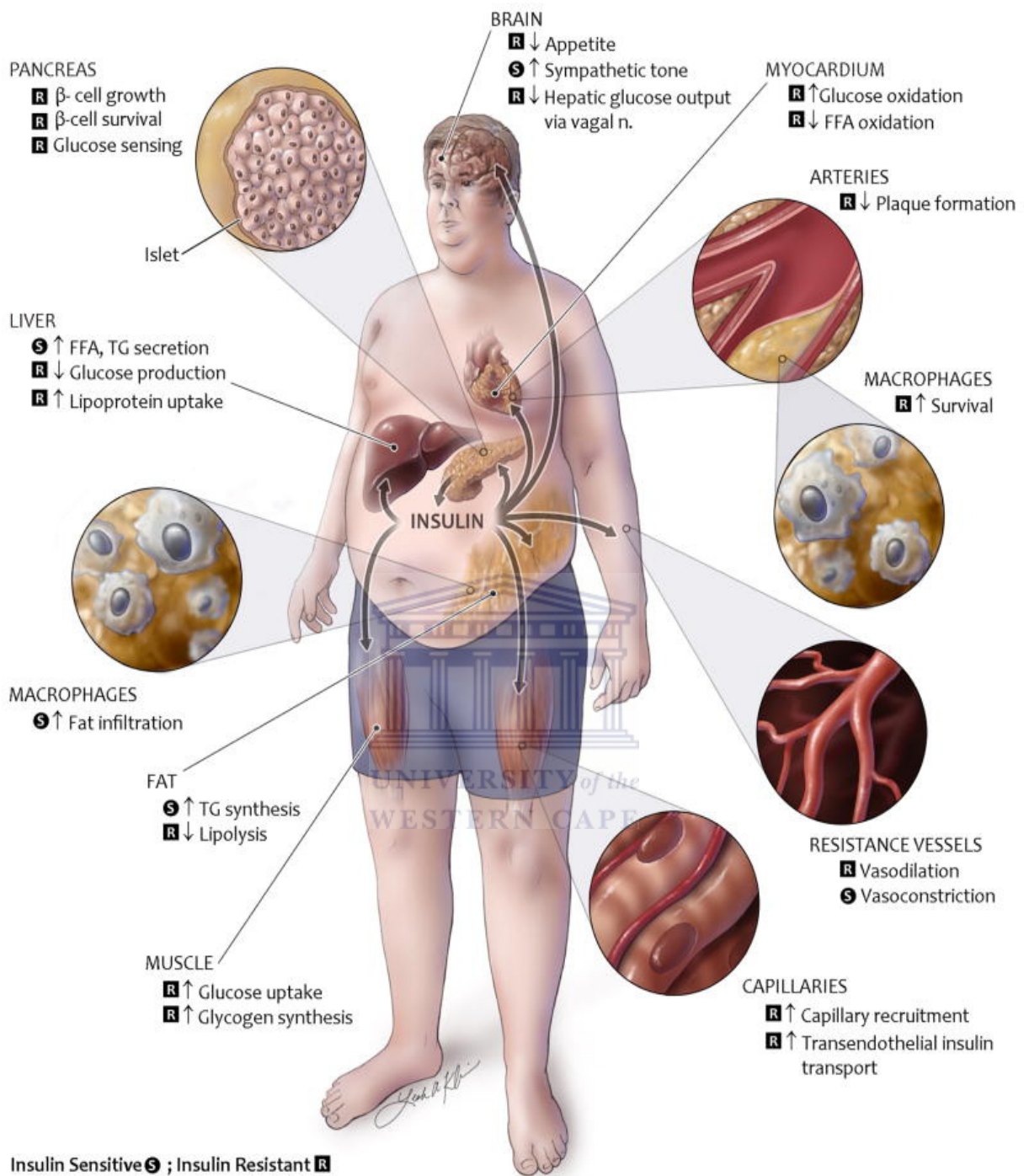


Figure 3: Insulin signalling in metabolic syndrome. Insulin actions which are insulin resistant in metabolic syndrome are labeled 'R', whereas insulin actions which remain insulin sensitive in metabolic syndrome are labeled 'S'.

Adapted from Rask-Madsen & Kahn (2012).

The basic concept of insulin resistance (IR) describes the phenomenon whereby adipose, hepatic or muscle cells do not respond to receptor mediated action of insulin, exacerbated by a dysregulation in various feedback mechanisms (Huang, 2009). IR can be described as a defect in the activity and action of insulin associated with fasting and even postprandial hyperinsulinaemia in order to maintain euglycaemia (Eckel et al., 2005). Insulin's action directly on vascular endothelium, atherosclerotic plaque macrophages, and in the heart, kidney, and retina has now been described, and impaired insulin signalling in these locations can alter progression of cardiovascular disease in the metabolic syndrome and affect development of microvascular complications of diabetes mellitus (Rask-Madsen & Kahn, 2012). This metabolic phenomenon is closely associated with hyperinsulinaemia and hyperglycaemia, and IR is therefore a powerful predictor of T2DM (Huang, 2009).

As a result of these associations, IR is generally the most accepted and unifying hypothesis to describe the underlying pathophysiology of MetS (Eckel et al., 2005; Gallagher et al., 2010). However, Cheal and colleagues (2004) also highlighted that although IR and/or hyperinsulinaemia is closely correlated with the diagnosis of MetS, their results indicated a sensitivity and positive predictive value of IR for MetS to be 46% and 76%, respectively. Obesity and increased WC is a predominant risk factor for the development of insulin IR, and appears to play a central role in the pathogenesis of atherosclerosis and T2DM (Liao et al., 2004; Zeyda & Stulnig, 2009). Although some obese patients do express the metabolic phenotype associated with CVD and T2DM, increasing IR is closely associated with MetS derangements such as higher blood pressure, triglyceride levels, low HDL cholesterol and glucose deregulation (Gallagher et al., 2010).

In IR, the PI3K-Akt pathway is affected, whereas MAP kinase pathway is not. This leads to a mismatch between these functions, and the balance between these pathways is affected (Huang, 2009). Inhibition of PI3K-Akt leads to reduced eNOS and thereby reduced nitric oxide (NO), resulting in endothelial dysfunction. It is also associated with reduced GLUT4 translocation, leading to reduced uptake of glucose by adipocytes and skeletal muscle (Huang, 2009). This pathway is also associated with a negative influence on the hypothalamic-pituitary –testis (HPT) axis (Acosta-

Martínez, 2012) (illustrated in Figure 4) and spermatozoa function post ejaculation (Andò & Aquila, 2005).

By contrast, the MAP-kinase pathway is overly active, leading to increased vasoconstriction, over expression of vascular cell adhesion molecules (which increases leukocyte interaction with blood vessel wall) and increases smooth muscle cell growth in blood vessels (Huang, 2009). IR thus leads to various vascular abnormalities that are closely associated with atherosclerosis (and hence CVD) and hyperglycaemia (and hence T2DM) (Eckel et al., 2005).

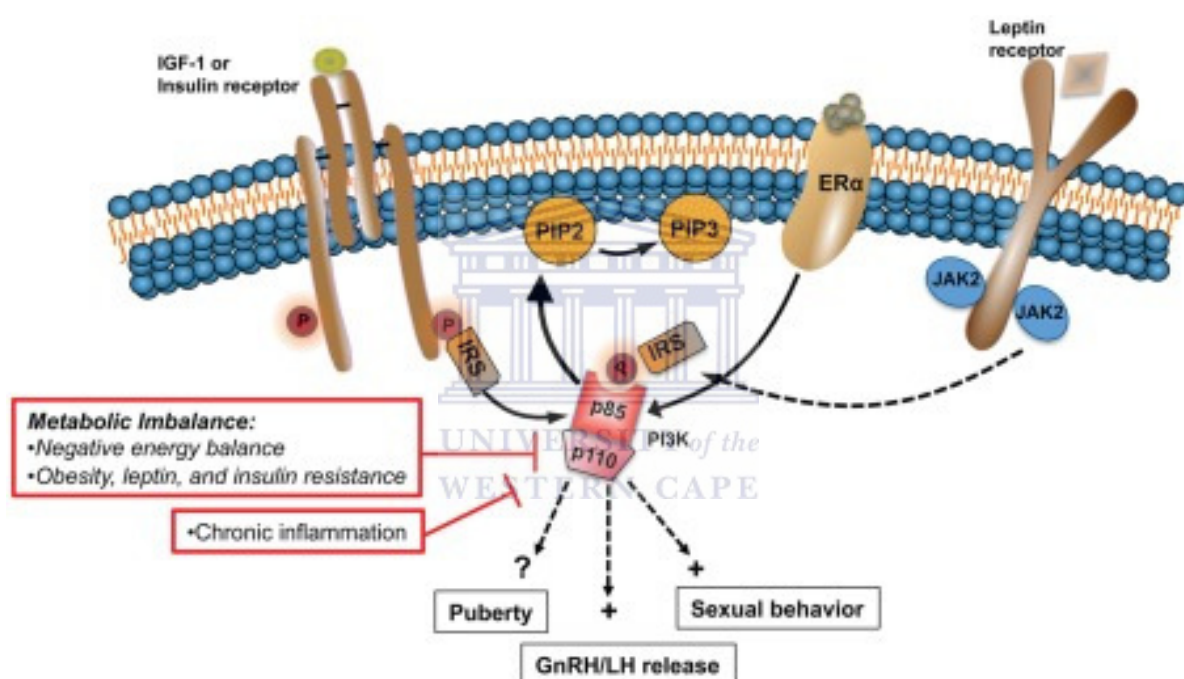


Figure 4: P13K pathway alterations in insulin and leptin resistance and effects on the hypothalamic-pituitary-testis axis during chronic metabolic disturbances. Alterations in the levels and sensitivity to peripheral hormones and metabolic signals, including insulin and leptin, play a major role in the dysfunction of the PI3K in this figure, altered PI3K signalling is linked to the negative effects that metabolic imbalance has on the HPG axis.

IRS: Insulin Receptor Substrate; PI3K: Phosphoinositide 3-kinase; PIP2: Phosphatidylinositol-diphosphate; PIP3: Phosphatidylinositol-triphosphate; IGF-1: Insulin-Like Growth Factor 1; ER α : Oestrogen Receptor alpha.

Adapted from Acosta-Martínez (2012).

Various metabolic factors play a role in the development of IR. This includes, but not limited to free fatty acids, inflammatory cytokines, adipokines, ROS, and mitochondrial dysfunction (Eckel et al., 2005; Huang, 2009; Gallagher et al., 2010). However, factors that can induce IR are also generally worsened by IR. This includes obesity itself, dyslipidaemia (such as increased triglycerides and VLDL and reduced HDL), glucose intolerance and inflammation (Eckel et al., 2005; Huang, 2009; Gallagher et al., 2010).

Various methods are used to estimate the sensitivity of cells to the action of insulin. The gold standard is considered the Euglycaemic Hyperinsulinaemic Clamp (EHC), as it directly measures the action of insulin on glucose metabolism in steady state conditions. However, this method is not suitable for clinical assessment (Rabasa-Lhoret et al., 2003). The Quantitative Insulin Sensitivity Check Index (QUICKI), proposed by Katz and colleagues (2000), is calculated from fasting blood glucose and insulin concentrations, with a decreased score indicative of decreased insulin sensitivity (increased insulin resistance) (Rabasa-Lhoret et al., 2003). Evidence indicates that the QUICKI is a useful assessment of insulin sensitivity, and that this score correlates closely with EHC across a wide range of glucose concentrations (i.e. is better correlated to EHC than other fasting-based index of insulin sensitivity in different insulin resistant states) (Hrebicek et al., 2002; Rabasa-Lhoret et al., 2003; Yokoyama et al., 2004).

1.2.4. Leptin

Work with genetically obese mouse models (*ob/ob*) lead to the identification of the *ob* gene and its protein product, leptin (from *leptos*, meaning thin) in 1994. The *ob/ob* mouse was found to lack this hormone. Leptin has been associated with reducing appetite and food consumption resulting in reduced body mass and body fat percent, with the maintenance of lean tissues. Injecting leptin into rodents is associated with increased energy expenditure, improvement in glycaemic control and even reproductive function (Bastard et al., 2006; Kwon & Pessin, 2009; Galic et al., 2010). Leptin is almost exclusively expressed by adipocytes, specifically visceral adipose tissues (Bastard et al., 2006). Serum leptin concentrations are correlated with several features of metabolic syndrome, and highly correlated with BMI and visceral

obesity, although the association with MetS and insulin resistance appears to be independent of rising BMI. This relationship is significantly mediated through the effects of central obesity (Esteghamati et al., 2009).

The protein structure of leptin is very similar to that of other pro-inflammatory cytokines, including interleukin-2 and IL6 (Ahima & Flier, 2000). Leptin receptors also belong to the class 1 receptor family, with also the indication that inflammation is raised in hyperleptinaemia without obesity (Loffreda et al., 1998; van Dielen et al., 2001). Therefore, although typically known for action on the central nervous system to regulate food intake and energy expenditure (Bastard et al., 2006), leptin can also mediate inflammation via receptor interactions (LepR_b) (Kwon & Pessin, 2009; Procaccini et al., 2012). It also activates monocytes to synthesise and secrete IL6 and TNF α , amongst other cytokines, in addition to Th1 cell stimulation (Kwon & Pessin, 2009; Procaccini et al., 2012). Almost all tissues exhibit leptin receptors, indicating a significant role in for leptin in overall physiology (Kwon & Pessin, 2009; Procaccini et al., 2012).

Leptin interacts with six types of receptors (Ob-Ra–Ob-Rf, or LepRa-LepRf), that in turn are encoded by a single gene, LEPR (Wang et al., 1996). Ob-Rb is the only receptor isoform that can signal intracellularly via the JAK2/STAT3 and MAPK signal transduction pathways (Malendowicz et al., 2006). As with insulin, biological effects of leptin are mediated via activation of the PI3K intracellular pathway (Figure 4) (Donato et al., 2010; Acosta-Martínez, 2012). As a breakdown in this pathway is central to the concept of insulin resistance (Huang, 2009), this too leads to a phenomenon associated with MetS termed leptin resistance (Acosta-Martínez, 2012).

1.2.5. Inflammation and inflammatory cytokines

MetS is closely associated with a low grade, asymptomatic, systemic and chronic inflammatory state (Monteiro & Azevedo, 2010; Fuentes et al., 2013). This is demonstrated by a subtle and detrimental increase in serum C-reactive protein (CRP), a biochemical marker of inflammation that can be easily assessed in laboratory investigations (Haffner, 2003; Tamakoshi et al., 2003; Monteiro &

Azevedo, 2010; Brooks et al., 2010). Synthesised in the liver, CRP production is up-regulated by the pro-inflammatory cytokines tumour necrosis factor alpha (TNF α) and interleukin-6 (IL6), with both cytokines raised in the serum of obese patients and those with MetS (Tamakoshi et al., 2003; Khaodhjar et al., 2004; Monteiro & Azevedo, 2010; Brooks et al., 2010). A chronic and subtly raised CRP concentration in serum, the so-called highly sensitive CRP (hs-CRP) (Brooks et al., 2010), is considered a non-specific inflammatory marker and predictor of T2DM, sub-clinical atherosclerosis, CVD and even some cancers (Pradhan et al., 2001; Malik et al., 2005; Reaven 2005; Haffner, 2006; Yuan et al., 2006; Hsing et al., 2007; Nakano et al., 2010).

Increased visceral obesity results in an altered secretion pattern of adipokines (Nawrocki & Scherer, 2004; Juge-Aubry et al., 2005; Kintscher et al., 2008; Nishimura et al., 2009; Fuentes et al., 2013). These adipokines, which originate adipocytes themselves as well as from adipose tissue-associated macrophages, have been found to play a significant role in multiple metabolic and inflammatory responses in human physiology and pathology, with a corresponding enhanced basal inflammatory tone (Nawrocki & Scherer, 2004; Juge-Aubry et al., 2005; Kintscher et al., 2008; Nishimura et al., 2009; Fuentes et al., 2013). This increased inflammatory setting is closely associated with the phenomenon of IR, which is considered by many researchers to be an underlying and central feature of the MetS (Nawrocki & Scherer, 2004; Kasturi et al., 2008, Kintscher et al., 2008; Phillips & Prins, 2008). Furthermore, all the parameters included in the definition and diagnosis of MetS are associated with a low-grade inflammatory state (Esposito & Giugliano, 2004), typically identified via a significant correlation with CRP (Pradhan et al., 2001; Malik et al., 2005; Haffner, 2006; Yuan et al., 2006; Nakano et al., 2010). As this pro-inflammatory state is directly linked with obesity, insulin resistance, diabetes and endothelial dysfunction (Esposito & Giugliano, 2004; Phillips & Prins, 2008), it is even suggested that MetS may be an adipose tissue disease different from obesity, and would thus be characterised by systemic inflammatory markers (Camera et al., 2008; Oda, 2008). This relationship is illustrated in Figure 5.

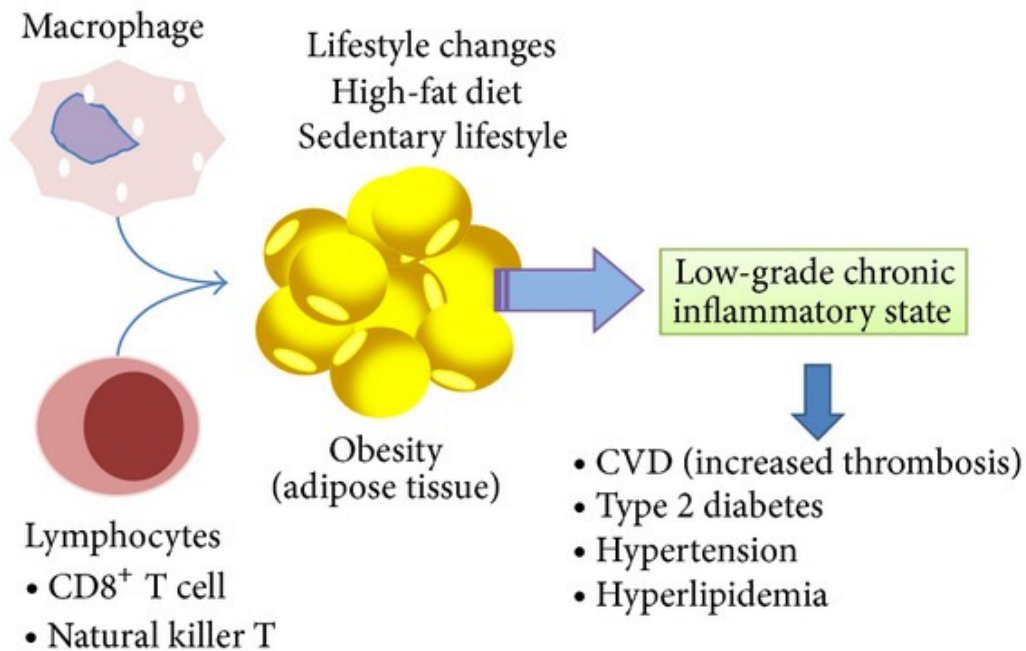


Figure 5: Leukocytes and adipose tissue inflammation associated with MetS. Macrophage and lymphocyte infiltration in adipose tissue may greatly contribute to obesity-related metabolic dysfunction and chronic inflammation, as well as adipocytes.

CVD: cardiovascular diseases. UNIVERSITY of the WESTERN CAPE

Adapted from Fuentes et al. (2013).

Immune cells are known to increasingly infiltrate adipose tissue in direct correlation to increased adipose tissue. Initial T-lymphocyte accumulation (Kintscher et al., 2008; Nishimura et al., 2009) is followed by macrophages (Lumeng et al., 2012), which increasingly secrete proinflammatory cytokines such as TNF α , IL6 and interleukin 1-beta (IL1 β) which contributes significantly to the induction of IR (Nawrocki & Scherer, 2004; Juge-Aubry et al., 2005; Bastard et al., 2006; Kintscher et al., 2008; Nishimura et al., 2009).

1.2.5.1. Tumour necrosis factor-alpha (TNF α)

TNF α was originally discovered as a protein that mediates tumour necrosis with a role in cancer cachexia, induced via endotoxin activity on macrophages (Kwon &

Pessin, 2009). It is a 26kDa transmembrane protein that is converted to a 17kDa soluble molecule via metalloproteinase. Although produced by a variety of cell types, it is mostly associated with macrophages and lymphocytes. Although adipocytes are capable of synthesising and secreting TNF α , this activity is weak in humans, and it is now thought that increased secretion associated with obesity is from M1-macrophages that infiltrate the adipose tissue (Bastard et al., 2006; Galic et al., 2010). TNF α has a wide range of inflammatory functions, with a variety of factors that can induce its production, and is thought to contribute to obesity associated complications (Wozniac et al., 2009).

TNF α has been well studied in the development of IR and MetS. However, the precise role and even origin of the protein requires further investigation. Numerous studies have shown that TNF α can impair insulin signalling in hepatocytes, adipose tissue and skeletal muscle (Galic et al., 2010). This is mediated via phosphorylation of insulin receptor substrate-1 (IRS-1), likely preventing the interaction of this protein with insulin receptor beta subunit and inhibiting this pathway (Bastard et al., 2006). In both cell culture and rodent models, TNF α administration induces IR, and neutralisation of TNF α improves insulin sensitivity (Kwon & Pessin, 2009) and improves insulin sensitivity in high fat diet induced IR in rodent models (Uysal et al., 1997). Long term TNF α -antagonist administration to obese patients with rheumatoid arthritis also improves insulin sensitivity (Kwon & Pessin, 2009). In obese patients with T2DM, this treatment does not improve hyperglycaemia or insulin sensitivity, but it does improve insulin sensitivity in obese patients without T2DM. However, this treatment in humans is highly controversial (Kwon & Pessin, 2009; Galic et al., 2010).

1.2.5.2. Interleukin 1-beta (IL1 β)

Interleukin 1-beta (IL1 β), also known as catabolin, is a member of the IL1 cytokine family. This cytokine is produced via cleavage of pro-IL1 β by NLRP3-caspase-1, which in turn is activated by a multiprotein complex called the inflammasome and mediates inflammatory activities including cell proliferation, differentiation and apoptosis (Mills & Dunne, 2009; Tack et al., 2012). Various lines of research suggest that IL1 β plays an important role in obesity associated inflammation and insulin

resistance (Tack et al., 2012). In NLRP3-caspase-1 deleted animal models, or inhibition of caspase 1, there is some protection offered against IR development induced by obesity (Stienstra et al., 2010; Vandanmagsar et al., 2011). In the IL1 receptor I (IL1RI) deficient mice fed a high fat diet, typically associated with the production of obesity and IR, the lack of IL1 β binding is also associated with IR protection and a reduced adipose tissue inflammatory response (McGillicuddy et al., 2012). IL1 β is also known to promote the inflammatory response, typically inducing production of other proinflammatory cytokines (Tanaka et al., 1999; Jager et al., 2007).

1.2.5.3. Interleukin 6 (IL6)

Interleukin 6 (IL6) is produced by many cell types, including monocytes and macrophages, fibroblasts and endothelial cells (Bastard et al., 2006). It is estimated that 15-30% of circulating IL6 is derived from adipose tissue in the absence of inflammation (Mohamed-Ali et al., 1997), with increased secretion associated with visceral adipose tissue rather than subcutaneous depots (Fain et al., 2004). In both non-obese and obese individuals, IL6 has a very close correlation with CRP levels, and IL6 is a predominant stimulator for hepatic CRP synthesis (Fain et al., 2004).

IL6 circulates at high plasma concentrations in MetS, and may represent a hormonal factor that induces muscle insulin resistance (Fernandez-Real et al., 2001; Esposito & Giugliano, 2004). As mentioned above, IL6 is typically secreted from activated macrophages and lymphocytes, but adipose tissue may be the source in non-acute inflammatory conditions such as MetS (Mohamed-Ali et al. 1997). IL6 levels correlate positively with BMI, hyperinsulinaemia (Fried et al., 1998), insulin sensitivity (Pradhan et al., 2001) and T2DM – although serum concentrations remain within normal limits (Fernandez-Real & Ricart, 2003). IL6 also appears to play a very prominent role in the link between obesity, MetS and coronary heart disease (Yudkin et al., 2000). TNF α is also a strong inducer of IL6 from adipocytes themselves (Rotter et al., 2003).

1.2.5.4. Interleukin 8 (IL8)

IL8, also known as neutrophil chemotactic factor, is a member of the CXC chemokine family, is produced predominantly by macrophages, but can be produced by any cell with toll like receptors (Baggiolini & Clark-Lewis, 1992). It is produced in response to activation of the innate immune system. IL8 predominantly has chemotactic (for granulocytes) and angiogenic properties, actively functioning in both acute inflammation and endothelial cell proliferation, and can also induce phagocytosis by granulocytes (Baggiolini & Clark-Lewis, 1992).

IL8 are secreted by adipocytes, and circulating concentrations are positively correlated with BMI, WC and IR, suggesting roles in obesity related phenomenon such as MetS (Kim et al., 2006b). TNF α has been shown to increase IL8 release in visceral adipose tissue (Bruun et al., 2001), with further studies indicating increased IL8 associated with increased waist-to-hip ratio and fat mass (Strackowski et al., 2002).

1.2.6. Hormonal changes and hypogonadism in males

Hormonal changes that are associated with MetS in males include reduced serum total testosterone, free testosterone and sex hormone binding globulin (SHBG), increased serum oestrogen, insulin (insulin resistance), leptin, FSH, LH and prolactin (Kasturi et al., 2008; Hofny et al., 2010). As described below, these hormones are also involved in male reproductive function, and therefore these changes are of interest in studies related to male infertility.

In adult males, MetS correlates with reduced serum testosterone concentrations and raised gonadotrophins, thus reflecting a state of primary hypogonadism (Pasquali, 2006; Guay, 2009; Saad & Gooren, 2009). As a more recent and very important development, it is emerging in the scientific literature that reduced serum testosterone in non-obese men, including those with asymptomatic androgen deficiency, increases the risk of developing MetS (Boyanov et al., 2003; Kupelian et al., 2006; Traish et al., 2009). This is further associated with further lines of evidence suggesting that the clinical administration of testosterone can improve many of the

characteristics associated with the syndrome, as well as T2DM, in male patients (Saad & Gooren, 2009).

Reduced testosterone associated with obesity and MetS is partly explained by increased activity of the aromatase cytochrome P450 enzyme which is over expressed in visceral adipose tissue associated with obesity. This results in increased peripheral conversion of testosterone into oestrogens (Roth et al., 2008). This is further associated with reduced LH and FSH production by the pituitary, leading to reduced testosterone synthesis (hypogonadotropic hypogonadism) and reduced spermatogenesis (Cabler et al., 2010).

1.3. Male reproduction and infertility

Infertility is defined by the World Health Organisation (WHO) as 'the inability of a couple to achieve conception or bring a pregnancy to term after 12 months or more of regular (at least three times per week), unprotected sexual intercourse' (WHO, 2010). It affects 15% (approximately one in seven) of couples trying to conceive (Kefer et al., 2009). Of these cases, 25-50% can be attributed partially or solely to the male partner (Lampiao & du Plessis, 2008a; Hamada et al., 2012a), with up to 7% of men affected by infertility during their reproductive lifetime (Behre & Nieschlag, 2000). Interestingly, it appears that sperm dysfunction is the single most common cause of infertility among couples of reproductive age (Barratt et al., 2011).

An assessment of sperm quality, based on WHO guidelines (2010), is normally used to estimate the fertilisation potential of the male partner. A decrease in sperm quality is considered a major reflection of the decreased ability of the male partner to contribute to fertilisation (Hamada et al., 2012a). As the incidence is increasing, male infertility represents a challenging and important area of laboratory and clinical science investigation, with a need to further improve diagnostics, mechanisms and possible treatments (Hamada et al., 2012a).

1.3.1. Causes and risks of male infertility

Male infertility arises from a variety of health problems, including genetic causes, organic pathology and lifestyle or environmental factors that can negatively influence the male fertility potential (Esteves et al., 2012).

Well defined genetic causes of infertility include Klinefelter syndrome, Kallmann syndrome and cryptorchidism ($\pm 2.7\%$ cases) (Dada et al., 2011; Hamada et al., 2011). The known acquired causes of male factor infertility include varicoceles ($\pm 25\%$ cases), urogenital infections ($\pm 10\%$ cases), immunogenic causes (such as antisperm antibodies), impotence or sexual/ejaculation inadequacy ($\pm 0.7\%$ cases), testicular failure ($\pm 1.1\%$ cases), other acquired urogenital abnormalities (e.g. structural complication following infections or inflammation, such as mumps orchitis), and various endocrine disorders ($\pm 1.5\%$ cases) (Dohle et al., 2005; Hamada et al., 2011; Esteves et al., 2012; Hamada et al., 2012a). Although numerous aetiologies and risks are associated with male infertility, approximately 20 - 50% of these cases have no known aetiology, termed 'idiopathic' (Hamada et al., 2012a), which is also termed idiopathic oligoasthenoteratozoospermia (iOAT) (Jungwirth et al., 2012). This term denotes unexplained abnormalities in sperm parameters that include low sperm concentration, reduced sperm motility and abnormal sperm morphology (Jungwirth et al., 2012).

Various environmental and lifestyle factors are increasingly associated with changes in male reproductive function, affecting fertilisation ability. Risks associated with male factor sub- or infertility include consumption of alcohol and tobacco, recreational drug use (such as marijuana and cocaine), exposure to excessive testicular heat (i.e. welders; bakers), prolonged urban driving and sitting (related to testicular heat), exposure to oestrogens and androgens, various environmental toxins known as endocrine disruptors (e.g. pesticides; phthalates), exposure to heavy metals (e.g. lead; cadmium), stress (both physiological and psychological), ionising radiation and even exposure to cell phone radiation (Dohle et al., 2005; Agarwal et al., 2008; Makker et al., 2009; Mendiola et al., 2009; Esteves et al., 2012). As discussed in more detail below, obesity (and conceivably MetS) is also associated with male infertility. Semen is therefore considered a sensitive indicator of environmental,

occupational and lifestyle exposures that can exert direct toxic effects and hormonal disruption (Mendiola et al., 2009).

1.3.2. Male fertility assessment

Male fertility and infertility should be investigated with a complete and detailed medical history and a thorough systemic and genital examination (Hamada et al., 2011; Esteves et al., 2012; Hamada et al., 2012a). Typically, a standard semen analysis is done (detailed below) in order to investigate sperm function (Aitken, 2006; Lewis, 2007; Hamada et al., 2012a). However, the predictive value of a normal semen test is approximately 60% (van der Steeg et al., 2010). In addition, hormonal analysis can be done, including testosterone, FSH, LH, oestrogen, prolactin and thyroid function (Hamada et al., 2012a). Additional analysis includes visual investigations such as ultrasounds, especially of the testes, vas deferens, seminal vesicles and prostate (Hamada et al., 2012a). Genetic testing is also required in many cases (Dada et al., 2011; Hamada et al., 2011; Esteves et al., 2012). In some cases, particularly cases that appear to be idiopathic (otherwise normal history, clinical and visual examinations, semen analysis and hormonal assessments), more elaborate analyses can be offered. This includes antisperm antibody determination, postcoital (sperm cervical fluid penetration) test, sperm DNA fragmentation and chromatin tests, sperm mitochondrial membrane potential, ROS assessment in seminal fluid and acrosome reaction tests (Aitken, 2006; Lewis, 2007; Dada et al., 2011; Hamada et al., 2011; Esteves et al., 2012). A more detailed flow diagram for male fertility assessment, as recommended by Hamada et al. (2011) is illustrated in Figure 6 below.

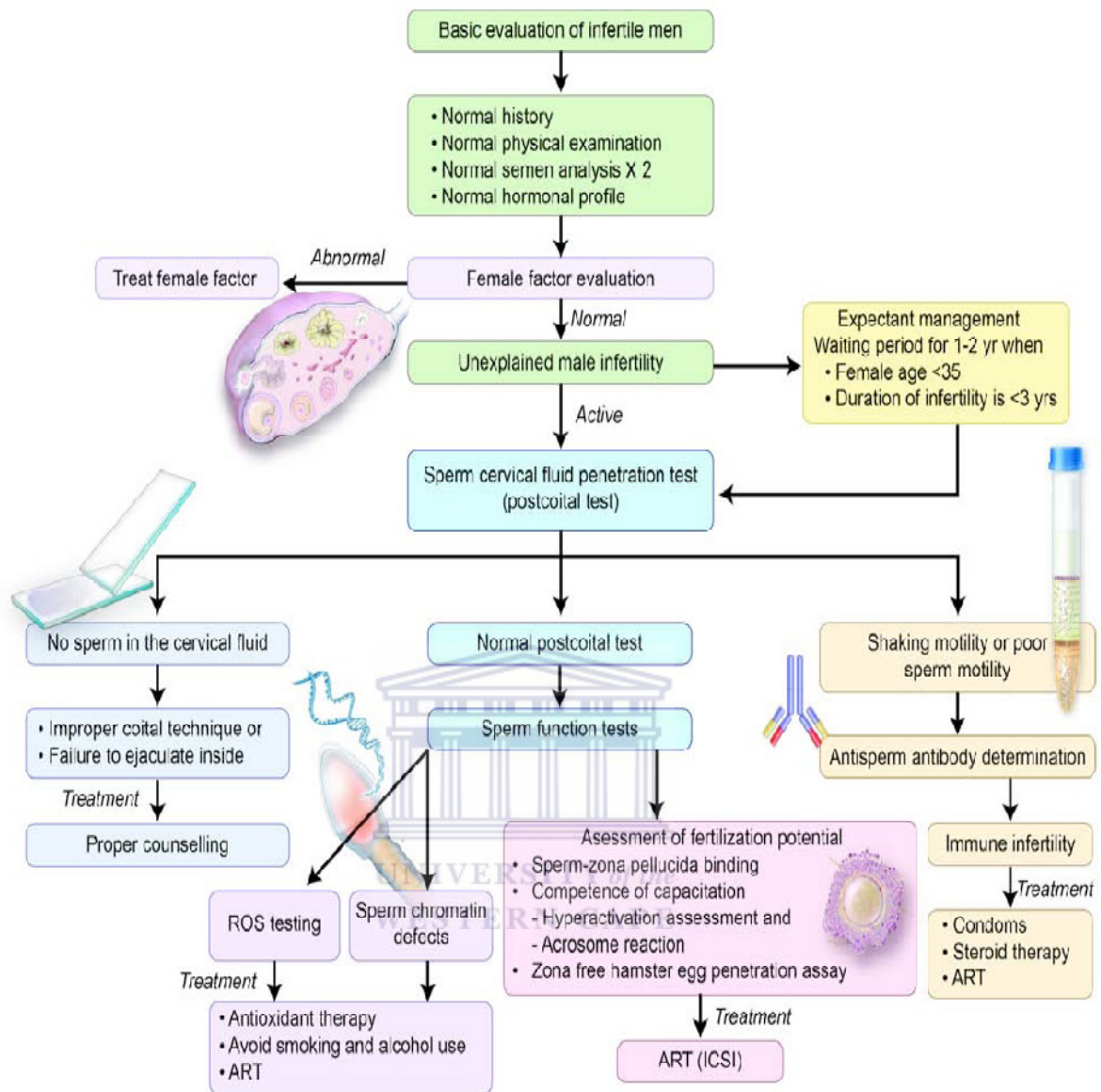


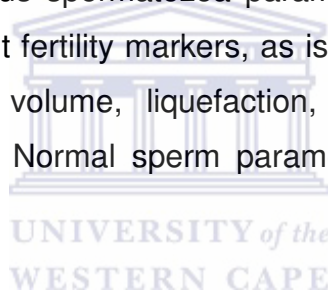
Figure 6: A detailed protocol for the clinical diagnostic approach to male fertility assessments as recommended by Hamada et al. (2011).

1.3.3. The semen analysis

Human semen, and particularly spermatozoa, is produced via complex and sophisticated biological processes, produced by specialised cells and tissues. Spermatozoa are produced over a 72 day cycle by germinal epithelium, and are very sensitive to changes in the regulatory mechanisms (Mendiola et al., 2009). These mechanisms include (but not limited to) hormonal factors such as testosterone,

insulin and leptin, and various cytokines (Dohle et al., 2003; Martinez et al., 2007; Lampiao et al., 2009). Damage or disruption of this cycle can occur at any stage, and may be either reversible or permanent (Mendiola et al., 2009). During ejaculation, semen is produced from a concentrated suspension of spermatozoa, stored in the paired epididymides, diluted and mixed by fluid secretions from the accessory sex organs, mostly the prostate and seminal vesicles (WHO, 2010). Semen therefore has two key factors, namely the number of spermatozoa (reflecting sperm production by the testes) and the total fluid volume (WHO, 2010).

The standard for male fertility assessment is outlined in the *Laboratory Manual for the Examination and Processing of Human Semen 5th Edition* (WHO, 2010). Notably, this latest edition lowers most normal values for male sperm parameters compared to the previous additions of the WHO guideline manuals (Esteves et al., 2012; Hamada et al., 2012a). Various spermatozoa parameters, such as motility, vitality and morphology, are important fertility markers, as is the quality and composition of the seminal fluid, such as volume, liquefaction, viscosity, pH and leukocyte concentration (WHO, 2010). Normal sperm parameter cut off values is listed in Table 2.



It must be noted, however, that although the semen analysis remains a standard test in male fertility assessments, its clinical value is limited as 5% of fertile men and 16% of infertile men display poor semen analyses (Lewis, 2007). There are also large variations between males from different geographical locations and countries, individuals within the same regions and even different samples from the same individuals (Lewis, 2007). In addition to the number of spermatozoa, the functional capacity of sperm is considered a more sensitive determinant of fertility potential (Aitkin, 2006). However, two factors are clear: firstly, samples with values below cut off values increases the risk of subfertility; secondly, values higher than cut off points do not provide any diagnostic information (Barratt et al., 2009).

Table 2: Cut off values for normal results associated with the standard semen analysis.

Parameter	Normal finding	Reference
Semen volume	> 1.5ml	WHO, 2010
Total sperm count	> 39 x 10 ⁶ /ejaculate	WHO, 2010
Sperm concentration	> 15 x 10 ⁶ /ml	WHO, 2010
Progressive Motility	> 32%	WHO, 2010
Total Motility	> 40%	WHO, 2010
Vitality	> 58%	WHO, 2010
Morphology	> 4% normal forms	WHO, 2010
Leukocytes	< 1 x 10 ⁶ leukocytes/ml	WHO, 2010

1.3.3.1. Sperm concentration and total sperm count

Sperm concentration, represented as number of spermatozoa per ml of seminal fluid (WHO 2010), is a predictor of conception and is closely related to time of pregnancy. Sperm concentration is not a direct measure of testicular sperm output, however, total sperm count is (Ng et al., 2004). Total sperm count is not synonymous with sperm concentration, but defined as the total number of spermatozoa in the entire ejaculate (product of sperm concentration and ejaculate volume) (WHO, 2010). However, both sperm concentration and total sperm count reflect testicular sperm productivity (Hamada et al., 2012a).

1.3.3.2. Sperm motility

The ability of sperm to move, the motility, is an important factor in fertilisation (Hamada et al., 2012a). Motility can be classified by WHO (2010) into three categories: namely progressive, non-progressive and immotile. Progressive motility (PM) is defined as spermatozoa moving actively, either linearly or in a large circle,

regardless of speed. Non-progressive motility (NP) is considered all other patterns of motility. Immobility is defined as spermatozoa with no movement (WHO, 2010). Total sperm motility is considered the sum of PM and NP (WHO, 2010). As with sperm concentration and total sperm count, the percentage of motile sperm, particularly PM, is closely related to male fertility potential and pregnancy rates (Hamada et al., 2012a).

1.3.3.3. Sperm vitality

Sperm vitality is simply a measure of the percentage of alive (viable) and dead spermatozoa in a sample, and expressed as a percentage of viable cells (WHO, 2010). Although often a routine parameter, it is especially important in the assessment of samples with less than 40% sperm motility (WHO, 2010). It is clinically important to know if immotile cells are dead or alive, and interpretation of vitality needs to be considered in light of motility (i.e. the percentage of viable cells should exceed that of total sperm motility) (WHO, 2010). Typically, the motility and vitality correlate with each other (Paoli et al., 2011). A large proportion of vital yet immotile cells is associated with flagellum defects (Chemes & Rawe, 2003), while immotile and non-viable cells (necrozoospermia) may indicate epididymal pathology (Correa-Pérez et al., 2004).

1.3.3.4. Morphology

Human spermatozoa morphology is highly variable, and it has proven difficult to identify and describe the normal shape of cells with fertilisation potential. With the application of strict criteria (Kruger et al., 1986; Menkveld et al., 1990), increased percentage of normal forms of morphology is associated with increased fertilisation ability, and parameters have been established that are important markers in prognosis of fertility (Menkveld et al., 1990; Eggert-Kruse et al., 1995; Obara et al., 2001). In a review by Coetzee and colleagues (1998), a large proportion of studies included indicated that normal sperm morphology and acrosome morphology is an important factor in male fertility potential. Application of the strict criteria is a reliable estimation of fertilization ability of human spermatozoa (Obara et al., 2001).

Normal spermatozoa consist of a smooth and regularly contoured oval shaped head with well defined acrosomal regions, a slender and regular midpiece (about the same length as the sperm head), a principle piece uniform in calibre and an endpiece. This can be simplified into a head (including neck) and tail (midpiece and principle piece), with both pieces needing to be normal (Mortimer & Menkveld, 2001). Head defects can include being too large or small, tapered, pyriform, round, amorphous, vacuolated, changes in the acrosomal area, double heads, or any combination of these. Neck and midpiece defects can include asymmetrical insertion of the midpiece into the head, thick or irregular, sharply bent, abnormally thin, or any combination of these. Principal piece defects include being short, multiple, broken, smooth hairpin bends, sharply angulated bends, of irregular width, coiled, or any combination of these (Kruger, 1993).

1.3.3.5. Leukocytes

Leukocytes, predominantly polymorphonuclear (PMN) leukocytes in the form of granulocytes, are present in ejaculated semen (Wolff, 1995; Martinez et al., 2007). It is important to differentiate these from spermatozoa on microscopy and especially with morphology staining techniques and assessments (Johanisson et al., 2000). Although visual differences between spermatozoa and leukocytes can be identified on microscopy, peroxidise reaction assays are frequently used as most PMN cells in semen are peroxidise positive granulocytes (Endtz et al., 1974; Politch et al., 1993, Wolff, 1995).

The total number of leukocytes in the ejaculate is correlated with the severity of the inflammation or infection (Wolff, 1995). The prevalence of leukocytospermia among male infertility patients is approximately 10% to 20%, with much controversy on the significance of leukocytes in semen (Wolff, 1995; Henkel, 2005). Reports of cut off values for peroxidise-positive leukocytes in fertile men range from $0.5 - 1 \times 10^6$ per ml of seminal fluid, with the WHO defining leukocytospermia as $> 10^6$ /ml in previous editions of the manual (WHO, 2010). This has been found by some to be too high, and others to be too low (Henkel, 2005). However, increasing numbers of leukocytes in the ejaculate is associated with infection, inflammation and poor semen quality and fertility endpoints (Henkel & Schill, 1998; Sanocka-Maciejewska et al., 2005).

Increased leukocytes and leukocyte activity associated with infections or inflammation are associated with reduced seminal volume and sperm cell count, impaired sperm motility and DNA integrity of spermatozoa (Kohn et al., 1998; Henkel, 2011a).

1.3.3.6. DNA fragmentation

Although the semen analysis remains the laboratory cornerstone in the assessment of male fertility potential, additional investigations have more recently been employed (Aitken, 2006). The inclusion of DNA damage in sperm can provide a more comprehensive analysis, as damaged sperm DNA is associated with numerous fertility checkpoints (Henkel et al., 2010; Schulte et al., 2009). This includes impaired fertilisation, reduced rate of early embryo development, reduced implantation, increased risk of spontaneous abortions and even birth defects (Lewis & Simon, 2010). Determination of DNA integrity in spermatozoa may be a primary predictor of the future health of the offspring, in addition to a predictor of semen function (such as morphology and motility), fertilisation, pregnancy complication risks, live birth rates and vitro fertilisation (IVF) outcomes (Henkel et al., 2004; Sergerie et al., 2005; Henkel et al., 2010; Sharma et al., 2013; Simon et al., 2013). Assessment of sperm DNA damage can also be useful in diagnostics associated with idiopathic cases of male infertility, with up to 80% of these cases displaying spermatozoa DNA fragmentation (> 25% of sample) (Simon et al., 2013).

Causes to damages in spermatozoa DNA are numerous, and importantly include oxidative stress due to ROS (Henkel, 2005; Sergerie et al., 2005; Henkel, 2011a). Apart from intrinsic production of ROS associated with mitochondrial energy production, increased ROS is associated with various factors, such as leukocyte activities, infections and inflammation, environmental pollutants and toxins, smoking and alcohol use, psychological and physiological stressors, nutritional deficiencies and advanced age, amongst other sources. Other causes of DNA fragmentation include aberrant spermatozoa maturation, apoptosis and radiation (Henkel, 2005; Sergerie et al., 2005; Henkel, 2011a; Sharma et al., 2013).

There are numerous methods to assess DNA integrity. TUNEL and COMET (in neutral conditions) assess molecular DNA strand breaks, whereby SCSA (sperm chromatic structure assay) and COMET (in alkaline conditions) assess susceptibility of DNA to damage, rather than damage itself (Sharma et al., 2013).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling (TUNEL) is considered an important technique used in the assessment of sperm DNA damage. The TUNEL staining technique is able to detect the percentage of spermatozoa in a given sample with either single or double-stranded DNA breaks (Henkel, 2005; Sharma et al., 2010; Sharma et al., 2013). There is a good correlation between percentages of sperm with DNA damaged found using optical microscopy versus flow cytometry, although actual percentages may be different (Domínguez-Fandos et al., 2007).

1.3.3.7. Mitochondrial membrane potential

Inner mitochondrial membrane potential (MMP) is considered an indicator of the mitochondrial energetic state in cells, as is considered the factor which most closely represents spermatozoal mitochondrial function (Paoli et al., 2011). In spermatozoa particularly, MMP is closely associated with motility (Paoli et al., 2011; Marchetti et al., 2012). In addition, sperm with high MMP are morphologically normal (MMP may be reduced in sperm with abnormal morphology), and can be a general indicator of sperm function overall (Marchetti et al., 2012). Increased percentage of sperm with abnormal MMP is associated with reduced fertility outcome, and particularly associated with reduced in vitro fertilisation (IVF) rates and embryo quality (Gallon et al., 2006; Marchetti et al., 2012). Marchetti and colleagues have published numerous articles related to MMP and IVF outcomes. In couples with >36% spermatozoa with reduced MMP, there was no chance of a pregnancy outcome (Marchetti et al., 2012).

1.3.4. Treatments for male infertility

Counselling and education for infertile men, including their partners, is an important approach to treatment. This can include various lifestyle modifications based on the detailed patient history (Esteves et al., 2012). Unfortunately, few male fertility

disorders have a pharmacological option as first line therapy. Although some known causes have targeted and effective options, other causes and iOAT are associated with non-specific and empirical therapeutic options with unknown efficacy (Hamada et al., 2012b). Therefore, treatments of male infertility can be divided into specific medical therapy (for known and clinically identified causes) and non-specific/empirical medical therapy (for idiopathic cases).

Specific medical treatments are aimed at well defined causes such as urogenital infections (e.g. antibiotics; non steroidal anti-inflammatory drugs; corticosteroids), primary hypogonadism (e.g. testosterone); secondary (hypogonadotropic) hypogonadism (e.g. gonadotropin releasing hormone), hyperprolactinaemia (e.g. dopamine agonists) and erectile dysfunction (e.g. phosphodiesterase-5 inhibitors). However, these therapies are estimated to be effective in approximately 20% of cases only (Hamada et al., 2012b).

Idiopathic cases remain a greater challenge in clinical practice, with many options having little effect. Treatments include various hormonal treatments (e.g. aromatase inhibitors; 5 α -reductase inhibitors; testosterone - although testosterone therapy is contraindicated in idiopathic infertility), anti-inflammatory medications and various antioxidants (e.g. vitamin C; vitamin E; carotenes; carnitine; selenium; zinc) (Snowell et al., 2011; Hamada et al., 2012b; Esteves et al., 2012). Anti-oxidant therapy in idiopathic infertility reportedly offers modest benefit, increased live birth rates with few side effects (Hamada et al., 2012b), although further studies are required.

1.4. Steroid hormones in male reproductive function

Lipophilic steroid hormones are produced via an enzymatic cascade in a process termed steroidogenesis. This can occur in various tissues, and most prominently in the gonads (testes or ovaries), adrenal medulla and adipose tissues (Sherbet et al., 2003; Midzak et al., 2009; Ye et al., 2011). Testosterone synthesis in males is most prominent in the Leydig cells within the testes (Midzak et al., 2009; Miller & Auchus 2011). The basic overview discussed below is illustrated in Figure 7.

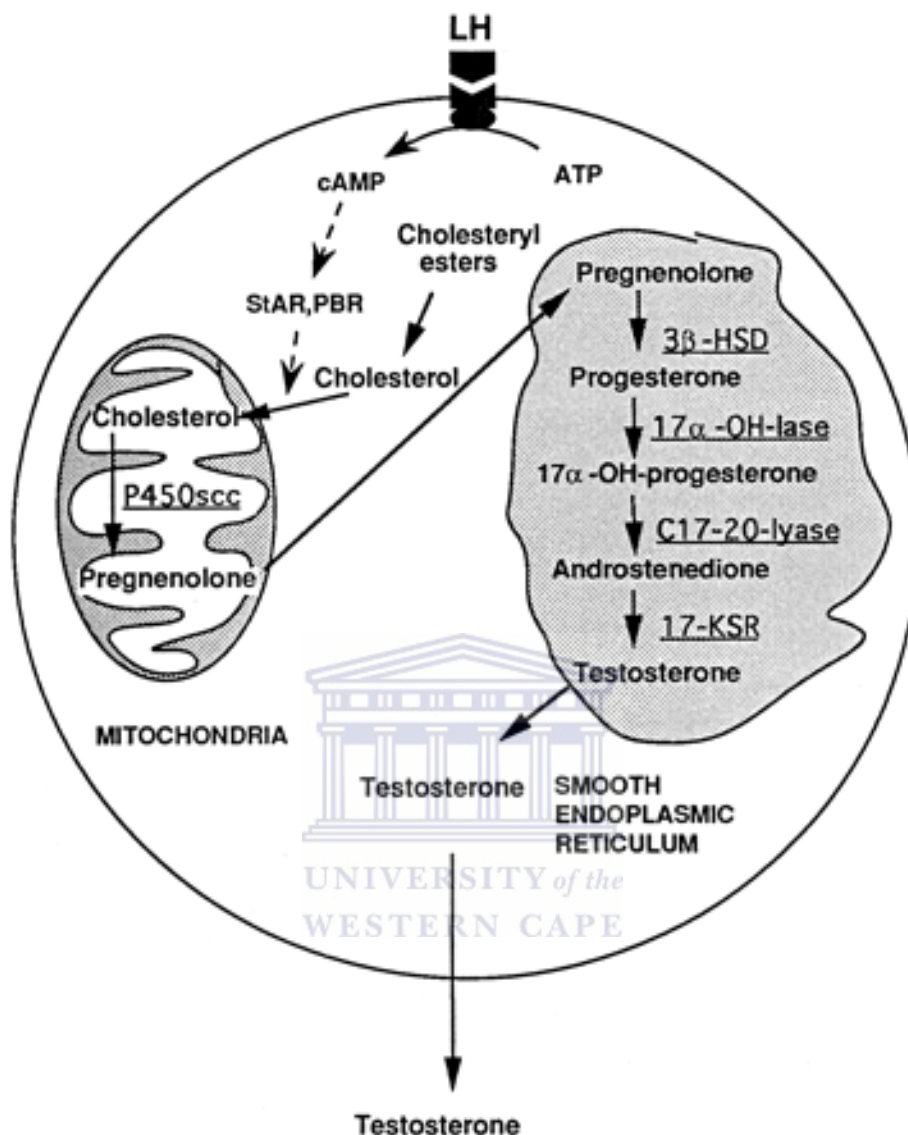


Figure 7: Steroidogenesis cascades in the synthesis of testosterone by the Leydig cell. Mediated by LH stimulation and intracellular cAMP activation, cholesterol is transport across the mitochondrial membrane by StAR and converted to pregnenolone. Pregnenolone is transported to the endoplasmic reticulum, where enzymatic activity produces various steroid based hormones. This figure illustrates the Δ^4 -steroid pathway.

StAR: steroidogenic acute regulatory protein; P450_{scc}: cholesterol side chain cleavage; 17 α -OH-lase: 17 α -hydroxylase; C17-20-lyase: 17,20 lyase; 3 β -HSD: 3 β -hydroxysteroid dehydrogenase; 17KSR: 17 β -hydroxysteroid dehydrogenase; Adapted from Zirkin & Chen (2000).

Steroid based hormones are all produced from cholesterol. Adult Leydig cell testosterone synthesis depends on pulsations of LH by the anterior pituitary gland (Midzak et al., 2009). On binding of LH to the Leydig cell membrane receptors, a cascade of intracellular events occurs. This includes LH receptor and G-protein coupling, activation of adenylate cyclase, increased intracellular cAMP followed by cAMP dependent phosphorylation of proteins through protein kinase A (Midzak et al., 2009).

Following LH stimulation, cholesterol is transported into the inner mitochondrial membrane. As cholesterol does not diffuse freely across this membrane, numerous enzymes are involved in this transport. The most prominent of these is the steroidogenic acute regulatory protein (StAR) (Midzak et al., 2009; Miller & Auchus 2011; Ye et al., 2011). Here, cholesterol is metabolised into pregnenolone via the cytochrome P450 cholesterol side chain cleavage (P450_{scC}/CYP11A1) enzyme (Midzak et al., 2009; Miller & Auchus 2011).

Pregnenolone is then transported to the endoplasmic reticulum for further metabolism (Midzak et al., 2009; Miller & Auchus 2011). Pregnenolone can either be metabolised into progesterone via the 3 β -hydroxysteroid dehydrogenase (3 β -HSD) or 17 α -OH-pregnenolone via cytochrome P450 17 α -hydroxylase (CYP17) (Sherbet et al., 2003; Midzak et al., 2009; Miller & Auchus 2011; Ye et al., 2011). Progesterone is hydrolysed into 17 α -hydroxyprogesterone (17 α -OH-P) also via the enzyme CYP17, and then into androstenedione via cytochrome P450 17,20-lyase action (Sherbet et al., 2003; Midzak et al., 2009; Miller & Auchus 2011; Ye et al., 2011). Androstenedione is then the immediate precursor to testosterone, a conversion catalysed by 17 β -hydroxysteroid dehydrogenase (17 β -HSD) (Midzak et al., 2009; Miller & Auchus 2011). This enzyme mediated cascade in testosterone synthesis is termed the Δ^4 -steroid pathway, in which progesterone is the entry (Sherbet et al., 2003). Mineralocorticoids and corticosteroids are also derived from progesterone via alternative pathways and the cytochrome P450 21-hydroxylase (Ye et al., 2011).

Alternatively, 17 α -OH-pregnenolone can be metabolised into dehydroepiandrosterone (DHEA) (via 17,20-lyase), then to androstenedione (via 3 β -HSD) and testosterone via the Δ^5 -steroid pathway (Sherbet et al., 2003; Midzak et al., 2009; Miller & Auchus 2011).

Testosterone can be further metabolised into 17 β oestradiol via cytochrome P450 aromatase, or into dihydrotestosterone (DHT) via 5 α -reductase (Payne & Youngblood, 1995; Miller & Auchus 2011; Ye et al., 2011).

1.4.1. Testosterone

Testosterone is a steroid hormone that is a major part of the androgen group, consisting of a 19 carbon chain (C-19). In males, testosterone is primarily produced by Leydig cells in the interstitial space of the testes (Dohle et al., 2003). The role of androgens, particularly testosterone, in male fertility is well defined, particularly via action on Sertoli cells to promote steroidogenesis (Singh et al., 1995). Androgens play a key role in the development of male reproductive organs, and are essential for male puberty and sexual function (Dohle et al., 2003). Testosterone levels are also 25 – 125 times higher in the testes (intratesticular) compared to the serum, for unknown yet essential purposes in spermatogenesis. In the absence of testosterone (or functional androgen receptors), males are infertile (Dohle et al., 2003; Walker, 2011). Although detailed mechanisms of the role of testosterone in spermatogenesis require further investigation, the major target for testosterone function are the Sertoli cells, where activation of androgen receptors directly changes gene expression (classical pathway), or testosterone activates kinases that regulate key processes in the maintenance of spermatogenesis (Walker, 2011). Additional roles for testosterone include muscle formation, body compositions and fat regulation, bone mineralisation and cognitive functions (Dohle et al., 2003).

Testosterone deficiency, known as hypogonadism (in males), can be a relatively common finding in the assessment of male infertility cases (Dohle et al., 2003). There are numerous potential causes of this, which can be classed as testicular failure (primary) or of hypothalamic or pituitary origin (secondary; hypogonadotropic) (Dohle et al., 2003). As discussed above, hypogonadotropic hypogonadism is well

established in obesity and MetS, both as a risk factor and a consequence (Kasturi, et al., 2008; Cabler et al., 2010). In ageing males, testosterone levels gradually decline up to 50% by age 60, closely associated with hypospermatogenesis on testicular biopsy (Dohle et al., 2003).

1.4.2. Progesterone

Progesterone is a 21 carbon (C-21) steroid hormone (Sherbet et al., 2003), the major naturally occurring hormone of a class known as progestogens, with well defined roles in the female menstrual cycle (particularly the luteal phase), pregnancy, fertilisation and embryogenesis (Andersen & Tufik, 2006; Pluchino et al., 2006). Although the role of progesterone in female reproduction physiology and pathology has been extensively elicited, and progesterone is even defined in textbooks as a 'female' hormone, there is minimal literature on progesterone in male reproductive function (Oettel & Mukhopadhyay, 2004; Andersen & Tufik, 2006). This is despite the fact that there is no great difference in serum progesterone concentrations between men and woman, except during the luteal phase of the menstrual cycle and during pregnancy (Oettel & Mukhopadhyay, 2004). Traditionally, progesterone in males has been viewed as an unimportant precursor hormone in male physiology, and has only recently begun to be recognised as an important modulator of male endocrine function (Sherbet et al., 2003; Oettel & Mukhopadhyay, 2004). Progesterone in men is produced by steroidogenesis in the adrenal glands and the testes, and is an essential precursor for all steroid hormones, including testosterone. Progesterone also regulates the hypothalamus and pituitary gland in the synthesis of gonadotropin releasing hormone (GnRH) and gonadotropins (LH & FSH), respectively. There is evidence that the hormone regulates sexual behaviour centrally (Oettel & Mukhopadhyay, 2004; Andersen & Tufik, 2006). Evidence also suggests that progesterone has various modulating functions in the central nervous system, and therefore affects mood, behaviour and cognitive functions (Oettel & Mukhopadhyay, 2004; Pluchino et al., 2006).

In the field of male contraception, a stronger focus on progesterone in the male reproductive system has been studied, with progestins suppressing gonadotropin releasing hormone (GnRH) and gonadotropin (LH; FSH) secretion by the

hypothalamus and the pituitary gland respectively. The addition of progestins with testosterone administration improves the rate of suppression of spermatogenesis in male hormonal contraception methods (McLachlan et al., 2004). Some progestins have been found to influence male sexual behaviour, and have even been reported to reduce a variety of deviant male sexual acts (such as paedophilia and rape) and hypersexuality in general, although these reports are highly contentious. However, this is partly why the role of testosterone in the determination of male libido seems to be overstated (Andersen & Tufik, 2006).

Progesterone action in seminal fluid has been well established, and is an essential requirement for numerous molecular processes leading to successful fertilisation after ejaculation of spermatozoa (Oettel & Mukhopadhyay, 2004). These functions include capacitation (Foresta et al., 1992), acrosome reaction (Meizel & Turner, 1991), increasing intracellular calcium concentration (Thomas & Meizel, 1989) and the stimulation of phospholipase activity and tyrosine phosphorylation of sperm proteins (Baldi et al., 2002). In addition, it is suggested that progesterone exerts a lipolytic action on spermatozoa, and increased glucose-6-phosphate activity, indicating a role in glucose metabolism via progesterone receptors (De Amicis et al., 2011). In sperm obtained from patients with varicocele, progesterone did not induce energy consumption, most likely through a decreased expression of these receptors on spermatozoa membranes (De Amicis et al., 2011).

Progesterone has also been found to influence various metabolic parameters in males. In 1986, Chen et al. reported that pharmacological doses of progesterone significantly reduced plasma concentrations of cholesterol, triglycerides, LDL-cholesterol and apolipoprotein B in men. More recently, Ma et al. (2009) found a negative association between serum progesterone and carotid artery atherosclerosis, determined via carotid intima-media thickness, in Chinese men over 60 years of age. This association remained after correcting for traditional atherosclerosis risk factors such as age, BMI, waist-to-hip ratio cholesterol and LDL levels, triglyceride levels, high-sensitive C-reactive protein and blood pressure. Incidentally, no such association was found in woman. Furthermore, these authors that progesterone negatively correlated with triglyceride concentrations, with no correlation with the other parameters listed above.

Ageing males have been shown to have increased progesterone concentrations in testicular tissues and the spermatic cord vein, associated with a reduction in testosterone concentrations (Pirke et al., 1980). Further indications from rat Leydig cell cultures imply that progesterone may inhibit testosterone production in ageing males, and hence may have a detrimental effect on Leydig cell function (Gruenewald et al., 1992)

Blanchette and colleagues (2006) reported a negative correlation between body weight, BMI and waist circumference with serum progesterone concentrations (as well as 17-hydroxyprogesterone, dehydroepiandrosterone sulphate, testosterone and dihydrotestosterone). Progesterone has therefore not been fully investigated in men who are obese or been diagnosed with MetS or T2DM.

1.4.3. Saliva measurement of steroid hormone

The accurate measurement of steroid hormones remains a challenge, and it is stated that inaccurate measurements are obtained by routine immunoassays (Goncharov et al., 2005; Goncharov et al., 2006). It also remains unresolved which parameter is best suited for assessment of activity, the total, bioavailable or free concentrations (e.g. total testosterone, bioavailable testosterone or free testosterone) (Goncharov et al., 2006). As steroid hormones are protein bound in serum, particularly sex hormone binding globulin (SHBG) and to a lesser degree albumin, the accurate assessment of the fraction of the free hormone is difficult to assess (Goncharov et al., 2006). Saliva is considered a source of steroid hormones, including progesterone and testosterone that is unbound to SHBG or albumin (Goncharov et al., 2006). Saliva levels of steroid hormones have been reported to correlate well with serum levels that represent an accurate measure of free hormone in peripheral tissues. In addition, it offers a non-invasive collection method that can be readily obtained over any time period (Brown et al., 2008).

1.5. Obesity, metabolic syndrome and male reproductive function

Although the effect of excess body fat on reproduction has been more extensively studied in females, there has been a recent increase in literature assessing the relationship between obesity and semen characteristics, male endocrine changes, male sexual function and male factor infertility. In a chapter entitled '*The health disadvantages of excessive weight*' in his *Canon of Medicine*, Avicenna wrote 'this [obese] human [man] has a cold temperament; this is why he is infertile, unable to impregnate [woman] and has low semen' (Avicenna, 1593).

Obesity, as a cardinal feature of MetS, is closely associated with an increased incidence of male factor infertility, with several patho-physiological mechanisms being implicated (Giagulli et al., 1994; Hammoud et al., 2008a; Kasturi et al., 2008; Hofny et al., 2009). Numerous studies have found an inverse correlation between increased obesity and semen quality that negatively affects male fertility, with an increased chance of subfertility among couples in which the male partner is obese. Hormonal changes that positively correlate with obesity include reduced serum total testosterone, free testosterone and sex hormone binding globulin (SHBG), and increased serum oestrogen (raised oestrogen:testosterone ratio), insulin (insulin resistance), leptin, FSH, LH and prolactin (Kasturi et al., 2008; Hofny et al., 2009; Cabler et al., 2010). Some physical mechanisms that have been implicated in obesity linked male infertility include erectile dysfunction (Cheng et al., 2007; Pauli et al., 2008), increased scrotal temperature (Hjollund et al., 2000) and sleep apnoea (Luboshitzky et al., 2005). Thus, the relationship between obesity and male infertility is multifactorial and complex (Hammoud et al., 2008b), and calls for a better understanding of the underlying mechanisms that result in abnormal sperm function in obese men (Fejes et al., 2005; Aggerholm et al. 2008; du Plessis et al., 2010; Hammoud et al., 2008a; Chavarro et al., 2010; Hofny et al., 2009).

Any negative effect of obesity on sperm parameters as determined by the WHO (2010) has not been conclusively resolved. Studies are not consistent, nor has there been a clear dose-response mechanism elicited (Hammoud et al., 2008a). Various studies have shown a reduction in sperm count and concentration, motility, vitality, morphology, and DNA integrity associated with obesity (Fejes et al., 2005, 2006;

Kort et al., 2006; Aggerholm et al., 2008; Hammoud et al., 2008b; Pauli et al., 2008; Chavarro et al., 2010; Hofny et al., 2009; Håkonsen et al., 2011; Rybar et al., 2011). In contrast, other researchers have not found similar relationships (Aggerholm et al., 2008; Chavarro et al., 2010; Rybar et al., 2011). A limited meta-analytical review by MacDonald et al. (2010) investigated the association of BMI with hormonal and semen parameters, and found no negative association between increased body weight and reduced semen parameters strong evidence for reduced testosterone with increased body mass index. In contrast, a more recent meta-analysis by Sermondade et al. (2013) found that obesity is associated with an increased risk for oligozoospermia or azoospermia. Although there is no clear evidence to show reduced sperm parameters in obese men, a disproportionately large number of men seeking infertility treatment are obese (du Plessis et al., 2010). Due to lack of research in this area, it is suggested that more controlled studies should be undertaken with increased focus on potential underlying mechanisms, in addition to increased focus on obesity as an aetiology of male infertility in the clinic (Cabler et al., 2010).

Further lines of a causal relationship between obesity and male reproductive dysfunction are suggested by a group of studies indicating that diet and/or exercise induced weight loss can improve various parameters, such as increasing testosterone and SHBG levels, decreasing insulin and leptin and improving semen parameters in obese men (Isidori et al., 1999; Kaukua et al., 2003; Niskanen et al., 2004; Kasturi et al., 2008; Chavarro et al., 2010), in addition to a reduction in peripheral inflammatory cytokine concentrations (Ziccardi et al., 2002; Sharman & Volek, 2004).

The effect of other parameters associated with MetS, such as dyslipidaemia, hypertension and hyperglycaemia, have only had limited scientific investigation (Kasturi et al., 2008). Ramírez-Torres et al. (2000), found no correlation between sperm abnormalities and hypertension, glucose intolerance and diabetes mellitus. However, the authors did indicate a relationship between dyslipidaemia and sperm abnormalities. Shalaby et al. (2004) reported a potential role for dyslipidaemia in the development of infertility in male rats fed a high cholesterol diet. Several studies have found an inverse relationship between blood pressure and total serum

testosterone concentrations, which may result in impaired reproductive potential (Kasturi et al., 2008). Palmer et al. (2012a) reported a positive correlation between glycaemia and sperm DNA fragmentation, with a negative correlation to normal morphological sperm, regardless of adiposity, in mice fed a high fat diet.

T2DM, a known consequence of MetS, has increasingly been associated with male factor infertility in recent years, with complex and multifactorial factors involved. Poor semen quality, such as reduced sperm concentration and motility, abnormal morphology, mitochondrial DNA damage, nuclear DNA damage and increased seminal plasma abnormalities have been reported (La Vignera et al., 2012a).

Mallidis and colleagues (2011) published a study in which animals fed a high fat diet induced metabolic changes characteristic of MetS, indicated that these changes may be associated with poor sperm quality and decreased spermatogenesis, although this was minimal. They hypothesised that this effect was primarily due to increased blood glucose as opposed to hypogonadism. In a recently published human cohort study, consisting of male partners of infertile couples, Lotti and colleagues (2013a) published results indicating that MetS is associated with poor sperm morphology and testes ultrasound inhomogeneity, in addition to hypogonadism, ED and depression, and hence declines sexual and overall health.

Hormonal changes that are associated with MetS and negatively affect various parameters of male fertility include reduced serum total testosterone (TT), free testosterone (FT) and sex hormone binding globulin (SHBG), increased serum oestrogen, insulin (insulin resistance), leptin, FSH, LH and prolactin, (Eckel et al., 2005). Conversely, the role of progesterone has not been extensively studied in males who are obese or diagnosed with MetS. Blanchette and colleagues (2006) reported a negative correlation between body weight, BMI and waist circumference with serum progesterone concentrations (as well as 17-hydroxyprogesterone, dehydroepiandrosterone sulphate, TT and dihydrotestosterone). Furthermore, the role of progesterone in male fertility has not been fully investigated, despite the fact that there is no great difference in serum progesterone concentrations between men and woman, except during the luteal phase of the menstrual cycle and during pregnancy (Oettel & Mukhopadhyay, 2004).

1.5.1. Insulin and male reproductive function

Insulin is considered a central regulator of gonadal function and spermatogenesis, although the role of insulin on the male reproductive system has not been fully elicited (Aquila et al., 2005a; Lampiao & du Plessis, 2008a; Lampiao et al., 2009). Furthermore, this well established metabolic hormone has been identified in human ejaculate, and human spermatozoa have been shown to synthesize and secrete insulin in an autocrine fashion in the ejaculate (Aquila et al., 2005a). Insulin also increased motility, nitric oxide concentrations and increases the acrosome reaction *in vitro* (Aquila et al., 2005a; Lampiao & du Plessis, 2008a). However, both increased and decreased serum levels of insulin have been shown to correlate negatively with male fertility (as do increased and decreased levels of leptin) (Lampiao et al., 2009). Ando and Aquila (2005) have suggested that the PI3K/Akt pathway is activated following insulin receptor stimulation by insulin, as illustrated in Figure 4.

Insulin has been reported as an important regulator of male reproduction via actions on the hypothalamus-pituitary-testes (HPT) axis, with modulating actions on gonadotropic releasing hormone (GnRH), LH and FSH centrally, and Leydig and Sertoli cell function locally (Lampiao et al., 2009). Pitteloud et al. (2005a) published *in vivo* evidence that insulin resistance is associated with decreased testosterone secretion from Leydig cells in males. However, the role of insulin in male (in)fertility has not been well elicited and remains poorly understood, especially in the context of insulin resistance.

1.5.2. Leptin and male reproductive function

Leptin, a well known central (hypothalamic) regulator of food intake and energy expenditure (Bastard et al., 2006), is also associated with metabolic and endocrine effects and a role in normal reproduction and sexual maturation (Wauters et al., 2000). This hormone also appears to have an important role in the puberty process, with mutations in the leptin (*ob*) gene associated with hypogonadism and no pubertal development in humans (Strobel et al., 1998).

The mechanisms by which leptin modulates reproductive potential is not clear, although evidence indicates effects through interaction with the hypothalamus and effects on GnRH production (Wauters et al., 2000; Lampiao et al., 2009). Reduced or absent leptin is associated with reduced GnRH, with effects on LH/FSH and testosterone levels, thereby influencing the HPG axis (Wauters et al., 2000; Lampiao et al., 2009). Higher serum leptin levels has also been associated with reduced motility and straight line velocity of sperm (Glander et al., 2002). However, the exact role of leptin in male and female reproduction requires further clarification (Lampiao et al., 2009).

Leptin is found in human seminal fluid, and leptin receptors are expressed on spermatozoa in addition to soluble receptors in seminal fluid (Jope et al., 2003). Interestingly, leptin (as well as insulin) appears to be synthesised and secreted by ejaculated spermatozoa, particularly mediating motility (Aquila et al., 2005b; Andò & Aquila, 2005). In uncapacitated samples, leptin is found within intracellular granules in the midpiece predominantly, and decreases significantly at capacitation, indicating a role alongside insulin in capacitation (Andò & Aquila, 2005). However, the source of is not well defined. Ando and Aquila (2005) have suggested that the PI3K/Akt pathway is activated following leptin receptor stimulation by leptin, similarly to insulin. Similarities and differences for both insulin and leptin action on intracellular mediators in spermatozoa is illustrated and summarised in Figure 8.

Leptin has been identified in the ejaculate (Aquila et al., 2005b), although the role of leptin levels on semen function has remained controversial and conflicting (Lampiao & du Plessis, 2008a). Some evidence indicates that leptin may modulate sperm motility, morphology, acrosome reaction and nitric oxide production, as well as sperm capacitation (Aquila et al., 2005b; Lampiao & du Plessis, 2008a)

Based on a series of experiments and literature reviews, Lampiao and colleagues (2009) hypothesised that leptin and insulin can mediate effects synergistically on post-ejaculated spermatozoa. The stimulation of respective receptors converge on the PI3K intracellular signalling pathway, leading to protein kinase B (PKB/Akt) phosphorylation, causing the translocation of GLUT8 and insertion into the cell membrane. This would then allow spermatozoa uptake of glucose for metabolism

and motility (Aiston & Agius, 1999; Lampiao et al., 2009). This pathway can also diverge and stimulate endothelial nitric oxide synthase (eNOS) of spermatozoa to increase NO production and influence acrosome reaction (Aiston & Agius, 1999; Lampiao et al., 2009).

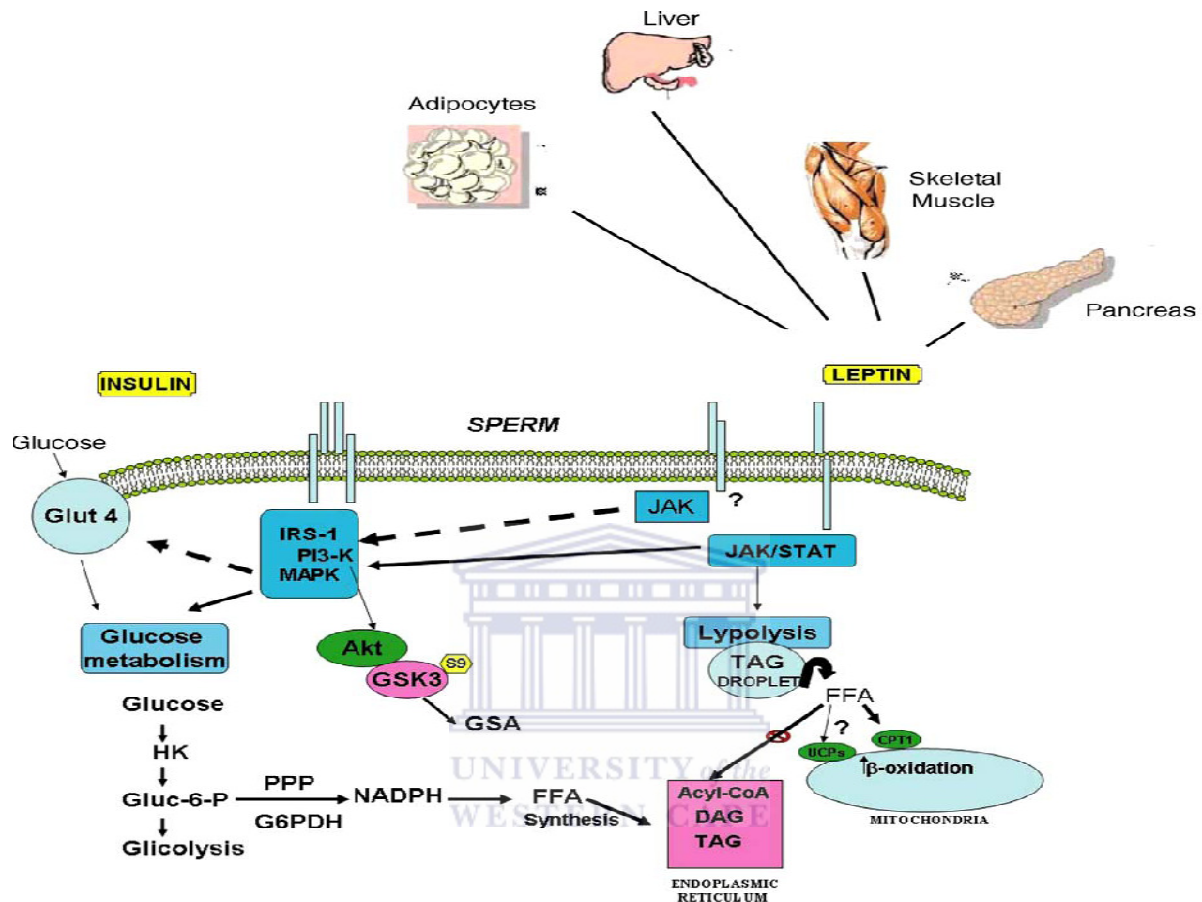


Figure 8: Hypothetic model of functional interaction between insulin and leptin in human ejaculated spermatozoa. Both hormones signalling converge on the PI3K/Akt pathway as observed in somatic cells. In sperm insulin and leptin show a similar positive action on GSK3 and G6PDH activities, however they sound to diverge in the β -fatty acid metabolism. As evidenced, insulin inhibits α -oxidation and makes easier lypogenesis while leptin antagonizes both actions and provides additional metabolic fuel through stimulation of FFA α -oxidation.

ϕ : blocked; TAG: triacylglycerol; FFA: free fatty acid; DAG: diacylglycerol; PPP: pentose phosphate pathway; CPT1: carnitine palmitoyl transferase 1; UCPs: uncoupling proteins; IRS-1: Insulin Receptor Substrate 1; Predicted signaling pathways depicted by broken arrows are as yet unknown.

Adapted from Ando & Aquila (2005).

1.5.3. Cytokines and male reproduction

The seminal plasma contains significant levels of various cytokines normally present in the male genital tract (Huleihel et al., 1996; Dousset et al., 1997; Martinez et al., 2007; Politch et al., 2007). It is generally thought that these proteins in the seminal plasma originate from Leydig cells, Sertoli cells, the epididymis and the prostate, with expressions modulated during the seminiferous epithelium cycle, but these origins are still a matter of scientific contention (Huleihel et al., 1999; Martinez et al., 2007).

Although several proinflammatory cytokines, such as TNF α , IL6 and IL8, may promote sperm membrane lipid peroxidation beneficial for fertilisation, increased concentrations in seminal fluid may negatively affect sperm fertility capability (Basu et al., 2004; Martinez et al., 2007). Studies have shown that increased inflammation in the male reproductive tract associated with leukospermia results in a negative effect on spermatogenesis and function of spermatozoa (Koçak et al., 2002; Basu et al., 2004; Eggert-Kruse et al., 2007; Martinez et al., 2007; Gallegos et al., 2008; La Vignera et al., 2012c). As with leptin and insulin presence in semen, several pro-inflammatory cytokines at physiological concentrations may have beneficial effects on male genital function. This is evident with increased lipid peroxidation of sperm membranes (mediated by cytokines) being important in the sperm fecundation process (Martinez et al., 2007), cytokine modulation of pro- and anti-oxidant systems (Sanocka et al., 2003) and the indication that some cytokines play a role in testicular function and modulation of steroid release from the testes (Eggert-Kruse et al., 2001). However, with increased cytokine concentration in the setting of inflammation/infection, these polypeptides appear to have a detrimental effect on male fertility (Gruschwitz et al., 1996; Eggert-Kruse et al., 2001). The potential negative effects of inflammatory cytokines on spermatozoa is not clear, however, numerous studies have indicated that an increase in seminal cytokines is associated with a reduction in sperm count, motility and reduced male fertility potential (Gruschwitz et al., 1996; Dousset et al., 1997; Koçak et al., 2002; Basu et al., 2004; Lampiao & du Plessis, 2008b; Tronchon et al., 2008).

Several lines of evidence indicate that various cytokines are involved in male fertility (Dousset et al., 1997). Elevated seminal plasma concentrations of several cytokines,

including IL6 and TNF α , have been associated with poor semen quality and male infertility (Naz & Kaplan, 1994; Gruschwitz et al., 1996; Dousset et al., 1997; Camejo et al., 2001; Eggert-Kruse et al., 2001). Many of the cytokines and immune factors present in semen of fertile men are involved in normal male reproductive biology, but an inflammation and/or infectious related rise in these immunological mediators is associated with an increased risk of male factor infertility (Hales et al., 1999; Hedger & Meinhardt, 2003). *In vitro*, cytokines have been shown to affect human sperm motility, increase the production of ROS by human spermatozoa, and reduce the ova penetrating ability of spermatozoa (Dousset et al., 1997). It is also suggested that an increase in cytokine expression may lead to an increased absorption onto sperm cells and a subsequent rise in regulatory activity (Dousset et al., 1997). Seshadri et al. (2009) showed significant higher concentrations of IL6 in mild and severe oligozoospermic men, higher concentrations of IL8 and IL10 in asthenozoospermic men, and higher concentrations of IL6, IL10 and TNF α in obstructed azoospermic men. The study also showed that concentrations of IL10 correlated closely with numerous other cytokines in both the obstructed azoospermic group and the asthenospermic group, indicating that the origin of these cytokines is outside of the testis. A study conducted by Poltich et al. (2007) indicated the presence of various cytokines and other immunological factors in the semen of healthy men. High concentrations of IL8 were present in all samples, with IL6, IL10 and TNF α among the cytokines detected in low concentrations. Furthermore, the study showed that polymorphonucleocyte (PMN) counts correlated significantly with IL6 and TNF α concentrations (amongst other cytokines). There is, however, controversy in the relationship between semen quality and elevated cytokine levels, with various studies supporting both sides of the argument (Kokab et al., 2010). More recently, Bialas and colleagues (2009) have shown that changes in testicular cytokine activity are related to male infertility. It is also suggested that increased cytokines associated with excessive white adipose tissue can exert toxic effects on spermatozoa via ROS (Fraczek & Kurpisz, 2007).

Male genital tract inflammation has been cited as a cause, or at least a contributing factor, of male infertility. However, there is difficulty in diagnosis due to the asymptomatic nature of the disease and the precise definition of male genital tract inflammation, which does not differentiate between infection as a cause and non-

infectious inflammation (Kopa et al., 2005). According to the World Health Organisation (WHO), leukocytospermia (more than 1 million leukocytes per ml of seminal fluid) is the most significant diagnostic indicator, and is assessed with the culture of pathogenic bacteria, increased seminal viscosity and/or abnormal biochemistry (WHO, 2010). Leukocytospermia significantly correlates with increased IL6, IL8, and TNF α in male semen, with IL8 and TNF α associated with simultaneous presence of leukocytes and IL6 more associated with presence of leukocytes (Martínez-Prado et al., 2010), and has been associated with decreased sperm numbers and impaired sperm motility (Wolff, 1995; Sanocka et al., 2003; Henkel et al., 2005).

1.6. Hypothesis of this thesis

On searching the scientific literature, it is apparent that the effect of MetS on male reproductive function and fertility potential has not been sufficiently investigated. With various components of MetS tentatively linked to a reduced fertility potential in males, it is hypothesised that male fertility may be compromised in the setting of this pathophysiological disorder. As both MetS and male infertility are dramatically on the increase globally, and that male hypogonadism appears to be a central risk for CVD and T2DM in males, this demands investigation into possible relationships between these various phenomenons. Although any negative relationship between MetS and male infertility is likely to be multifactorial and complex, a clear association first needs to be suggested by case controlled investigations. Prior to the onset of this study, there has not been as case controlled investigation into any possible impact of MetS on male fertility parameters.

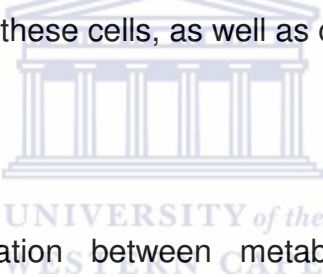
It is conceivable that an additional source of increased inflammatory cytokines in the male reproductive tract may be related to the systemic pro-inflammatory changes associated with MetS. Increased serum concentrations may potentially cross into the testicular tissue via the blood testes barrier, and/or gain access to seminal fluid through secretions of the seminal vesicles and prostate during ejaculation, potentially reducing sperm concentration, motility and fertilising capabilities. This would provide a novel explanation for reduced fertility potential in obese, MetS and T2DM males. It is hypothesised that MetS will have a negative influence on seminal

parameters as compared to healthy males in a case controlled study. In addition, it is hypothesised that seminal concentrations of insulin, leptin, TNF α , IL1 β , IL6 and IL8 will be increased and positively correlate to well establish serum changes.

It is well known that testosterone is reduced in MetS males, so it is predicted that this will hold true in this study. In addition, it is hypothesised that progesterone concentrations will also be reduced indicating a compromised steroidogenesis cascade. Although hypogonadism has been well established in obese and MetS males, these studies have all been done on serum total free concentrations. There are no studies in the literature assessing salivary testosterone or progesterone in MetS males.

As insulin and cytokines are known to regulate steroidogenesis in Leydig cells, it is predicted that insulin, leptin, TNF α , IL1 β , IL6 and IL8 will affect both progesterone and testosterone production in these cells, as well as cell viability.

1.7. Aims of the study

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- a. Investigate an association between metabolic syndrome and seminal parameters in males, including sperm concentration, motility, vitality, morphology, mitochondrial membrane potential and DNA fragmentation;
 - b. Investigate changes in seminal fluid insulin, leptin, TNF α , IL1 β , IL6 and IL8 in metabolic syndrome males, and compare with serum changes;
 - c. Investigate changes in free progesterone in males with metabolic syndrome in light of free testosterone concentrations obtained from saliva;
 - d. Investigate effects of insulin, TNF α , IL1 β , IL6 and IL8 on cell viability, protein production and testosterone and progesterone concentrations in an hCG (human chorionic gonadotropin) stimulated TM3 cell line.

CHAPTER 2 - MATERIALS AND METHODS

2.1. Study design overview

The study consists of two arms: a case controlled investigation that included volunteer participants and a TM3 Leydig cell culture model exposed to various concentrations of insulin and inflammatory cytokines.

The case control study assessed male patients with metabolic syndrome (MetS) against a control group (CG) of males. Various parameters were assessed, which can be divided into clinical, biochemical and seminal. Clinical and biochemical assessments were used to define and diagnose participants in terms of MetS and insulin resistance (IR). Further biochemical assays assessed serum cytokine concentrations and leptin. Biochemical analysis of saliva was used to assess free testosterone (FT) and free progesterone (FP) concentrations. Standard semen analysis according to the World Health Organisation (WHO, 2010) was carried out, in addition to the determination of abnormal mitochondrial membrane potential (MMP) and DNA fragmentation (DF) of spermatozoa. Furthermore, seminal fluid was assayed for cytokines, insulin and leptin concentrations. Comparisons were made between the groups for the various variables, and correlations were made of the entire cohort to investigate associations between variables.

The cell culture model essentially exposed the TM3 Leydig cell line to various concentrations of insulin and cytokines for 48 hours, all co-cultured with 25 mIU/ml human Chorionic gonadotropin (hCG). Cell viability and protein was determined, and supernatant was assayed for testosterone and progesterone concentrations.

2.2. Recruitment of participants

This clinical case controlled study was approved by the Ethics Committee of the University of the Western Cape, Bellville, South Africa. Participants were recruited randomly via word of mouth and advertisements distributed by hand, electronically and in local community newspapers, between October 2010 and February 2013. Therefore, the cohort represents those confined to the Western Cape region of

South Africa, with multiple ethnic and cultural backgrounds. Accepted participants attended one consultation and were individually counselled on the study background and design, and all participants signed an informed consent form in order to undergo a full medical consultation and clinical examination, and allow for sample collection and relevant biochemical testing. All participants received detailed feedback on the results via email, telephone or a follow up consultation, with detailed report of clinical results for their records. They were further advised appropriately on the recommended treatments or further investigations that may be required. All participants had direct access to the clinician during the study period.

Subjects were expected to be fasting for a minimum of 8 hours before blood collection, and abstain from sexual activity for a minimum of three and maximum of five days before collection of a semen sample. Further instructions related to sample collections included was not consume caffeine or tobacco, chew gum or brush teeth 1 hour prior to the consultation. All samples were collected in the morning between 7h00 and 10h00. All of the relevant biological samples were collected at the consultation, specifically blood (via venous puncture), passive saliva (via 'drooling) and semen (via masturbation into a sterile container). A total of 78 participants were included in the study for data analysis, divided into a MetS group (n=34) and control group (n=44) based on the MetS diagnostic criteria outlined by Alberti et al. (2009) (Table 1).

2.2.1. Inclusion and exclusion factors

Eligibility criteria included being an adult male of between 18 and 70 years of age. On pre-clinical screening via telephonic or electronic communication, interested participants with a vasectomy, diagnosed with any disorder of the prostate, reproductive system or systemic pathology, or on any hormonal therapy (e.g. testosterone or insulin) in the last six months were excluded from entering the study. In order to reduce possible selection bias, all other interested participants were accepted to enter the study. The details of this clinical interview and sample collections are described below.

Following clinical investigation and sample assays, participants with clinically apparent reproductive disorders (e.g. varicocele), leukocytospermia (conservatively defined for this study as $>0.5 \times 10^6/\text{ml}$) or with a known or clinically detected acute or chronic inflammatory condition were excluded from study. Further exclusion factors based on medical history and examination included a surgery or hospital admission for any reason within the last 6 months. Participants with unexplained (idiopathic) azoospermia (n=4; 2 participants from each group) had semen parameters removed from the study, but clinical, biochemical and hormonal parameters were included for data analysis. Participants with a history of unexplained infertility between the male and female partner were included in the study. Participants on medication related to MetS, smokers, and those diagnosed with T2DM were included in the study. A summary of specific and general exclusion criteria is tabulated in Table 3 below:

Table 3: Specific and general exclusion criteria for the study.

Specific Exclusion Criteria	General Exclusion Criteria
Vasectomy.	Surgery or hospitalisation in the last six months.
Leukocytospermia ($> 0.5 \times 10^6/\text{ml}$).	Inability to supply a semen sample by masturbation at the consultation in which serum and saliva samples were collected.
Existing diagnosis of any testicular, prostate or other reproductive tract pathology.	Existing diagnosis of a known local or chronic systemic inflammatory disease.
Clinical or laboratory indication of any testicular, prostate or other reproductive tract pathology.	Clinical or laboratory detection of a known local or chronic systemic inflammatory disease.
Insulin, testosterone or thyroid medications in the last six months.	Recreational illicit drug use in the last six months.

2.2.2. Clinical interview and sample collection

All participants accepted for participation following initial screening attended a clinical history, medical examination and sample collection at specified clinics at the Bellville Campus of the University of the Western Cape (Natural Medicine Clinic) or in Stellenbosch (Natural Health Centre). The medical history was recorded by a trained clinician following a medical history template. The outline of the history is Table 4 below.

Following an intensive medical history, the participants underwent a full clinical examination, including genital examinations. The examination aspects recorded included are tabulated below in Table 5. The genital examination did not routinely include a rectal and prostate exam (digital rectal examination), except where clinically indicated based on recommended screening guideline, medical history and/or physical examinations.

Following medical history and physical examination, biological samples were collected. Saliva samples were collected via drooling. Serum samples were collected via appropriate venopuncture techniques on arm (antecubital fossa) or hand veins (when arm veins not able to be located). Semen samples were collected via masturbation into a sterile container. All samples were collected within a maximum window of 30 minutes. Further details are provided where relevant below.

Table 4: Medical history guideline for clinical case taking.

Category	Specific Details
General	- personal information and demographics
Main or current medical complaints	- detailed history were applicable
Medical and surgical history	- previous hospitalisation and/or surgery - known allergies
Current medications	- prescribed medicines - nutritional supplements
Family history	- heart disease and diabetes - cancer - general
Psycho-social history	- smoking and tobacco consumption - caffeine use - alcohol use - recreational drug use
Fertility history	- number of children biologically fathered - year of birth of youngest child (if applicable) - personal or couple related fertility complaints
Systems review	- head, neck and ENT (ear, nose and throat) - chest (including cardiovascular and respiratory systems) - abdomen (including gastrointestinal system) - genito-urinary system - musculoskeletal system - skin - endocrine system - neurological and psychiatric system

Table 5: Clinical examination used for patient consultations.

Category	Specific Details
General assessment	- overview of patient's demeanour and appearance
Vital signs	- height & weight (body mass index) - waist circumference - temperature - pulse and respiration rate and rhythm - blood pressure
Lymphadenopathy	- cervical, axilla and inguinal
Systems examination	- head, neck, oropharyngeal and ENT - chest (including cardiovascular and respiratory systems) - abdomen (including gastrointestinal system) - genito-urinary system - musculoskeletal system - skin - endocrine system - neurological and psychiatric system

2.3. Clinical data collection

Relevant clinical data collected by a trained clinician included age, body mass index (BMI), waist circumference (WC) and blood pressure. Height was recorded by a standardised wall chart, and weight was recorded on a digital scale to the nearest decimal point. BMI was calculated by body weight (kilograms to nearest decimal point) divided by height (meters) squared (kg/m^2). The WC was measured in centimetres around the abdomen at the midpoint between the lowest point of the costal margin and the highest point of the iliac crest with the patient standing and relaxed. Blood pressure was measured on the dominant arm after a rest period of at least 15 minutes, and recorded as systolic (SBP) and diastolic (DBP). All

measurements above were taken twice, with the mean between the two samples taken as the recorded parameter.

The fasting blood samples were analysed for high density lipoprotein (HDL) cholesterol, triglycerides, glucose, insulin, leptin and inflammatory cytokines (TNF α ; IL1 β ; IL6; IL8). Saliva underwent analysis for FT and FP concentrations. Semen was analysed for ejaculate volume, sperm concentration, total sperm count, progressive motility, total motility, vitality, abnormal mitochondrial membrane potential, DNA fragmentation and seminal leukocyte concentration. Seminal fluid further underwent investigation for glucose, insulin and inflammatory cytokines (TNF α ; IL1 β ; IL6; IL8). Each participant was provided a unique alpha numeric code in which all samples were labelled.

All laboratory assays were conducted by an adequately trained technician. Samples sent to outside laboratories for analysis were blinded to clinical data and identification of the patients. Standard semen analysis done at the UWC laboratory were not blinded to clinical data and history of the participant, but was blinded to further biochemical data. Samples frozen or fixed to slides and assayed in batched analysis at future dates allowed blinding to clinical, biochemical and other seminal parameters previously recorded via sample codes. Selection bias was limited as no participants who met the criteria were excluded from data analysis. Participants with idiopathic azoospermia (n=4; 2 participants in each group) had data removed from the seminal analysis for statistical analysis. Further potential confounding variables not included in detail by the data collection process and analysis includes lifestyle factors (diet, physical activity and alcohol use), educational status, and socioeconomic background.

Based on the clinical and laboratory data, participants were assessed for MetS according to the diagnostic criteria outlined in Table 1 (Alberti et al., 2009). Various parameters were compared between the groups.

2.3.1. Serum lipogram, triglycerides, insulin and glucose

Blood was collected in sodium fluoride and serum separating tubule (SST) vacutainers[®] (BD Biosciences, Franklin Lakes, New Jersey, USA), and transported immediately to PathCare Laboratories (Pathcare Park, Goodwood, South Africa) for analysis. PathCare Laboratories are a private commercial pathology laboratory servicing clinical practice and research sectors in South Africa. HDL, triglycerides (lipogram) and glucose were assayed using the timed endpoint method. Highly sensitive C-reactive Protein (CRP) was done using the Beckman Coulter LX system (Brea, California, USA).

2.3.2. Saliva hormones: testosterone and progesterone

Saliva samples were collected by passive drooling into a sterile Eppendorf container and immediately stored at -20°C until sampling. After thawing, samples were centrifuged at 2500xg prior to analysis. Testosterone and progesterone assays were performed using commercial ELISA kits (IBL International GMBH, Hamburg, Germany). A minimum of 1.5 ml of saliva fluid was required for appropriate assays. Participants who were not able to produce adequate saliva sample were permitted in the study (n=10; 6 in the control group and 4 in the MetS group), and had these parameters removed from data analysis. All available samples were assayed in duplicate, with a mean value recorded for data analysis. These assays were conducted at Synexa Life Sciences (Milnerton, Cape Town, South Africa).

The Testosterone Saliva ELISA (IBL International GMBH, Hamburg, Germany) is an enzyme immunoassay for *in-vitro* diagnostic quantitative determination of free testosterone in human saliva. The reportable range is 2–760 pg/ml with intra- and inter-assay coefficients of variation (CV) of 4.2–15.1% and 5.5–6% respectively. The Progesterone Saliva ELISA (IBL International GMBH, Hamburg, Germany) is an enzyme immunoassay for the *in-vitro* diagnostic quantitative determination of free progesterone in human saliva. The reportable range is 3.8–5000 pg/ml with CV of 4.7–7.6% and 5.3–7.7% respectively. Samples were assayed on an ELISA reader obtained from Biotek (Winooski, VT, USA).

2.4. Semen analysis

Semen was collected via masturbation into sterile wide-mouthed containers and transferred to a 37°C incubator within 30 minutes of collection. Seminal fluid was left for 60 minutes to liquefy (combination of room temperature and incubator). Participants with samples that did not liquefy appropriately would be excluded from the study. Participants with excess viscosity for adequate analysis (n=3; 2 in the control group and 1 in the MetS group) had minute (spatula tip) amounts of α -chymotrypsin (Sigma-Aldrich, St. Louis, MO, USA) added to seminal fluid for analysis.

2.4.1. Medium used in semen analysis

Phosphate Buffered Saline (PBS) was used as a standard buffer and dilutant were applicable in seminal analysis and cell culture experiments. Concentrated PBS tablets were acquired from Oxoid Microbiology Products (Basingstoke, United Kingdom). Tablets were dissolved in distilled water as instructed by the manufacture for use in laboratory assays.



Human tubal fluid medium (HTFM) was prepared according to the method outlined by Quinn and colleagues (1985) and adjusted to an osmolarity of 280 mOsmol/Kg. Before use, 10 mg/ml Human Serum Albumin (HSA) (Sigma-Aldrich, St. Louis, MO, USA) was added. Aliquots were prepared and frozen at -20°C for future use. HTFM use throughout the thesis refers to the final solution used in laboratory procedures.

2.4.2. Seminal volume and total sperm count

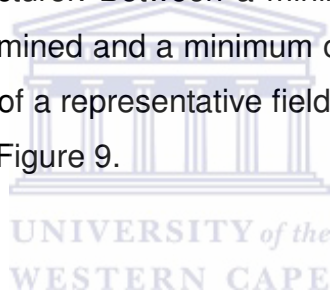
Seminal volume was assessed by the transfer of the sample into a measuring cylinder with a wide mouth. The volume was visually read directly after liquefaction with an accuracy of 0.1 ml. Total sperm count was assessed by the multiplication of seminal volume with the sperm concentration (WHO, 2010).

2.4.3. Sperm concentration and motility (CASA system)

Sperm count and motility was assessed using the Motility/Concentration module of the Sperm Class Analyzer[®] (SCA) CASA system version 4.1.0.1 (Microptic S.L., Barcelona, Spain).

For analysis, a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa) equipped with phase contrast optics and a heated stage was used. Assays were conducted within 30 minutes of liquefaction, kept at 37°C as outlined by WHO (2010). Sperm concentrations of $>50 \times 10^6/\text{ml}$ were diluted with HTFH to below this threshold for accurate motility assessments.

Appropriate disposable counting chambers were used with software specifications recommended by the manufacturer. Between a minimum of 5 and maximum of 10 representative fields were examined and a minimum of 200 spermatozoa included in the assessment. An example of a representative field from one participant (placed in the control group) is shown in Figure 9.



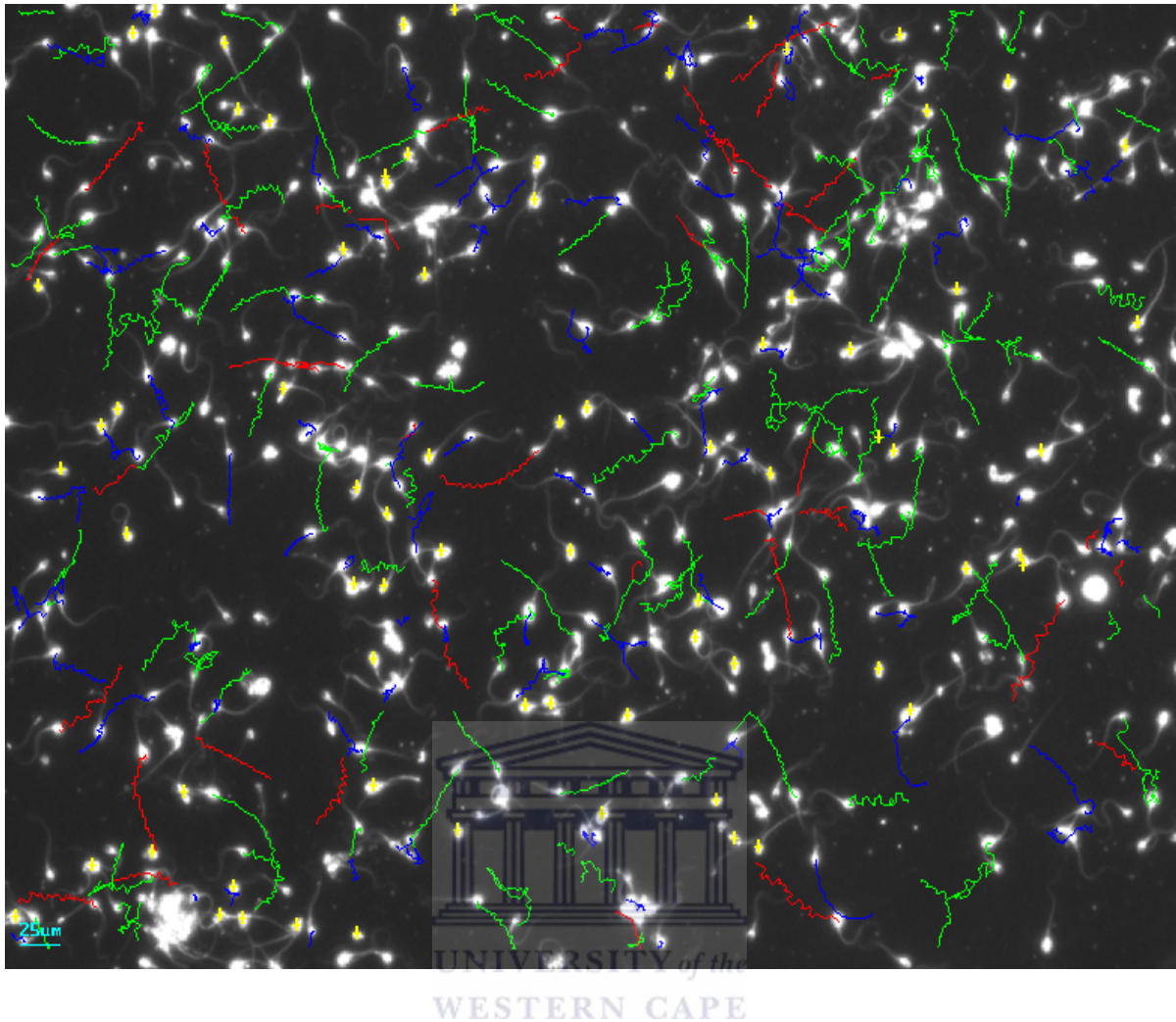


Figure 9: Typical determination of sperm concentration and motility by means of the Sperm Class Analyzer[®] (SCA) CASA system version 4.1.0.1 (Microptic S.L., Barcelona, Spain). This figure specifically indicates motility, with red and green comprised of progressively motile sperm, blue representing non-progressively motile sperm and yellow representing immotile sperm.

2.4.4. Sperm vitality

Sperm vitality was assessed using the eosin-nigrosin staining technique within 30 minutes following liquefaction as outlined by WHO (2010). Eosin-nigrosin stain was prepared by dissolving 0.67 g eosin Y (Sigma-Aldrich, St. Louis, MO, USA) and 0.9 g sodium chloride in 100 ml purified water.

Following this, 10 g of nigrosin (Sigma-Aldrich, St. Louis, MO, USA) was added to the solution, boiled, and left to cool to room temperature. The stain was filtered and stored in a sealed dark glass container at room temperature for use in the assays. After mixing the semen sample, 20 μ l was removed and mixed with 20 μ l eosin–nigrosin suspension in an Eppendorf vial. After 30 seconds, a smear of the suspension was made on a glass slide and allowed to air dry at room temperature. This was done in duplicate.

Immediately after drying, each slide with examined with bright field optics at \times 1000 magnification. A minimum of 100 (those with poor sperm count) and maximum of 200 spermatozoa were counted on each slide, recorded as stained (dead) vs unstained (alive) cells, and an average between both slides determined when the difference between the two slides was considered acceptable (WHO, 2010).

The vitality is represented as percentage of vital (unstained) cells. . An example of a representative field from one participant (placed in the control group) is shown in Figure 10.

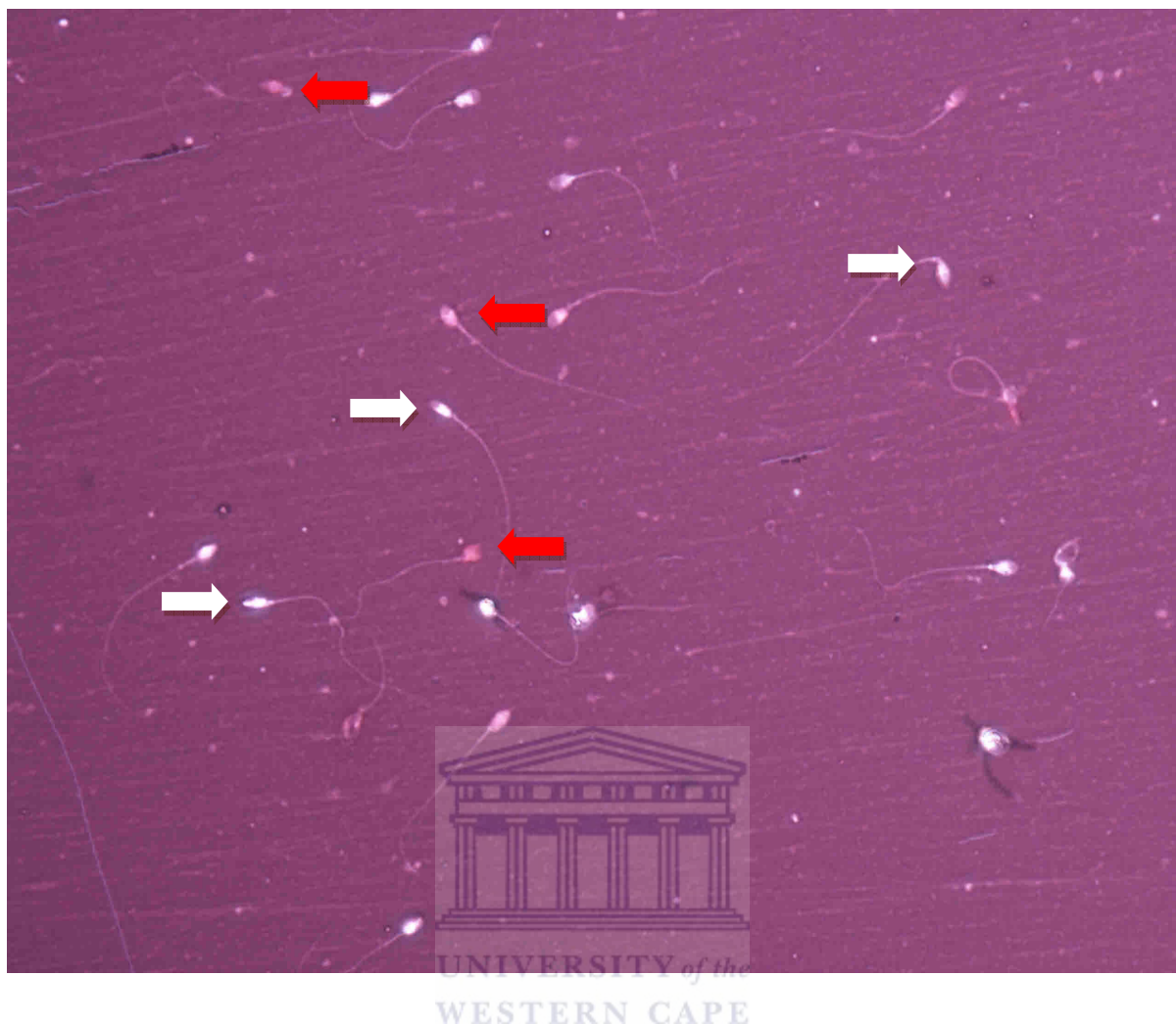


Figure 10: Typical determination of sperm vitality by means the eosin-nigrosin staining technique. The unstained (alive) cells are depicted by white arrows pointing to the right. The stained cells (dead) are depicted by red arrows pointing to the left.

2.4.5. Morphology

Morphology was assessed by the preparation of a smear and the application of the Papanicolaou staining method as outlined by WHO (2010). A smear of semen on frosted slides was made after mixing sample. To do this, 10 μ l semen was placed on the non-frosted end of the slide. A slide cover slip was used to pull the semen sample along the surface of the slide. The slides were allowed to air dry and stored in a cool dark cupboard for later staining and counting. They were transported in dark slide holding containers to be evaluated by Professor R. Menkveld (Department

of Obstetrics and Gynaecology, Stellenbosch University, Tygerberg Academic Hospital, Tygerberg), according to strict criteria (Menkveld et al., 1990).

2.4.6. Leukocytes

Leukocytes concentration was determined using the peroxidase staining technique as described by Politch and colleagues (Politch et al., 1993; Politch et al., 2007). An amount of 125 mg Benzidine (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 50 ml distilled water and 50 ml 96% (v/v) ethanol, mixed and prepared in aliquots of 4 ml each. These were stored in the dark at 4°C for a maximum of 6 months, and labelled 'stock solution'. In order to conduct the assay, a fresh 'working solution' was made by adding 5 µl 30% hydrogen peroxidise (H_2O_2) to the stock solution at room temperature. After liquefaction, 20 µl of ejaculate was mixed with 20 µl working solution and incubated for 5 minutes at room temperature. Following this, 160 µl phosphate buffer solution (PBS) was then added. Counting was done using a Neubauer chamber at 400x magnification with a dilution factor of 1:10 considered. Clearly stained brown cells were considered peroxidase positive. For every 1 peroxidase positive cell counted, a score of 100 000 leukocytes per ml semen (0.1×10^6 /ml) was recorded.

2.4.7. Mitochondrial membrane potential (MMP)

MMP was assessed as described previously (Henkel et al., 2011b) using a Zeiss fluorescence microscope (Oberkochen, Germany) for analysis after staining sperm with DePsipher staining kit (R&D Systems Inc., Minneapolis, MN, USA). DePsipher is a lipophilic cation (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolyl carbocyanin iodide) used as a mitochondrial marker.

A sample of liquefied semen was diluted 1:5 ratio with HTFM and centrifuged for 10 minutes at 500xg. The supernatant was discarded, the pellet re-suspended in DePsipher staining solution and incubated for 20 minutes at 37°C in the dark. The DePsipher sperm suspension was then centrifuged at 500xg, the supernatant was discarded, and the pellet re-suspended in 100µl pre-warmed 1X reaction buffer. The cells were observed immediately with fluorescence microscopy at 1000-times

magnification. Sperm exhibiting a green fluorescence within their mid pieces were regarded as having disturbed MMP, while those sperm showing red fluorescence were regarded as having intact MMP (as shown in Figure 11). A minimum of 100 (those with poor sperm count) and maximum of 200 spermatozoa were counted on each slide. The percentage of sperm with disturbed (abnormal) MMP was calculated and recorded.

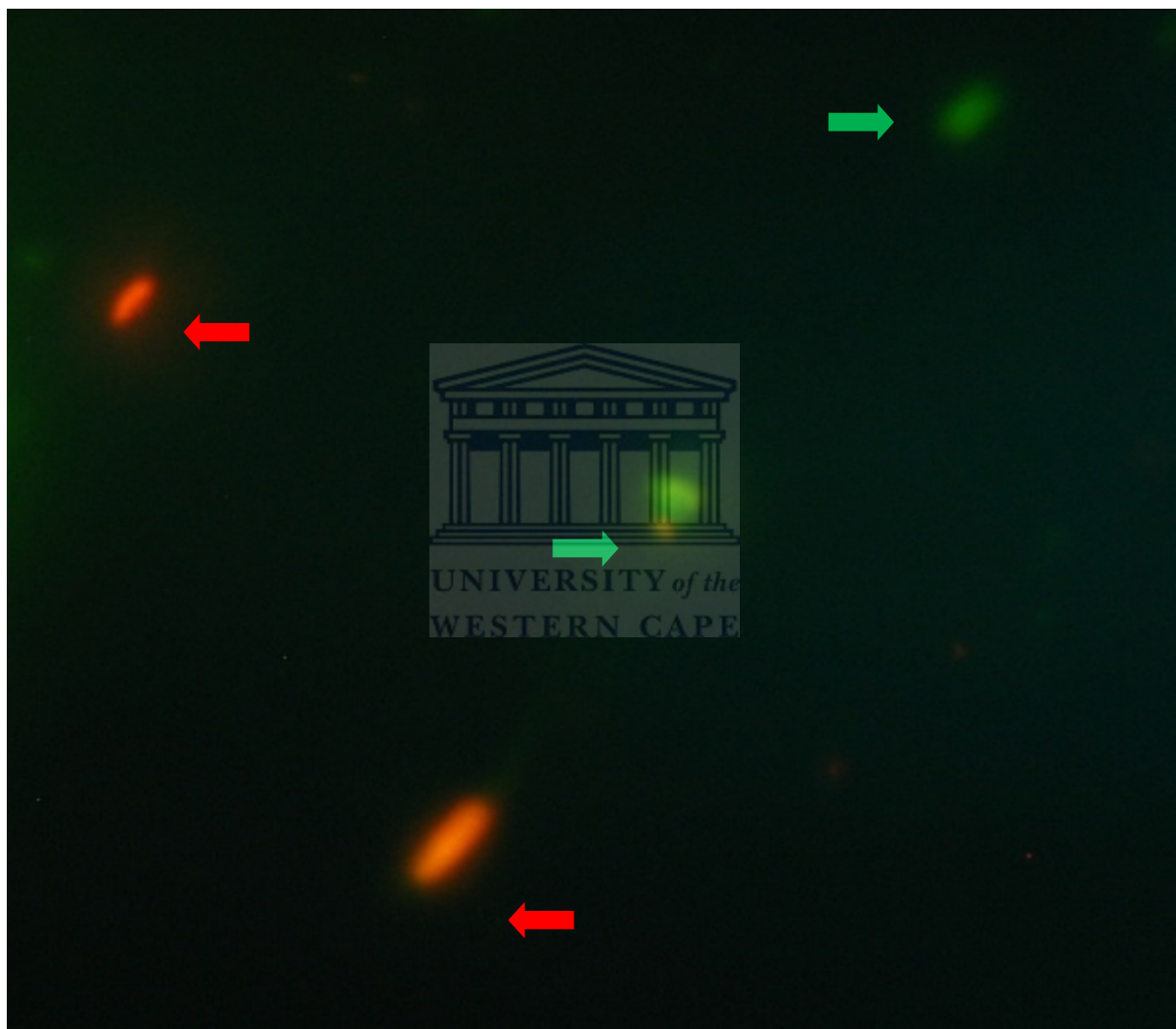


Figure 11: Typical determination of mitochondrial membrane potential (MMP) in spermatozoa stained with the DePsipher staining solution and observed under fluorescent microscopy. Sperm showing red fluorescence, indicated by the red arrow pointing to the left, was regarded as having intact MMP. Sperm exhibiting a green fluorescence within their mid pieces, indicated by the green arrow pointing to the right, were regarded as having disturbed MMP.

2.4.8. DNA fragmentation

DNA fragmentation was assessed by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay according to Henkel et al. (2004). The DeadEnd™ Colorimetric TUNEL System (Promega Corp., Madison, WI, USA) end labels the fragmented DNA of apoptotic cells using a modified TUNEL assay.

A sample of liquefied semen was diluted in a 1:5 ratio with HTFM and centrifuged for 10 minutes at 500xg. The pellet was re-suspended in PBS (Oxoid, Basingstoke, Hampshire, UK). A smear on a Superfrost® slide (Mentzel, Braunschweig, Germany) was made and allowed to air dry and accumulated for future analysis. All slides were analysed within 6 weeks of preparation.

Prepared slides were fixed in 4% methanol-free formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 25 minutes at 4°C. Slides were washed in fresh PBS for 5 minutes at room temperature, then sperm cells permeabilized in 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 5 minutes. After adequate rinsing of slides in fresh PBS, cells were allowed to equilibrate using the equilibration buffer (100µl added to each slide) for 10 minutes. Based on number of samples requiring assessment, the TdT incubation buffer was prepared in the dark according to manufacturer instruction. Slides were blotted around the equilibrated areas and 20 µl TdT incubation buffer was added to an area of 5 cm² and covered with plastic slips. This was incubated in the dark at 37°C for 60 minutes and terminated using SSC diluted appropriately with deionised water for 15 minutes. The slides were washed in fresh PBS at room temperature 5 times of 5 minutes each, before draining excess water. Immediately following washing, DNA fragmentation was assessed by manual counting done using a Zeiss fluorescence microscope (Oberkochen, Germany). A minimum of 100 (those with poor sperm count) and maximum of 200 spermatozoa were counted on each slide and the results expressed as a percentage of cells showing green fluorescence indicating fragmented DNA (TUNEL-positive cells). An example is of a field is shown in figure 12.



Figure 12: Typical determination of TUNEL positive cells using fluorescence microscopy techniques. The cells showing bright green fluorescence 'glow', as highlighted by the green arrows, was considered to have damaged DNA content.

2.5. Serum and seminal fluid cytokines

Cytokines in serum and seminal fluid investigated included TNF α , IL1 β , IL6 and IL8. Blood was collected in serum separating tubule vacutainers[®] (BD Biosciences, Franklin Lakes, New Jersey, USA) and centrifuged at 5000xg for 10 minutes. Serum was transferred to a sterile Eppendorf container and stored at -20°C until sampling. These assays were conducted at Synexa Life Sciences (Milnerton, Cape Town, South Africa).

Both serum and seminal fluid were assessed using the BD[™] Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (BD Biosciences, Franklin Lakes, New Jersey, USA), which is a multiplexed assay using the sensitivity of amplified fluorescence detection by flow cytometry to detect soluble analytes in a particle based immunoassay. The reportable range for cytokines assessed has an upper quantifiable limit of 5000 pg/ml. The lower quantifiable limits are variable; TNF α = 3.7 pg/ml, IL1 β = 7.2 pg/ml, IL6 = 2.5 pg/ml and IL8 = 3.6 pg/ml. Intra-assay CV are 6–10% for TNF α , 4–7% for IL1 β , 5–8% for IL6 and 2–5% for IL8. Inter-assay CV is 8–15% for TNF α , 4.9–13% for IL1 β , 9–10% for IL6 and 4–7% for IL8. All samples were assayed in duplicate, with a mean value recorded for data analysis. Samples were assayed on a flow cytometry reader obtained from BD Biosciences (Franklin Lakes, New Jersey, USA).

2.6. Seminal fluid insulin

Seminal insulin was assayed using the Human Insulin ELISA Kit (RayBiotech, Inc., Norcross, Georgia, USA). This is an *in-vitro* ELISA based assay for the quantitative measurement of insulin. Following liquefaction and seminal analysis, remaining semen was centrifuged at 5000xg for 10 minutes. Seminal fluid was transferred to Eppendorf containers and frozen at -20°C until sampling. All reagents and samples were thawed and brought to room temperature for analysis. The lower quantitative limit is 4 μ IU/ml, with an intra- and inter-assay CV of <10% and <12% respectively. These assays were conducted at Synexa Life Sciences (Milnerton, Cape Town, South Africa). All samples were assayed in duplicate, with the mean value recorded

for data analysis. Samples were assayed on an ELISA reader obtained from BioTek (Winooski, VT, USA).

2.7. Serum and seminal fluid leptin

Serum and seminal leptin was assayed using the Human Leptin ELISA Kit (RayBiotech, Inc., Norcross, Georgia, USA). This is an *in-vitro* ELISA based assay for the quantitative measurement of leptin. Blood was collected serum separating tubule vacutainers[®] (BD Biosciences, Franklin Lakes, New Jersey, USA), centrifuged at 5000xg for 10 minutes, with serum transported Eppendorf containers and frozen at -20°C until sampling. Following liquefaction and seminal analysis, remaining semen was centrifuged at 5000xg for 10 minutes. Seminal fluid was transferred to Eppendorf containers and frozen at -20°C until sampling. All reagents and samples were thawed and brought to room temperature for analysis. The lower quantitative limit is 2 pg/ml, with an intra- and inter-assay CV of <10% and <12% respectively. These assays were conducted at Synexa Life Sciences (Milnerton, Cape Town, South Africa). All samples were assayed in duplicate, with the mean value recorded for data analysis. Samples were assayed on an ELISA reader obtained from BioTek (Winooski, VT, USA).

2.8. Seminal fluid glucose

Seminal glucose was assayed using the Glucose (HK) Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). This is an *in-vitro* ELISA based assay for the quantitative measurement of leptin. Following liquefaction and seminal analysis, remaining semen was centrifuged at 5000xg for 10 minutes. Seminal fluid was transferred to Eppendorf containers and frozen at -20°C until sampling. All reagents and samples were thawed and brought to room temperature for analysis. The CV of the kit based on correspondence with the supplier is 2.0%. If the duplicate samples are within 2% of each other, these were considered accurate and the mean value recorded for data analysis. These assays were conducted at Synexa Life Sciences (Milnerton, Cape Town, South Africa). All samples were assayed in duplicate, with the mean value recorded for data analysis. Samples were assayed on an ELISA reader obtained from BioTek (Winooski, VT, USA).

2.9. TM3-Leydig cell culture experiments

A hCG stimulated TM3-Leydig cell culture model was used in order to investigate the effect of insulin and cytokines on cell viability, protein concentration and steroid hormone (testosterone and progesterone) production. The TM3 immortalised mouse (*Mus musculus*) Leydig cells (ATCC, Middlesex, United Kingdom) are obtained from testicular tissues of 11- 13 day old mice, with epithelial morphology and adherent culture properties (ATCC CRL-1714). Passages between 19 and 24 were used for the experiments. Cells were cultured in optimal conditions described below. For each experiment, approximately 25 000 cells were seeded in a sterile 96-well plate for 48 hours. Medium was then removed, and cells were further cultured with experimental medium at varying concentrations for a further 48 hours, with 25 mIU/ml hCG co-culture for stimulation of steroidogenesis. After termination of the experiment, the following parameters were assessed: cell viability (XTT assay), protein concentration, testosterone concentration and progesterone concentration. All experiments were done in triplicate, and repeated six times.

2.9.1. Cell culture conditions

TM3-Leydig cells were cultured using standard sterile cell culture techniques. Cell culture medium used was Dulbecco's Modified Eagle Medium F-12 (DMEM/F-12) (Gibco, Johannesburg, South Africa) with 5% Horse Serum (Gibco, Johannesburg, South Africa), 2.5% Fetal Bovine Serum (Gibco, Johannesburg, South Africa) and 1% Penicillin-Streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured in 75 ml culture flasks and incubated at 37°C with 5% CO₂. When confluent, and for experimental preparations, cultured cells were detached using 0.25% Trypsin/EDTA (Gibco, Johannesburg, South Africa).

2.9.2. Preparation of cells for experiments

Cultured cells were allowed to reach confluence in 75 ml cell culture flasks for experiments. Medium was removed and flask washed with sterile PBS briefly. An amount of 2 ml 0.25% Trypsin/EDTA was added for 10 seconds before removal. The

flask was then allowed to sit flat for 2-3 minutes before 2 ml medium was added and briefly swirled. The cell rich medium was removed and placed in a sterile test tube. The medium was centrifuged at 2500xg and the pellet resuspended in 2 ml culture medium.

TM3 cells in suspension were counted using Trypan Blue stain (Sigma-Aldrich, St. Louis, MO, USA) under light microscopy on a haematocytometer at 20x magnification. Based on the cell count, approximately 25 000 cells were seeded under optimal and sterile conditions in 96-well plates with 300 µl of medium and incubated for 48 hours in preparation for the experiments.

2.9.3. Cell culture experiments

After 48 hours of preparation, the cells were co-cultured for a further 48 hours with 25 µl/ml hCG (hCG stimulated cells) and 4 concentrations of a specific hormone or cytokine being investigated. Experiments included a positive (25 µl/ml hCG) and a negative (hCG negative) control. In preparation of each experiment, hCG medium was prepared fresh using aliquots of 5000 mIU/ml hCG (Sigma-Aldrich, St. Louis, MO, USA) frozen at -20°C. Frozen aliquots of 5 µl were allowed to thaw in a water bath at 37°C. As 995 µl medium added to 5 µl determined a concentration of 25 mIU/L (dilution factor of 1:200), appropriate dilutions was determined for the preparation of adequate amount of medium required for the experiment. All experiments had a positive (hCG 25 mIU/ml) and a negative (medium) control.

2.9.3.1. Insulin

Recombinant insulin appropriate for mouse cell culture experiments was obtained from Sigma-Aldrich (St. Louis, MO, USA) as a lyophilised powder. As per manufacturer instructions, 25 mg insulin was reconstituted by adding 1250 µl 0.01M hydrochloric acid (HCl) solution to achieve 20 mg/ml concentration. This solution was further diluted at 1:1000 with culture medium in order to achieve 20 pg/ml insulin stock solution. Aliquots of this stock solution were frozen at -20°C until required for experiments.

For each experiment, four concentrations of insulin were used:

- 10 ng/ml
- 1 ng/ml
- 0.1 ng/ml
- 0.01 ng/ml

From 20 µg/ml stock solution, a dilution of 1:1000 was made with prepared 25 mIU/L hCG medium to achieve 20 ng/ml. This was again diluted at 1:2 to achieve 10 ng/ml. Serial dilutions of 1:10 with hCG medium was done to achieve 1, 0.1 and 0.01 ng/ml concentrations.

After preparation of various insulin concentrations in hCG medium, prepared cell culture experimental plates were visually inspected. Medium was removed in sterile conditions, and the four concentrations were added at 300 µl to each well as appropriate, in addition to 300 µl of positive and negative controls. An exposure of 48 hours was allowed before termination of the experiment.

2.9.3.2. Tumour necrosis factor-alpha (TNF α)

Recombinant TNF α appropriate for mouse cell culture experiments was obtained from Sigma-Aldrich (St. Louis, MO, USA) as a lyophilised powder. As per manufacturer instructions, 10 µg TNF α was reconstituted by adding 100 µl distilled and sterilised water to achieve 100 µg/ml solution. This solution was further diluted at 1:10 with culture medium in order to achieve 10 µg/ml TNF α stock solution. Aliquots of this stock solution were frozen at -20°C until required for experiments.

For each experiment, four concentrations of TNF α were used:

- 100 ng/ml
- 10 ng/ml
- 1 ng/ml
- 0.1 ng/ml

From 10 µg/ml stock solution, a dilution of 1:10 was made with prepared 25 mIU/L hCG medium to achieve 1000 ng/ml. Further serial dilutions of 1:10 with hCG medium was done to achieve 100, 10, 1 and 0.1 ng/ml concentrations for the experiments.

After preparation of various TNF α concentrations in hCG medium, prepared cell culture experimental plates were visually inspected. Medium was removed in sterile conditions, and the four concentrations were added at 300 µl to each well as appropriate, in addition to 300 µl of positive and negative controls. An exposure of 48 hours was allowed before termination of the experiment.

2.9.3.3. Interleukin 1-beta (IL1 β)

Recombinant IL1 β appropriate for mouse cell culture experiments was obtained from Sigma-Aldrich (St. Louis, MO, USA) as a lyophilised powder. As per manufacturer instructions, 5 µg IL1 β was reconstituted by adding 50 µl distilled and sterilised water to achieve 100 µg/ml solution. This solution was further diluted at 1:10 with culture medium in order to achieve 10 µg/ml IL1 β stock solution. Aliquots of this stock solution were frozen at -20°C until required for experiments.

For each experiment, four concentrations of IL1 β were used:

- 100 ng/ml
- 10 ng/ml
- 1 ng/ml
- 0.1 ng/ml

From 10 µg/ml stock solution, a dilution of 1:10 was made with prepared 25 mIU/L hCG medium to achieve 1000 ng/ml. Further serial dilutions of 1:10 with hCG medium was done to achieve 100, 10, 1 and 0.1 ng/ml concentrations for the experiments.

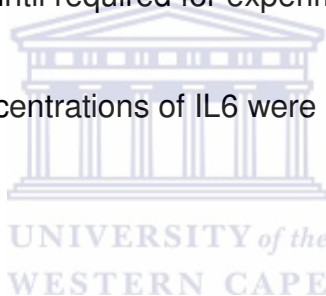
After preparation of various IL1 β concentrations in hCG medium, prepared cell culture experimental plates were visually inspected. Medium was removed in sterile conditions, and the four concentrations were added at 300 μ l to each well as appropriate, in addition to 300 μ l of positive and negative controls. An exposure of 48 hours was allowed before termination of the experiment.

2.9.3.4. Interleukin 6 (IL-6)

Recombinant IL6 appropriate for mouse cell culture experiments was obtained from Sigma-Aldrich (St. Louis, MO, USA) as a lyophilised powder. As per manufacturer instructions, 5 μ g IL6 was reconstituted by adding 50 μ l distilled and sterilised water to achieve a 100 μ g/ml solution. This solution was further diluted at 1:10 with culture medium in order to achieve a 10 μ g/ml IL6 stock solution. Aliquots of this stock solution were frozen at -20°C until required for experiments.

For each experiment, four concentrations of IL6 were used:

- 100 ng/ml
- 10 ng/ml
- 1 ng/ml
- 0.1 ng/ml



From 10 μ g/ml stock solution, a dilution of 1:10 was made with prepared 25 mlU/L hCG medium to achieve 1000 ng/ml. Further serial dilutions of 1:10 with hCG medium was done to achieve 100, 10, 1 and 0.1 ng/ml concentrations for the experiments.

After preparation of various IL6 concentrations in hCG medium, prepared cell culture experimental plates were visually inspected. Medium was removed in sterile conditions, and the four concentrations were added at 300 μ l to each well as appropriate, in addition to 300 μ l of positive and negative controls. An exposure of 48 hours was allowed before termination of the experiment.

2.9.3.5. Interleukin 8 (IL-8)

Recombinant IL8 appropriate for mouse cell culture experiments was obtained from Sigma-Aldrich (St. Louis, MO, USA) as a lyophilised powder. As per manufacturer instructions, 10 µg IL8 was reconstituted by adding 100 µl distilled and sterilised water to achieve a 100 µg/ml solution. This solution was further diluted at 1:10 with culture medium in order to achieve a 10 µg/ml IL8 stock solution. Aliquots of this stock solution were frozen at -20°C until required for experiments.

For each experiment, four concentrations of IL8 were used:

- 100 ng/ml
- 10 ng/ml
- 1 ng/ml
- 0.1 ng/ml

From 10 µg/ml stock solution, a dilution of 1:10 was made with prepared 25 mIU/L hCG medium to achieve 1000 ng/ml. Further serial dilutions of 1:10 with hCG medium was done to achieve 100, 10, 1 and 0.1 ng/ml concentrations for the experiments.

After preparation of various IL8 concentrations in hCG medium, prepared cell culture experimental plates were visually inspected. Medium was removed in sterile conditions, and the four concentrations were added at 300 µl to each well as appropriate, in addition to 300 µl of positive and negative controls. An exposure of 48 hours was allowed before termination of the experiment.

2.9.4. Experimental assays

After 48 hours exposure to experimental concentrations, experiments were terminated. Cell viability, protein concentration and steroid hormone (testosterone and progesterone) concentrations were assayed for statistical analysis.

2.9.4.1. Cell viability and proliferation determination

Cell viability was assessed using the Cell Proliferation Kit II (XTT) (Roche, Illovo, Johannesburg, South Africa). XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene sulfonic acid hydrate)) is cleaved by living viable cells from a yellow tetrazolium salt to an orange formazan dye, which can be quantified using standard ELISA readers.

In order to conduct the assay, under sterile conditions, XTT labelling reagent and the electron coupling reagent were thawed at 37°C and each mixed thoroughly, and 1 µl electron coupling reagent was added to 50 µl XTT labelling reagent (or in required amounts based on this ratio). A total of 200 µl of experimental culture medium was removed and stored in Eppendorf containers at -20°C for future analysis if required. 50 µl XTT labelling mixture was added to the remaining 100 µl experimental cell culture fluid to achieve a final XTT concentration of 0.3 mg/ml. This was incubated for 5-6 hours in the dark at 37°C in the cell culture incubator. Following incubation, the plate was read with an ELISA reader (Labtech, East Sussex, UK) at a wavelength of 450 nm and recorded. Cell viability was expressed as a percentage of XTT binding as compared to the hCG control group.

2.9.4.2. Protein concentration determination

Protein concentration was determined using the Bio-Rad Protein Assay Dye Reagent Concentrate, a colorimetric assay. The assay is based on the reaction of proteins with an alkaline copper tartrate solution and Folin reagent. Materials used for the assay includes Protein Assay Reagent A (Bio-Rad, Hercules, California, USA) and Protein Assay Reagent B (Bio-Rad, Hercules, California, USA).

In order to perform the assay, a lysis solution was prepared by adding 250 mg sodium dodecyle sulphate (SDS) (Sigma-Aldrich, St. Louis, MO, USA) and 10 mg sodium hydroxide pellets (NaOH) with 250 ml distilled water and thoroughly dissolved. Following this, 300 µl of supernatant was removed from cell culture plate wells and transferred to an Eppendorf container to be stored at -20°C to be used for steroid hormone determination. The plate was hit on dry paper to remove all excess

fluid. An amount of 200 μl lysis solution was added to each well and incubated at room temperature for 30 minutes. After this, 20 μl was transferred in duplicate to another 96-well plate, along with standard dilutions of Bovine Serum Albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) diluted with lysis solution (1400 $\mu\text{g/ml}$; 1000 $\mu\text{g/ml}$; 600 $\mu\text{g/ml}$; 200 $\mu\text{g/ml}$ and pure lysis solution as 0 $\mu\text{g/ml}$). An amount of 200 μl Reagent B and 25 μl Reagent A was added to each well and incubated at room temperature for a further 30 minutes. This was then read at 690 nm on a standard ELISA reader (Labtech, East Sussex, UK). Protein concentration was quantified based on the standard curve determined by BSA concentrations, an example of which is illustrated in Figure 13.

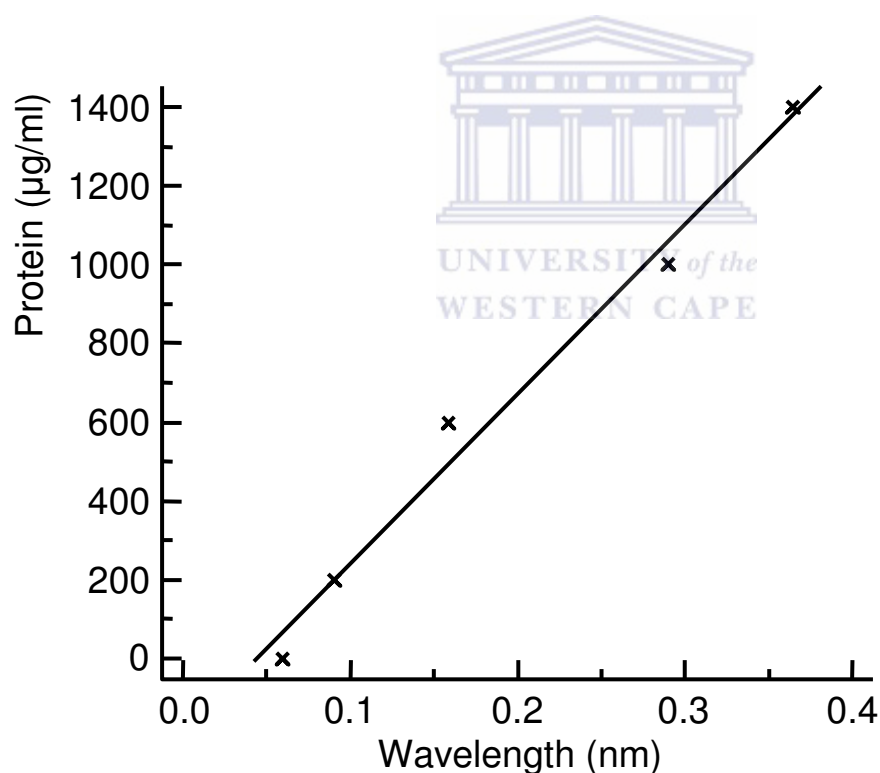


Figure 13: A standard curve based on Bovine Serum Albumin (BSA) concentrations with a correlation coefficient (r^2) of 0.9821. The regression equation used for conversion of light wave to protein concentrations of samples was $y = -197.8895 + 4347.3318x$.

2.9.4.3. Testosterone

Testosterone concentrations were determined from experimental cell culture supernant stored in Eppendorf containers from the wells used for protein determination. Supernant was stored at -20°C until being assayed. The testosterone ELISA kits (DRG International, Inc., Springfield, New Jersey, USA), an enzyme based immunoassay for quantitative *in vitro* assays, was used to determine testosterone concentrations. The sensitivity of the assay is 0.083-16 ng/ml with an intra- and inter-assay CV of 3.28-4.16% and 4.73-9.94% respectively. All assays were carried out in duplicate, with the mean value recorded as the testosterone concentration. Testosterone concentration was quantified based on the standard curve determined by supplied testosterone concentrations, an example of which is illustrated in Figure 14.

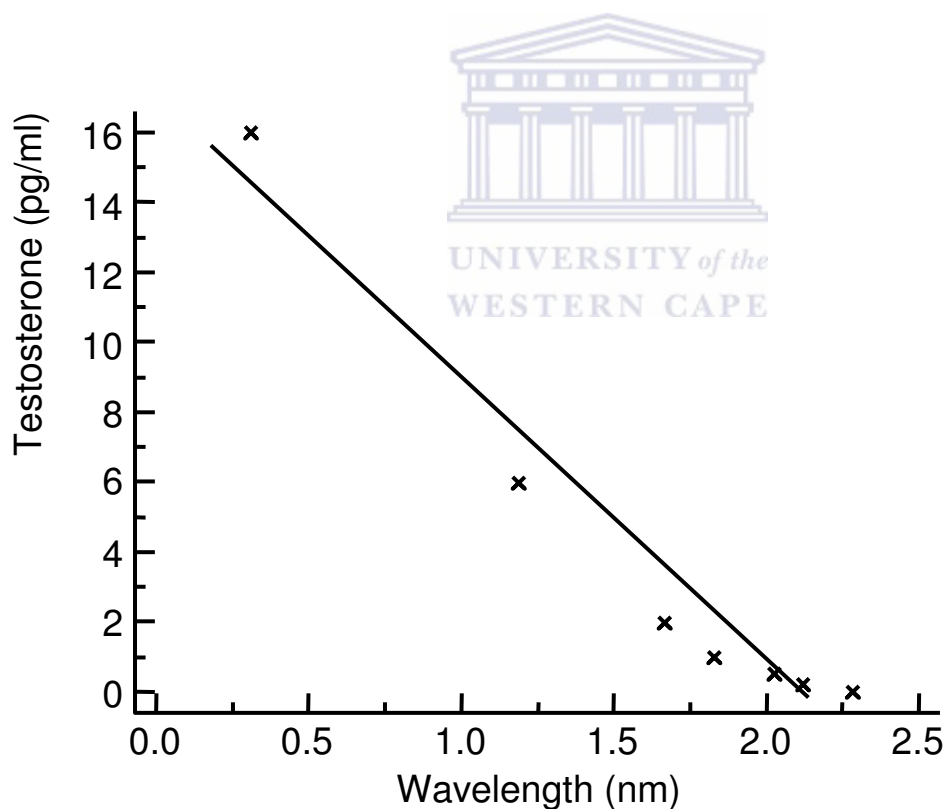


Figure 14: A standard curve based on testosterone standard dilutions supplied with the ELISA kit. The correlation coefficient (r^2) of this example was 0.9551, and the formula used for conversion of light wave to testosterone concentrations of samples was $y=17.2287+-8.3024x$.

2.9.4.4. Progesterone

Progesterone concentrations were determined from experimental cell culture supernant stored in Eppendorf containers from the wells used for protein determination. Supernant was stored at -20°C until being assayed. The progesterone ELISA kits (DRG International, Inc., Springfield, New Jersey, USA), an enzyme based immunoassay for quantitative *in vitro* assays, was used to determine progesterone concentrations. The sensitivity of the assay is 0-40 ng/ml with an intra- and inter-assay CV of 5.4-6.99% and 4.34-9.96% respectively. All assays were done in duplicate, with the mean value recorded as the progesterone concentration. Progesterone concentration was quantified based on the standard curve determined by supplied progesterone concentrations, an example of which is illustrated in Figure 15.

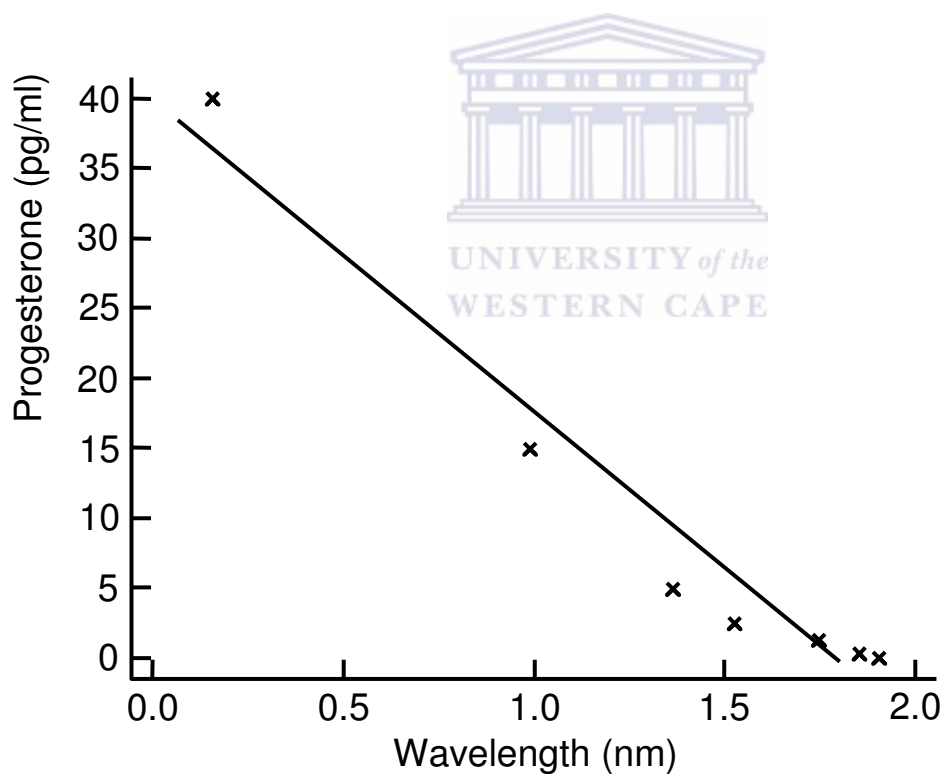


Figure 15: A standard curve based on progesterone standard dilutions supplied with the ELISA kit. The correlation coefficient (r^2) of this example was 0.9549, and the formula used for conversion of light wave to progesterone concentrations of samples was $y=40.4473+-22.9516x$.

2.10. Statistical analysis

Statistical analysis was performed using the MedCalc software (Version 12.0; Mariakerke, Belgium). After testing for normal distribution using the Kolmogorov-Smirnov test, appropriate statistical tests, either parametric (Pearson correlation, independent samples t-test) or non-parametric (Spearman Rank correlation, Mann-Whitney test) were performed. A P-value of <0.05 was considered as significant. Correlations were determined using Rank correlation coefficients, with $P < 0.05$ considered significant and the correlation coefficient expressed as r^2 .



CHAPTER 3 – RESULTS

3.1. Case controlled study cohort

A total number of 78 participants have been included in the study after supplying the appropriate biological samples and not reaching any of the exclusion criteria determined prior to onset of the study Table 3. Although a total of 85 participants requested to take part in the study, seven participants were excluded based on these criteria. The majority were excluded at the electronic or telephonic communication phase, with three participants due to reported hormonal therapy (insulin or testosterone treatments) and three due to a vasectomy history. One participant was excluded after supplying samples due to leukocytospermia (he was subsequently referred to an appropriate practitioner for further evaluation and treatment, with full disclosure of all clinical and biochemical results obtained). An additional three participants were originally excluded based on a recent history of inflammatory pathology (n=2) or surgery (n=1), but allowed to enter the study after a period of six months following full recovery. Selection bias was limited as no participants who met the criteria were excluded from data analysis, nor were participants invited to the study. Based on the diagnostic criteria for metabolic syndrome as outlined in Table 1 (Alberti et al., 2009), 44 participants were placed in the control group (CG), and 34 participants were placed in the metabolic syndrome group (MetS).

3.1.1. Potential confounding factors

Numerous potential confounding variables were identified in the study. These included participant age, body mass index (BMI), demographics, number of MetS components, specific MetS components in phenotypic expression, underlying MetS components (e.g. insulin resistance/hyperinsulinaemia; T2DM), medications related to MetS and smoking. Details of these factors present in the cohort are in Table 6 and Table 7. Further factors recorded included recent fertility history (where applicable) and semen analysis characteristics (Table 8). P-value differences between the groups were calculated using Fisher's Exact Test. As there is no statistical difference between the groups for most of these factors, statistical correction of data using regression analysis was not conducted.

Although many of these factors were asked in a clinical history, not all potential confounding factors were recorded in detail for statistical analysis. These include additional lifestyle factors (such as frequency and/or type of exercise, nutrition, nutritional and herbal supplements and alcohol use), education, socio-economic status, occupation, potential exposure to toxins and endocrine disruptors, exposure to excessive testicular heat or irradiation (e.g. cell phones) and sexual (dys)function (e.g. poor libido; erectile dysfunction; premature ejaculation).

Table 6: Details of potential confounders such as age, BMI, demographics and smoking in the full cohort, with comparisons between the control and MetS groups. Variables are represented as percentages rounded to the nearest decimal point. P-value was determined using Fisher's Exact Test.

Variable	Cohort (n=78)	Control (n=44)	MetS (n=34)	P-value
Age (years)				
18-29	11.5%	11.4%	11.8%	1.000
30-39	46.2%	54.5%	35.1%	0.315
40-49	26.9%	22.7%	32.4%	0.622
50-59	12.8%	9.1%	17.8%	0.502
60-70	2.6%	2.3%	2.9%	1.000
BMI				
18-24.9 (normal weight)	25.6%	40.9%	5.8%	0.008
25-29.9 (over-weight)	34.6%	50.0%	14.7%	0.022
30-34.9 (obese)	19.2%	9.1%	32.4%	0.048
>35 (morbidly obese)	20.6%	0%	47.1%	<0.001
Demographics				
Caucasian	66.7%	72.7%	58.8%	0.590
Coloured	25.6%	15.9%	38.3%	0.131
Black	7.7%	11.4%	2.9%	0.393
Asian	-	-	-	-
Smokers	15.4%	11.4%	20.5%	0.369

Table 7: Potential confounding parameters such as the metabolic syndrome diagnostic criteria, individual metabolic syndrome components, associated metabolic features and related medications in the full cohort, with comparisons between the control and MetS groups. Variables are represented as percentages rounded to the nearest decimal point. P-value was determined using Fisher's Exact Test. Insulin resistance is based on a QUICKI cut off value of <0.357. Low grade inflammation is based on an hs-CRP value of >1.00mmol/L.

Variable	Cohort (n=78)	Control (n=44)	MetS (n=34)	P-value
MetS diagnostic criteria				
0 criterion	17.9%	31.8%	-	-
1 criterion	28.2%	50.0%	-	-
2 criterions	10.3%	18.2%	-	-
3 criterions	29.5%	-	67.7%	-
4 criterions	10.2%	-	23.5%	-
5 criterions	3.8%	-	8.8%	-
MetS components:				
Increased WC	56.4%	36.7%	82.4%	0.039
Hypertension	41.0%	18.2%	70.6%	0.003
Reduced HDL cholesterol	42.3%	20.5%	70.6%	0.006
Hypertriglyceridaemia	19.2%	4.6%	38.2%	0.004
Increased serum glucose	15.4%	2.3%	32.4%	0.004
Insulin resistance (QUICKI)	47.4%	18.2%	85.3%	<0.001
T2DM	3.8%	-	8.8%	0.091
Low grade inflammation (hs-CRP)	66.6%	43.2%	97.1%	0.032
MetS medications	21.8%	2.3%	47.1%	<0.001
Hypertension	17.9%	2.3%	38.2%	<0.001
Cholesterol	10.3%	-	23.5%	0.002
Triglycerides	-	-	-	-
Glucose	3.8%	-	8.8%	0.091
Other medications				
COX inhibitors	3.8%	0%	8.8%	0.091

Table 8: Details of recent fertility history (where applicable) and semen analysis characteristics in the study cohort and comparisons between the control and metabolic syndrome groups. Variables are represented as percentages rounded to the nearest decimal point. P-value was determined using Fisher's Exact Test. Recent history of couple infertility was defined as an inability to achieve a conception with regular sexual intercourse over last 12 months. Proven fertility was defined as a live birth within the last 2 years of the consultation date. The following definitions were used: hypospermia = < 1.5ml ejaculate; azoospermia = no sperm in the ejaculate; oligozoospermia = < 15×10^6 spermatozoa per ml ejaculate; normozoospermia = $> 15 \times 10^6$ spermatozoa per ml ejaculate; asthenozoospermia = progressive motility < 32% and/or total motility < 40% spermatozoa; necrozoospermia = < 58% viable spermatozoa; teratozoospermia = < 4% normal form (morphology) spermatozoa; leukospermia = $> 0.5 \times 10^6$ leukocytes per ml ejaculate; Percentage of sperm with fragmented DNA (DF) = $> 25\%$ spermatozoa damaged; Percentage of spermatozoa with damaged mitochondria (MMP) = $> 36\%$ spermatozoa (WHO, 2010; Marchetti et al., 2012; Simon et al., 2013).

Variable	Cohort (n=78)	Control (n=44)	MetS (n=34)	P-value
Recent history of couple infertility	15.4%	9.1%	35.3%	0.028
Recent history of proven fertility	15.4%	25.0%	2.9%	0.024
Sperm parameters:				
Hypospermia	14.1%	6.8%	23.5%	0.106
Azoospermia	5.1%	4.5%	5.8%	1.000
Oligozoospermia	16.7%	6.8%	29.4%	0.035
Normospermia	78.2%	88.7%	64.8%	0.389
Asthenozoospermia	58.9%	47.8%	73.5%	0.268
Necrozoospermia	23.1%	22.7%	52.9%	0.078
Teratospermia	58.9%	52.3%	67.6%	0.576
Leukocytosis	-	-	-	-
DF	21.8%	6.8%	41.2%	0.006
MMP	52.5%	38.6%	70.6%	0.128

3.1.1.1. Cohort age and age distributions

Mean ages (years) between the groups did not significantly differ (CG = 37.8 ± 8.8 ; MetS = 40.7 ± 9.8), with a range of 24 – 67 years for the entire cohort Table 9. Age groups were based on decades as outlined in Table 6. The majority of participants were in the 30 – 39 years old age group for the whole cohort and each specific group (a common age group for fathering a child). No statistical difference was observed between the groups for any of the age group subsets. Therefore, the impact of age on the results and statistical analysis is limited.

3.1.1.2. Cohort body mass index (BMI) distribution

Mean BMI was significantly higher ($P < 0.0001$) in the MetS group (25.7 ± 3.0) compared to the control group (33.8 ± 5.2) (Table 9). BMI categories assessed are normal weight (BMI = 18 – 24.9), over-weight (BMI 25 – 29.9), obese (BMI = 30 – 34.9) and morbidly obese (BMI > 35) (Kort et al., 2006; Ervin, 2009). Participants in each BMI category are detailed in Table 6. The majority of participants in the study were either normal weight (25.6%) or over-weight (34.6%), with fewer participants obese (19.2%) or morbidly obese (20.6%). Generally, more participants in the MetS group were obese or morbidly obese. Significantly more CG participants were normal weight ($P = 0.008$) or over-weight ($P = 0.022$) compared to MetS participants. Conversely, more MetS participants were obese ($P = 0.048$) or morbidly obese ($P < 0.001$) compared to CG participants.

3.1.1.3. Cohort demographic distribution

The majority of participants in the study were Caucasians (66.7%), with coloured (25.6%) and black (7.7%) participants less represented. No Asians or other demographic backgrounds were in the study cohort. There was no difference between CG and MetS group participants for any of these demographics. Details are tabulated in Table 6.

Table 9: Clinical and biochemical parameters compared between the control group (n=44) and metabolic syndrome group (n=34). All statistical analysis using the Student's t-test, and values expressed as mean±SD (range), except * = Mann-Whitney tests expressed as median (interquartile range). MMP = mitochondrial membrane potential. BMI = body mass index; HDL = high density lipoprotein; QUICKI = quantitative insulin sensitivity check index.

Parameter	Control	MetS	P-value
Age (years)	37.8 ± 8.8 (24 – 67)	40.7 ± 9.8 (26 – 63)	0.1702
BMI (kg/m ²)	25.7 ± 3.0 (18.6 – 31.5)	33.8 ± 5.2 (22.3 – 44.0)	<0.000 1
Waist circumference (cm)	92.3 ± 9.1 (73 – 108)	116.6 ± 12.1 (89 – 142)	<0.000 1
Systolic BP (mmHg)*	120.0 (110.0 – 125.0)	135.0 (130.0 – 140.0)	<0.000 1
Diastolic BP (mmHg)*	80.0 (72.5 – 80.0)	90.0 (85.0 – 90.0)	<0.000 1
Serum HDL Cholesterol (mmol/L)	1.24 ± 0.33 (0.57 – 2.24)	0.95 ± 0.26 (0.58 – 2.04)	0.0001
Serum Triglycerides (mmol/L)	0.96 ± 0.39 (0.42 – 2.7)	1.69 ± 0.98 (0.47 – 5.33)	0.0002
Serum Glucose (mmol/L)	5.03 ± 0.59 (3.8 – 7.0)	5.79 ± 1.70 (4.4 – 9.7)	0.0018
Serum Insulin (mIU/ml)	5.73 ± 2.27 (2.4 – 14.3)	12.77 ± 5.45 (3.8 – 32.0)	<0.000 1
QUICKI Index (Insulin Resistance)	0.373 ± 0.024 (0.319 – 0.436)	0.327 ± 0.025 (0.276 – 0.391)	<0.000 1
hs-CRP (mmol/L)	1.04 ± 0.64 (0.2 – 2.96)	2.55 ± 0.7 (1.0 – 3.64)	<0.000 1

3.1.1.4. Smokers in the cohort

Smokers were allowed to enter the study (Table 6). A total of 12 smokers (15.4%) were in the cohort. Of these, 5 were in the CG (11.4% of the CG) and 7 in the MetS group (20.5% of the MetS group). This difference was not statistically significant ($P=0.369$).

3.1.1.5. Metabolic syndrome criteria, components, medications and related metabolic disorders in the cohort

The cohort ($n=78$) consisted of 44 (56.4%) participants in the CG and 34 (43.6%) participants in the MetS group. Based on the Fishers Exact test, this was not a significant difference ($P=0.406$). The number of diagnostic criteria in the MetS is related to the severity of the syndrome and an increasing risk of complications and chronic disease. Of participants representing the full cohort, 17.9% did not fulfill any criterion, 28.2% fulfilled 1 criterion and 10.3% fulfilled 2 criterions. MetS is defined in the study as 3 or more of the criterion outlined in Table 1. A proportion of 29.5% of the cohort fulfilled 3 criterions, with 10.2% fulfilling 4 criterions and 3.8% fulfilling of all 5 criterion. The majority of participants in the CG group (50%) fulfilled 2 diagnostic criterions, with the majority of participants in the MetS group (67.7%) fulfilling 3 diagnostic criterions. It is not possible to determine the difference between the groups. Details are summarised in Table 7.

In terms of individual components of MetS (Table 1), the majority of the cohort (56.4%) had an increased waist circumference (WC). This is followed by reduced HDL cholesterol (42.3%), hypertension (41%), hypertriglyceridaemia (19.2%) and increased serum glucose (15.4%). As mentioned above, 17.9% of the cohort did not full any of these criterions, and numerous participants had more than one component. After group comparisons, the MetS group had significantly increased proportion of participants with each of these components compared to the CG. The details are summarised in Table 7. It is important to note that medications related to MetS are also part of the diagnostic criteria.

Participants on any medication related to the metabolic syndrome are significantly increased in the MetS group ($p < 0.001$). Hypertensive, cholesterol (statins) and triglyceride (fibrate) medications were found to be significantly higher in the MetS group ($P < 0.001$, $P < 0.001$ & $P = 0.002$, respectively). Glucose regulating medication use was increased in the MetS group, but this was not significant ($P = 0.091$). Although not addressing the MetS criterion, non-steroidal anti-inflammatory drugs (COX-inhibitors) were used by 3.8% of the cohort; all of them in the MetS group. However, this difference did not reach statistical significance. No other medications were used by participants in the study. It is not known if these medications have a positive or negative impact on fertility and hormonal parameters in males. The details are summarised in Table 7.

Insulin resistance (IR) is closely related to obesity, MetS and T2DM, and can be accurately determined based on the QUICKI (Katz et al., 2000). Using a QUICKI ratio of < 0.357 as a marker of IR supplied by PathCare Laboratories (Pathcare Park, Goodwood, South Africa), 47.4% of the cohort was diagnosed with IR. This was distributed between the groups with 18.2% of the CG and 85.3% of the MetS group having IR. This increased number of participants with IR in the MetS group was statistically significant ($P < 0.001$). Low grade inflammation is also a feature of MetS (Monteiro & Azevedo, 2010), with hs-CRP concentrations of > 1.00 mmol/L as a marker of low grade inflammation (PathCare Laboratories, Pathcare Park, Goodwood, South Africa).

Based on this definition, 66.6% of the cohort displayed low grade systemic inflammation. The MetS group had a significantly increased ($P = 0.032$) number of participants with inflammation (97.1%) compared to those in the control group (43.2%). Participants with T2DM not on insulin were included in the study. Although it is well established that T2DM has a negative association with male fertility (La Vignera et al., 2012), patients with diabetes do fulfil the MetS diagnostic criteria and have been included for analysis. A total of 3 participants (3.8%) were included in the study, all of them in the MetS group (8.8%). However, the number of participants with T2DM included in the study did not differ significantly from the control group ($p = 0.091$). The details are summarised in Table 7.

3.1.1.6. Reproductive potential of the cohort

Recent history of couple infertility was defined as an inability to achieve a conception with regular sexual intercourse over last 12 months without any form of contraception (WHO, 2010). Although 15.4% of the cohort fulfilled this definition, not all participants were currently attempting to have children. Furthermore, there was no analysis of the female partner in this assessment. However, within the study, there was a statistically significant ($P=0.028$) increased number in the MetS group (35.3%) compared to the CG (9.1%) with a recent history of infertility. For purposes of this study, proven fertility was defined as a live birth within the last 2 years of the consultation date. In the cohort, 15.4% had recently had children, with statistically significant ($P=0.024$) increased numbers in the control group (25%) compared to the MetS group (2.9%). These are summarised in Table 8.

3.1.1.7. Semen analysis and abnormalities in the cohort

Abnormality semen analysis definitions are outlined in Table 2 based on WHO (2010). DNA fragmentation and MMP values are based on Simon and colleagues (2013) and Marchetti and colleagues (2012), respectively.

Assessing the cohort for ejaculate volume, 14.1% had hypospermia (<1.5ml). Although 23.5% in the MetS group had hypospermia compared to 6.8% in the CG, this difference was not significant ($P=0.106$). In each group, 2 participants had azoospermia (5.1% of the total cohort). This was not significantly different between the groups. Within the total cohort, 78.2% had normozoospermia ($> 15 \times 10^6$ spermatozoa per ml), with 16.8% participants with oligozoospermia ($< 15 \times 10^6$ spermatozoa per ml). Although the CG had an increased percentage of participants considered normozoospermia (88.7%) compared to the MetS group (64.8%), this was not significant ($P=0.389$). However, the percentage of participants with oligozoospermia in the MetS group (29.4%) compared to the CG (6.8%) was significantly increased ($P=0.035$). Within the total cohort, asthenozoospermia (progressive motility $< 32\%$ and/or total motility $< 40\%$ spermatozoa) was identified in 58.9% of participants, necrozoospermia ($< 58\%$ viable spermatozoa) was identified in 23.1% of participants, teratozoospermia ($< 4\%$ normal form

(morphology) spermatozoa) was identified in 58.9% of participants, DNA fragmentations (> 25% spermatozoa damaged) identified in 21.8% of participants and abnormal MMP (> 36% spermatozoa) identified in 52.5% of participants. Although all these parameters were generally more frequent in the MetS group compared to CG, only DNA fragmentations was significantly different ($P=0.006$). These are summarised in Table 8.

3.2. Clinical and biochemical parameter results

Predictable differences are found between the groups of all parameters. MetS patients had significantly increased body mass index ($P<0.0001$), waist circumference ($P<0.0001$), systolic blood pressure ($P<0.0001$), diastolic blood pressure ($P<0.0001$), triglycerides ($P=0.0002$) and glucose ($P=0.0018$), with decreased HDL-cholesterol ($P=0.0001$). Serum insulin concentrations were significantly increased in MetS ($P<0.0001$), with the QUICKI significantly decreased ($P<0.0001$). hs-CRP was significantly increased ($P<0.0001$) in MetS. Details of mean, standard deviation and range are summarised in Table 9.

3.3. Semen analysis and sperm function results

A total of 4 participants in the cohort were diagnosed with idiopathic azoospermia (Table 8). As they did not meet any other exclusion criteria, they were entered into the study. This included 2 participants from the CG group, and 2 participants in the MetS group. This difference was not statistically significant based on the Fisher's Exact Test ($P=1.0$). However, these participants had all semen parameters reported in this section removed from analysis, with all other parameters in the study included (including other seminal fluid assays for insulin, leptin and inflammatory cytokines). Therefore, 74 participants had data entered for semen analysis, with 42 in the CG and 32 the MetS group. In addition, not all samples were assayed for DNA fragmentation (DF) or abnormal mitochondrial membrane potential (MMP), with a total number of samples assayed being 55 and 67, respectively. This was due to inadequate sampling, low ejaculate volume and/or low sperm counts. For DF, 28 samples from CG and 27 samples from MetS group were obtained. For MMP, 36

samples from CG and 31 samples from MetS group were obtained. Results for semen analysis are summarised in Table 10.

Ejaculation volume was significantly ($P=0.008$) decreased in the MetS group, with sperm concentration also significantly decreased ($P=0.0005$) in the MetS group compared to the CG. As the product of volume and concentration, total sperm count was also significantly ($P=0.0002$) decreased in the MetS group. Sperm functions were significantly decreased in MetS group, including progressive motility ($P=0.0225$), total motility ($P=0.0033$) and vitality ($P=0.0006$). Although there was a decreased percentage of sperm with abnormal morphology in the MetS group ($2.72 \pm 2.38\%$) compared to the CG ($3.9 \pm 3.17\%$), this was not statistically significant ($P=0.0817$). Percentage of sperm with abnormal MMP ($P=0.0007$) and DF ($p=0.004$) were significantly increased in MetS patients. The results are summarised in Table 10.

Although most parameters were significantly different between the groups, the mean or median values for most parameters in both groups were above the WHO (2010) (Table 2) recommended thresholds for a normal semen analysis, except for vitality ($50.0 \pm 23.2\%$) and progressive motility ($20.0 \pm 17.1\%$) in the MetS group. Although there is no statistical difference in morphology between the groups, the mean values in both groups are below the WHO (2010) recommended thresholds (CG: $3.9 \pm 3.17\%$; MetS: $2.72 \pm 2.38\%$). Based on a threshold for abnormal MMP recommended by Marchetti et al. (2012), both the CG and MetS group means are above this value ($42.1 \pm 25.8\%$ & $63.1 \pm 22.2\%$). Based on the threshold for DF recommended by Simon et al. (2013), only the MetS group was above this threshold ($26.9 \pm 19.7\%$).

Table 10: Seminal parameters compared between the control group (n=42) and participants diagnosed with the metabolic syndrome (n=32), except MMP (n=36 and n=31 respectively) and DNA fragmentation (n=28 and n=27 respectively). All statistical analysis using the Student's t-test, and values expressed as mean±SD (range), except * = Mann-Whitney tests expressed as median (interquartile range). MMP = mitochondrial membrane potential.

Parameter	Control	MetS	P-value
Ejaculation Volume (ml)*	2.55 (1.95 – 3.5)	2.0 (1.2 – 2.5)	0.0080
Sperm Concentration (million/ml)	43.7 ± 24.6 (6.4 – 110.8)	26.7 ± 15.8 (3.2 – 58.7)	0.0005
Total Sperm Count (million)*	103.6 (65.6 – 139.5)	48.1 (25.5 – 65.8)	0.0002
Sperm Vitality (% sperm alive)	67.3 ± 15.4 (29 – 92)	50.0 ± 23.2 (6 – 88)	0.0006
Progressive Motility (% motile)	29.4 ± 17.2 (0.0 – 59.5)	20.0 ± 17.1 (0.0 – 70.1)	0.0225
Total Motility (% motile)	57.5 ± 20.8 (18.5 – 90.1)	42.9 ± 19.9 (1.1 – 78.4)	0.0033
Morphology (% normal)	3.9 ± 3.17 (1 – 14)	2.72 ± 2.38 (0 – 12)	0.0817
MMP (% abnormal)	42.1 ± 25.8 (3 – 95)	63.1 ± 22.2 (21 – 100)	0.0007
DNA Fragmentation (% abnormal)	13.9 ± 9.8 (3 – 45)	26.9 ± 19.7 (4 – 83)	0.0040

3.4. Saliva steroid hormone concentrations

All 78 participants supplied saliva samples for analysis. However, a total of 10 samples were not assayed (6 in the CG & 4 in the MetS group). This was due to either an inadequate sample size (5 in the CG and 1 in the MetS group) or values below the lower limit of quantification (LLOQ) (1 in the CG and 3 in the MetS group). Therefore, samples included in data analysis represented 38 participants in the CG and 30 participants in the MetS group.

Free testosterone ($P=0.0001$) and free progesterone ($P=0.0011$) concentrations were significantly reduced in participants with MetS compared to controls. Results are summarised in Table 11.

Table 11: Saliva hormone parameters compared between the control group ($n=38$) and participants diagnosed with the metabolic syndrome ($n=30$). All statistical analysis using the Student's t-test, and values expressed as mean \pm SD (range).

Parameter	Control	MetS	P-value
Free Testosterone (pg/ml)	272.5 \pm 162.4 (74.0 – 744.2)	144.7 \pm 87.4 (32.5 – 415.8)	0.0001
Free Progesterone (pg/ml)	95.2 \pm 45.3 (29.4 – 212.8)	65.4 \pm 24.9 (24.6 – 122.7)	0.0011

3.5. Serum and seminal glucose and insulin concentrations

Serum glucose ($P=0.0018$) and insulin ($P<0.0001$) concentrations were increased in participants with MetS compared to the control group, as illustrated in Table 9.

Of 78 semen samples received, 61 samples were assayed accurately for seminal glucose concentrations, with 37 in the CG and 24 in the MetS group. In the CG, 2 samples did not yield adequate seminal fluid for analysis, and 5 samples were recorded as below the lowest level of quantification (LLOQ) .

Of the MetS group, 7 samples did not yield adequate seminal fluid for analysis, and 3 samples were recorded as LLOQ. Seminal glucose concentrations showed a trend to be decreased in the MetS group, however, this did not reach statistical significance ($P=0.0531$).

A total of 42 semen samples were assayed for insulin concentrations, with 20 in the CG and 22 in the MetS group. This was based on the first 42 participants who supplied samples and did not reach any exclusions. Seminal insulin concentrations are significantly higher in the MetS group ($P<0.0001$). In addition, seminal insulin is highly concentrated as compared to serum concentrations. Results are summarised alongside serum insulin and glucose concentrations in Table 12.

Table 12: Seminal concentrations for glucose and insulin compared between the control group ($n=37$ & $n=20$ Respectively) and participants diagnosed with the metabolic syndrome ($n=24$ & $n=22$ respectively). Serum glucose and insulin (CG: $n=44$; MetS $n=34$) have been included for comparative purposes. All statistical analysis using the Student's t-test, and values expressed as mean \pm SD (range), except * = Mann-Whitney tests expressed as median (interquartile range).

Parameter	Control	MetS	P-value
Serum Glucose (mmol/L)	5.03 \pm 0.59 (3.8 – 7.0)	5.79 \pm 1.70 (4.4 – 9.7)	0.0018
Seminal Glucose (μ g/ml)	472.1 \pm 245.3 (48.1 – 990.0)	357.1 \pm 207.1 (90.6 – 781.7)	0.0531
Serum Insulin (mIU/ml)	5.73 \pm 2.27 (2.4 – 14.3)	12.77 \pm 5.45 (3.8 – 32.0)	<0.0001
Seminal Insulin (mIU/ml)	164.7 \pm 75.6 (128.7 – 368.0)	539.8 \pm 245.7 (175.6 – 1059.9)	<0.0001

3.6. Serum and seminal inflammatory cytokine concentrations

Of the 78 serum and semen samples received, all were assayed for cytokine concentrations. However, some results were below the lowest level of quantification (LLOQ), and other results were not considered reliable for data analysis. Results are summarised in Table 13.

Serum tumor necrosis factor-alpha (TNF α) had results for 36 samples from the CG, and 29 samples from the MetS group analysed. Within the CG, 8 samples were LLOQ. Within the MetS group, 5 samples were LLOQ. Seminal TNF α had 33 samples from the CG, and 26 samples from the MetS group. Within the CG, 5 samples were LLOQ, and 6 removed due to unreliable results. Within the MetS group, 8 samples were LLOQ. The MetS group had significantly increased TNF α in serum ($P<0.0001$) and seminal ($P=0.0001$) samples.

Serum interleukin 1-beta (IL1 β) had results for 32 samples from the CG, and 25 samples from the MetS group analysed. Within the CG, 10 samples were LLOQ, and 2 removed due to unreliable results. Within the MetS group, 8 samples were LLOQ, and 1 removed due to unreliable results. Seminal IL1 β had 35 samples from the CG, and 27 samples from the MetS group. Within the CG, 8 samples were LLOQ. Within the MetS group, 5 samples were LLOQ, and 2 removed due to unreliable results. The MetS group had significantly increased IL1 β in serum ($P<0.0001$) and seminal ($P=0.0008$) samples.

Serum interleukin 6 (IL6) had results for 31 samples from the CG, and 28 samples from the MetS group analysed. Within the CG, 11 samples were LLOQ. Within the MetS group, 6 samples were LLOQ. Seminal IL6 had 32 samples from the CG, and 33 samples from the MetS group. Within the CG, 12 samples were LLOQ. Within the MetS group, 1 sample was LLOQ. The MetS group had significantly increased IL6 in serum ($P<0.0001$) and seminal ($P<0.0001$) samples.

Serum interleukin 8 (IL8) had results for 34 samples from the CG, and 27 samples from the MetS group analysed. Within the CG, 8 samples were LLOQ, and 2 removed due to unreliable results. Within the MetS group, 7 samples were LLOQ.

Seminal IL1 β had 33 samples from the CG, and 20 samples from the MetS group. All samples excluded from data analysis for seminal IL8 from both groups was due to results being above the upper level of quantification (ULOQ). The MetS group had significantly increased IL8 in serum (P=0.0001) and seminal (P=0.0007) samples. In addition, seminal IL-8 is highly concentrated compared to serum levels.

Table 13: Serum and seminal inflammatory cytokines compared between the control group and participants diagnosed with the metabolic syndrome. All statistical analysis using Mann-Whitney tests, and values expressed as median (interquartile range).

Parameter	Control	MetS	P-value
Serum TNF α (pg/ml)	5.8 (5.6 – 8.0)	19.7 (15.9 – 23.5)	<0.0001
Seminal TNF α (pg/ml)	6.8 (6.1 – 11.6)	16.2 (11.7 – 21.1)	0.0001
Serum IL-1 β (pg/ml)	12.5 (8.7 – 16.4)	28.5 (17.9 – 32.9)	<0.0001
Seminal IL-1 β (pg/ml)	22.3 (14.2 – 30.3)	42.3 (20.7 – 99.4)	0.0008
Serum IL-6 (pg/ml)	5.8 (5.4 – 6.3)	16.5 (15.8 – 33.5)	<0.0001
Seminal IL-6 (pg/ml)	9.8 (5.6 – 21.9)	30.0 (18.9 – 59.1)	<0.0001
Serum IL-8 (pg/ml)	10.9 (7.4 – 14.8)	21.6 (14.9 – 29.5)	0.0001
Seminal IL-8 (pg/ml)	1266.8 (836.0 – 2037.8)	2324.9 (1598.1 – 3658.7)	0.0007

3.7. Serum and seminal leptin results

A total of 78 serum and semen samples were available for leptin assays. Of these, 54 serum and 23 semen samples provided accurate data for statistical analysis. Within the serum samples, 30 were in the CG and 24 in the MetS group. Serum samples not included in data analysis was due to results either below the lowest level of quantification (LLOQ) (n=12 in the CG and n=3 in the MetS group) or above the upper level of quantification (ULOQ) (n=2 in the CG and n=7 in the MetS group). Within the semen samples, 10 were in the CG and 13 in the MetS group. Semen samples not included in data analysis was due results either below the lowest level of quantification (LLOQ) (n=2 in the CG and n=5 in the MetS group) or above the upper level of quantification (ULOQ) (n=2 in the CG and n=0 in the MetS group), or due to semen sample being too small (n=12 in the CG and n=16 in the MetS group). Both serum (P=0.0049) and seminal (P=0.0002) leptin was significantly increased in the MetS group compared to the CG group. Results are summarised in Table 14.

Table 14: Serum and seminal leptin compared between the control group (n=30 and n=10 respectively) and participants diagnosed with the metabolic syndrome (n=24 and n=30 respectively). All statistical analysis using the Student's t-test, and values expressed as mean±SD (range).

Parameter	Control	MetS	P-value
Serum Leptin (ng/ml)	1.23 (0.43 – 2.23)	6.97 (0.86 – 12.1)	0.0049
Seminal Leptin (ng/ml)	6.8 (4.5 – 8.0)	18.0 (12.4 – 21.7)	0.0002

3.8. Correlations between variables in the case controlled study

Correlations between variables were analysed in three components. The full cohort was assessed (combined CG and MetS groups), as was the CG and MetS groups separately. All correlation details (P-value and correlation coefficient) are tabulated. The abbreviations for the various parameters have not been included in the table legends. Statistically significant correlations ($P < 0.05$) are included, with non-significant correlations indicated with NS.

3.8.1. Clinical and biochemical correlations

Correlation details for the clinical and biochemical variables are summarised in Tables 15, 16 and 17.

Age correlated positively with waist circumference (WC) in the cohort and CG (but not MetS group), fasting blood glucose (FBG) in the cohort and MetS group (but not the CG) and free testosterone (FT) in all three groups. Age correlated negatively with insulin resistance (IR) in the cohort, but not in the CG or MetS groups. Although there was no significant correlation with age and high density lipoprotein (HDL) cholesterol in the cohort and MetS groups, there was a significant positive correlation in CG. There were no significant correlations between age and body mass index (BMI), systolic blood pressure (sBP), diastolic blood pressure (dBP), triglycerides (TG), fasting blood insulin (FBI), C-Reactive protein (CRP) and free progesterone (FP) in all three groups.

BMI correlated positively with WC in the cohort, CG and MetS groups, with sBP and dBP in the cohort only, with TG in the cohort and CG (but not the MetS group), with FBI in all three groups and CRP in the cohort only. BMI negatively correlated with HDL, FT and FP in the cohort only, and IR in all three groups. There was no correlation between BMI and FBG in all three groups.

WC correlated positively with dBP, sBP, FBI and CRP, and negatively with HDL, IR, FT and FP in the cohort, but not the CG and MetS groups. There was no significant correlation of WC with TG or FBG in any of the three groups.

sBP and dBP significantly positively correlated in all three groups. Both of the variables correlated positively with TG, FBI and CRP, and negatively with IR in the cohort, but not CG and MetS groups. There was no significant correlation between sBP or dBP and HDL, FT and FP for all three groups. HDL correlated positively with IR and FP, and negatively with TG, FBI and CRP in the cohort, but not CG and MetS groups. There was no HDL correlation with FBG or FT in any of the groups. TG correlated positively with FBI and negatively with IR in the cohort and CG (but not the MetS group). TG further correlated positively with CRP and negatively with FT and FP in the cohort, but not the CG and MetS groups. There was no significant correlation with TG and FBG in any of the groups. FBG correlated positively with CRP in all three groups. Furthermore, FBG correlated positively with FBI and negatively with IR in the cohort and MetS groups (but not the CG). There was no significant FBG correlation with FT and FP in any of the groups.

FBI correlated negatively with IR in all three groups. There was a positive correlation between FBI and CRP in the cohort and CG (but not the MetS group), and with FP in the control group (but not the cohort nor MetS groups). There was no significant correlation between FBI and FT in any of the groups. However, IR correlated positively with FT in the cohort, but not the CG nor MetS groups. There was a negative correlation between IR and CRP in the cohort and CG (but not the MetS group), with no correlation between IR and FP in any of the groups. CRP correlated negatively with FT in the cohort, but not the CG or MetS groups, with no correlation between CRP and FP in any of the groups. FT and FP positively correlated in the cohort and CG, with no correlation in the MetS group.

Legend for Table's 15, 16 and 17: NS = not significant. BMI = body mass index; WC = waist circumference; SBP = systolic blood pressure; DBP = diastolic blood pressure; HDL = high density lipoprotein cholesterol; TG = triglycerides; FBG = fasting blood glucose; FBI = fasting blood insulin; IR = insulin resistance (based on QUICKI); CRP = highly sensitive C-reactive protein; FT = free testosterone; FP = free progesterone.

Table 15: Clinical and biochemical parameter correlations for the cohort.

		BMI	WC	SBP	DBP	HDL	TG	FBG	FBI	IR	CRP	FT	FP
Age	$r^2=$ P=	NS	0.231 0.0416	NS	NS	NS	NS	0.385 0.0005	NS	-0.229 0.0435	NS	-0.444 0.0001	NS
BMI	$r^2=$ P=	-	0.912 <0.0001	0.241 0.0335	0.307 0.0062	-0.427 0.0001	0.234 0.0396	NS	0.706 <0.0001	-0.692 <0.0001	0.562 <0.0001	-0.303 0.0119	-0.307 0.0108
WC	$r^2=$ P=		-	0.320 0.0043	0.400 0.0003	-0.403 0.0003	NS	NS	0.650 <0.0001	-0.661 <0.0001	0.608 <0.0001	-0.395 0.0008	-0.345 0.0040
SBP	$r^2=$ P=			-	0.846 <0.0001	NS	0.308 0.0061	NS	0.328 0.0033	-0.332 0.0030	0.386 0.0005	NS	NS
DBP	$r^2=$ P=				-	NS	0.322 0.0041	NS	0.400 0.0003	-0.427 0.0001	0.411 0.0002	NS	NS
HDL	$r^2=$ P=					-	-0.249 0.0279	NS	-0.372 0.0008	0.413 0.0002	-0.326 0.0036	NS	0.268 0.0274
TG	$r^2=$ P=						-	NS	0.477 <0.0001	-0.436 0.0001	0.325 0.0037	-0.247 0.0422	-0.257 0.0343
FBG	$r^2=$ P=							-	0.218 0.0493	-0.422 0.0001	0.317 0.0046	NS	NS
FBI	$r^2=$ P=								-	-0.889 <0.0001	0.571 <0.0001	NS	NS
IR	$r^2=$ P=									-	-0.644 <0.0001	0.259 0.0332	NS
CRP	$r^2=$ P=										-	-0.239 0.0494	NS
FT	$r^2=$ P=											-	0.498 <0.0001

Table 16: Clinical and biochemical parameter correlations for the control group.

		BMI	WC	SBP	DBP	HDL	TG	FBG	FBI	IR	CRP	FT	FP
Age	$r^2=$ P=	NS	0.391 0.0086	NS	NS	0.317 0.0362	NS	NS	NS	NS	NS	-0.332 0.0418	NS
BMI	$r^2=$ P=	-	0.757 <0.0001	NS	NS	NS	0.349 0.0204	NS	0.412 0.0055	-0.478 0.0010	NS	NS	NS
WC	$r^2=$ P=		-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
SBP	$r^2=$ P=			-	0.780 <0.0001	NS	NS	NS	NS	NS	NS	NS	NS
DBP	$r^2=$ P=				-	NS	NS	NS	NS	NS	NS	NS	NS
HDL	$r^2=$ P=					-	NS	NS	NS	NS	NS	NS	NS
TG	$r^2=$ P=						-	NS	0.366 0.0145	0.366 0.0144	NS	NS	NS
FBG	$r^2=$ P=							-	NS	NS	0.309 0.0414	NS	NS
FBI	$r^2=$ P=								-	-0.882 <0.0001	0.454 0.0020	NS	0.336 0.0392
IR	$r^2=$ P=									-	-0.406 0.0063	NS	NS
CRP	$r^2=$ P=										-	NS	0.462 0.0035
FT	$r^2=$ P=											-	0.427 0.0076

Table 17: Clinical and biochemical parameter correlations for the MetS group.

		BMI	WC	SBP	DBP	HDL	TG	FBG	FBI	IR	CRP	FT	FP
Age	$r^2=$ P=	NS	NS	NS	NS	NS	NS	0.470 0.0050	NS	NS	NS	-0.610 0.0003	NS
BMI	$r^2=$ P=	-	0.858 <0.0001	NS	NS	NS	NS	NS	0.463 0.0058	-0.362 0.0351	NS	NS	NS
WC	$r^2=$ P=		-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
SBP	$r^2=$ P=			-	0.843 <0.0001	NS	NS	NS	NS	NS	NS	NS	NS
DBP	$r^2=$ P=				-	NS	NS	NS	NS	NS	NS	NS	NS
HDL	$r^2=$ P=					-	NS	NS	NS	NS	NS	NS	NS
TG	$r^2=$ P=						-	NS	NS	NS	NS	NS	NS
FBG	$r^2=$ P=							-	NS	-0.403 0.0182	NS	NS	NS
FBI	$r^2=$ P=								-	-0.849 <0.0001	NS	NS	NS
IR	$r^2=$ P=									-	NS	NS	NS
CRP	$r^2=$ P=										-	NS	NS
FT	$r^2=$ P=											-	0.458 0.0032

3.8.2. Seminal analysis correlations

Correlation details for the clinical and biochemical variables are summarised in Tables 18, 19 and 20.

Age correlated negatively with ejaculation volume (EV) in the cohort and CG (but not the MetS group), progressively motility (PM), total motility (TM) and vitality in the cohort and MetS group (but not the CG). There was a positive correlation between age and DNA fragmentation (DF) in the cohort and MetS group (but not the CG). No correlation of age with sperm concentration (SC), total sperm count (TSC), morphology and mitochondrial membrane potential (MMP) in any of the groups was found.

EV correlated positively with TSC in all three groups, and with PM, TM and vitality in the cohort (but not the CG or MetS group). There was a negative correlation between EV and MMP for the cohort and CG (but not the MetS group). No significant correlation of EV was found with SC, morphology and DF in any of the groups. SC correlated positively with TSC, PM, TM and vitality in all three groups, and morphology in the cohort (but not the CG or MetS group).

SC correlated negatively with MMP in the cohort and MetS group (but not the CG) and with DF in the cohort (but not the CG or MetS group). TSC correlated positively with PM, TM and vitality in all three groups, and morphology in the cohort and MetS groups (but not the CG). There was a negative correlation between TSC and MMP in all three groups, and no correlation between TSC and DF in all three groups.

PM and TM correlated positively in all three groups. Both PM and TM correlated positively with vitality, negatively with MMP and not at all with morphology in all three groups. There was no correlation between PM and DF in all three groups, however, TM correlated negatively with DF in the cohort only.

Vitality correlated positively with morphology in the cohort only and negatively with MMP in all three groups. Vitality and DF correlated negatively with DF in the cohort and MetS groups (but not the CG). There was a negative correlation between

morphology and DF in the cohort (but not the CG or MetS group), with no significant correlation between morphology and MMP in any of the groups. MMP and DF did not correlate in any of the groups.

Legend for Table's 18, 19 and 20: NS = not significant. EV = ejaculation volume; SC = sperm concentration; TSC = total sperm count; PM = progressive motility; TM = total motility; Morph = normal morphology; MMP = abnormal mitochondrial membrane potential; DF = DNA fragmentation.

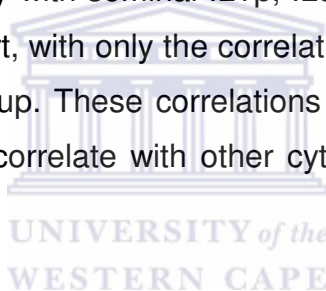


3.8.3. Cytokine, leptin, seminal insulin and glucose correlations

Correlation details for the clinical and biochemical variables are summarised in Tables 21, 22 and 23.

Age did not correlate with any of the variables in any group. Serum TNF α strongly correlated positively with all serum and seminal cytokines, and negatively with serum glucose, in the cohort. A correlation between serum TNF α and seminal TNF α , serum and seminal IL6, and serum leptin was observed in both the CG and MetS groups in addition to the cohort. Serum TNF α also correlated with seminal IL8 in the MetS group. Seminal TNF α correlated positively with seminal IL1 β , IL6 and IL8 in the cohort and MetS groups, and also with serum leptin in the CG.

Serum IL1 β correlated strongly with seminal IL1 β , IL8, leptin and insulin, and serum IL6, IL8 and leptin in the cohort, with only the correlation with serum IL6 and seminal IL8 observed in the MetS group. These correlations were not observed in the CG. Seminal IL1 β did not further correlate with other cytokines, leptin or insulin in any group.



Serum IL6 correlated positively with serum IL8 and leptin, and with seminal IL6, IL8, leptin and insulin in the cohort, but not in the CG or MetS groups. Seminal IL6 further correlated with serum and seminal IL8 in the cohort, seminal IL8 and the CG and seminal leptin in the MetS group.

Serum IL8 further correlated positively with seminal insulin in the cohort only, and the CG or MetS groups. Seminal IL8 also correlated with seminal leptin in the cohort and MetS group, but not in the CG.

Serum leptin strongly correlated with seminal leptin in the cohort, control and MetS groups. Seminal leptin further correlated with seminal insulin in the cohort, but not the CG or MetS groups.

Legend for Table's 21, 22 and 23: NS = not significant. TNF α = tumour necrosis factor-alpha; IL1 β = Interleukin 1-beta; IL6 = interleukin 6; IL8=interleukin 8.

3.8.4. Clinical, biochemical and seminal parameters correlations

There were no significant correlations between any of these parameters for the CG Table 25. This group will therefore not be further reported in under this sub-heading. Within the MetS group, there was a positive correlation between SC and HDL only. FBG negatively correlated with PM and TM within the MetS group. There were no other significant correlations within the MetS group (Table 26). This group will not further reported in under this sub-heading.

Within the entire cohort, the following significant correlations were observed. These are tabulated in Table 24.

BMI and WC correlated negatively with SC, TSC, TM and vitality, and positively with MMP and DF. There was no BMI correlation with EV, PM and morphology. There was no correlation with sBP and any seminal parameters. dBP correlated negatively with SC, TM and vitality only. HDL and TG did not correlate with any semen parameters (as reported above, HDL correlated positively with SC in the MetS group only). FBG correlated negatively with PM, TM (as well as in the MetS group) and vitality, and negatively with DF.

FBI correlated negatively with EV, SC, TSC, PM, TM and vitality, and positively with MMP. IR correlated positively with EV, SC, TSC, PM, TM and vitality, and negatively with MMP and DF. CRP correlated negatively with SC, TSC, PM, TM and vitality, and positively with MMP and DF. FT correlated positively with SC, TSC, PM, TM and vitality. FP did not correlate with any semen parameter assessed.

Legend for Table's 24, 25 and 26: NS = not significant. BMI = body mass index; WC = waist circumference; SBP = systolic blood pressure; DBP = diastolic blood pressure; HDL = high density lipoprotein cholesterol; TG = triglycerides; FBG = fasting blood glucose; FBI = fasting blood insulin; IR = insulin resistance (based on QUICKI); CRP = highly sensitive C-reactive protein; FT = free testosterone; FP = free progesterone; EV = ejaculation volume; SC = sperm concentration; TSC = total sperm count; PM = progressive motility; TM = total motility; Morph = morphology; MMP = mitochondrial membrane potential; DF = DNA fragmentation.

Table 24: Clinical, biochemical and seminal parameters correlations for the cohort.

		BMI	WC	SBP	DBP	HDL	TG	FBG	FBI	IR	CRP	FT	FP
EV	$r^2=$ P=	NS	NS	NS	NS	NS	NS	NS	-0.239 0.0372	0.263 0.0215	NS	NS	NS
SC	$r^2=$ P=	-0.385 0.0008	-0.372 0.0013	NS	-0.295 0.0120	NS	NS	NS	-0.368 0.0015	0.384 0.0009	-0.319 0.0063	0.310 0.0142	NS
TSC	$r^2=$ P=	-0.352 0.0024	-0.367 0.0015	NS	NS	NS	NS	NS	-0.362 0.0018	0.377 0.0011	-0.283 0.0160	0.317 0.0120	NS
PM	$r^2=$ P=	NS	NS	NS	NS	NS	NS	-0.318 0.0066	-0.310 0.0355	0.234 0.0484	-0.240 0.0420	0.309 0.0147	NS
TM	$r^2=$ P=	-0.275 0.0195	-0.344 0.0031	NS	-0.267 0.0232	NS	NS	-0.330 0.0047	-0.390 0.0392	0.308 0.0085	-0.236 0.0463	0.288 0.0231	NS
Vitality	$r^2=$ P=	-0.381 0.0010	-0.452 0.0001	NS	-0.262 0.0262	NS	NS	-0.321 0.0060	-0.245 0.0383	0.385 0.0009	-0.299 0.0106	0.255 0.0458	NS
Morph	$r^2=$ P=	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
MMP	$r^2=$ P=	0.336 0.0063	0.386 0.0015	NS	NS	NS	NS	NS	0.290 0.0190	-0.340 0.0057	0.354 0.0038	NS	NS
DF	$r^2=$ P=	0.306 0.0231	0.368 0.0057	NS	NS	NS	NS	0.300 0.0258	NS	-0.339 0.0114	0.262 0.0432	NS	NS

3.8.5. Clinical, biochemical, cytokine, insulin and glucose correlations

Correlation details for the clinical and biochemical variables are summarised in Table's 27, 28 and 27.

BMI and WC correlated with all serum and seminal cytokines except serum IL8, as well as serum and seminal leptin and serum insulin in the cohort. Only the correlation between BMI and WC was observed in the CG, and none of these correlations observed in the MetS group.

Systolic and diastolic blood pressure correlated with serum TNF α , IL1 β , IL6 and IL8, and seminal insulin, in the cohort only.

HDL negatively correlated with serum TNF α , IL1 β and IL6, and seminal TNF α and insulin in the cohort only.

TG correlated positively with serum TNF α and IL1 β , and seminal insulin in the cohort only. TG correlated negatively with seminal glucose in the cohort.

Fasting blood glucose correlated negatively with seminal glucose in the cohort, but no in the CG or MetS groups. Blood glucose further correlated positively with serum and seminal TNF α in the cohort and MetS groups, and serum IL1 β in the cohort only.

Serum insulin (FBI) correlated very strongly with seminal insulin and negatively with QUICKI in the cohort, CG and MetS groups. Serum insulin and QUICKI further correlated with seminal glucose negatively and positively respectively in the cohort, but not the Cg and MetS group.

Serum insulin and QUICKI correlated negatively with each other. Both these parameters further correlated positively and negatively respectively with serum TNF α , IL1 β , IL6, IL8 and leptin, and seminal IL8 and leptin in the cohort, but not the CG or MetS groups.

hs-CRP correlated strongly and positively with serum and seminal TNF α , IL1 β , IL6, IL8 and leptin in the cohort, in addition to seminal insulin. There was a negatively correlation with hs-CRP and seminal glucose in the cohort. These correlations were not observed in the CG or MetS group.

FT correlated with serum TNF α , IL1 β and IL6 and seminal IL8 in the cohort. FT also correlated negatively with serum seminal IL8 in the MetS group. FP negatively correlated with serum and seminal TNF α in the cohort, serum and seminal IL1 β in the CG, serum TNF α and seminal IL6 in the MetS group.

Legend for Table's 27, 8 and 29: NS = not significant. BMI = body mass index; WC = waist circumference; SBP = systolic blood pressure; DBP = diastolic blood pressure; HDL = high density lipoprotein cholesterol; TG = triglycerides; FBG = fasting blood glucose; FBI = fasting blood insulin; IR = insulin resistance (based on QUICKI); CRP = highly sensitive C-reactive protein; FT = free testosterone; FP = free progesterone; TNF α = tumour necrosis factor-alpha; IL1 β = Interleukin 1-beta; IL6 = interleukin 6; IL8=interleukin 8.

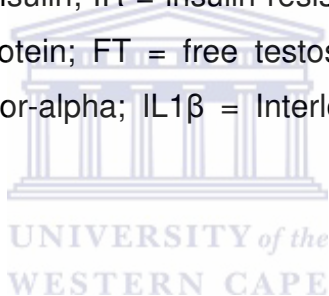


Table 27: Clinical, biochemical, cytokine and seminal insulin and glucose correlations for the cohort.

		BMI	WC	SBP	DBP	HDL	TG	FBG	FBI	IR	CRP	FT	FP
Serum	$r^2=$	0.579	0.619	0.317	0.387	-0.441	0.302	0.446	0.539	-0.579	0.653	-0.284	-0.330
TNF α	P=	<0.0001	<0.0001	0.0102	0.0015	0.0002	0.0144	0.0002	<0.0001	<0.0001	<0.0001	0.0264	0.0095
Seminal	$r^2=$	0.371	0.376	NS	NS	-0.303	NS	0.385	NS	NS	0.331	NS	-0.292
TNF α	P=	0.0042	0.0037	NS	NS	0.0209	NS	0.0028	NS	NS	0.0112	NS	0.0305
Serum	$r^2=$	0.411	0.462	0.297	0.371	-0.259	0.270	0.410	0.327	-0.377	0.506	-0.272	NS
IL1 β	P=	0.0015	0.0003	0.0249	0.0045	0.0485	0.0426	0.0015	0.0131	0.0038	0.0001	0.0407	NS
Seminal	$r^2=$	0.293	0.303	NS	NS	NS	NS	NS	NS	NS	0.504	NS	NS
IL1 β	P=	0.0219	0.0177	NS	NS	NS	NS	NS	NS	NS	<0.0001	NS	NS
Serum	$r^2=$	0.378	0.405	0.264	0.339	-0.255	NS	NS	0.315	-0.304	0.328	-0.264	NS
IL6	P=	0.0031	0.0015	0.0437	0.0087	0.0490	NS	NS	0.0150	0.0191	0.0112	0.0450	NS
Seminal	$r^2=$	0.269	0.275	NS	NS	NS	NS	NS	NS	NS	0.406	NS	NS
IL6	P=	0.0314	0.0277	NS	NS	NS	NS	NS	NS	NS	0.0025	NS	NS
Serum	$r^2=$	NS	NS	0.375	0.332	NS	NS	NS	0.253	-0.290	0.429	NS	NS
IL8	P=	NS	NS	0.0032	0.0097	NS	NS	NS	0.0490	0.0247	0.0006	NS	NS
Seminal	$r^2=$	0.358	0.374	NS	NS	NS	NS	NS	0.283	-0.287	0.415	-0.304	NS
IL8	P=	0.0086	0.0058	NS	NS	NS	NS	NS	0.0401	0.0372	0.0020	0.0425	NS
Serum	$r^2=$	0.362	0.372	NS	NS	NS	NS	NS	0.423	-0.403	0.523	NS	NS
Leptin	P=	0.0357	0.0368	NS	NS	NS	NS	NS	0.023	0.0223	0.0053	NS	NS
Seminal	$r^2=$	0.581	0.563	NS	NS	NS	NS	NS	0.525	-0.522	0.723	NS	NS
Leptin	P=	0.0036	0.0052	NS	NS	NS	NS	NS	0.01	0.0107	0.0001	NS	NS
Seminal	$r^2=$	0.651	0.564	0.336	0.334	-0.436	0.341	NS	0.832	-0.770	0.515	NS	NS
Insulin	P=	<0.0001	0.0001	0.0294	0.0306	0.0039	0.0271	NS	<0.0001	<0.0001	0.0005	NS	NS
Seminal	$r^2=$	NS	NS	NS	NS	NS	-0.279	-0.271	-0.265	0.351	-0.240	NS	NS
glucose	P=	NS	NS	NS	NS	NS	0.0297	0.0344	0.0394	0.0056	0.0627	NS	NS

3.8.6. Seminal, cytokine and seminal insulin and glucose correlations

Correlation details for the clinical and biochemical variables are summarised in Table's 30, 31 and 32.

Serum and seminal TNF α correlated negatively with sperm concentration and total sperm count, but not ejaculation volume, in the cohort. These cytokines further negatively correlated with total motility (but not progressive motility), vitality and normal sperm morphology, and positively with DF, in the cohort. These correlations were not observed in the CG. In the MetS group, serum TNF α correlated negatively with total motility, and both serum and seminal TNF α correlated positively with DF in this group. Similarly, serum and seminal leptin correlated negatively with sperm concentration, total sperm count, progressive and total motility and abnormal sperm morphology, in addition to a positive correlation with DF in the cohort only.

Serum IL1 β correlated negatively with sperm concentration in the cohort only. Serum and seminal IL1 β correlated negatively with normal sperm morphology in the MetS group only. Serum and seminal IL6 correlated negatively with normal sperm morphology and positively with DF, and seminal IL6 correlated negatively with sperm concentration in the cohort. These correlations were not observed in the CG or MetS groups. Serum and seminal IL8 did not correlate with any sperm parameters in any group.

Seminal insulin correlated negatively with sperm concentration in the cohort only, and progressive and total motility in the cohort, CG and MetS groups. Seminal glucose correlated negatively with progressive and total motility and positively with abnormal MMP in the MetS group only.

Legend for Table's 30, 31 and 32: NS = not significant. EV = ejaculation volume; SC = semen concentration; TSC = total sperm count; PM = progressive motility; TM = total motility; Morph = morphology; MMP = mitochondrial membrane potential; DF = DNA fragmentation; TNF α = tumour necrosis factor-alpha; IL1 β = Interleukin 1-beta; IL6 = interleukin 6; IL8=interleukin 8.

Table 32: Seminal, cytokine and seminal insulin and glucose correlations for the MetS group.

		EV	SC	TSC	PM	TM	Vitality	Morph	MMP	DF
Serum TNF α	$r^2=$ P=	NS	NS	NS	NS	-0.435 0.0337	NS	NS	NS	0.677 0.0005
Seminal TNF α	$r^2=$ P=	NS	NS	NS	NS	NS	NS	NS	NS	0.291 0.0491
Serum IL1 β	$r^2=$ P=	NS	NS	NS	NS	NS	NS	-0.400 0.0483	NS	NS
Seminal IL1 β	$r^2=$ P=	NS	NS	NS	NS	NS	NS	-0.385 0.0473	NS	NS
Serum IL6	$r^2=$ P=	NS	NS	NS	NS	NS	NS	NS	NS	NS
Seminal IL6	$r^2=$ P=	NS	NS	NS	NS	NS	NS	NS	NS	NS
Serum IL8	$r^2=$ P=	NS	NS	NS	NS	NS	NS	NS	NS	NS
Seminal IL8	$r^2=$ P=	NS	NS	NS	NS	NS	NS	NS	NS	NS
Serum Leptin	$r^2=$ P=	NS	NS	NS	NS	NS	NS	NS	NS	NS
Seminal Leptin	$r^2=$ P=	NS	NS	NS	NS	NS	NS	NS	NS	NS
Seminal Insulin	$r^2=$ P=	NS	NS	NS	0.449 0.0405	0.335 0.0490	NS	NS	NS	NS
Seminal glucose	$r^2=$ P=	NS	NS	NS	-0.329 0.0464	-0.361 0.0422	NS	NS	0.445 0.0138	NS

3.9. Cell culture experiments

3.9.1. Insulin

TM3 cell viability was significantly increased for the 0.01 ($P=0.0016$), 0.1 ($P=0.0288$) and 1 pg/ml ($P=0.0466$) concentrations. There was no significant impact with the 10 pg/ml concentration. The ANOVA repeated measures analysis of variance was not significant ($P=0.802$). Nevertheless, the one-way analysis of variance did reach statistical significance ($P=0.022$). Results are shown in Figure 16. Protein concentrations were significantly increased for 0.1 ($P=0.0003$), 1 ($P=0.0279$) and 10 ($P<0.0001$) pg/ml concentrations, but not 0.01 pg/ml concentrations. The ANOVA repeated measures analysis of variance was statistically significant ($P=0.0033$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 17. Testosterone was significantly increased for 0.01 ($P=0.0002$), 0.1 ($P<0.0001$), 1 ($P=0.0104$) and 10 ($P<0.0001$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was statistically significant ($P=0.0154$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 18. The testosterone-to-protein ratio was significantly increased for 0.01 ($P=0.0005$) pg/ml concentration, but not for 0.1 ($P=0.111$), 1 ($P=0.0557$) and 10 ($P=0.2535$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was not significant ($P=0.1925$). The ANOVA one-way analysis of variance was significant ($P<0.001$). Results are shown in Figure 19. Progesterone concentrations were significantly decreased for 0.01 ($P<0.0001$), 0.1 ($P<0.0001$), 1 ($P<0.0001$) and 10 ($P<0.0001$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was statistically significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 20. The progesterone-to-protein ratio was significantly decreased for the 0.01 ($P=0.0245$), 0.1 ($P<0.0001$), 1 ($P=0.0006$) and 10 ($P<0.0001$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was statistically significant ($P=0.0002$). The ANOVA one-way analysis of variance was significant ($P<0.001$). Results are shown in Figure 21.

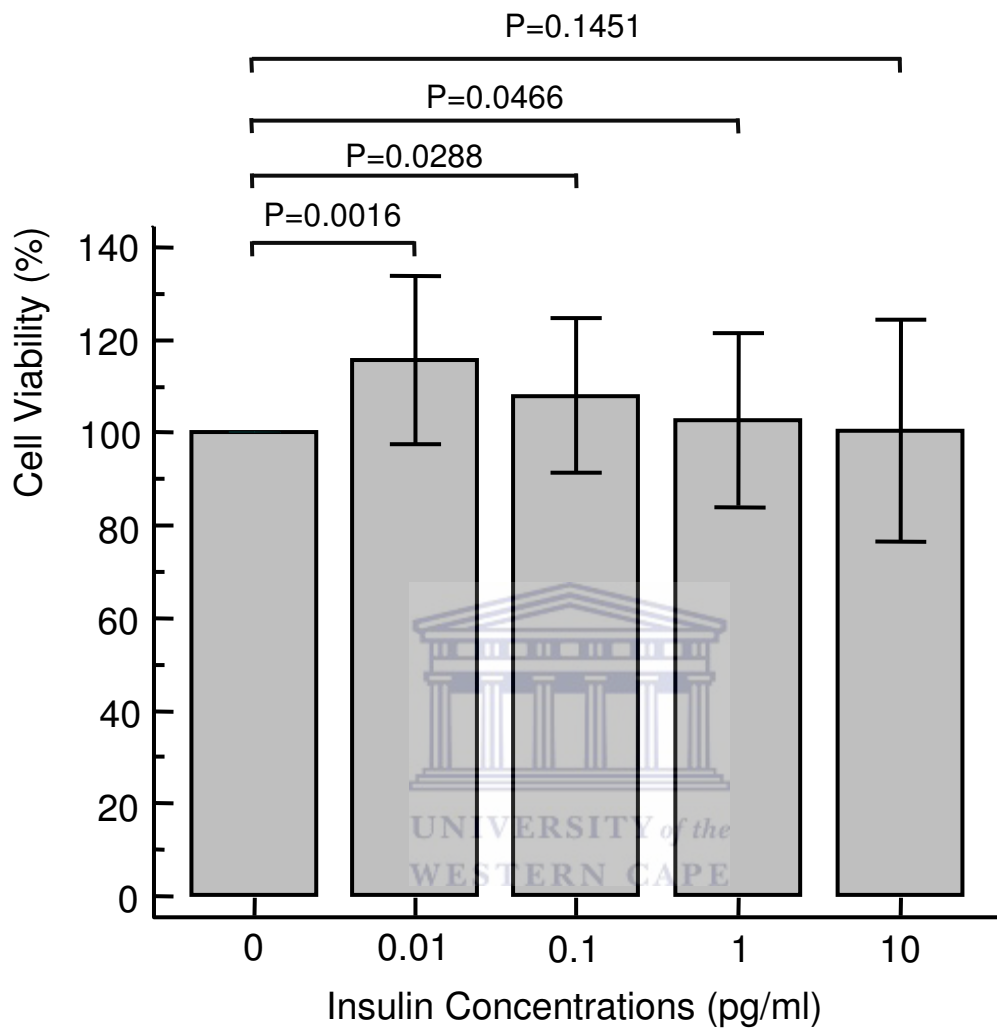


Figure 16: Cell viability results for TM3 cell exposure to various insulin concentrations. Cell viability was significantly increased for the 0.01, 0.1 and 1 pg/ml concentrations. ANOVA repeated measures analysis of variance was not significant ($P=0.802$). The one-way analysis of variance did reach significance ($P=0.022$).

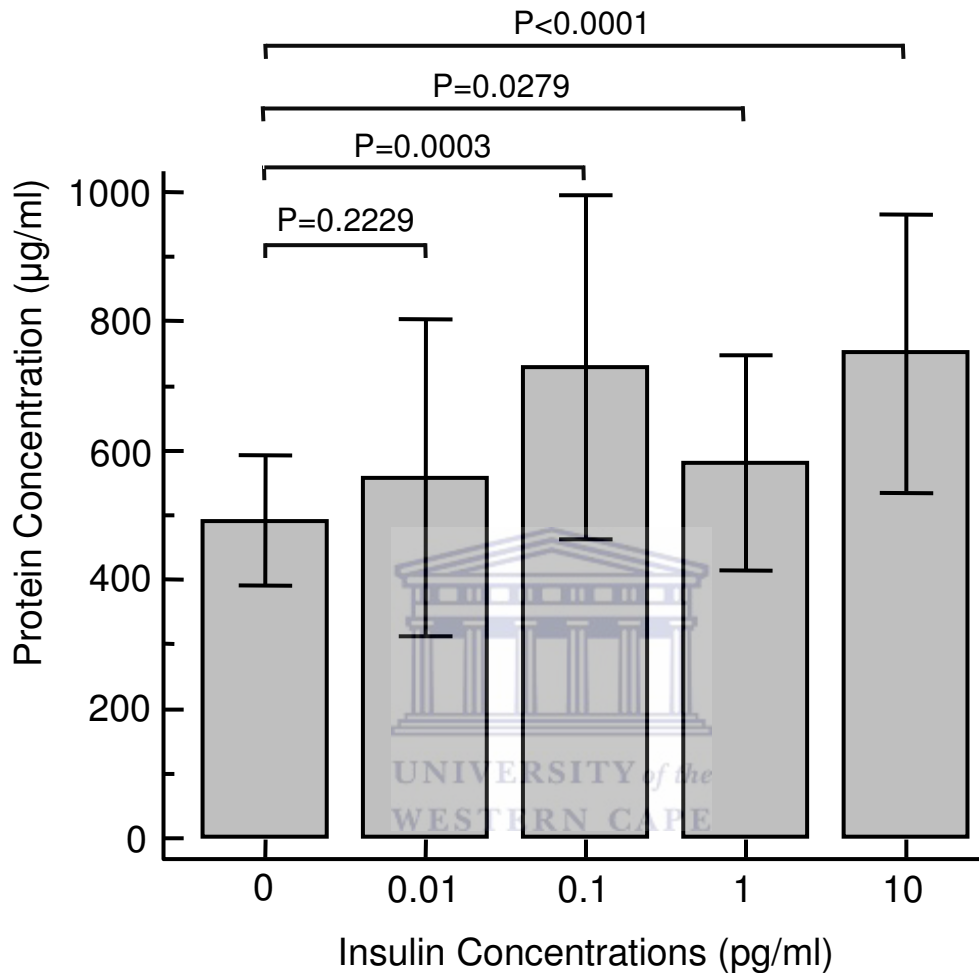


Figure 17: Protein concentration results for TM3 cell exposure to various insulin concentrations. Protein concentrations were significantly increased for 0.1, 1 and 10 pg/ml concentrations, but not 0.01 ng/ml concentrations. ANOVA repeated measures analysis of variance was significant ($P=0.0033$), as was the one-way analysis of variance ($P<0.001$).

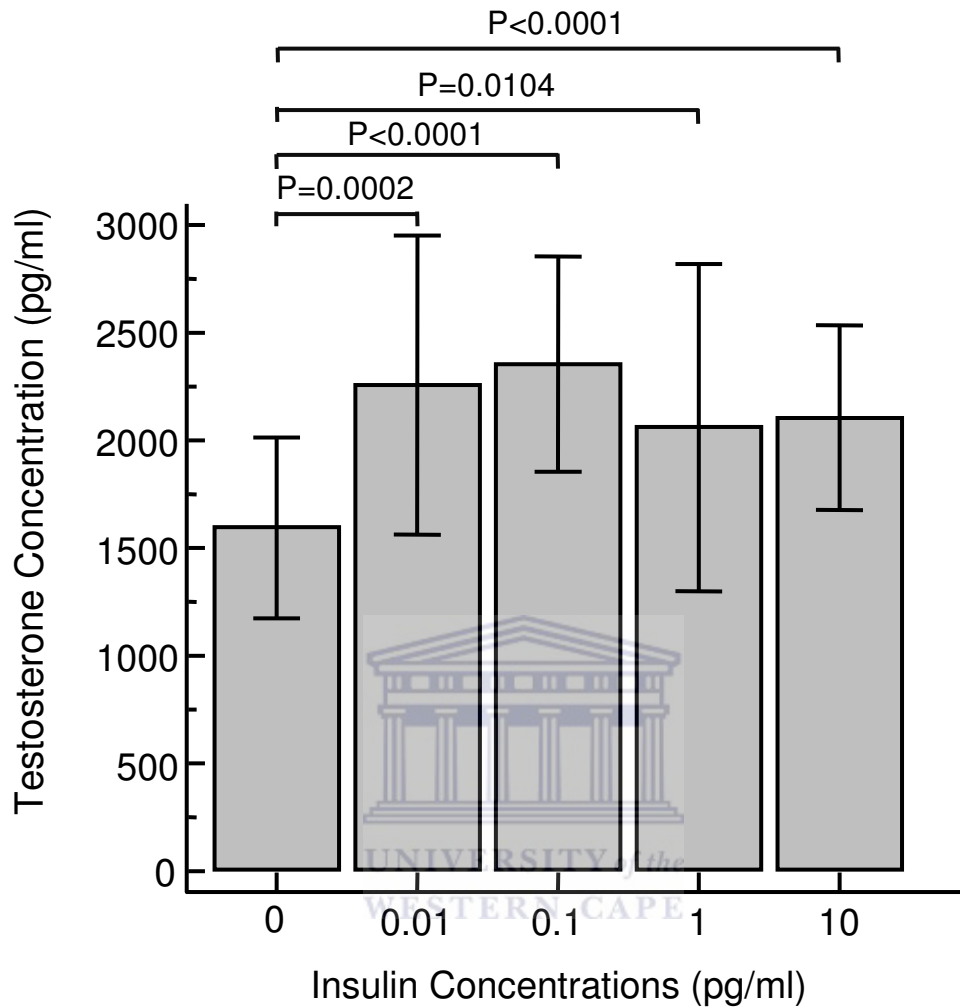


Figure 18: Testosterone concentration results for TM3 cell exposure to various insulin concentrations. Testosterone concentrations were significantly increased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P=0.0154$), as was the one-way analysis of variance ($P<0.001$).

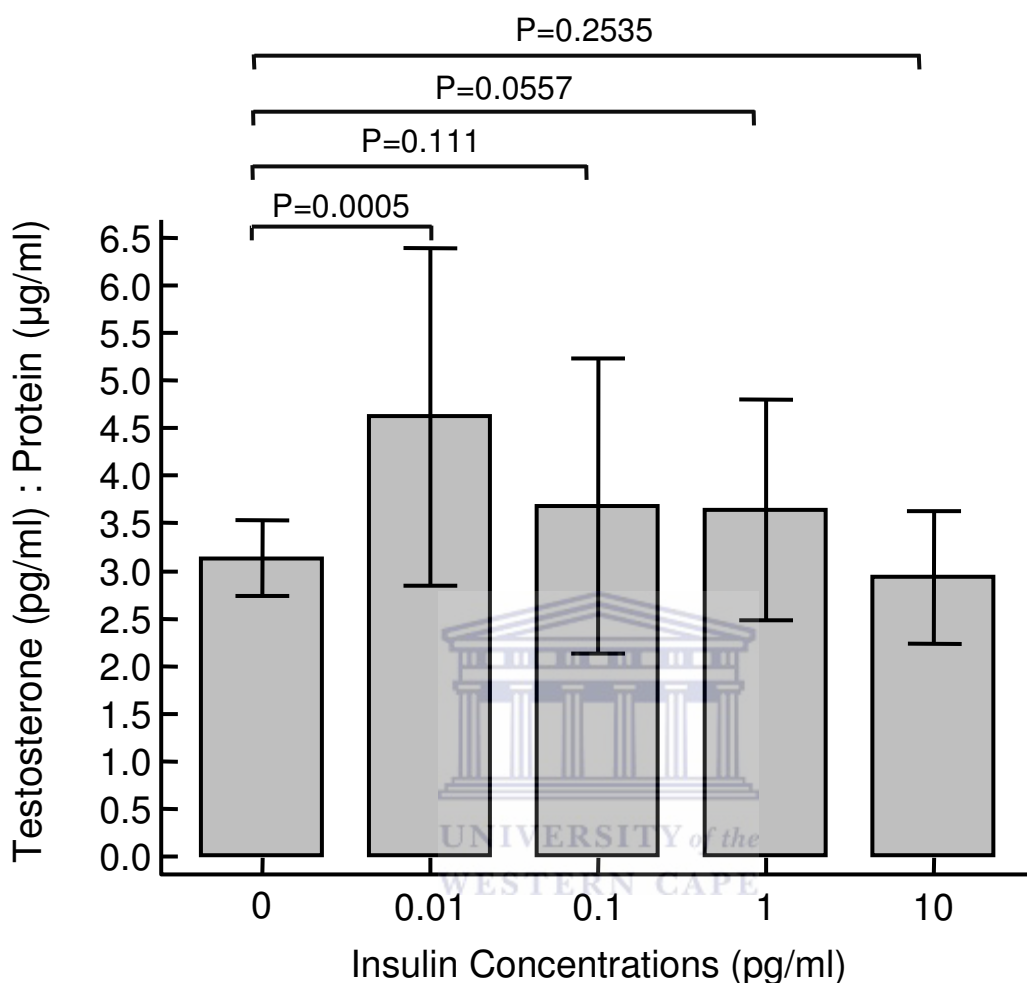


Figure 19: Testosterone-to-protein ratio results for TM3 cell exposure to various insulin concentrations. The ratio was significantly increased for 0.01pg/ml concentration, but not for 0.1, 1 and 10 pg/ml concentrations. ANOVA repeated measures analysis of variance was not significant (P=0.1925). The ANOVA one-way analysis of variance was significant (P<0.001).

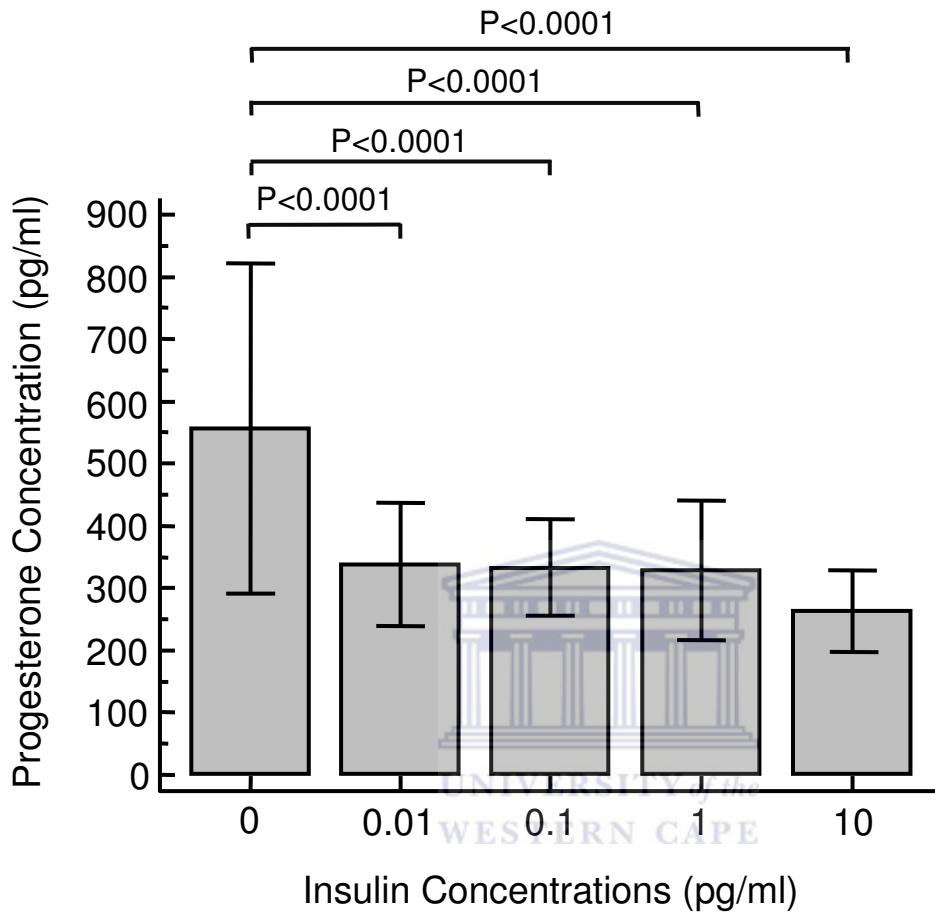


Figure 20: Progesterone concentration results for TM3 cell exposure to various insulin concentrations. Progesterone concentrations were significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was statistically significant ($P < 0.0001$), as was the one-way analysis of variance ($P < 0.001$).

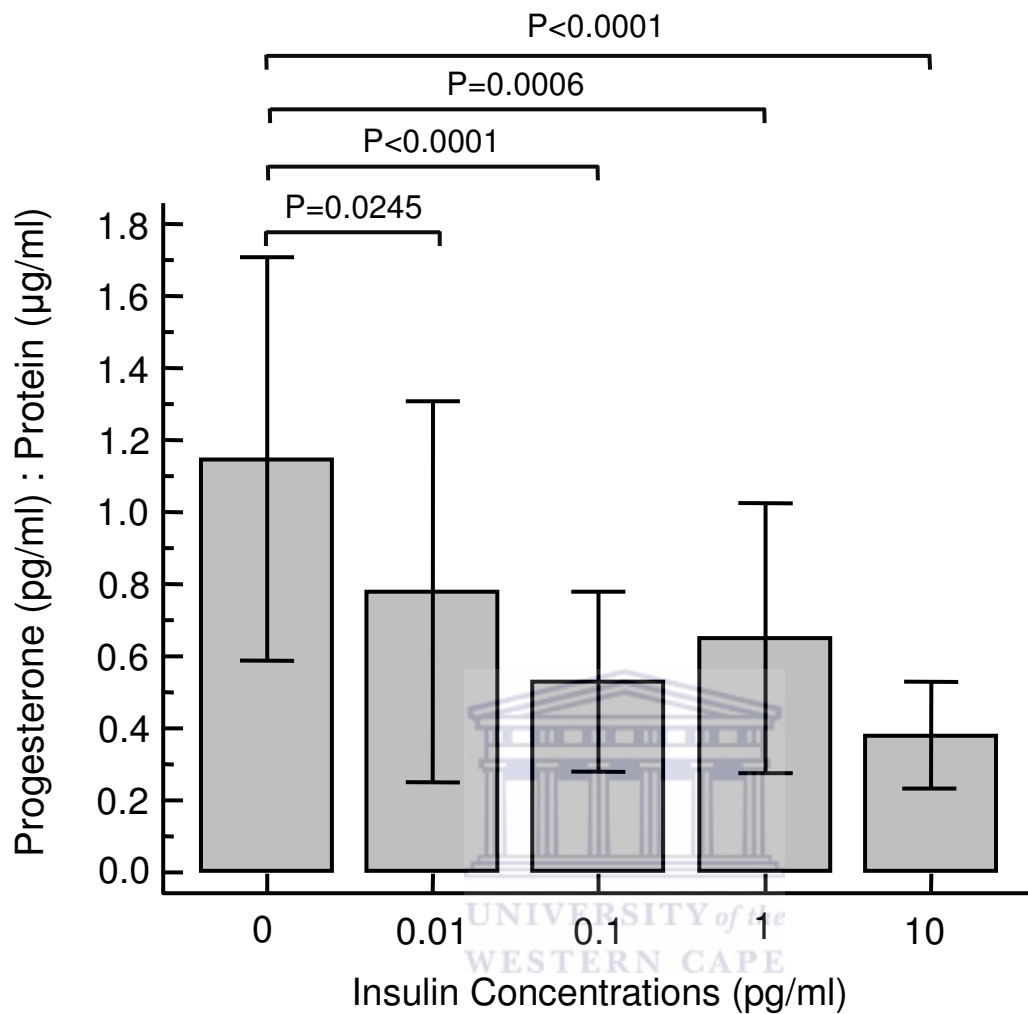


Figure 21: Progesterone-to-protein ratio results for TM3 cell exposure to various insulin concentrations. The ratio was significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P=0.0002$), as was the ANOVA one-way analysis of variance was significant ($P<0.001$).

3.9.2. Tumour necrosis factor-alpha

TM3 cell viability was significantly decreased for the 0.1 ($P=0.0359$), 1 ($P=0.0145$), 10 ($P<0.0001$) and 100 ($P<0.0001$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 22. Protein concentrations were significantly decreased for all concentrations. The ANOVA repeated measures analysis of variance was statistically significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$). Protein concentrations were significantly decreased the 0.1 ($P<0.0001$), 1 ($P<0.0001$), 10 ($P<0.0001$) and 100 ($P<0.0001$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was statistically significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 23. Testosterone concentrations were significantly decreased for the 0.1 ($P<0.0001$), 1 ($P<0.0001$), 10 ($P<0.0001$) and 100 ($P<0.0001$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was statistically significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 24. The testosterone-to-protein ratio was significantly increased for the 0.1 ($P=0.0215$) concentration, and significantly decreased for the 10 ($P=0.0393$) and 100 ($P<0.0001$) pg/ml concentrations. The 1 pg/ml concentration was decreased, but this did not reach statistical significance ($P=0.0648$). The ANOVA repeated measures analysis of variance was statistically significant ($P<0.0001$), as was the ANOVA one-way analysis of variance ($P<0.001$). Results are shown in Figure 25. Progesterone concentrations were significantly decreased for the 0.1 ($P<0.0001$), 1 ($P<0.0001$), 10 ($P<0.0001$) and 100 ($P<0.0001$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was statistically significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 26. The progesterone-to-protein ratio was significantly increased for 0.1 ($P=0.0036$) pg/ml concentration and significantly decreased for the 100 pg/ml concentration ($P=0.0054$). There was no significant change for the 1 ($P=0.3272$) and 10 ($P=0.6593$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was not statistically significant ($P=0.1797$). The ANOVA one-way analysis of variance was significant ($P<0.001$). Results are shown in Figure 27.

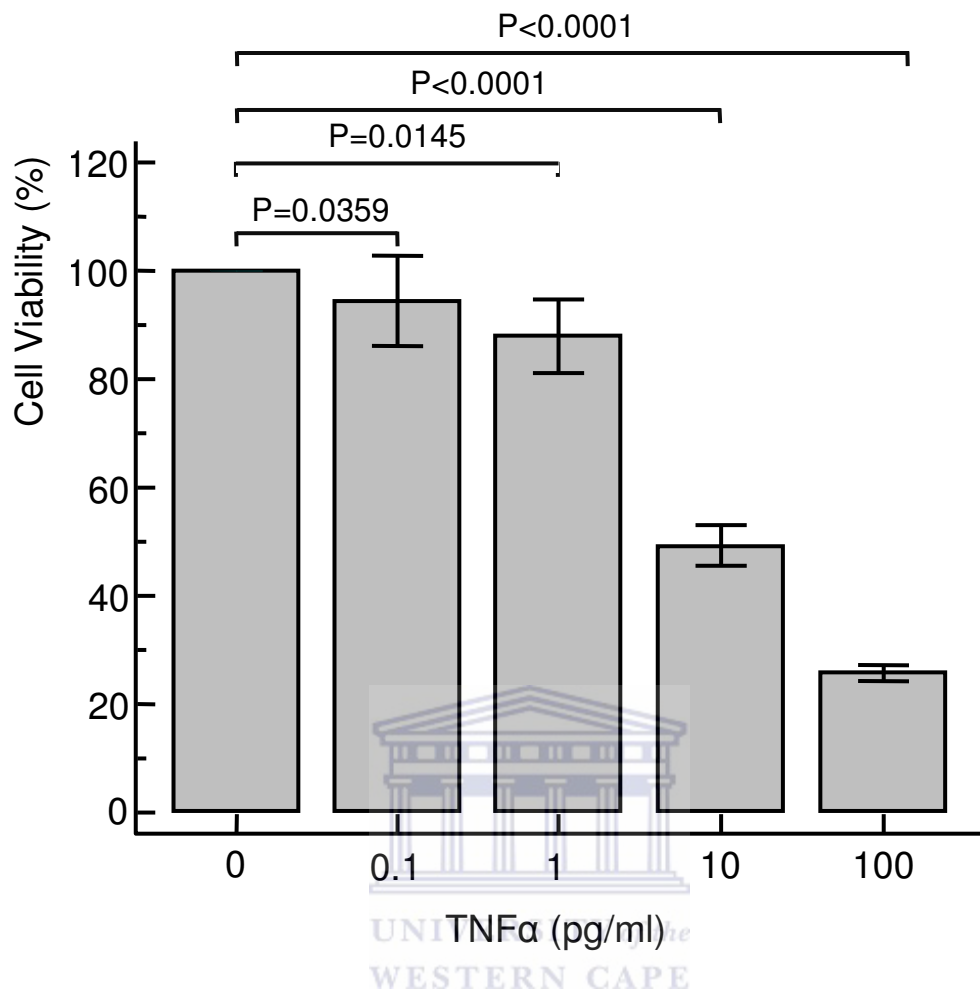


Figure 22: Cell viability results for TM3 cell exposure to various tumour necrosis factor-alpha (TNFα) concentrations. Cell viability was significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$).

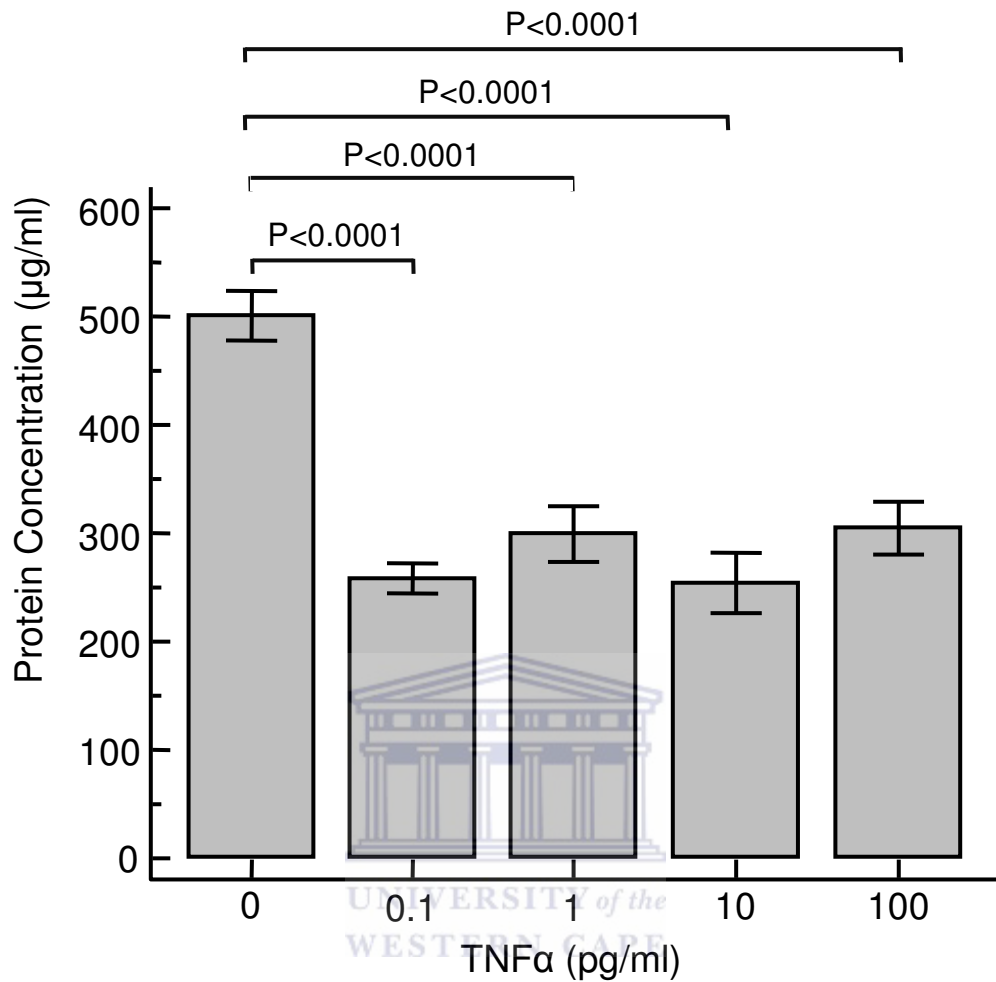


Figure 23: Protein concentration results for TM3 cell exposure to various tumour necrosis factor-alpha (TNFα) concentrations. Protein concentrations were significantly decreased for all concentrations. The ANOVA repeated measures analysis of variance was significant ($P < 0.0001$), as was the one-way analysis of variance ($P < 0.001$).

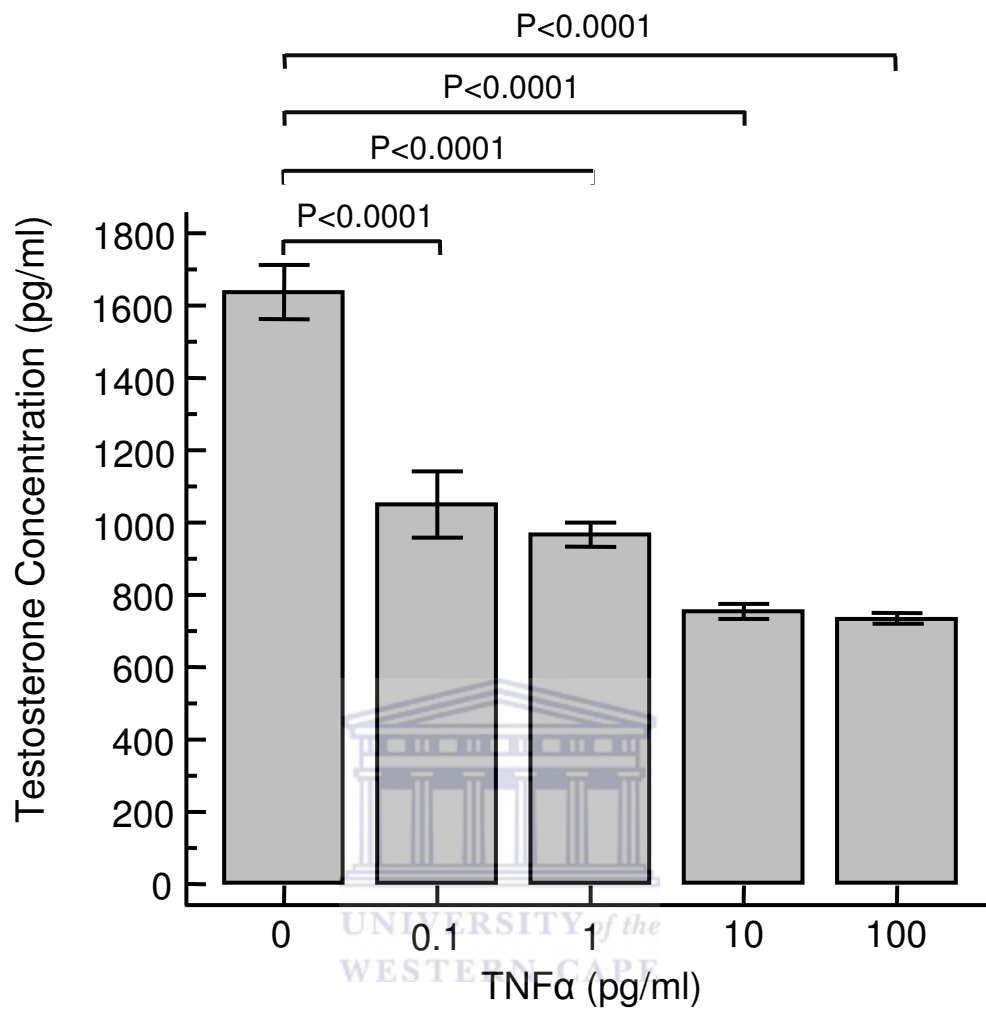


Figure 24: Testosterone concentration results for TM3 cell exposure to various tumour necrosis factor- α (TNF α) concentrations. Testosterone concentrations were significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P < 0.0001$), as was the one-way analysis of variance ($P < 0.001$).

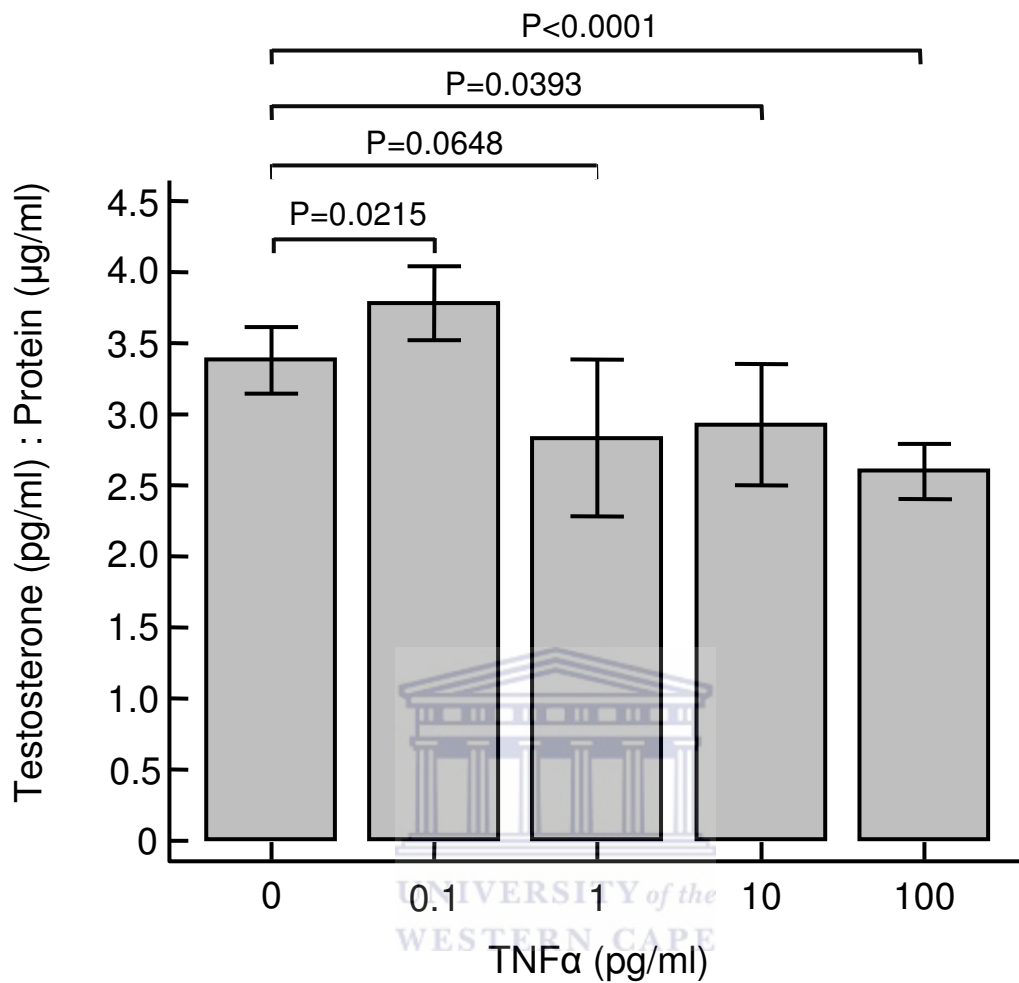


Figure 25: Testosterone-to-protein ratio results for TM3 cell exposure to various tumour necrosis factor-alpha (TNFα) concentrations. The ratio was significantly increased for 0.1 concentrations, and significantly decreased for the 10 and 100 pg/ml concentrations. The 1 pg/ml concentration was decreased, but this was not significant. ANOVA repeated measures analysis of variance was significant ($P<0.0001$), as was the ANOVA one-way analysis of variance ($P<0.001$).

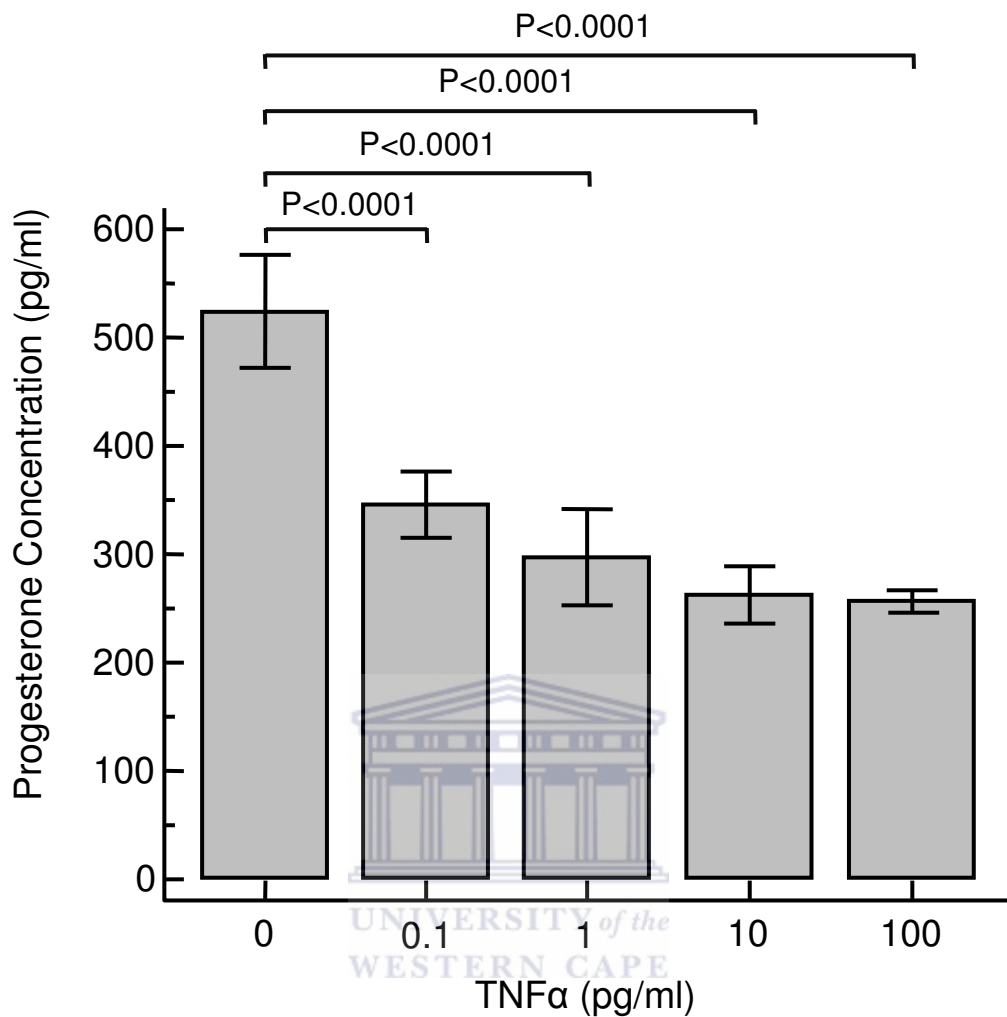


Figure 26: Progesterone concentration results for TM3 cell exposure to various tumour necrosis factor-alpha (TNFα) concentrations. Progesterone concentrations were significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$).

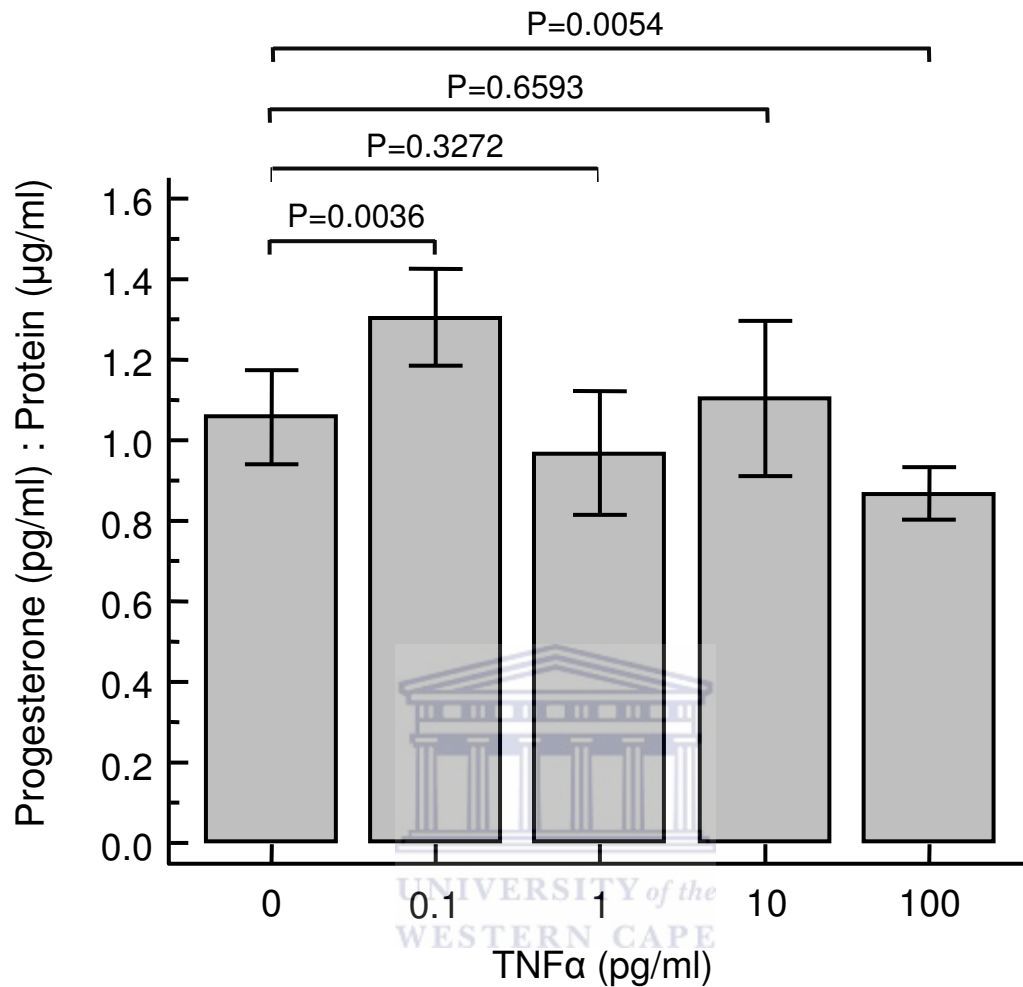


Figure 28: Progesterone-to-protein ratio results for TM3 cell exposure to various tumour necrosis factor-alpha (TNFα) concentrations. The ratio was significantly increased for 0.1 pg/ml concentration and significantly decreased for the 100 pg/ml concentration, but no significant change for the 1 and 10 pg/ml concentrations. ANOVA repeated measures analysis of variance was not significant (P=0.1797). However, ANOVA one-way analysis of variance was significant (P<0.001).

3.9.3. Interleukin 1-beta

TM3 cell viability was significantly decreased for the 0.1 ($P=0.0001$), 1 ($P<0.0001$), 10 ($P<0.0001$) and 100 ($P<0.0001$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 29. Protein concentrations were significantly decreased for the 0.1 ($P<0.0001$), 1 ($P<0.0001$), 10 ($P<0.0001$) and 100 ($P<0.0001$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 30. Testosterone concentrations were significantly decreased for the 0.1 ($P=0.0001$), 1 ($P=0.0013$), 10 ($P<0.0001$) and 100 ($P=0.001$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was not statistically significant ($P=0.1589$), nor was the ANOVA one-way analysis of variance ($P=0.256$). Results are shown in Figure 31. The testosterone-to-protein ratio was not significantly different for the 0.1 ($P=0.6457$) and 1 ($P=0.1046$) pg/ml concentrations, and significantly increased for the 10 ($P<0.0001$) and 100 ($P=0.001$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was not statistically significant ($P=0.093$). The ANOVA one-way analysis of variance was statistically significant ($P<0.001$). Results are shown in Figure 32. Progesterone concentrations were significantly decreased for the 0.1 ($P<0.0001$), 1 ($P<0.0001$), 10 ($P<0.0001$) and 100 ($P<0.0001$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 33. The progesterone-to-protein ratio was significantly decreased for the 0.1 ($P=0.0001$), 1 ($P=0.0001$), 10 ($P=0.0039$) and 100 ($P=0.0003$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was not statistically significant ($P=0.0694$). The ANOVA one-way analysis of variance was significant ($P<0.001$). Results are shown in Figure 34.

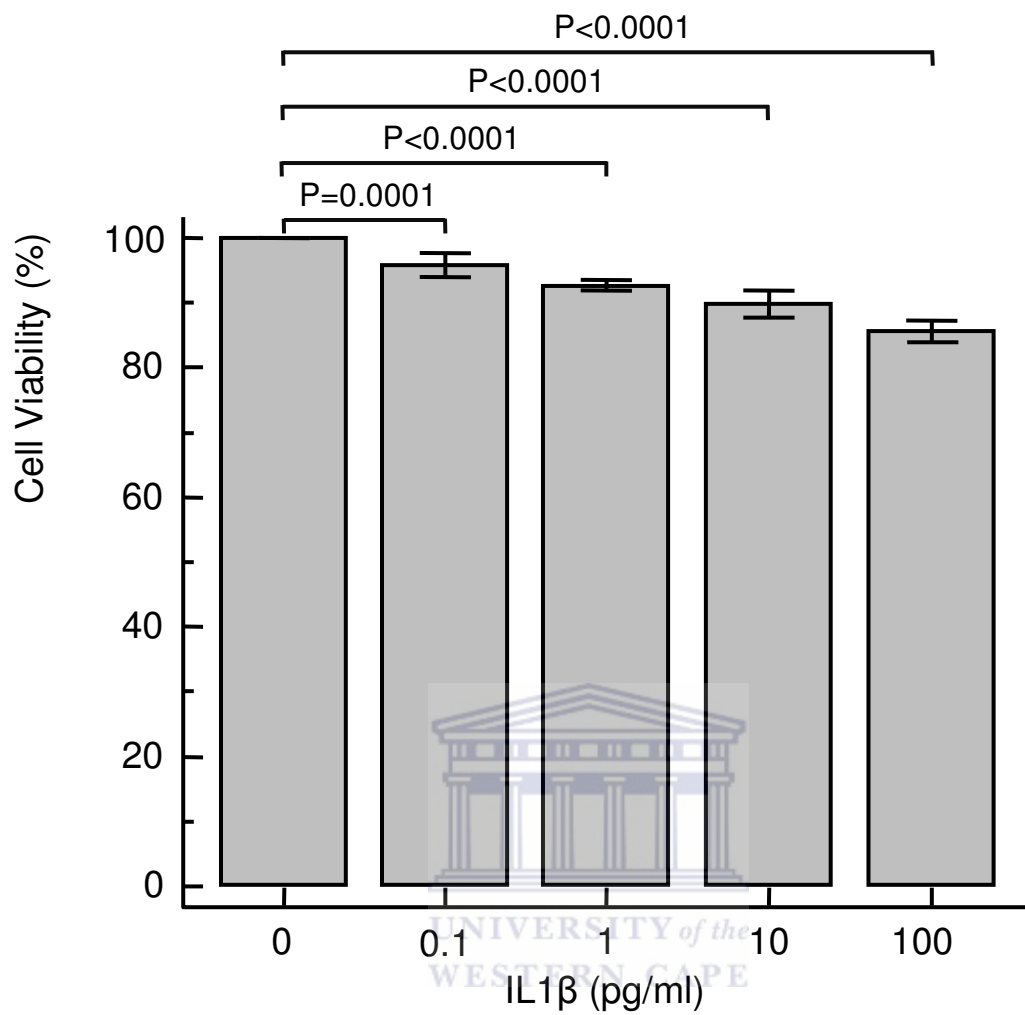


Figure 29: Cell viability results for TM3 cell exposure to various interleukin 1-beta (IL1β) concentrations. Cell viability was significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$).

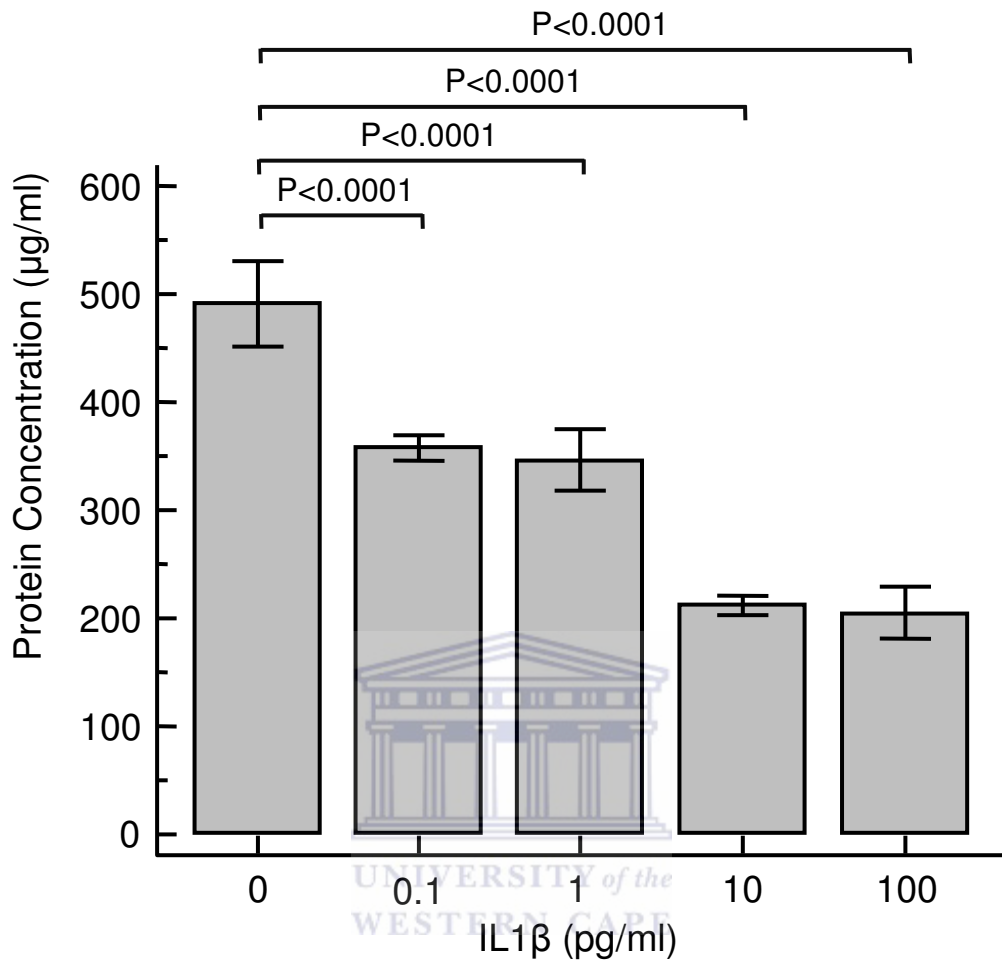


Figure 30: Protein concentration results for TM3 cell exposure to various interleukin 1-beta (IL1 β) concentrations. Protein concentrations were significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P < 0.0001$), as was the one-way analysis of variance ($P < 0.001$).

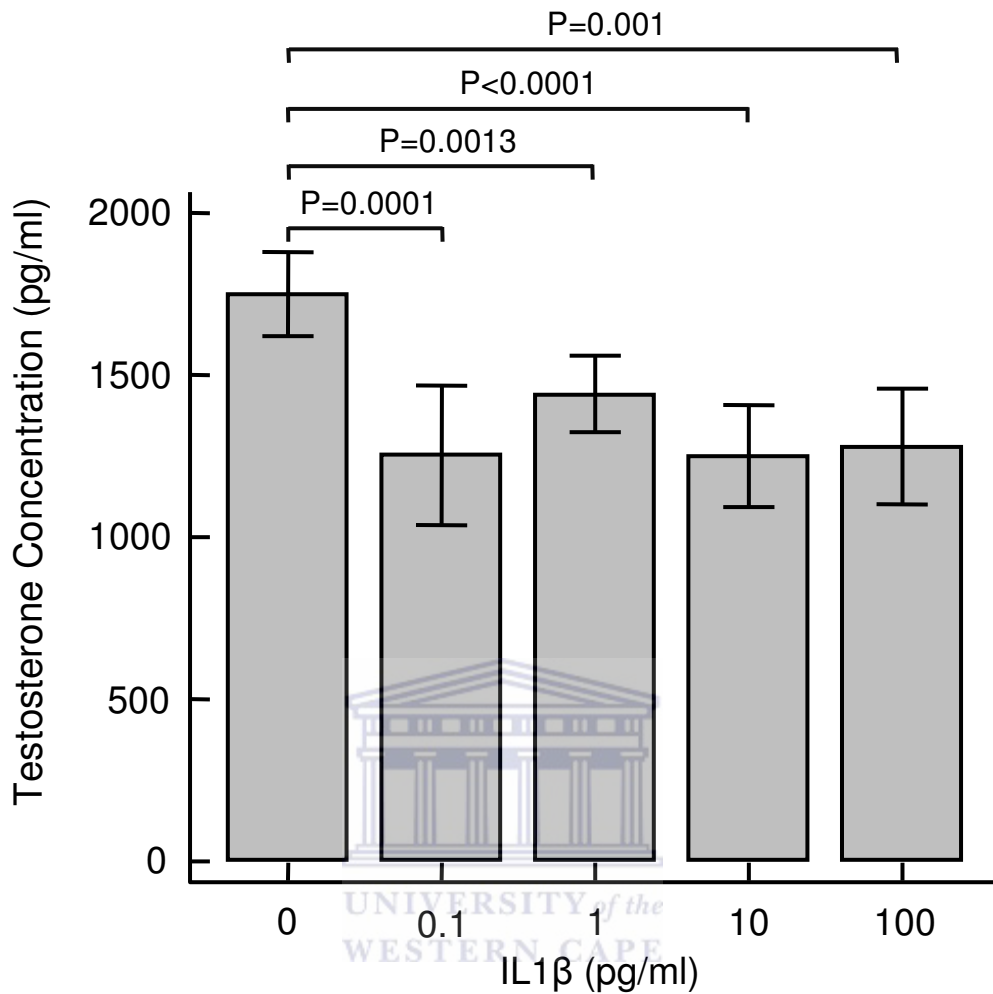


Figure 31: Testosterone concentration results for TM3 cell exposure to various interleukin 1-beta (IL1 β) concentrations. Testosterone concentrations were significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was not significant ($P=0.1589$), nor was the ANOVA one-way analysis of variance ($P=0.256$).

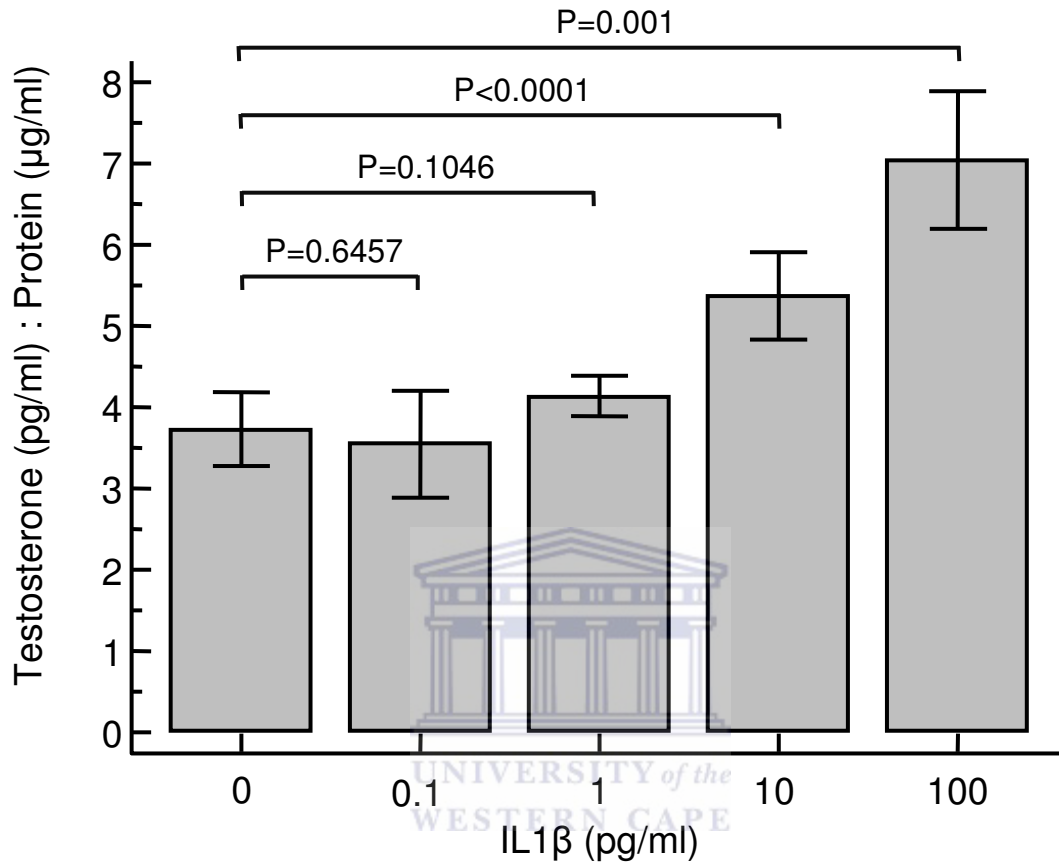


Figure 32: Testosterone-to-protein ratio results for TM3 cell exposure to various interleukin 1-beta (IL1 β) concentrations. The ratio not was significantly different for 0.1 and 1 pg/ml concentrations, and significantly increased for the 10 and 100 pg/ml concentrations. ANOVA repeated measures analysis of variance was not significant (P=0.093). ANOVA one-way analysis of variance was significant (P<0.001).

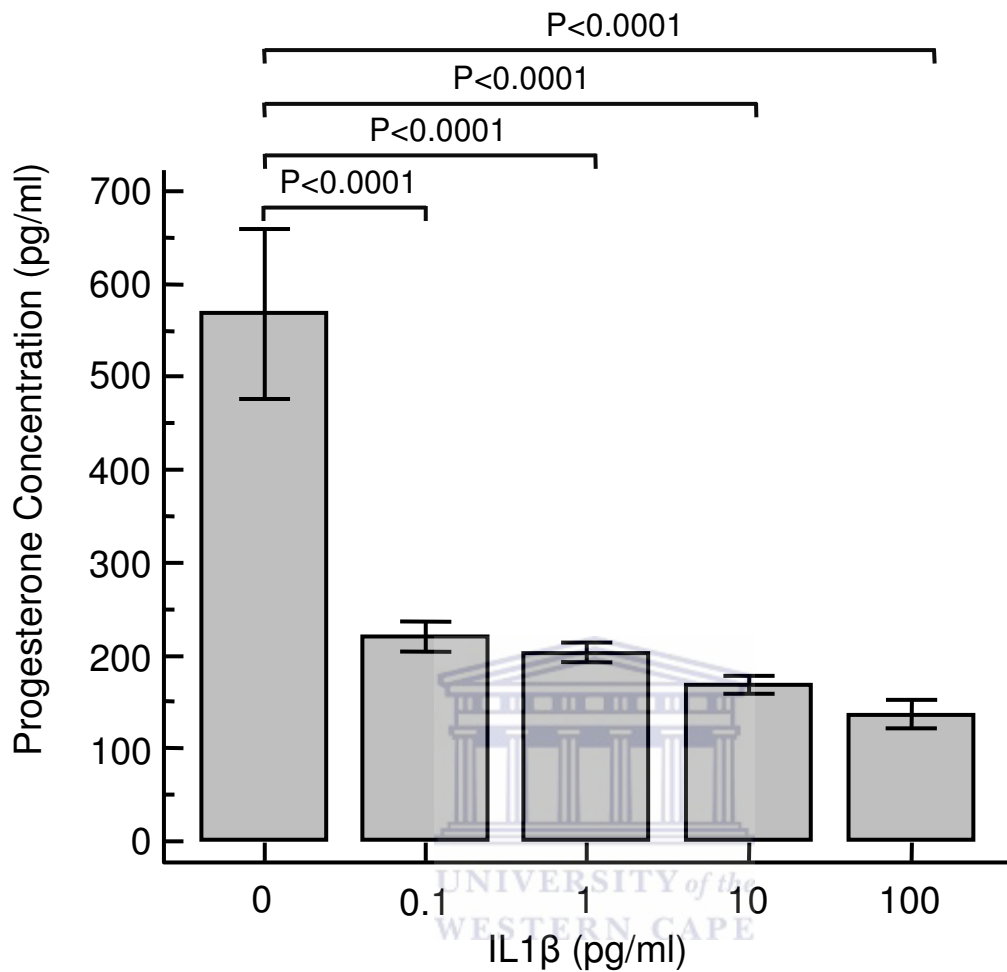


Figure 33: Progesterone concentration results for TM3 cel exposure to various interleukin 1-beta (IL1 β) concentrations. Progesterone concentrations were significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P < 0.0001$), as was the one-way analysis of variance ($P < 0.001$).

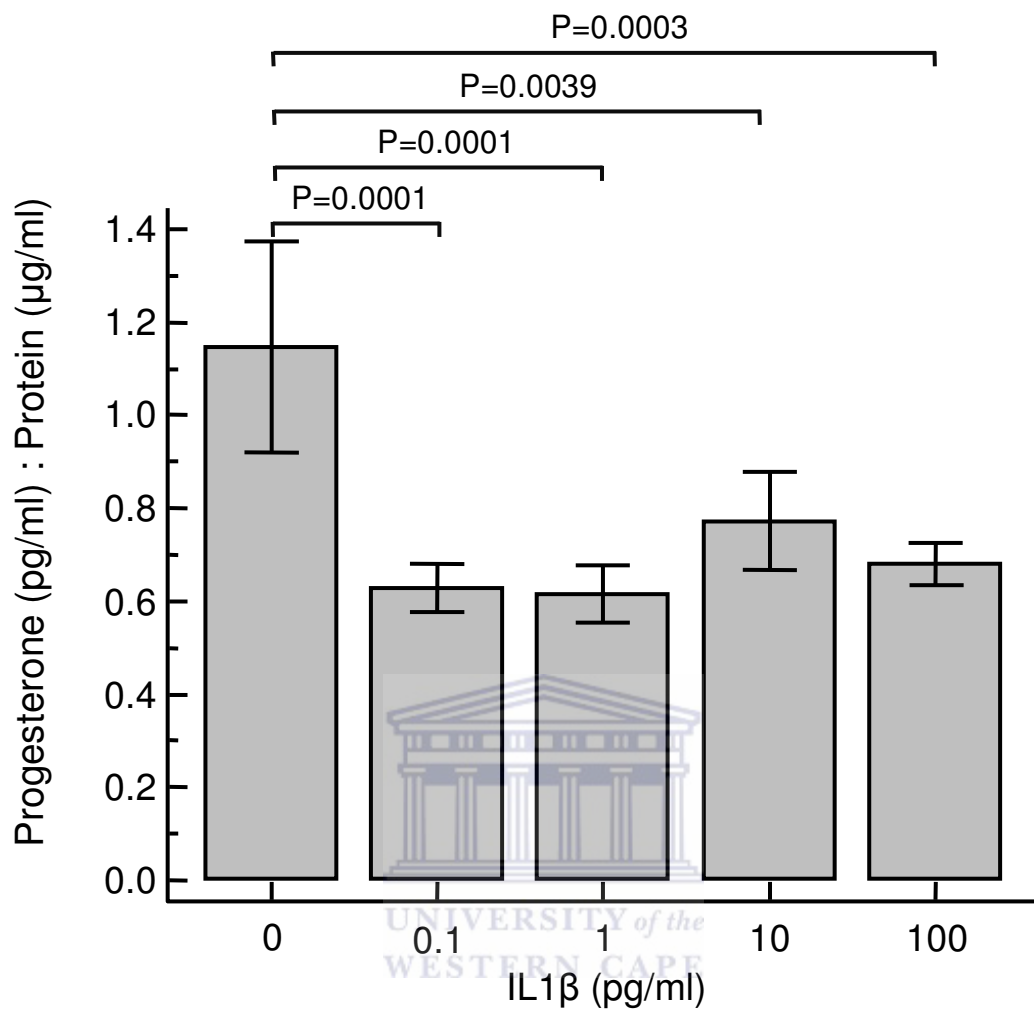


Figure 34: Progesterone-to-protein ratio results for TM3 cell exposure to various interleukin 8 (IL8) concentrations. The ratio was significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was not significant ($P=0.0694$). ANOVA one-way analysis of variance was significant ($P<0.001$).

3.9.4. Interleukin 6

TM3 cell viability was significantly decreased for the 0.1 ($P=0.0001$), 1 ($P<0.0001$), 10 ($P<0.0001$) and 100 ($P<0.0001$) concentrations. The ANOVA repeated measures analysis of variance was significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 35. Protein concentrations were not significantly different for the 0.1 ($P=0.4284$), 1 ($P=0.9094$) and 10 ($P=0.6738$) pg/ml concentrations, but were significantly decreased ($P=0.0414$) for the 100 pg/ml concentrations. The ANOVA repeated measures analysis of variance was not statistically significant ($P=0.1039$). The one-way analysis of variance was significantly different ($P<0.001$). Results are shown in Figure 36. Testosterone concentrations were significantly decreased for the 0.1 ($P=0.0031$), 1 ($P=0.0025$), 10 ($P=0.0134$) and 100 ($P=0.0003$) concentrations. The ANOVA repeated measures analysis of variance was statistically significant ($P<0.0001$), as was the one-way analysis of variance ($P=0.009$). Results are shown in Figure 37. The testosterone-to-protein ratio was not significantly different for the 0.1 ($P=0.8893$), 1 ($P=0.3134$), 10 ($P=0.8799$) and 100 ($P=0.9587$) concentrations. The ANOVA repeated measures analysis of variance was not statistically significant ($P=0.7268$). The ANOVA one-way analysis of variance was statistically significant ($P<0.001$). Results are shown in Figure 38. Progesterone concentrations were significantly decreased for the 0.1 ($P<0.0001$), 1 ($P<0.0001$), 10 ($P<0.0001$) and 100 ($P<0.0001$) concentrations. The ANOVA repeated measures analysis of variance was significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 39. The progesterone-to-protein ratio was significantly decreased for the 0.1 ($P<0.0001$), 1 ($P<0.0001$), 10 ($P<0.0001$) and 100 ($P<0.0001$) concentrations. The ANOVA repeated measures analysis of variance was statistically significant ($P=0.003$), as was the ANOVA one-way analysis of variance was significant ($P<0.001$). Results are shown in Figure 40.

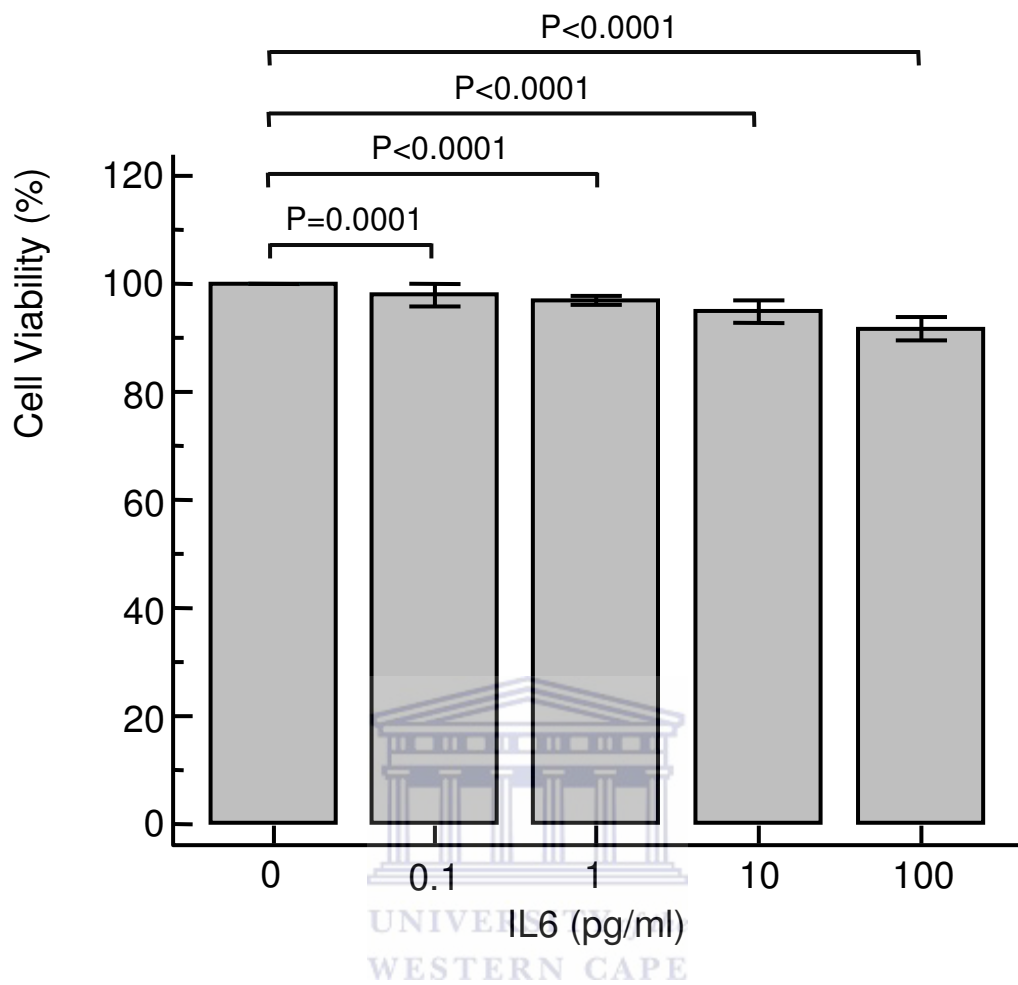


Figure 35: Cell viability results for TM3 cell exposure to various interleukin 6 (IL6) concentrations. Cell viability was significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P<0.0001$), as was the one-way analysis of variance ($P<0.001$).

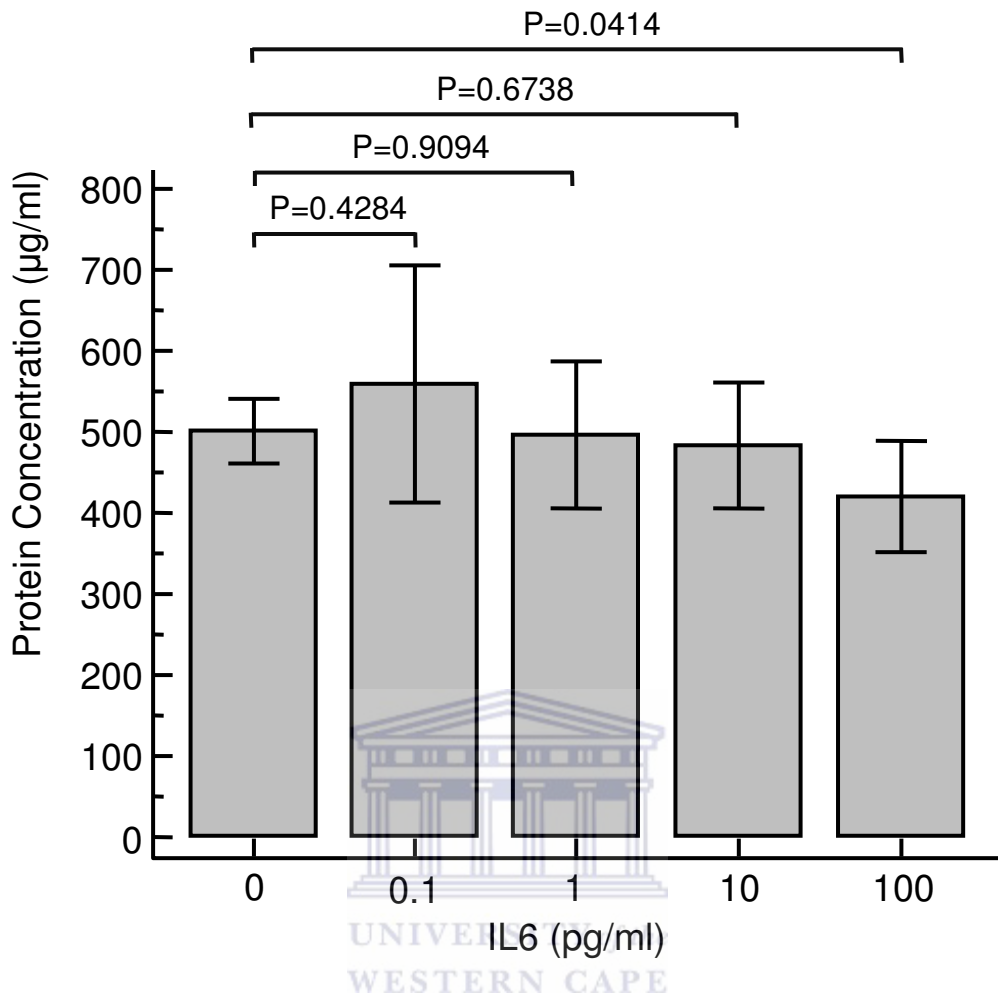


Figure 36: Protein concentration results for TM3 cell exposure to various interleukin 6 (IL6) concentrations. Protein concentrations were not significantly different for 0.1, 1 and 10 pg/ml concentrations, but were significantly decreased for the 100 pg/ml concentrations. ANOVA repeated measures analysis of variance was not significant ($P=0.1039$). ANOVA one-way analysis of variance was significantly different ($P<0.001$).

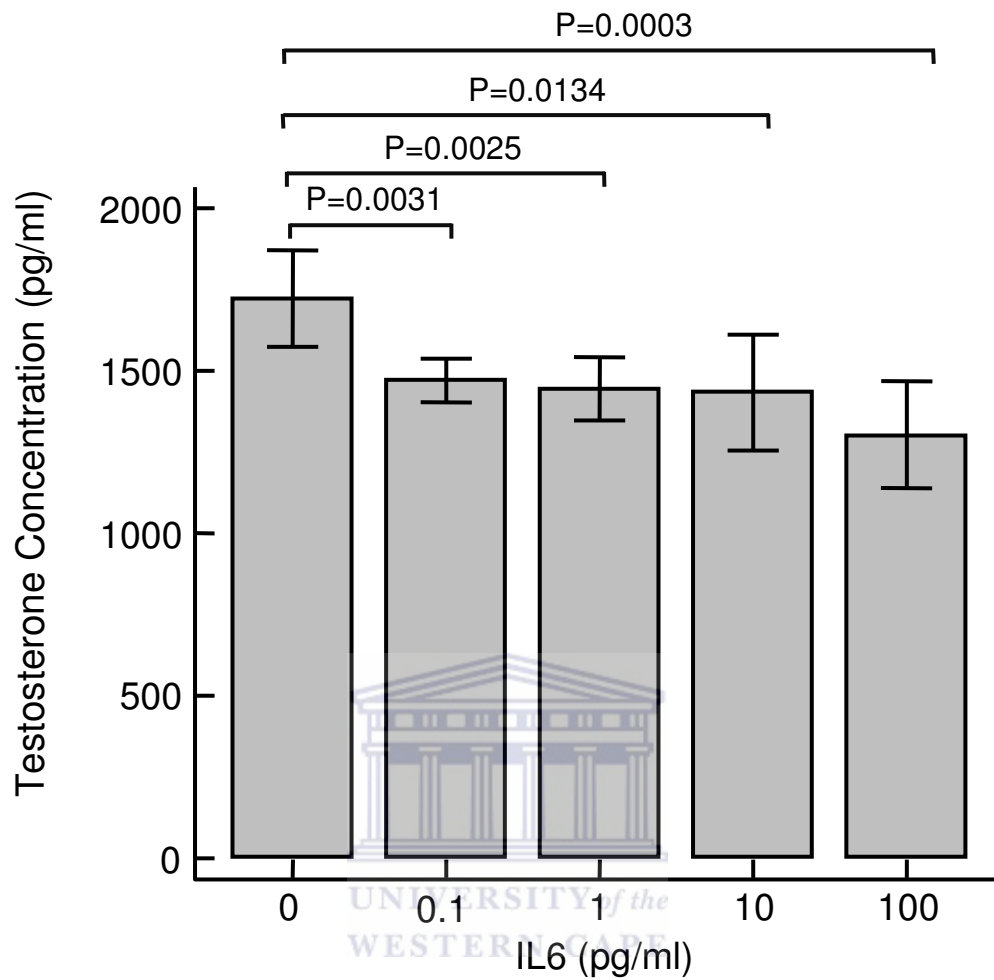


Figure 37: Testosterone concentration results for TM3 cell exposure to various interleukin 6 (IL6) concentrations. Testosterone concentrations were significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P < 0.0001$), as was the one-way analysis of variance ($P = 0.009$).

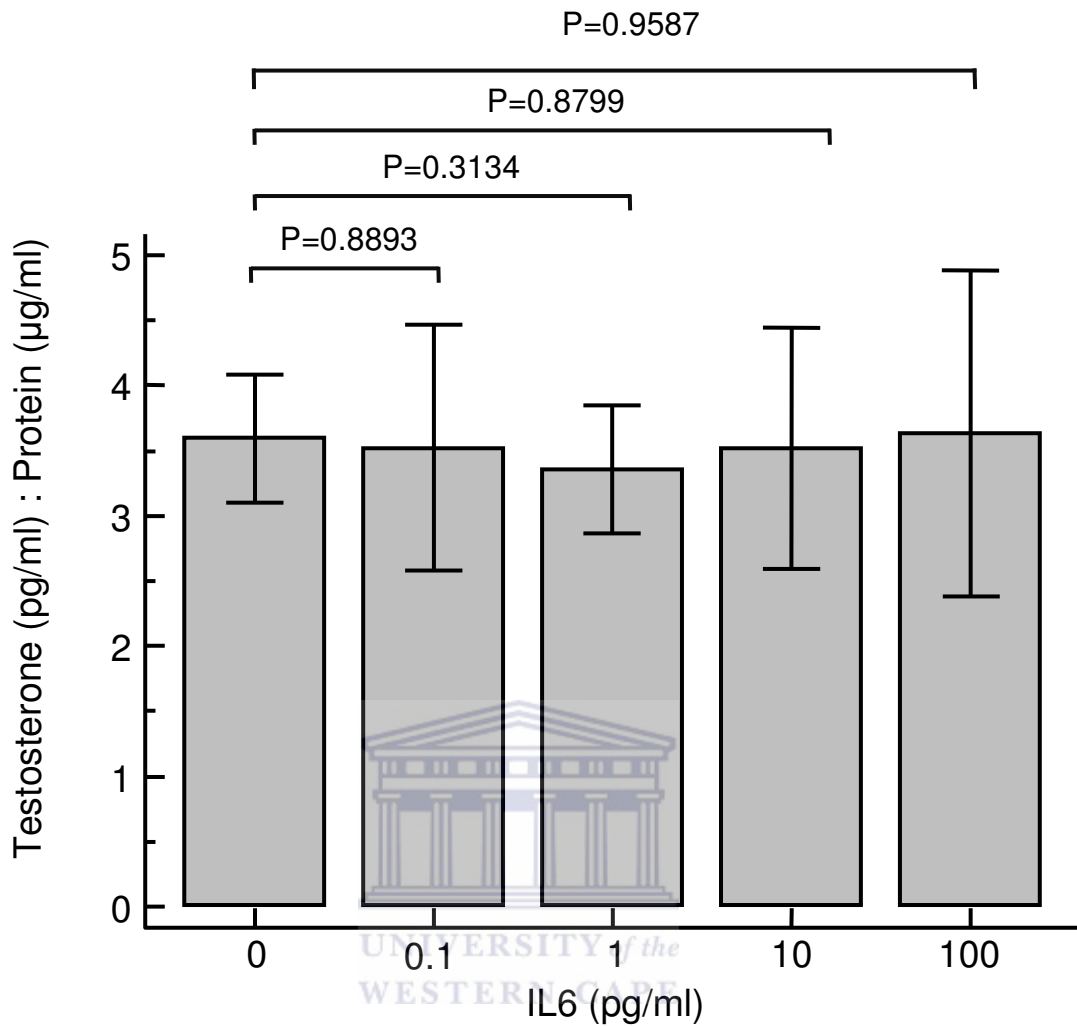


Figure 38: Testosterone-to-protein ratio results for TM3 cell exposure to various interleukin 6 (IL6) concentrations. The ratio not was significantly different for all concentrations. ANOVA repeated measures analysis of variance was not statistically significant ($P=0.7268$). ANOVA one-way analysis of variance was statistically significant ($P<0.001$).

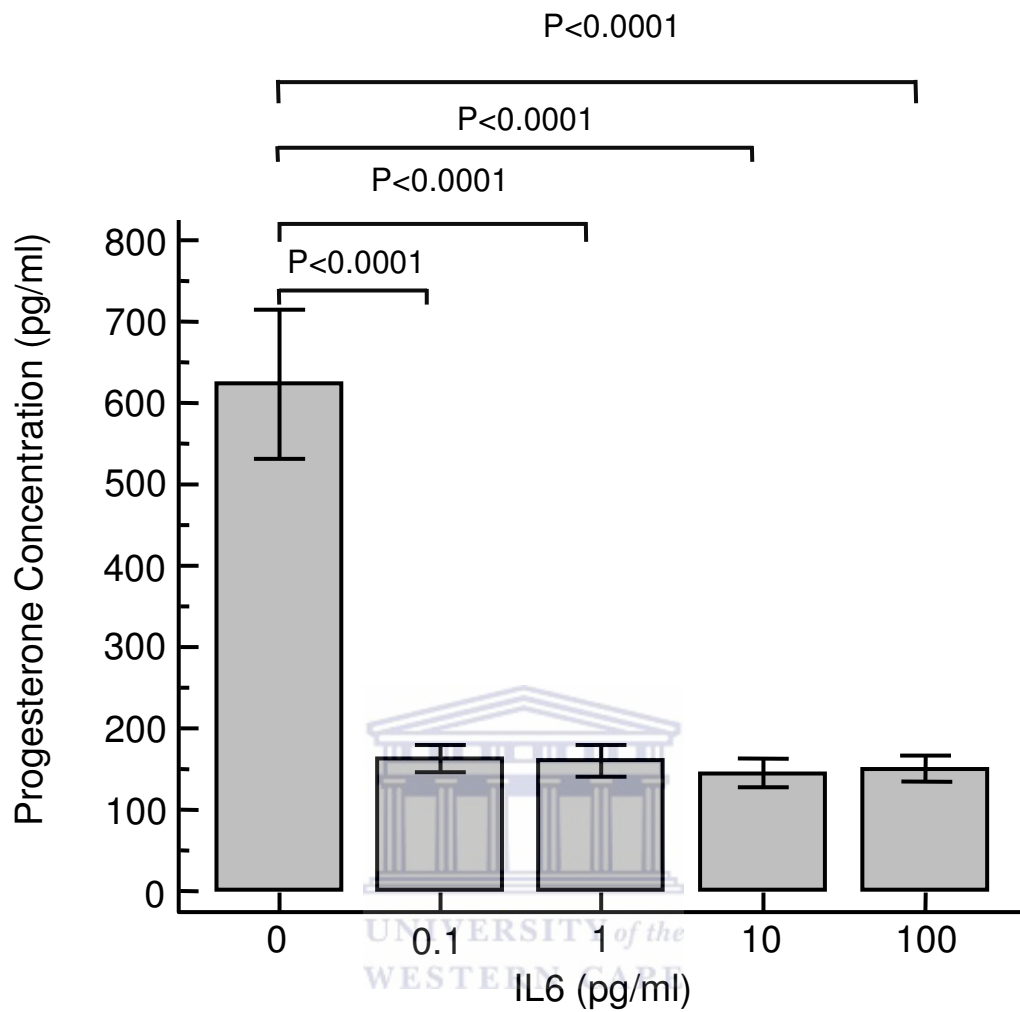


Figure 39: Progesterone concentration results for TM3 cell exposure to various interleukin 6 (IL6) concentrations. Progesterone concentrations were significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P < 0.0001$), as was the one-way analysis of variance ($P < 0.001$).

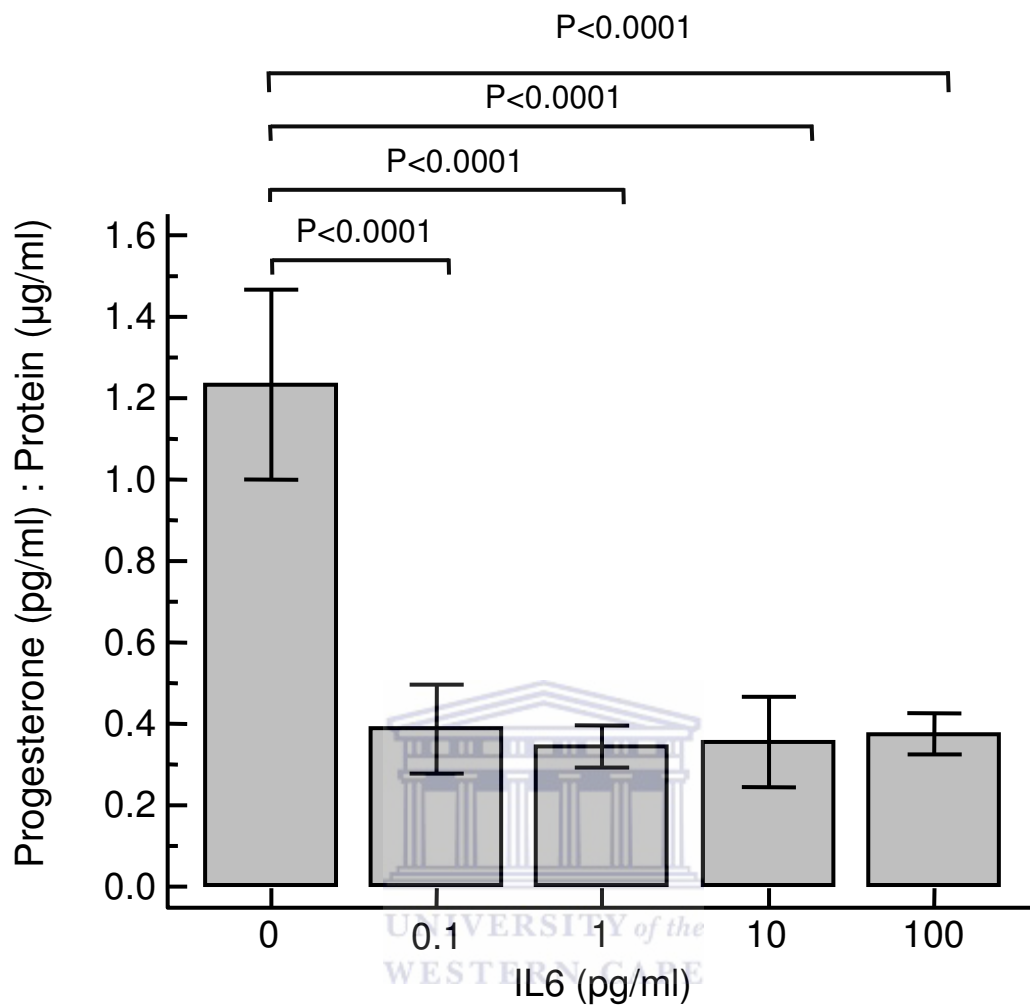


Figure 40: Progesterone-to-protein ratio results for TM3 exposure to various interleukin 6 (IL6) concentrations. The ratio was significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P=0.003$), as was the ANOVA one-way analysis of variance was significant ($P<0.001$).

3.9.5. Interleukin 8

TM3 cell viability was significantly increased for the 0.1 ($P=0.0016$), 1 ($P=0.0359$), 10, ($P<0.0001$) and 100 ($P<0.0001$) concentrations. The ANOVA repeated measures analysis of variance was significant ($P=0.0014$), as did the one-way analysis of variance ($P=0.004$). Results are shown in Figure 41. Protein concentrations were significantly ($P=0.0037$) decreased for 10 pg/ml and significantly ($P=0.0037$) increased for 100 pg/ml, with no significant difference for the 0.1 ($P=0.1249$) and 1 ($P=0.6556$) pg/ml concentrations. The ANOVA repeated measures analysis of variance was not statistically significant ($P=0.1048$). The ANOVA one-way analysis of variance did reach statistical significance ($P<0.001$). Results are shown in Figure 42. Testosterone concentrations were not significantly affected for the 0.1 ($P=0.0958$), 1 ($P=0.6763$), 10 ($P=0.6314$) and 100 ($P=0.5334$) concentrations. The ANOVA repeated measures analysis of variance was not statistically significant ($P=0.0524$), nor was the ANOVA one-way analysis of variance ($P=0.187$). Results are shown in Figure 43. The testosterone-to-protein ratio was significantly increased for the 0.1 ($P<0.0001$) and 10 ($P=0.0099$) pg/ml concentrations, but the 1 ($P=0.3593$) and 100 ($P=0.2690$) pg/ml concentrations did not reach significance. The ANOVA repeated measures analysis of variance was not statistically significant ($P=0.1895$). The ANOVA one-way analysis of variance was significant ($P<0.001$). Results are shown in Figure 44. Progesterone concentrations were significantly decreased for the 0.1 ($P=0.0007$), 1 ($P=0.0074$), 10, ($P=0.0022$) and 100 ($P=0.0076$) concentrations. The ANOVA repeated measures analysis of variance was statistically significant ($P=0.0024$), as was the one-way analysis of variance ($P<0.001$). Results are shown in Figure 45. The progesterone-to-protein ratio was significantly decreased for 0.1 ($P=0.0492$), 1 ($P=0.019$) and 100 ($P=0.0059$) pg/ml concentrations, but not for 10 pg/ml concentration ($P=0.1295$). The ANOVA repeated measures analysis of variance was statistically significant ($P=0.0032$). The ANOVA one-way analysis of variance was significant ($P=0.004$). Results are shown in Figure 46.

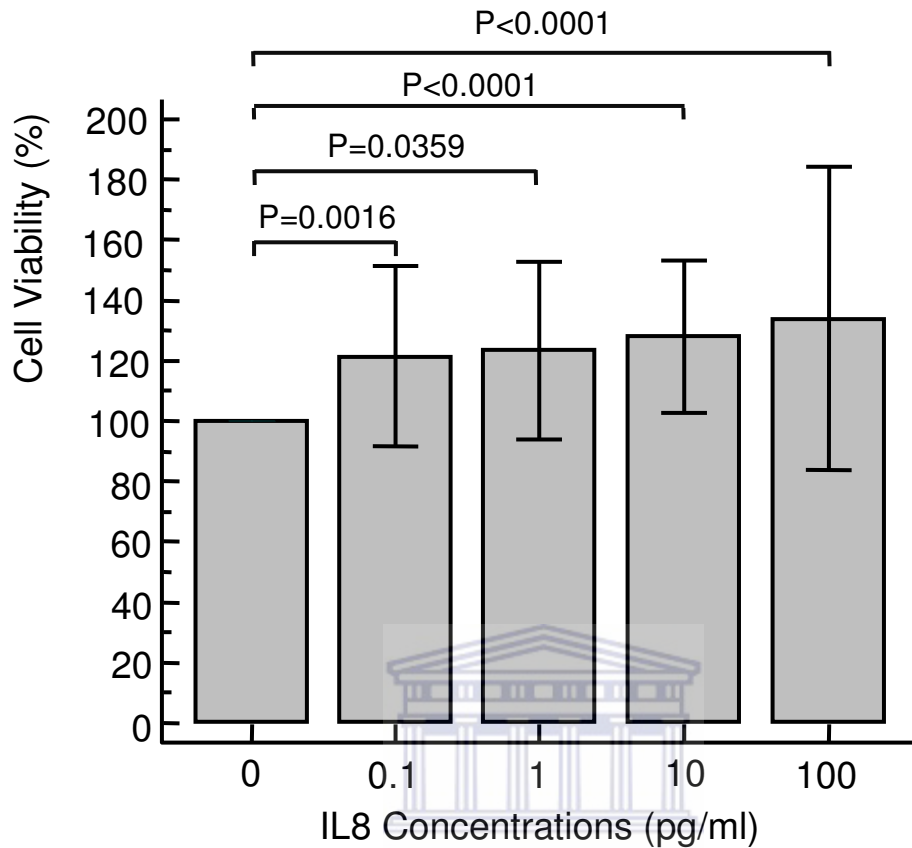


Figure 41: Cell viability results for TM3 cell exposure to various interleukin 8 (IL8) concentrations. Cell viability was significantly increased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P=0.0014$), as was the one-way analysis of variance ($P=0.004$).

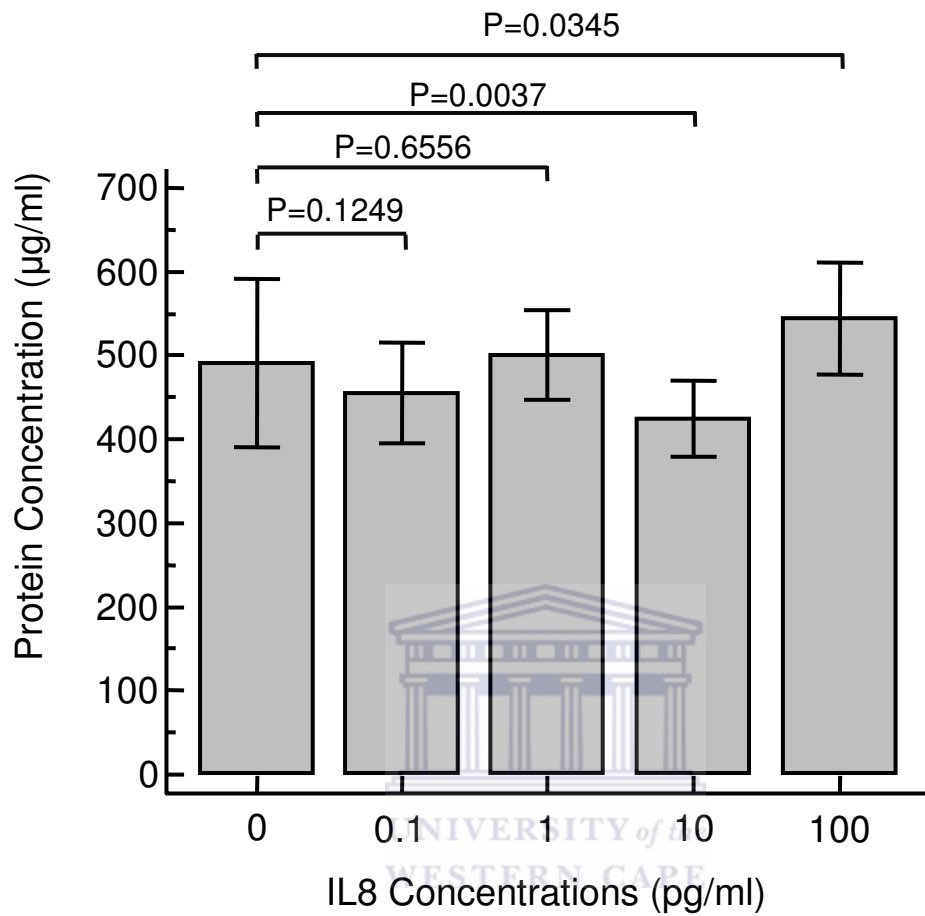


Figure 42: Protein concentration results for TM3 cell exposure to various interleukin 8 (IL8) concentrations. Protein concentrations were significantly decreased for 10 pg/ml and significantly increased for 100 pg/ml, with no significant difference for 0.1 and 1 pg/ml concentrations. ANOVA repeated measures analysis of variance was not statistically significant ($P=0.1048$). ANOVA one-way analysis of variance did reach statistical significance ($P<0.001$).

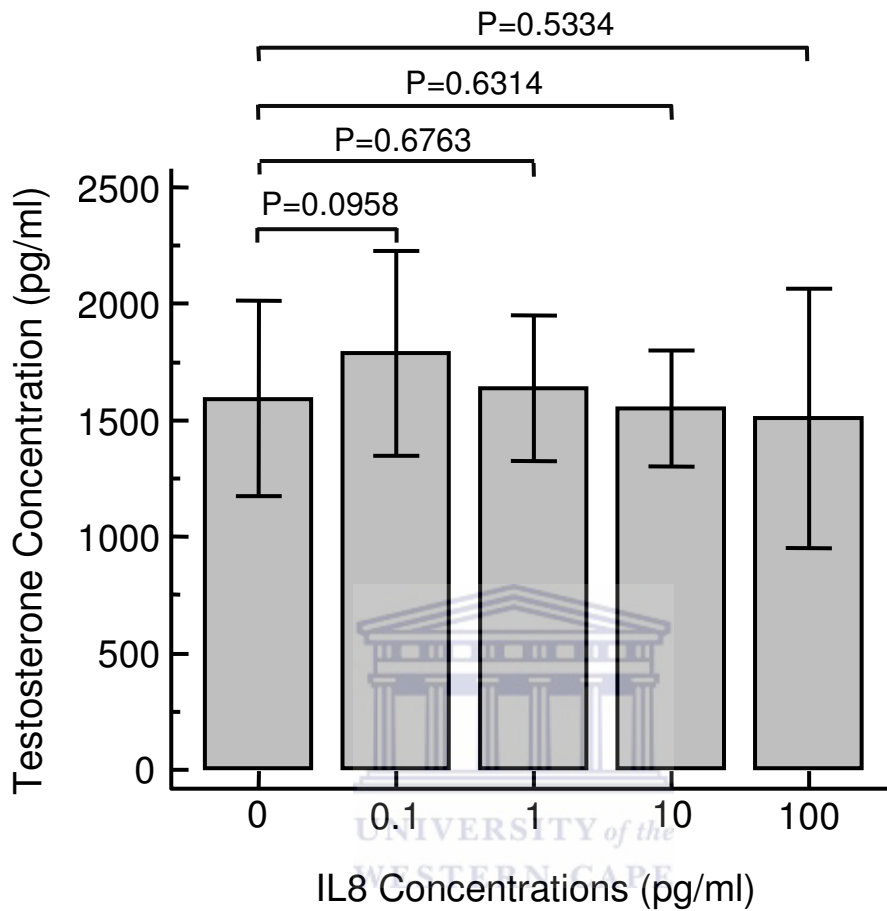


Figure 43: Testosterone concentration results for TM3 cell exposure to various interleukin 8 (IL8) concentrations. Testosterone concentrations were not significantly affected for all concentrations. ANOVA repeated measures analysis of variance was not statistically significant ($P=0.0524$), nor was the ANOVA one-way analysis of variance ($P=0.187$).

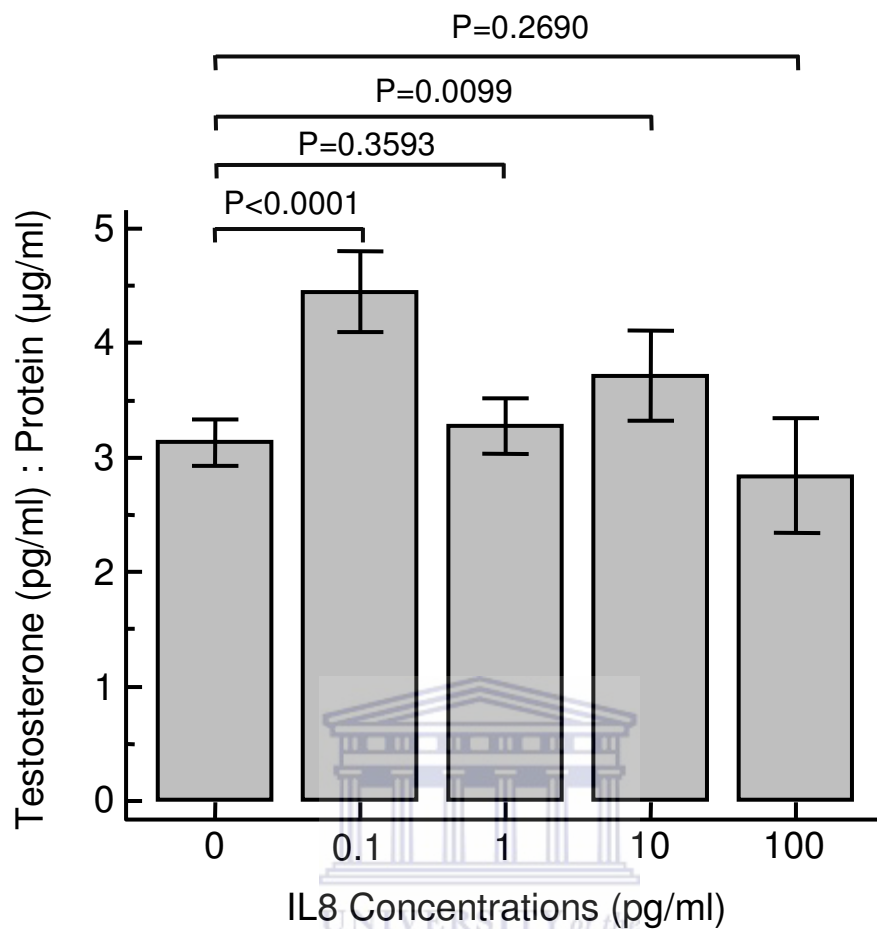


Figure 44: Testosterone-to-protein ratio results for TM3 cell exposure to various interleukin 8 (IL8) concentrations. The ratio was significantly increased for 0.1 and 10 pg/ml concentrations, but 1 and 100 pg/ml concentrations did not reach significance. ANOVA repeated measures analysis of variance was not statistically significant ($P=0.1895$). ANOVA one-way analysis of variance was significant ($P<0.001$).

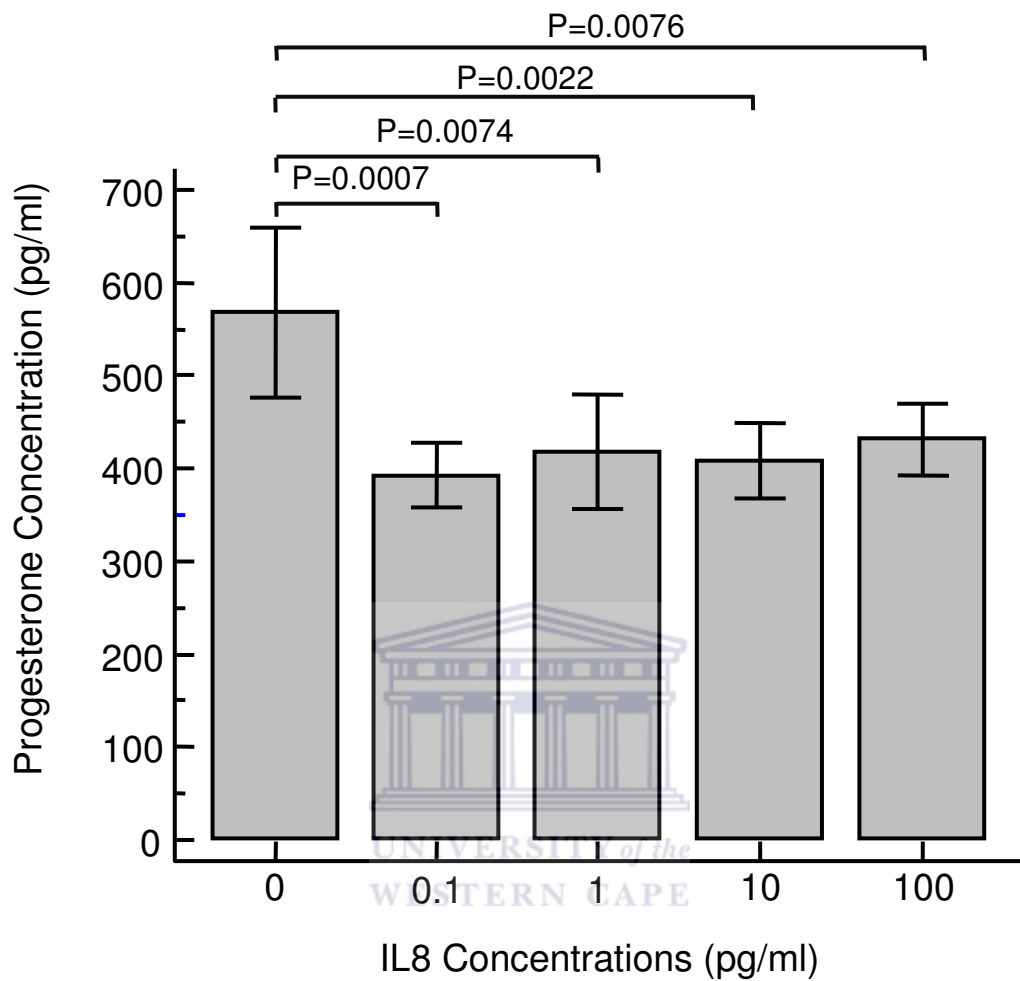


Figure 45: Progesterone concentration results for TM3 cell exposure to various interleukin 8 (IL8) concentrations. Progesterone concentrations were significantly decreased for all concentrations. ANOVA repeated measures analysis of variance was significant ($P=0.0024$), as was the one-way analysis of variance ($P<0.001$).

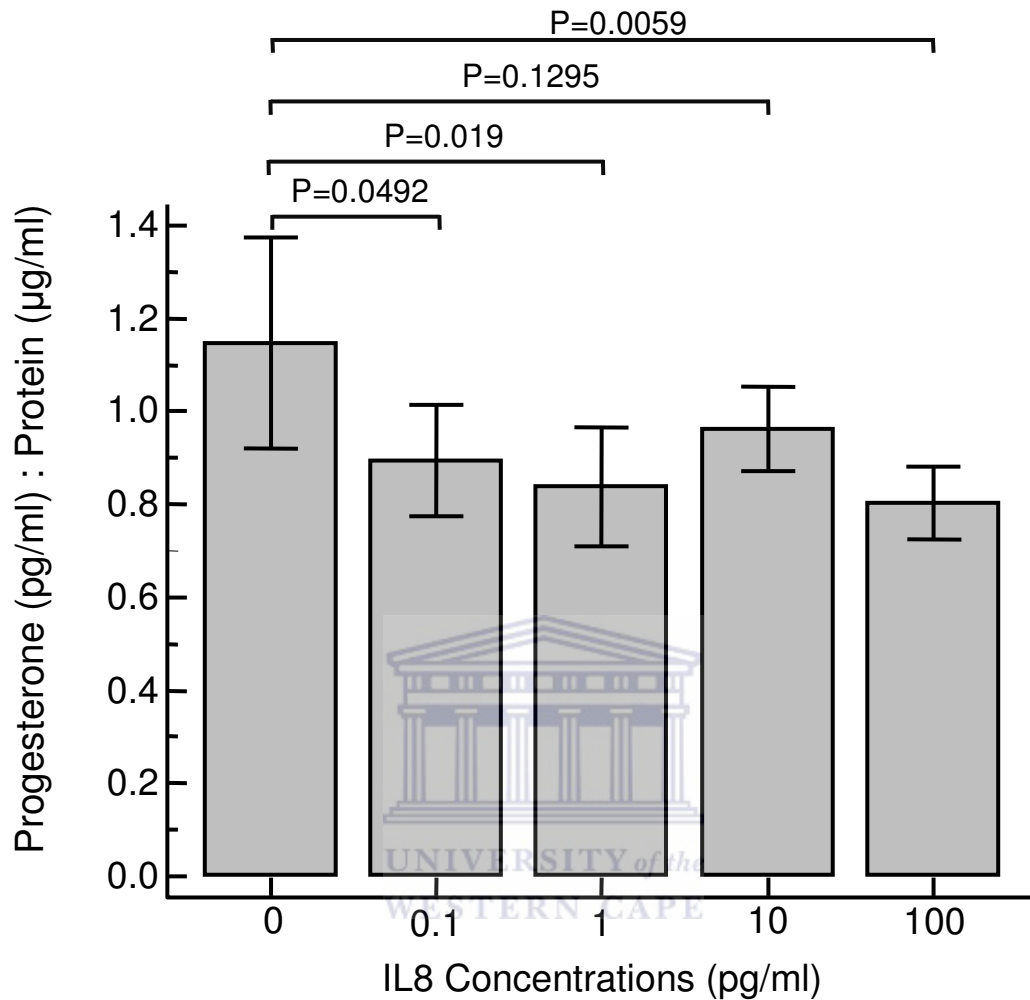


Figure 46: Progesterone-to-protein ratio results for TM3 cell exposure to various interleukin 8 (IL8) concentrations. The ratio was significantly decreased for 0.1, 1 and 100 pg/ml concentrations, but not for 10 pg/ml concentration. ANOVA repeated measures analysis of variance was statistically significant ($P=0.0032$). ANOVA one-way analysis of variance was significant ($P=0.004$).

CHAPTER 4 – DISCUSSION

4.1. Introduction

The metabolic syndrome (MetS) is a poorly understood pathophysiological phenomenon, associated with complex metabolic, hormonal and immune dysfunction, resulting in various deleterious effects on patients such as cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) (Alberti et al., 2009). The syndrome is characterised by abdominal obesity, hypertension, dyslipidaemia and poor glucose regulation (Eckel et al., 2005; Kasturi et al., 2008; Huang, 2009; Taslim & Tai, 2009). Further phenomena associated with MetS and pertinent to this study include hyperinsulinaemia and insulin resistance, hyperleptinaemia and leptin resistance, a low grade, chronic, systemic inflammation and in males, hypogonadism (Eckel et al., 2005; Kasturi et al., 2008; Huang, 2009; Taslim & Tai, 2009). The impact of MetS on male fertility has not been previously evaluated in a case controlled epidemiological based study, and therefore remains relatively unknown. This study aimed to assess the impact of MetS on male fertility parameters, free testosterone and progesterone concentrations, serum and seminal inflammatory cytokines, insulin and leptin concentrations. In addition, a cell culture model has been used to further investigate the effect of insulin and inflammatory cytokines on testosterone and progesterone synthesis in the TM3 Leydig cell line.

The results of this study, consisting of a cohort of males of reproductive age, have reinforced the known clinical and biochemical features of MetS. As expected, highly significant differences between the control group (CG) and and metabolic syndrome (MetS) group was found for BMI, abdominal adiposity (waist circumference), systolic and diastolic blood pressure, serum HDL cholesterol, serum triglycerides and serum glucose. Phenomena closely related to the pathophysiology of MetS was also found to be significantly different between the groups, specifically serum insulin concentrations and fasting blood insulin, insulin resistance (based on the QUICKI), serum CRP and free testosterone. The individual components of MetS also generally correlated with each other in the cohort, except for fasting blood glucose which showed no correlation with the other components of MetS. These are all well defined

aspects of MetS in the scientific literature, with the results of this study correlating with the extensive literature defining the syndrome.

4.2. The metabolic syndrome and male fertility potential and sperm function

Results show statistically significant differences in various sperm parameters analysed in males in the CG and those clinically diagnosed with MetS. This was in the absence of reproductive tract inflammation and leukocytospermia. Mean ejaculation volume and sperm concentration was significantly reduced in males with MetS, as was the total sperm count. It should be noted, however, that the mean sperm concentration in the MetS group was 26.7 ± 15.8 million/ml, which is above the 15 million/ml threshold and therefore considered normal according to parameters defined by WHO (2010). This is also seen with the total sperm count in the MetS group (median = 48.1 million spermatozoa in the ejaculate), which is above the 40 million threshold outlined by WHO (2010). Patients in the MetS group also showed significantly reduced spermatozoa vitality, progressive motility and total motility. The mean values for vitality ($50.0 \pm 23.2\%$) and progressive motility ($20.0 \pm 17.1\%$) are both below the threshold levels set by WHO (2010) (58% and 32%, respectively), with the mean value for total motility ($42.9 \pm 19.9\%$) above the suggested cut-off value of 40% (WHO, 2010) in the MetS group. The MetS group showed a decrease in percentage of spermatozoa with normal morphology, however, this did not reach statistical significance. Interestingly, both groups had a mean value of normal morphological forms below the WHO (2010) recommended threshold of 4%, with $3.9 \pm 3.17\%$ in the CG and $2.72 \pm 2.38\%$ in the MetS group, with both groups showing a wide variation in percentages (indicated by the large standard deviations). In terms of classic semen analysis parameters, males with MetS in this study have a reduced fertility potential compared to the CG.

In addition to the total number of spermatozoa introduced to the female reproductive tract, and the ability of a large percentage of these cells to swim in a progressive manner towards the fallopian tubes, numerous additional characteristics of spermatozoa and the quality of seminal fluid are required to ensure a successful fertilisation with an oocyte (Aitken, 2006). These parameters include seminal fructose concentration (Said et al., 2009), oxidant-antioxidant balance (Henkel, 2005;

Henkel, 2011a), normal sperm head morphology (Menkveld et al., 2001), capacitation and acrosome reaction (Aitken, 2006), normal sperm mitochondrial membrane potential (MMP) (Paoli et al., 2011; Marchetti et al., 2012) and DNA integrity (Henkel et al., 2010; Lewis and Simon, 2010). The results of this study strongly suggest that males with MetS have significantly increased damage to mitochondrial function and spermatozoa DNA.

Damage to the mitochondria is suggested to negatively affect oxidative phosphorylation, reducing ATP synthesis and thus energy availability for motility (Paoli et al., 2011; Marchetti et al., 2012). Percentage of sperm with abnormal mitochondrial membrane potential (MMP) was negatively correlated with vitality and total and progressive motility in the entire cohort, as well as the CG and MetS groups, supporting this well defined relationship. Cut-off values for MMP are not well defined. Xia et al. (2008) identified 31 fertile control males to have a mean MMP of 24.1%, with 32 infertile counterparts having a mean of 46%. Hu et al. (2009) found a mean MMP in a healthy male cohort to be 23.8%, with increased means in males with grade 1 (43.7%), grade 2 (55%) and grade 3 (68.4%) varicoceles. La Vignera et al. (2012c) found normal weight men to have a mean MMP of 5%, with overweight and obese males having a mean of 27% and 44%, respectively. In two separate publications, males with varicocele (La Vignera et al., 2012c) and male accessory gland infection (MAGI) (La Vignera et al., 2011) had a mean MMP of 28%. However, it has been suggested by Marchetti et al. (2012) that the percentage of sperm with abnormal (compromised) MMP should be less than 36%. The mean values of MMP in both the CG and MetS groups in this study were above that of these studies, with $42.1 \pm 25.8\%$ and $63.1 \pm 22.2\%$, respectively. Reasons for the higher values in both groups are not clear based on the data. It may be related to genetics, may be geographical, or possibly due to the methodology used in the study (DePsipher staining kit), as JC-1 methods were used in the above mentioned studies.

Percentage of sperm with DNA fragmentation (DF) was also significantly increased in the MetS group. As with MMP, DF cut off values for risks are not well defined. Literature reports for healthy fertile males range from 7.5% - 25.7%. Values associated with poor fertility outcomes and various male reproductive disorders range from 14.8% - 64.9% (Evenson & Wixon, 2005; Fernández et al., 2005; Enciso

et al., 2006; Kort et al., 2006; Chavarro et al., 2010; Rybar et al., 2011; Venkatesh et al., 2011; Zini & Dohle, 2011). Recently, Simon et al. (2013) has suggested that DF should be less than 25%. The MetS group showed a mean percentage of spermatozoa DF above this suggested threshold ($26.9 \pm 19.7\%$), but not the CG. However, results indicate that MetS is associated with an increase in percentage of spermatozoa with DF, and thus potentially decreasing fertility potential in males.

There are few publications in the literature which support the results of this study. Lotti and colleagues (2013a) associated MetS with poor sperm morphology and testes ultrasound inhomogeneity, in addition to hypogonadism, erectile dysfunction (ED) and depression and a significant decline in sexual and overall health. This study found no significant relationship between other sperm parameters. This may be explained by the fact that the cohort recruited by Lotti et al. (2013a) involved males already attending a fertility clinic due to a history of couple infertility (there was a mean duration of infertility of 1.9 years in the entire cohort), and were then placed into a MetS group or group without MetS. Of a cohort of 351 participants, only 27 (7.7%) were in the MetS group. Furthermore, within the entire cohort, there was a mean sperm concentration of 13 million/ml and total sperm count of 36 million per ejaculate (both below WHO threshold), 14% were diagnosed with azoospermia (only 33% were normospermic), a mean progressive motility of 36.5%, a mean of 5% normal morphological forms and 8.7% participants had leukocytospermia. Therefore, generally, the entire cohort had reduced semen parameters from the outset. The results of this study are taken from the general population, and not necessarily males with already established couple infertility.

An interesting study by Ozturk and colleagues (2012) aimed to investigate the impact of MetS on varicocele surgery success. Of patients that underwent surgical vein ligation, they were retrospectively divided into two groups, those with and without MetS. Preoperatively, those with MetS had a mean sperm concentration of 17.03 ± 7.6 million/ml, mean motile spermatozoa of $16.3 \pm 6.6\%$ and mean normal sperm morphological forms of $5.04 \pm 7.05\%$. There was no significant difference between the non-MetS group, with 18.01 ± 1.88 million/ml, mean motile spermatozoa of $17.9 \pm 6.6\%$ and mean normal sperm morphological forms of $7.01 \pm 7.05\%$. The lack of significant difference at baseline between the groups may be due to the diagnosis

of varicocele within all patients. Both groups had a significant improvement in all three parameters assessed following surgery. Interestingly, 3 months post-surgical treatment, the MetS group had a significantly decreased sperm concentration and motility compared to the non-MetS group, but no difference for normal morphological forms. The post-surgical MetS group showed a mean sperm concentration of 30.1 ± 4.7 million/ml, mean motile spermatozoa of $20.6 \pm 5.5\%$ and mean normal sperm morphological forms of $10.5 \pm 4.21\%$. These are similar means for sperm concentration (26.7 ± 15.8 million/ml) and progressive motility ($20.0 \pm 17.1\%$) found in the MetS group of this study. As part of the conclusion, the authors stated that MetS may be an independent predictor for poor sperm parameters, and that patients with MetS and varicocele improved sperm parameters after surgery, but not as well as those without MetS. The number of patients was not reported, but the authors mentioned it as 'low'. Furthermore, detailed sperm analyses according to WHO (2010) guidelines, as well as the criteria used to define MetS, are not provided in this study.

According to the *Laboratory Manual for the Examination and Processing of Human Semen 5th Edition* (WHO, 2010), the semen parameters are important markers for fertility. However, it is difficult to assess fertility potential based on the semen parameters, particularly as these were a once off semen sample that have not been repeated. Lewis (2007) has described that only 16% of infertile men and 5% of fertile men have a poor semen analysis. Therefore, these results do not accurately reflect the fertility potential of the males in the cohort. However, a decrease in sperm concentration and motility are closely associated with a decrease in male fertility potential (Hammoud et al., 2008b). It is therefore clear that this data indicates the MetS is associated with a general decrease in sperm quantity and quality, potentially decreasing the chances of achieving a successful fertilisation and live birth.

Although not a central component of this thesis, and caution should be expressed in the interpretation of this data, it is interesting to note that there is a significant increase in a clinical history of males with recent couple infertility in the MetS group. However, this does not include any potential female factor infertility, only male partners of couples with infertility as defined by the WHO (2010), and thus caution is required in the interpretation of this significance. Similarly, the CG had a significant

increase in males with a live birth within the last two years of the consultation date. These interesting statistics may indicate a reduced fertility potential in males with MetS, and certainly warrant that studies related to fertility rates and live births in couples in which the male partner has MetS be undertaken.

Many semen parameters correlated with each other as expected. However, there was little significant correlation within the cohort between clinical and biochemical parameters associated with MetS and semen parameters. This was partially evident in very few correlations in the CG and MetS groups. Within the entire cohort, age correlated negatively with ejaculation volume, progressive and total motility and vitality and positively with DNA fragmentation.

4.2.1. Semen parameters and body weight

The effect of BMI and sperm parameters has been investigated in numerous studies. In a systemic review of the literature, over 10 000 articles, including duplicates, are reportedly available (Sermendade et al., 2013). However, the effect of BMI on sperm characteristics remains controversial (Sermendade et al., 2013). Within the cohort, BMI and waist circumference (reflecting abdominal obesity) correlated negatively with sperm concentration, total sperm count, total motility and vitality, and positively with MMP and DF. These correlations were not seen in the CG or MetS groups. These correlations are not always supported in the scientific literature.

Hofny and colleagues (2009) negatively correlated BMI with sperm concentration and motility, but also with abnormal morphological forms, in male partners of couples attending a fertility clinic. In a similar cohort of males seeking fertility treatment, Hammound and colleagues (2008b) found an increasing incidence of oligospermia and reduced motility correlated to increasing BMI. Investigating normal weight, overweight and obese individuals, Fejes and colleagues (2006) reported a negative correlation with BMI and sperm concentration. Prior to this publication, Fejes and colleagues (2005) found that an increasing waist circumference (and hip circumference) negatively correlated with sperm concentration and motility in a cohort of male patients presenting with infertility. In Danish men recruited for the military, Jensen and colleagues (2004) found a negative correlation between sperm

count and concentration with BMI. These studies generally agree with the correlations between BMI, waist circumference and semen parameters observed in this study, and further studies from various authors (Kort et al., 2006; Pauli et al., 2008; Chavarro et al., 2010; Håkonsen et al., 2011).

A meta-analysis by Sermondade et al. (2013) found that obesity is associated with an increased risk for oligozoospermia or azoospermia. However, many authors have found no relationship between BMI and sperm profiles in males (Aggerholm et al., 2008; Chavarro et al., 2010; Rybar et al., 2011). Aggerholm and colleagues (2008) found interesting yet mixed results, with a marginally negative effect of BMI on sperm quality, despite a significant effect on sex hormones. A similar conclusion was published by MacDonald et al. (2010), reporting no negative association between increased body weight and reduced semen parameters alongside strong evidence for reduced testosterone with increased body mass index in a limited meta-analysis. Interestingly, there was no correlation between poor sperm morphology and BMI in this study, which is consistent with many studies (Fejes et al., 2005, Qin et al., 2007, Pauli et al., 2008; Chavarro et al., 2010; Rybar et al., 2011, Tunc et al., 2011, Fariello et al., 2013), but in contrast to other studies indicating a relationship (Jensen et al., 2004; Kort et al., 2006; Hammoud et al., 2008b; Hofny et al., 2009; Kriegel et al., 2009; Paasch et al., 2010). The inconsistency in results may be due to various potential confounders, such as lifestyle, nutrition and genetic backgrounds. Further factors that might have to be considered are the numerous metabolic, endocrine, immunological and physical factors associated with obesity and the MetS that have not been fully elicited.

Although the molecular structure of spermatozoa is a well accepted parameter in order to achieve a successful pregnancy (Palmer et al., 2012a), a more limited number of studies have assessed correlations between obesity and DF and MMP. BMI and waist circumference (WC) were both positively correlated with MMP and DF in the cohort, but not the CG and MetS groups. The negative impact of BMI on DF and MMP reported in this study has been observed in previous studies (Kort et al., 2006; Agbaje et al. 2007; Kriegel et al., 2009; Bakos et al., 2010; Chavarro et al., 2010; Fabriello et al., 2012; La Vignera et al., 2012b). Although it can be hypothesised that MetS related phenomena, such as inflammation and oxidative

stress may mediate damage to spermatozoa mitochondria and DNA integrity, the mechanisms of these relationships require further information, however.

4.2.2. Semen parameters and metabolic syndrome components

Relationships found between WC, a component of MetS, and sperm parameters have been discussed above. Correlations between the additional components of MetS, namely hypertension, dyslipidaemia and glucose intolerance are further discussed here. The scientific literature has very limited studies investigating these relationships independently, reviewed in detail by Kasturi et al. (2008). Within this study, correlations between MetS components and sperm parameters indicated few relationships within the cohort.

Hypertension is a pertinent feature of MetS, and closely related to an increased risk of CVD. Hypertension has also been established as an independent risk factor for erectile dysfunction. However, there are no conclusive studies linking hypertension with reduced fertility potential in males (Kasturi et al., 2008). Results of a study by Ramírez-Torres and colleagues (2000) reported no correlation between sperm abnormalities and hypertension. More recently, hypertension has been strongly associated with increased DF in a small pilot study (Muciaccia et al., 2012). This study found no correlation between blood pressure and DF, and most other sperm parameters assessed. Limited and relatively weak negative correlations were found for diastolic blood pressure and sperm concentration, total motility and vitality within the entire cohort only.

Although Ramírez-Torres et al. (2000) reported no correlation between sperm abnormalities and hypertension, they did report a relationship between dyslipidaemia and sperm abnormalities. Non-obstructive azoospermia has also been associated with dyslipidaemia in human males (Bobjer et al., 2012). Shalaby et al. (2004) reported a potential role for dyslipidaemia in the development of infertility in male rats fed a high cholesterol diet, with improvements in fertility parameters on simvastatin and α -tocopherol treatment. Hypercholesterolaemia induced by diet in rabbits was associated with reduced semen volume, motility and normal morphology (Saez Lancellotti et al., 2012). In a Japanese population, increased triglycerides have also

been associated with poor semen quality and androgen levels (Haqiuda et al., 2012). The data in this study found no direct correlation between HDL cholesterol or triglycerides with any semen parameter. However, the association with hypertension and dyslipidaemia in various studies may not be an independent factor, but rather associated features with common underlying pathophysiological features associated with MetS, such as insulin resistance, inflammation and oxidative stress. This is further indicated by Shalaby et al. (2004), where a small but significant improvement in semen characteristics was observed with cholesterol lowering medications (simvastatin), but highly remarkable improvements were seen in a subgroup of animals given both simvastatin and α -tocopherol (a potent antioxidant), implying a role of oxidation in the setting of dyslipidaemia as an important mechanism of decreased fertility potential.

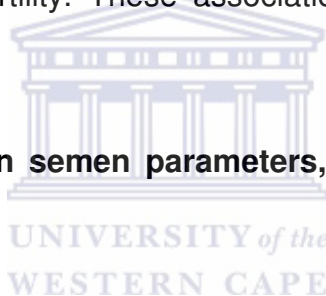
Correlations with fasting blood glucose and semen parameters were more prominent in this study, showing negative correlations with progressive motility, total motility and vitality and a positive correlation with DF within the entire cohort, and a negative correlation with progressive and total motility in the MetS group. However, Ramírez-Torres et al. (2000) found no correlation between glucose and sperm abnormalities. The correlation between blood sugar on DF does agree with a recent publication by Palmer et al. (2012a), which reported a positive correlation between glycaemia and sperm DF regardless of adiposity, in mice fed a high fat diet. This study found a negative correlation for dysglycaemia to normal morphological sperm, which was not observed in this study, which is contrary to Palmer et al. (2012a).

Mallidis and colleagues (2011) induced MetS characteristics in male rabbits fed a high fat diet for 12 weeks, although this study did not assess the impact of the syndrome itself on reproductive function. Within the context of induced hypogonadotropic hypogonadism, experimental rabbits did not find increased DF in spermatozoa, with no histological changes in the testes or epididymal histology. They concluded that there are minimal effects on spermatogenesis and sperm quality, and attributed these effects to increased blood glucose rather than hypogonadism. This conclusion would agree with the correlations found in this study pertaining to blood glucose and hypertension on sperm parameters. T2DM, a known consequence of MetS characterised by high blood glucose, has increasingly been

associated with male factor infertility in recent years (La Vignera et al., 2012a). Poor semen quality, such as reduced sperm concentration and motility, abnormal morphology, mitochondrial DNA damage, nuclear DNA damage and increased seminal plasma abnormalities have been reported (Amaral et al., 2008; La Vignera et al., 2012a). However, if this is directly related to glucose intolerance or the complex underlying metabolic dysfunction associated with MetS is not well described nor investigated.

Neither the scientific literature nor the data in this study support any cause and effect hypothesis. Any relationship between individual components of MetS and semen parameters may be due to common underlying mechanisms. However, there is the potential that components of the MetS expressed in patients may further negatively impact reproductive potential in males, and hence may provide a compounding detrimental effect on male fertility. These associations require further clarification from further research.

4.2.3. Relationships between semen parameters, blood insulin and systemic inflammation



Fasting blood insulin and insulin resistance (IR), as established by the Quantitative Insulin Check Index (QUICKI) (Katz et al., 2000; Hrebicek et al., 2002; Rabasa-Lhoret et al., 2003), strongly correlated with each other as expected in the cohort, as well as the CG and MetS groups. Both serum insulin and IR further strongly correlated as expected with BMI, WC, systolic blood pressure, diastolic blood pressure, HDL, triglycerides and fasting glucose in the cohort. Serum hs-CRP, as a reflection of inflammation (Brooks et al., 2010), was also correlated as expected with BMI, WC, systolic blood pressure, diastolic blood pressure, HDL, triglycerides and fasting glucose in the cohort. These are expected correlations within the context of MetS.

Fasting blood insulin correlated negatively with ejaculation volume, semen concentration, total sperm count, motility and vitality in the cohort, with a positive correlation with abnormal MMP. Similarly, IR correlated positively with ejaculation volume, semen concentration, total sperm count and vitality, in addition to

progressive and total motility, in the cohort, with a negative correlation with abnormal spermatozoa MMP and DF. There was no correlation with morphology. On reviewing the literature, there are no studies indicating correlations between serum insulin and IR with sperm parameters. hs-CRP correlated negatively with semen concentration, total sperm count, total motility, progressive motility, and vitality in the cohort, with a positive correlation with abnormal MMP and DF. Similarly, there are no reports identified in the literature in which to contrast these findings.

These correlations, although weak, suggest an important influence between insulin and systemic inflammation and spermatogenesis. As insulin directly and indirectly modulates reproductive function (Pasquali et al., 1995; Pasquali et al., 1997; Andò & Aquila, 2005; Lampiao et al., 2009), this is a plausible consideration discussed in further detail below. These serum markers could also be considered as potential markers for reduced male fertility if these possible correlations can be further researched and confirmed by additional studies.

4.3. Metabolic syndrome and salivary steroid hormones in males

Hypogonadism has been well established in adult males with MetS (Pasquali 2006; Kasturi et al., 2008; Guay 2009; Saad & Gooren, 2009). In addition, it is emerging that reduced serum testosterone in non-obese men, including those with asymptomatic androgen deficiency, increases the risk of developing obesity and MetS, as well as developing T2DM and CVD (Boyanov et al., 2003; Kupelian et al., 2006; Traish et al., 2009), with further lines of evidence suggesting that the clinical administration of testosterone can improve many of the characteristics associated with the syndrome (Saad & Gooren, 2009). Therefore, in males, testosterone appears to be a significant hormone involved in the aetiology and pathogenesis of MetS.

The results of this study indicate that free testosterone, determined via saliva samples of the male participants, is significantly decreased in the MetS group compared to the controls. Although there are no reports identified in the literature investigating the effect of MetS on free testosterone measured in saliva, this study

agrees with the well described literature of male hypogonadism associated with MetS (Pasquali 2006; Kasturi et al., 2008; Guay 2009; Saad & Gooren, 2009).

Changes in free progesterone concentrations associated with MetS in males have not been previously investigated. A significant reduction in progesterone alongside reduced testosterone in the MetS group when compared to the CG is a novel finding. As progesterone is a well defined precursor hormone in testosterone synthesis (Sherbet et al., 2003), this finding suggests that steroidogenesis cascades may be compromised in males with MetS.

Progesterone, as a well defined 'female' and pregnancy hormone has been traditionally ignored in male physiology and pathology. This is highlighted by very few detailed and comprehensive studies assessing progesterone in the male available on searching the scientific literature. This is particularly interesting, as adult men and woman generally have similar concentrations of serum progesterone except during the luteal phase of the menstrual cycle and during pregnancy (Oetell & Mukhopadhyay, 2004; Andersen & Tufik, 2006). The majority of research in this field has focused on male contraception (McLachlan et al., 2004; Wang et al., 2010), the treatment of male hypersexuality (Andersen & Tufik, 2006) and the role of progesterone in fertilisation (Thomas & Meizel, 1991; Foresta et al., 1992; Emilioza et al., 1996; Meizel & Turner, 1996; Baldi et al., 2002; De Amicis et al., 2011). The general view of progesterone in males is therefore limited to an essential precursor for testosterone synthesis via its immediate metabolite, 17α -hydroxprogesterone (17α -OH-P), which is further hydrolysed into androstenedione, which is itself hydrolysed into testosterone following the Δ^5 -steroid pathway (as opposed to the Δ^4 -steroid pathway via dehydroepiandrosterone) (Sherbet et al., 2003; Oettel & Mukhopadhyay, 2004; Midzak et al., 2009; Ye et al., 2011).

Progesterone has not been completely studied in men who are overweight or obese, been diagnosed with MetS or T2DM. The results of this study indicate that saliva concentrations of free progesterone are significantly reduced in MetS positive males. Considered with the reduced testosterone concentrations in saliva, it would be logical, albeit premature, to suggest that testosterone production is reduced due to a restriction in the progesterone precursor supply based on the Δ^4 -steroid pathway.

This would agree with Isidori et al. (1999), who found that serum 17α -OH-P is reduced in obese patients compared to controls, and moderately obese (BMI 30 – 40) patients compared to those who were described as massively obese (BMI > 40). However, testosterone may be sourced via the Δ^5 -steroid pathway, bypassing the need for progesterone and 17α -OH-P.

In rats fed a high fat diet, both progesterone and testosterone decreased in both serum and testes, which would agree with the results found in this male cohort (Ahn et al., 2013). This would imply that cholesterol conversion into pregnenolone may be affected by MetS, although the molecular mechanisms associated with these results are unlikely to be this simplistic.

These previous studies, in conjunction with results in this study, indicate that steroidogenesis cascades may be compromised, especially via the Δ^4 -steroid pathway for testosterone synthesis. FT and FP strongly correlated with each other within the cohort, as well as the CG and MetS groups, providing some evidence that the steroidogenic pathways may be compromised in patients with MetS. However, testosterone can be synthesised via the Δ^5 -steroid pathway, which would then bypass progesterone (Sherbet et al., 2003). Future research would need to investigate the effect of dehydroepiandrosterone (DHEA) to further explore a potential impact on this alternative pathway.

The mechanisms of these relationships require further investigation. Obesity and MetS in males is associated with both a decrease in testosterone and an increase in oestrogen (Kasturi et al., 2008; Guay, 2009). This is due to an increase cytochrome P450 aromatase activity, particularly in white adipose tissues, with increased expression in increasing obesity and MetS (Cabler et al., 2010). The activity of aromatase would increase oestrogen while drawing on the pool of testosterone. Another metabolite of testosterone, dihydrotestosterone (DHT), is converted from testosterone via the enzyme 5α -reductase. DHT in obesity and MetS has not been fully investigated, but appear to be decreased in obesity (Blanchette et al., 2006). It is therefore well accepted that peripheral testosterone metabolism into 17β -oestradiol via an upregulation on aromatase in adipose tissues is a well-defined mechanism for hypogonadism associated with MetS (Kasturi et al., 2008; Guay,

2009). This would not necessarily explain why testosterone levels are not maintained by upregulating steroidogenesis via negative feedback mechanisms, nor would this explain the decreased progesterone observed in the male cohort with MetS (and obesity as described by Blanchette et al. (2006). Levels of LH appear to be low or normal in males with obesity and/or MetS, with a better defined decrease in LH-pulse amplitude. There is also an associated decrease in gonadotropin secretion from the hypothalamus and a breakdown of the hypothalamus-pituitary-testis (HPT) axis (Kasturi et al., 2008; Guay, 2009). A decline in gonadotropin stimulation of the pituitary, and/or a decrease in LH stimulation of Leydig cell steroidogenesis would explain a decrease in both progesterone and testosterone observed in this study.

Insulin (Lampiao et al., 2009), leptin (Lampiao et al., 2009), inflammatory cytokines (Hales et al., 1999; Bornstein et al., 2004) and oxidative stress (Diemer et al., 2003) are all proposed to directly modulate Leydig cell steroidogenesis, with all of these phenomena associated with an increase in serum in males with MetS (Kasturi et al., 2008). It can be hypothesised that these phenomena may provide a novel explanation for reduced steroidogenesis (progesterone and testosterone), in addition to effects on semen parameters. However, the mechanisms for this relationship will require further investigation.

As the decrease in progesterone and testosterone indicates a collapse of steroidogenesis in the Δ^4 -steroid pathway, testosterone could still be synthesised via the Δ^5 -steroid pathway. Firstly, insulin, leptin, cytokine or oxidative stress induced decreased in LH mediated signalling, StAR activation and/or P450_{scc} regulation would collapse both pathways. However, this is not clear in the data obtained in this study, with 17 α -pregnenolone and DHEA as important hormones in the Δ^5 -steroid pathway. DHEA is a known modulator of endothelial function, inflammation, insulin sensitivity, blood flow, cellular immunity, body composition, bone metabolism, sexual function, and physical strength in frailty and provides neuroprotection, improves cognitive function, and memory enhancement (Traish et al., 2011). DHEA-sulphate is negatively correlated with body weight in males, and progesterone and DHEA-sulphate are closely correlated with each other, and both negatively correlated with BMI, waist circumference and subcutaneous adipose diameter (hip circumference) (Blanchette et al., 2006). However, DHEA-sulphate is a derivative of DHEA, and any

decline may not be associated with a decrease in DHEA itself. This is, however, biologically plausible, and the impact of decreased DHEA on male reproduction, as well as within MetS and its consequences, requires further study by scientists.

4.3.1. Relationship between steroid hormones clinical and biochemical parameters

Age was negatively correlated with FT in the cohort, as well as the CG and MetS groups. Androgen decline is well established in ageing males (Dohle et al., 2003). FP, however, was not significantly associated with age in either group. This is in contrast to the study by Pirke et al. (1980), in which progesterone may be increased in ageing males, possibly inhibiting testosterone synthesis and Leydig cell function (Gruenewald et al., 1992). FT and FP was negatively correlated with BMI and WC in the cohort, but not in the CG or MetS groups. Androgens are well accepted to be decreased in obese males, as well as MetS (Kasturi et al., 2008). A decline in FP with obesity has been suggested by Blanchette and colleagues (2006), alongside 17-OH-P, DHEA-sulphate, testosterone and dihydrotestosterone (DHT), further indicating steroidogenesis collapse associated with obesity.

Several studies have found an inverse relationship between blood pressure and total serum testosterone concentrations, which may result in impaired reproductive potential (Kusturi et al., 2008). Results of this study found no relationship between blood pressure and FT within the entire cohort, or either group. No relationship was found between FP and blood pressure either. Interestingly, HDL cholesterol was correlated with FP in the cohort, but not FT. Both FT and FP negatively correlated with triglycerides. There are no studies identified investigating relationships between lipids and FP in males. However, numerous studies assessing obesity and MetS in males correlate dyslipidaemia with hypogonadism (Kasturi et al., 2008). Neither FT nor FP correlated with fasting blood glucose.

Several studies inversely correlate testosterone with BMI and insulin, and hyperinsulinaemia may be a common aetiological factor for both hypogonadism and onset of T2DM (as reviewed by Kasturi et al., 2008). Increasing insulin administration has also been associated with decreased Leydig cell testosterone secretion

independent of changes at the hypothalamus or pituitary (Pitteloud et al., 2005). Within the cohort, QUICKI (but not fasting blood insulin) was negatively correlated with FT in the cohort, indicating that testosterone declines with increasing IR. This was not observed in with FP. Similarly, systemic inflammation and hypogonadism are well correlated in males with MetS (Kasturi et al., 2008). Within the cohort, FT correlated negatively with CRP.

However, in this study there was no correlation between FP and hs-CRP (although a weak negative correlation between serum and seminal TNF α was found). The correlations between testosterone, insulin and CRP were not as strong as would be expected. Furthermore, these correlations were absent within the MetS group. A relatively small cohort may be a reason for this. However, as this is the first study assessing FT in saliva samples, these correlations may not be as strong as serum samples possibly are.

4.3.2. Relationship between steroid hormones and semen parameters

FT correlated with semen concentration, total sperm count, progressive and total motility and vitality in the cohort. These correlations were not observed in the CG or MetS groups. There was no correlation in any group for FT and ejaculation volume, normal morphology, MMP or DF. FP did not correlate with any semen parameter assessed in any group. As testosterone is an essential hormone in the maintenance of spermatogenesis, it has a well established close association with sperm concentration and sperm count, as observed in the cohort. However, this testosterone is within the reproductive tract, where it is generally concentrated 25-125 times compared to that of serum levels (Dohle et al., 2003; Walker, 2011). There is no data in this study to suggest a decrease in androgens, or progesterone, within the reproductive tract.

It is also known that progesterone influences spermatogenesis within the testes (Oettel & Mukhopadhyay, 2004). Similarly, there is no data to indicate that this is negatively influenced in this study. However, a decrease in both testosterone and progesterone in the reproductive tract is certainly plausible. Furthermore, considering the essential actions of progesterone on sperm function after ejaculation, as well as

fertilisation (Thomas & Meizel, 1991; Foresta et al., 1992; Emilioza et al., 1996; Meizel & Turner, 1996; Baldi et al., 2002; De Amicis et al., 2011), it will be of importance to establish if there is a related change in progesterone within seminal fluid. Some studies have indicated that sperm obtained from oligospermic males have decreased sensitivity to progesterone stimulation, suggesting that progesterone has a key role in sperm development (Falsetti et al., 1993; Oehninger et al., 1994). It could be hypothesised that decreased progesterone within the male reproductive tract may negatively influence spermiogenesis, in addition to reduced androgen stimulation, in males with MetS opening a novel avenue for molecular study. Some studies have suggested that a reduced or absent action of progesterone on ejaculated sperm can be a sole reason for infertility, especially in idiopathic cases (Tesarik & Mendoza, 1992). The confirmation and establishment of these plausible biological mechanisms requires further investigation.

4.4. The effect of metabolic syndrome on glucose and insulin concentrations

As discussed above, serum insulin and glucose was significantly increased in the MetS group, as was expected. However, changes in seminal plasma concentrations of glucose or insulin in patients with MetS has not previously been investigated. As glucose (Truta et al., 2010) and insulin (Lampiao et al., 2009) are found in human semen, and utilised in sperm function and/or fertilisation, any alterations in concentrations or function of these molecules may influence reproduction in males.

4.4.1. Serum and seminal glucose in metabolic syndrome

Glucose has been identified in human semen (Truta et al., 2010), with more than half the sugar consumed by ejaculated spermatozoa being in the form of glucose (Diamandis et al., 1999). In addition, although various sugars are used by spermatozoa post ejaculation, glucose is generally used more as time progresses (Diamandis et al., 1999). Although there have been glucose assessments in human semen for over 100 years, reports on normal concentrations vary widely, from 183 µg/ml – 1020 µg/ml (Truta et al., 2010). The study carried out by Truta et al. (2010) identified a mean concentration of 471.7 µg/ml glucose in seminal fluid from 30 normozoospermic males. However, Diamandis et al. (1999) reported a mean

concentration of 1180 µg/ml, with ranges as low as 36 µg/ml and as high as 2700 µg/ml, in 202 semen samples from healthy donors (there was no difference between normozoospermic, oligozoospermic and azoospermic males). Seminal glucose concentrations in this study showed a mean of 472.1 µg/ml in the CG and 357.1 µg/ml in the MetS group. There was a range across the entire cohort of 48.1 – 990.0 µg/ml, with both the lowest and highest concentration in the CG. Although there was a lower mean in the CG compared to the MetS group, this did not reach statistical significance ($P=0.0531$). The mean values and range of seminal glucose concentrations was found to be similar to previous reports outlined by Truta et al. (2010), but lower than that reported by Diamandis et al. (1999).

Mean serum glucose in the CG was 5.03 mmol/L, which is equivalent to 905.4 µg/ml, with seminal glucose mean at 472.1 µg/ml. This indicates a general lower concentration of seminal glucose compared to serum concentrations (a seminal to serum ratio of 0.52 based on the means). Similarly in the MetS group, mean serum glucose was 5.79 mmol/L, which is equivalent to 1042.2 µg/ml, and seminal glucose was 357.1 µg/ml (a seminal to serum ratio of 0.34 based on the means). This decreased seminal glucose compared to serum may be due to a tight control mechanism for glucose to pass from the peripheral circulation into the reproductive tract through the blood testis barrier (BTB), reducing glucose concentrations in order to optimally support and maintain spermatogenesis. Testicular cells have glucose sensing machinery which enable them to react and adapt to hormonal fluctuations and counteract hyper- or hypoglycaemic events, as reviewed in a recent publication by Alves and colleagues (2013).

Spermatogenesis maintenance *in vivo* is dependent on adequate glucose metabolism. The BTB tightly controls transport of glucose to germ cells and Sertoli cells, which are relevant cells for the functions of the BTB. Glucose transport across the BTB is mediated by various glucose transport molecules (GLUT's), such as GLUT1, GLUT3 and GLUT8, and are sensitive to various hormones (including insulin), inflammatory cytokines and growth factors (Alves et al., 2013). With changes in glucose or insulin, glucose transport machinery adapts in order to maintain lactate production (Alves et al., 2013). Insulin deprived Sertoli cells in culture show decreased glucose uptake via the BTB barrier (Alves et al., 2013).

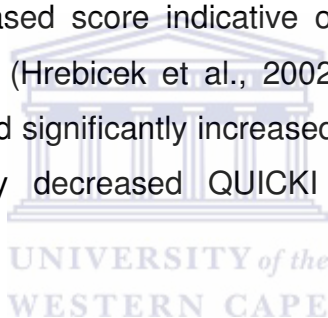
Therefore, it may be biologically plausible that insulin resistance in the setting of MetS may be associated with a decrease in glucose uptake across the BTB. This is however hypothetical, and further research in the physiology and pathophysiology of the BTB in relation to glucose, insulin sensitivity and inflammation is required.

Seminal glucose correlated negatively to serum concentrations of triglycerides, glucose, insulin and hs-CRP, and positively with insulin sensitivity in the cohort. These correlations were not observed in either the CG or MetS groups. These seemingly counter intuitive results are likely due to a non-significant decrease in seminal glucose observed in the MetS group compared to CG. Based on a biologically plausible hypothesis that glucose transport across the BTB may be comprised in the setting of insulin resistance, larger studies may find seminal glucose to be significantly decreased in males with MetS. Although the data does not support this speculation, if potentially true, this would likely be associated with negative correlations to MetS parameters such as triglycerides, serum glucose, insulin and hs-CRP observed in this study.

There is a positive correlation suggested in the literature between seminal glucose and sperm motility (Truta et al., 2010). However, other studies did not find such a correlation (Diamandis et al., 1999). Seminal glucose was negatively correlated with progressive and total motility, and positively correlated with sperm MMP in the MetS group, but not in the CG, nor in the cohort. These correlations support data published by Truta and colleagues (2010). ATP levels in sperm are maintained by several substrates, and both glycolysis and mitochondrial respiration are active in human sperm (Alves et al., 2013). Sperm capacitation is known to be stimulated by glucose, and also via oxidative stress possibly mediated via oxidative phosphorylation of glucose (Alves et al., 2013). A potential decrease in seminal glucose in MetS and T2DM may be a novel mechanism by which a decrease in motility and mitochondrial function is mediated, especially as glucose is an essential fuel for sperm activity and motility (Diamandis et al., 1999). Ejaculated sperm cell function may therefore be negatively influenced by a decrease in optimal concentrations of glucose, a hypothesis that would require further investigation, especially in the setting of MetS and T2DM.

4.4.2. Serum and seminal insulin in metabolic syndrome

Insulin is well described as the key hormone involved in the regulation of glucose and free fatty acid uptake by tissue cells, with roles in the promotion and regulation of growth, differentiation and metabolism (Kim et al., 2006a; Karnieli & Armoni, 2008). The concept of insulin resistance is used to describe the process whereby target tissues develop impaired sensitivity to the action of the hormone, particularly in adipose tissue, liver and skeletal muscle (Eckel et al., 2005; Huang, 2009; Gallagher et al., 2010). Obesity and increased WC is a predominant risk factor for the development of insulin resistance, and appears to play a central role in the pathogenesis of atherosclerosis and type 2 diabetes mellitus (Liao et al., 2005; Zeyda & Stulnig 2009; Gallagher et al., 2010). The Quantitative Insulin Sensitivity Check Index (QUICKI) is calculated from fasting blood glucose and insulin concentrations, with a decreased score indicative of decreased insulin sensitivity (increased insulin resistance) (Hrebicek et al., 2002; Yokoyama et al., 2004). As discussed, the MetS group had significantly increased serum insulin as compared to the CG, with a significantly decreased QUICKI (indicating increased insulin resistance).



Insulin has been reported as an important regulator of male reproduction via actions on the HPT axis, with modulating actions on gonadotropin releasing hormone (GnRH), LH and FSH centrally, and Leydig and Sertoli cell function locally (Lampiao et al., 2009). Furthermore, Pitteloud et al. (2005) published *in vivo* evidence that insulin resistance is associated with decreased testosterone secretion from Leydig cells in males. Furthermore, insulin is present in human semen, and insulin receptors are expressed on spermatozoa cell membranes, having important regulatory roles for sperm function and in fertilisation (Aquila et al., 2005; Lampiao et al., 2009). Interestingly, leptin as well as insulin, appears to be synthesised and secreted by ejaculated spermatozoa (Aquila et al., 2005b; Andò & Aquila, 2005). Insulin produced in uncapacitated sperm is found in granules predominantly on the midpiece and tail. This is associated with a massive intracellular decline and extracellular increase at capacitation, indicating a role in capacitation (Andò & Aquila, 2005). However, the role of insulin in male (in)fertility has not been well elicited and remains poorly understood.

The results of this study indicated a significantly increased insulin concentration in the semen in the MetS group compared to the CG. Serum and seminal insulin strongly correlated with each other, and seminal insulin inversely correlated with QUICKI (as did serum insulin). These correlations were found in the cohort, CG and MetS group. This suggests that insulin in the semen gains access to the reproductive tract via the BTB, seminal vesicles or prostate. Insulin and insulin-like peptides in human semen have previously been suggested to be secreted by the seminal vesicles (Paz et al., 1977; Stahler et al., 1987). García-Díez and colleagues (1991) concluded that insulin appears to freely cross the BTB into the reproductive tract, and thereby supports findings of concentrated seminal insulin in this study.

Insulin was found to be highly concentrated in human semen as compared to serum. Based on the mean serum and seminal insulin concentrations, insulin is 28.7 times more concentrated in seminal fluid than in serum in the CG, and 42.7 times more concentrated in the MetS group. There are a few studies in the literature which compare the seminal insulin concentrations too. Hicks et al. (1973) found that insulin was more than twice as concentrated in human semen as compared to serum in non-diabetic men (19 ± 3 $\mu\text{U/ml}$ and 7.5 ± 1.5 , $\mu\text{U/ml}$ respectively). In a Portuguese publication, Pova Junior et al. (1973) observed higher seminal concentrations of 45.8 ± 15.1 $\mu\text{U/ml}$ in human semen of normozoospermic men. García-Díez and colleagues (1991) reported insulin to be concentrated in human semen compared to serum in human males with type 1 diabetes mellitus. This was across numerous groups, including fertile and infertile normoglycaemic subjects, carbohydrate intolerant subjects and excretory and secretory azoospermic subjects.

Seminal insulin, alongside serum insulin and insulin resistance, correlated with BMI, WC, blood pressure, triglycerides HDL (negative correlation) and hs-CRP in the cohort. Seminal insulin correlated with BMI and WC in the CG, but not the MetS group. Numerous metabolic and immunological features of MetS are therefore associated with increased seminal insulin, and it can be postulated that this has a negative influence of sperm function and fertilisation capability in males with MetS.

Seminal insulin correlated negatively with sperm concentration in the cohort, but not CG and MetS groups, and motility (progressive and total) in the cohort, CG and MetS groups.

Although García-Díez and colleagues (1991) reported no correlation between seminal or serum insulin and sperm parameters in males with type 1 diabetes mellitus, Lampiao & du Plessis (2008a) showed that ejaculated healthy sperm exposed to 10 μ U insulin significantly increased total and progressive motility, acrosome reaction and nitric oxide production *in vitro* after 1, 2 and 3 hours of incubation. Further lines of evidence (Pasquali et al., 1995; Andò & Aquila, 2005; Pitteloud et al., 2005) support the notion that insulin concentrations in serum and semen can directly and indirectly modulate the HPT axis, spermatogenesis and sperm function.

The data available from this study indicates an important role for seminal insulin in the sperm function. Although acute increase in insulin exposure may increase motility and acrosome reaction in the spermatozoa (Andò & Aquila, 2005; Lampiao & du Plessis, 2008a), this study found a negative correlation with seminal insulin and motility. As the increased seminal insulin is in the setting of insulin resistance and MetS, increased insulin exposure during spermatogenesis may develop insulin resistance in the spermatozoa themselves. Evidence to support this hypothesis may be found in the intracellular molecular cascades associated with insulin receptor stimulation in these cells. Insulin exert its effect on spermatozoa via the PI3K/Akt intracellular signalling pathway, leading to protein kinase B (PKB) phosphorylation (Aiston & Agius, 1999; Andò & Aquila, 2005), which may mediate beneficial effects on ejaculated spermatozoa (Lampiao et al., 2009). This pathway ultimately increases cellular nitric oxide production (Aiston & Agius, 1999; Andò & Aquila, 2005; Lampiao et al., 2009). In human tissues, particularly hepatocytes, adipocytes and skeletal muscle cells, this intracellular pathway is negatively influenced in insulin resistance (Huang, 2009). Therefore, over the spermatogenic cycle, it is conceivable that spermatozoa may induce insulin resistance in a manner similar to other tissue cell. This hypothesis would provide an explanation as to the potential negative association between increased seminal insulin and reduced motility of ejaculated sperm. This requires further detailed investigation.

Insulin-dependent (type 1) diabetic males have severe structural and motility abnormalities with sperm, indicating the important role in morphology and function of insulin (Baccetti et al., 2002). Although this occurs in patients with low insulin, insulin resistance could result in a similar end point. As this data indicates that serum and seminal insulin is negatively associated with sperm motility, this may provide some evidence that spermatogenesis is subjective to negative effect of insulin resistance. However, there was no correlation with normal morphology and insulin in this study.

4.5. The effect of metabolic syndrome on serum and seminal leptin

Leptin, a predominantly adipocyte-derived polypeptide, has a significant role in the regulation of body weight, appetite and energy expenditure, mediating many of these actions via the hypothalamus (Bastard et al., 2006; Kwon & Pessin, 2009; Wozniac et al., 2009; Galic et al., 2010). Furthermore, leptin exerts numerous effects on the immune, endocrine, metabolic and reproductive systems, playing a key and complex psycho-neuro-immuno-endocrine function (Casabiell et al., 2001). Leptin therefore has a direct and independent effect on the HPT axis, influencing spermatogenesis and steroidogenesis at the very least (Casabiell et al., 2001). Leptin has been strongly associated with a role in the pathophysiology of MetS, although this role has not been well understood or described (Bastard et al., 2006; Esteghamati et al., 2009).

The results of this study agree with the literature in general, with the MetS group showing significantly increased serum leptin. Previously unreported in the literature, seminal leptin concentrations were also significantly increased in the MetS group. Serum leptin strongly correlated with seminal leptin in the cohort, as well as the CG and MetS group. These correlations suggest that leptin may freely cross into seminal plasma, either across the blood-testes barrier, or alternatively via the seminal vesicles or prostate directly. However, results should be interpreted carefully, due to the generally lower number of samples analysed.

Various studies have assessed leptin concentrations in male serum, although an adequate reference range appears elusive. Hanafy et al. (2007) reported serum

leptin mean of 6.8 ng/ml in fertile male control patients, and a significantly increased concentration of 16.3 ng/ml in oligozoospermic infertile males. These serum ranges agree with concentrations found in the male cohort of this study. Niskanen et al. (1997) found mean serum leptin concentrations of 19.8 ng/ml in obese males. Zitzmann et al. (2005) reported mean serum leptin concentrations of 3.0 ng/ml in 27 healthy males. Zorn et al. (2007) reported serum concentrations of 12.4 – 18.2 ng/ml in a male cohort with fertility complaints. Serum leptin concentrations in the cohort of this study therefore generally agree with concentrations found in the literature.

Based on the results of this study, seminal leptin appears to be concentrated compared to serum levels. However, Thomas and colleagues (2013), comparing serum and seminal leptin in normal weight to overweight men, found serum levels to be higher than seminal levels. In 41 normal weight males, a median of 2.36 ng/ml was found in serum, and 0.91 ng/ml in semen. In 55 overweight or obese males, a median of 8.69 ng/ml was found in serum, and 0.83 ng/ml in semen (Thomas et al., 2013). These are comparable to serum concentrations in this study as indicated above, but seminal concentrations are much lower in comparison. Camiña et al. (2002) reported a mean seminal leptin concentration of 0.95 ng/ml in 40 healthy male donors, also much lower than the concentrations found in the CG of this study. Glander et al. (2002) found seminal leptin concentrations of 1.5 ng/ml in normozoospermic infertile men, and 3.2 ng/ml in pathozoospermic infertile men. These previous studies all reported lower seminal concentrations of leptin than reported in the cohort of this study.

4.5.1. Correlations of seminal and serum leptin with clinical and biochemical parameters

Age did not correlate with serum or seminal leptin in the cohort, or either group. Both serum and seminal leptin positively correlated with BMI, WC, serum insulin and hs-CRP, with a negative correlation with QUICKI (insulin sensitivity). These correlations were not found in the CG or MetS groups.

These results agree with the literature suggesting that serum leptin is associated with abdominal obesity (BMI and WC) and serum insulin and insulin resistance

(Isidori et al., 1999; Esteghamati et al., 2009). Although it appears that leptin has a role in MetS independent of BMI, it exerts detrimental effects through the setting of obesity (Esteghamati et al., 2009). In fact, the five MetS participants with a BMI < 30 (normal weight or overweight, not obese) and with a recorded serum leptin result, had a mean leptin concentration of 21.3 ng/ml. Thus, this is more comparable to the CG than MetS group. This subset sample is too small for statistical analysis, but would indicate that increased leptin in MetS may be related to the abdominal obesity component specifically, agreeing with Esteghamati et al. (2009). In a study of normal weight compared to overweight males (Thomas et al., 2013), leptin was significantly increased in serum of the overweight males as would be expected. However, seminal concentrations of leptin were not significantly different, with no correlation between serum and seminal concentrations (Thomas et al., 2013). These authors would disagree with results in this study, correlated seminal and serum leptin with BMI and WC.

The close association between insulin and insulin resistance is reportedly independent of BMI, indicating that leptin exerts independent detrimental consequences within the aetiology and or pathophysiology of MetS (Esteghamati et al., 2009). Leptin is known to be associated with inflammation. The correlation with hs-CRP within the cohort of this study agrees with previous research (Meyers et al., 2005). Leptin is considered an acute phase protein, and is further correlated in the literature to cytokines such as TNF α , IL6 and IL1 in acute and chronic inflammatory states (Meyers et al., 2005). Therefore, leptin, insulin and inflammation are closely associated with each other within the cohort of this study.

Serum leptin is generally associated with male hypogonadism, particularly in obesity, in the scientific literature (Isidori et al., 1999). Interestingly, the results in this study did not show a correlation between serum or seminal leptin and either testosterone or progesterone as expected. This may be due to the low numbers used in the correlation analyses. However, increased concentrations of leptin have been closely associated with a decrease in testosterone production at the level of the testes (Tena-Sempere et al., 2001; Tena-Sempere et al., 2002).

4.5.2. Impact of seminal and serum leptin on sperm parameters

Leptin is found in human seminal fluid, and leptin receptors are expressed on spermatozoa in addition to soluble receptors in seminal fluid (Jope et al., 2003). Interestingly, leptin (as well as insulin) appears to be synthesised and secreted by ejaculated spermatozoa, particularly mediating motility (Aquila et al., 2005b; Andò & Aquila, 2005). In uncapacitated samples, leptin is found within intracellular granules in the midpiece predominantly, and decreases significantly at capacitation, indicating a role alongside insulin in capacitation (Andò & Aquila, 2005). However, the source of seminal leptin is not known based on the data.

Both serum and seminal leptin negatively correlated with semen concentration, total sperm count, total motility, progressive motility and morphology, with a positive correlation with DF. These correlations were not found in the CG or MetS groups. Glander and colleagues (2002) reported an association between increased seminal leptin and a reduced sperm function, particularly a negative correlation with motility. These findings agree with the correlations in this study, where motility correlated negatively with seminal (and serum) leptin. However, Zorn et al. (2007) found no correlation between serum leptin and any sperm parameters investigating males with infertility problems (including azoospermia).

Injecting 8 – 10 week old rats with increasing dosages (5, 10 and 30 $\mu\text{g}/\text{Kg}$) of leptin over 42 days, Abbasihormozi and colleagues (2013) recently reported a dose and time dependent negative effect on progressive motility, as well as an increase in seminal reactive oxygen species (ROS) and DF using the TUNEL method. This would agree with results obtained in this study, with DF showing a positive correlation and progressive motility showing a negative correlation with both serum and seminal leptin and DF in the male cohort. Abbasihormozi et al. (2013) allude to an inflammatory activity of leptin based on an increase in seminal ROS. In addition, leptin injections induced a dose- and time-dependent decrease in testosterone (with increases in LH and FSH) (Abbasihormozi et al., 2013). There was no correlation between testosterone and leptin in this study. In the study by Thomas et al. (2013), serum leptin correlated negatively with sperm count and poor sperm morphology, which agrees with observations in this study. However, seminal leptin correlated

negatively with sperm volume, but positively with sperm motility (which would disagree with results in this study).

Alongside an investigation into the effects of insulin on human sperm function, Lampiao & du Plessis (2008a) published a study in which washed human spermatozoa from normozoospermic donors were exposed to 10 nmol leptin. Leptin was shown to significantly increase total and progressive motility, acrosome reaction and nitric oxide production *in vitro* at 1, 2 and 3 hours after incubation. This study indicates an important role for seminal leptin in the fertilisation process. Leptin appears to exert its effect via the PI3K/Akt intracellular signalling pathway, leading to protein kinase B (PKB) phosphorylation (Andò & Aquila, 2005). This is similar to that seen for insulin and leptin in other cell types, particularly hepatocytes, skeletal muscle and adipocytes, and leptin may mimic insulin action on glycogen synthase (Aiston & Agius, 1999). Ultimately, a key end point in this pathway is the mediation of nitric oxide, and hence a positive effect on motility in spermatozoa (Lampiao et al., 2009). Interestingly, this is the same intracellular pathway that is negatively affected with insulin resistance, associated with decreased nitric oxide production (Huang, 2009). These hypotheses would provide an explanation as to the potential negative association between increased seminal leptin, induction of leptin resistance in developing spermatozoa, and reduced motility of ejaculated sperm. Although Lampiao & du Plessis (2008a) found an increase in motility of ejaculated spermatozoa exposed to leptin, this was not found by Li et al. (2008), nor was there an effect on capacitated or acrosome reacted cells. The effect of leptin on various sperm parameters and fertilisation in physiology therefore needs further investigation, as does the potential detrimental effects of increased seminal leptin associated with MetS.

As leptin is akin in structure to the inflammatory cytokine IL6, it is biologically plausible that it may exert similar detrimental effects as previously reported by IL6 (discussed below). This may therefore directly implicate increased leptin in decreased Leydig cell steroidogenesis, impaired spermatogenesis, and impaired sperm morphology and increased DF. Alternatively, as leptin is closely correlated to IL6, these correlations may be due to increased levels of IL6, and not as direct influence of leptin itself. However, as Leydig cells, Sertoli cells and spermatozoa express

receptors for leptin (Aquila et al., 2005b; Lampiao & du Plessis, 2008a), it is likely that increased leptin concentrations in serum and semen directly influence sperm production and function, as well as steroidogenesis. This would require further research to elicit these associations and pathways (Ahima & Flier, 2000).

Seminal insulin and seminal leptin strongly correlated in the cohort. With an associated increase in serum, as well as potential overlaps on function in both of these hormones on function of ejaculated sperm (Andò & Aquila, 2005; Lampiao et al., 2009), these seminal increases offer novel and potentially critical areas of research for the impact of MetS, as well as obesity, on male reproduction, sperm function and fertilisation ability.

4.6. The effect of metabolic syndrome on serum and seminal inflammatory cytokines

MetS is associated with a low grade, systemic and chronic inflammatory state, demonstrated by a subtle and detrimental increase in serum CRP (Haffner, 2003; Tamakoshi et al., 2003; Brooks et al., 2010). This is associated with increased serum inflammatory cytokines, particularly tumour necrosis factor-alpha (TNF α), interleukin 1-beta (IL1 β), interleukin-6 (IL6) and interleukin 8 (IL8) (Haffner 2003; Tamakoshi et al., 2003; Khaodhiar et al., 2004; Kim et al., 2006b; Brooks et al., 2010; Tack et al., 2012).

As expected, the results of this study agree with the well defined literature (Haffner 2003; Tamakoshi et al., 2003; Khaodhiar et al., 2004; Kim et al., 2006b; Brooks et al., 2010; Tack et al., 2012), indicating that serum TNF α , IL1 β , IL6 and IL8 are all significantly increased in MetS males compared to the control group. Furthermore, within the cohort, the serum cytokines correlated strongly and positively with each other, as well as serum leptin (with the exception of serum IL8 and leptin). These correlations were not always preserved when analysing the CG and MetS correlations. This may be due to the smaller numbers within the correlation equations not providing a strong enough correlation for significance. Nevertheless, this data indicates a strong pro-inflammatory relationship between the serum cytokines and leptin within the cohort.

Within the cohort, BMI and WC (abdominal obesity) were strongly correlated with serum TNF α , IL1 β and IL6, but not IL8, in addition to serum leptin and insulin as discussed elsewhere. Furthermore, hs-CRP was strongly correlated to all cytokines in addition to leptin in the cohort. These correlations again agree with the well-defined literature, which suggests that adipocytes and immune activity within white adipose tissue are a major source of cytokines and inflammation in obesity (Nawrocki et al., 2004; Juge-Aubry et al., 2005; Kintscher et al., 2008; Nishimura et al., 2009; Fuentes et al., 2013).

Further features of MetS as serum cytokines showed correlations, such as blood pressure positively correlating with all serum cytokines, HDL correlating negatively with serum TNF α , IL1 β and IL6, triglycerides correlating positively with serum TNF α and IL1 β , suggesting common underlying metabolic features associated with the MetS. Serum insulin and insulin resistance, based on the QUICKI, was also strongly correlated with all serum cytokines in the cohort, in addition to serum leptin and seminal insulin as discussed elsewhere. Again, it is well defined that inflammation, hyperinsulinaemia/insulin resistance and hyperleptinaemia are common phenomenon that underlies the poorly understood pathophysiology of MetS (Nawrocki et al., 2004; Kasturi et al., 2008, Kintscher et al., 2008; Phillips & Prins, 2008), and these correlations were expected.

4.6.1. Serum cytokine concentrations

A range of 3.8 – 12.5 pg/ml was found for serum TNF α in the CG, with 8 samples undetectable. The MetS group had a range of 5.7 – 45.7 pg/ml, with 5 samples undetectable. Although previous studies assessing serum TNF α in health and disease has shown a variation in concentrations, the medians (reported) and means (unreported) in addition to the ranges, generally agree with previous studies assessing serum TNF α concentrations. González et al. (2001) found 15 healthy subjects to have a concentration of 3.14 pg/ml. In 24 lean (BMI < 25) subjects, Strackowski et al. (2002) found a concentration of 5.34 pg/ml, in contrast to 5.76 pg/ml in 30 obese (BMI > 30) subjects. Khaodhiar et al. (2004) published a concentration of 0.3 pg/ml in 9 non-obese (BMI < 30) patients and 3.2 pg/ml in 41 obese subjects. Bahceci et al. (2007) found a concentration of 6.8 pg/ml in 30 lean

patients, 12.6 pg/ml in 30 non-diabetic obese patients, 19.6 pg/ml in 20 diabetic obese patients and 11.2 pg/ml non-obese diabetic patients. Healthy normal weight control participants (n=23) in a psoriasis study were found to have a concentration of 11.2 pg/ml with a range of 0 – 32.5 pg/ml (Arıcan et al., 2005). Maes et al. (2011) reported a concentration of 7.41 pg/ml in a group of 20 healthy control participants in a study assessing inflammatory correlates with chronic fatigue syndrome. Interestingly, Naz & Kaplan (1994) reported TNF α to be undetected in 20 healthy male participants.

Within the CG, a range of 7.6 – 24.7 pg/ml was found for serum IL1 β , with 10 samples undetectable. The MetS group had a range of 16.2 – 80.2 pg/ml, with 8 samples undetectable. Although previous studies assessing serum IL1 β in health and disease has shown a variation in concentrations, the medians (reported) and means (unreported) in addition to the ranges, generally agree with previous studies assessing serum IL1 β concentrations. Naz & Kaplan (1994) found a concentration of 20.1 pg/ml (range: 11 – 42 pg/ml) in 10 fertile (not defined) males, and 38.3 pg/ml (range: 0 – 111 pg/ml) in 10 males with infertility due to antisperm antibodies. González et al. (2001) found 15 healthy subjects to have a concentration of 0.67 pg/ml. Maes et al. (2011) reported a concentration of 3.6 pg/ml in a group of 20 healthy control participants in a study assessing inflammatory correlates with chronic fatigue syndrome.

A range of 4.5 – 14.0 pg/ml was found for serum IL6 in the CG, with 11 samples undetectable. The MetS group had a range of 6.1 – 100.6 pg/ml, with 6 samples undetectable. Although previous studies assessing serum IL6 in health and disease has shown a variation in concentrations, the medians (reported) and means (unreported) in addition to the ranges, generally agree with previous studies assessing serum IL6 concentrations. Naz & Kaplan (1994) found a concentration of 6.9 pg/ml (range: 0 – 18 pg/ml) in 10 fertile (not defined) males, and 28.8 pg/ml (range: 0 – 105 pg/ml) in 10 males with infertility due to antisperm antibodies. Fernandez-Real et al. (2001) found a concentration of 6.4 pg/ml in 132 normal weight males and 5.8 pg/ml in 96 normal weight females, and correlated IL6 concentrations with blood pressure, serum insulin and insulin resistance. Khaodhriar et al. (2004) published a concentration of 0.1 pg/ml in 9 non-obese subject (BMI <

30) and 1 pg/ml in 41 obese (BMI > 30) subjects. Bahceci et al. (2007) found a concentration of 6.6 pg/ml in 30 lean (BMI < 25) patients, 11.7 pg/ml in 30 non-diabetic obese patients, 15.9 pg/ml in 20 diabetic obese patients and 10.4 pg/ml non-obese diabetic patients, with IL6 correlating positively with hs-CRP and TNF α . Healthy normal weight control participants (n=23) in a psoriasis study were found to have a concentration of 4.2 pg/ml, with a range of 0 – 12.7 pg/ml (Arıcan et al., 2005).

Within the CG, a range of 4.3 – 20.9 pg/ml was found for serum IL8, with 2 samples undetectable. The MetS group had a range of 3.5 – 12.7 pg/ml, with 7 samples undetectable. Although previous studies assessing serum IL8 in health and disease has shown a variation in concentrations, the medians (reported) and means (unreported) in addition to the ranges, generally agree with previous studies assessing serum IL8 concentrations. González et al. (2001) found 15 healthy subjects to have a concentration of 3.68 pg/ml. In 24 lean (BMI < 25) subjects, Strackowski et al. (2002) found a concentration of 3.24 pg/ml, in contrast to a concentration of 4.31 pg/ml in 24 obese (BMI > 30) subjects. This was positively correlated to TNF α . Healthy normal weight control participants (n=23) in a psoriasis study were found to have a concentration of 12.9 pg/ml, with a range of 0 – 50.4 pg/ml (Arıcan et al., 2005).

With serum cytokine concentrations supported by previous studies, in the setting of a significant increase in inflammatory markers in the MetS group as compared to the CG, a further discussion and investigation of cytokines in seminal fluid of males with MetS is warranted.

4.6.2. Seminal cytokines and metabolic syndrome

Human seminal plasma contains significant levels of various cytokines normally present in the male genital tract (Huleihel et al., 1996; Dousset et al., 1997; Martinez et al., 2007; Politch et al., 2007). They are secreted by different parts of the male genital tract and may exert effects on steroidogenesis, spermatogenesis and sperm functions (Huleihel et al., 1996; Dousset et al., 1997; Martinez et al., 2007; Politch et al., 2007). In addition to beneficial roles within the HPT axis, inflammatory cytokines

in physiological concentrations in ejaculated semen are considered to be beneficial for fertility, and may promote sperm membrane lipid peroxidation beneficial for fertilisation (Basu et al., 2004; Martinez et al., 2007).

As peripheral inflammatory cytokines, associated with inflammatory disease, as well as local inflammatory cytokines associated with reproductive tract infections, are associated with a negative impact on male reproductive health and fertility, it is conceivable that low grade chronic inflammation associated with MetS may also have a negative influence on male reproduction. It is plausible that increased serum cytokines may be associated with increased seminal cytokines. In order to further investigate any relationship between MetS, serum and seminal inflammatory cytokines (i.e. inflammation), inflammatory cytokines in seminal fluid were assessed alongside serum cytokine concentrations in the male cohort.

All cytokines were found to be significantly increased in seminal fluid of the MetS groups as compared to the CG. This is a novel and important finding that has not been previously reported. This reflects a local reproductive tract inflammatory state in the absence of leukocytospermia or other clinical or biochemical causes of local inflammation. This increase is associated with an increase in serum cytokines concentrations, as well as increased serum and seminal insulin.

There was some correlation between seminal cytokines and other seminal fluid parameters. Seminal TNF α strongly correlated with seminal IL1 β , IL6 and IL8, but not seminal leptin or glucose, in the cohort and MetS groups. IL6 further correlated with seminal IL8 and insulin in the cohort and CG, and with seminal leptin in the MetS group only. However, seminal IL1 β did not correlate with any IL6, IL8, leptin nor glucose in the semen of any group.

There were strong correlations between most serum and seminal cytokines. Serum and seminal TNF α correlated strongly in all groups. Serum and seminal IL1 β correlated strongly in the cohort, but this was not observed in the CG or MetS groups. Similarly, serum and seminal IL6 correlated in the cohort, but not in the CG or MetS group. However, there was no correlation between serum and seminal IL8 in any group. This may be due to IL8 being highly concentrated in seminal fluid as

compared to serum, with a wide variation in concentrations. However, seminal IL8 concentrated strongly with seminal leptin in the cohort and MetS group, but not the CG. This strongly supports the concept that cytokines do not act in isolation, but rather in a network with other cytokines (Eggert-Kruse et al., 2001).

Furthermore, serum and seminal cytokines correlated generally with other cytokines in serum and seminal fluid. TNF α correlated with seminal IL1 β , IL6, IL8, leptin and insulin, as well as negatively with seminal glucose in the cohort only. Serum IL1 β correlated with seminal IL8, leptin and insulin in the cohort, with the correlation between serum IL1 β and seminal IL8 also observed in the MetS group. Serum IL6 further correlated with seminal IL8, leptin and insulin in the cohort, serum IL8 correlated with seminal insulin. Alongside correlations between hs-CRP and serum cytokines, all seminal cytokines strongly correlated with hs-CRP in the cohort, as did serum and seminal leptin and insulin. This suggests that hs-CRP may be a useful marker of reproductive tract inflammation in males with obesity and MetS, and may be of benefit in fertility assessments in these patients.

The source of these cytokines in the reproductive tract is not clear based on the scientific literature. Cytokines in seminal plasma have been suggested to originate from Leydig cells, Sertoli cells, the epididymis and the prostate, with expressions modulated during the seminiferous epithelium cycle (Huleihel et al., 1999; Martinez et al., 2007). Leydig cells function optimally in close proximity and in conjunction with unique macrophages which produce cytokines, amongst other communication proteins (Hales, 2002). An additional and previously unexplored consideration would be the role of epididymal adipose tissue itself, which, similarly to abdominal adipose tissue, may up-regulate expression of cytokines in MetS. The correlations reported in this study suggest that the inflammation associated with MetS is dependent on multiple inflammatory cytokines acting together, as well as leptin and insulin being associated with this inflammatory response. It is conceivable that the increase in reproductive tract inflammation may be, at least in part, due to serum cytokines crossing into the reproductive tract from an increase in serum concentrations. However, as Sertoli cells, Leydig cells and testicular macrophages are able to synthesis and secrete these cytokines, a change in cellular function within these cells may also account for the reproductive tract inflammation. However, high TNF α

concentrations in males with obstructive azoospermia (Seshadri et al., 2009) suggests that the testis may not be the only source of seminal TNF α , at least. An interesting consideration is the potential physiological and pathological role of epididymal fat in cytokine secretion, as change in these adipocyte structure and function may be akin to that of abdominal adipocytes in MetS, with detrimental consequences on sperm and testosterone. This hypothesis would require further investigation, as there is no evidence in the literature to support this notion currently. However, regardless of the source, it is likely that an increase in seminal cytokines provide a novel mechanism for infertility related to not only MetS, but obesity in general, as there is a strong correlation between all seminal cytokines and BMI and waist circumference (alongside similar correlations between serum cytokines and BMI/WC).

Much of the published literature related to cytokines in the male reproductive tract has been in the setting of genital tract infections (GTI), as GTI are associated with leukocytospermia and increased inflammatory cytokines (Koçak et al., 2002; Basu et al., 2004; Eggert-Kruse et al., 2007; Martinez et al., 2007; Politch et al., 2007; Gallegos et al., 2008; La Vignera et al., 2011a). This is associated with infertility in males (Politch et al., 2007). Numerous lines of evidence have indicated a reduction in sperm count, motility and reduced male fertility potential as a result of increased inflammation and cytokine activity (Gruschwitz et al., 1996; Dousset et al., 1997; Koçak et al., 2002; Basu et al., 2004; Lampiao & du Plessis, 2008b; Tronchon et al., 2008). Increased cytokines in the male reproductive tract are therefore considered to have detrimental consequences on spermatogenesis and the function of ejaculated spermatozoa, and elevated seminal plasma cytokine concentrations have been closely associated with fertility problems (Koçak et al., 2002; Basu et al., 2004; Eggert-Kruse et al., 2007; Martinez et al., 2007; Gallegos et al., 2008; Seshadri et al., 2009; La Vignera et al., 2011a).

In addition to local effects of reproductive tract inflammation on sperm production and function, peripheral inflammation and increased serum cytokines have also been suggested to negatively influence male reproduction. Changes in serum cytokines may have direct detrimental effects on the HPT axis affecting steroidogenesis, as well as the Sertoli cells affecting spermatogenesis (Hales, 2002). Both local

(reproductive tract) or systemic inflammation and inflammatory cytokine expression during injury, illness, infection or chronic inflammatory disease may contribute to the disruption of testicular function and fertility that frequently accompanies these conditions (Hales et al., 1999; Hedger & Meinhardt, 2003). Acute systemic inflammatory disease is associated with a transient decrease in spermatogenesis and the HPT axis, and chronic inflammatory disease is associated with impaired HPT function and sperm concentration (Hales et al., 1999; Hales, 2002; Hedger & Meinhardt, 2003). As MetS is considered a systemic inflammatory disease, albeit low grade inflammation (Haffner, 2003; Tamakoshi et al., 2003; Brooks et al., 2010), this warrants investigation of the effect of this inflammation on male reproductive health.

The data of this study does not support any argument on the source of the cytokines. Although the source, physiological roles and pathophysiological mechanisms are still a matter of contention and debate, mechanisms associated with increased inflammatory cytokines in serum and semen of males with MetS requires further investigation. However, this study appears to be the first to report an association between non-infectious, asymptomatic and chronic reproductive tract inflammation in males and the MetS. Although the cytokines are further discussed individually, it is important to note that these cytokines rarely act in isolation, but as a complex interacting network with other cytokines and proteins that may positively or negatively influence sperm function (Seshadri et al., 2009).

4.6.3. Tumour necrosis factor-alpha and male reproduction

TNF α is a key and critical mediator of inflammation, with increased levels in serum, tissues or semen associated with increased inflammation (Alexander et al., 1998; Eggert-Kruse et al., 2007). Furthermore, based on receptors for TNF α in almost all cells, increased serum or seminal concentration may negatively influence reproduction in males (Eggert-Kruse et al., 2007). A single nucleotide polymorphism (SNP) in the gene encoding TNF α (-308 polymorphism), which is associated with obesity and MetS risks, has also been associated with infertile males (based on altered sperm parameters or motility) (Tronchon et al., 2008). Patients with this SNP have higher basal serum TNF α compared to those without this SNP (Tronchon et al., 2008). This study further indicates an important role of this cytokine in male fertility.

Increased TNF α associated with chronic pelvic pain syndrome, irrespective of leukocytes (Alexander et al., 1998).

Within the CG, a range of 5.3 – 24 pg/ml was found for serum TNF α , with 5 samples undetectable. A range of 5.3 – 106.1 pg/ml was found for serum TNF α in the MetS group, with 8 samples undetectable. Although previous studies assessing serum TNF α in health and disease has shown a variation in concentrations, the medians (reported) and means (unreported) in addition to the ranges, generally agree with previous studies assessing serum TNF α concentrations. Gruschwitz et al. (1996) reported a concentration of 2.4 pg/ml in 8 normospermic males, 62.5 pg/ml in 8 subfertile males with positive sperm culture and 4.0 pg/ml in 9 subfertile males with negative sperm culture. Koçak et al. (2002) reported a concentration of 4.4 pg/ml (range: 1.2 – 14.3 pg/ml) in 24 fertile males, 5.1 pg/ml (range: 1.4 – 13.7 pg/ml) in 23 infertile males diagnosed with varicocele and 11 pg/ml (range: 2.3 – 61.3 pg/ml) in 10 infertile males with male accessory gland infection. Eggert-Kruse et al. (2005) reported a concentration of 18.7 pg/ml (range: 0.1 – 144.4 pg/ml) in 148 asymptomatic men from subfertile couples. In a cohort of 59 healthy and fertile men, Politch et al. (2007) reported a generally lower concentration of 1.5 pg/ml (range: 0 – 40.3 pg/ml). Penna et al. (2007), however, reported higher concentrations (33 pg/ml; interquartile range: 22 – 65 pg/ml) in 20 healthy males, with 68 pg/ml (interquartile range: 34 – 200 pg/ml) in 23 men with symptomatic benign prostatic hyperplasia, 58 pg/ml (interquartile range: 37 – 95 pg/ml) in 9 males with chronic prostatitis without leukocytospermia, and 48 pg/ml (interquartile range: 34 – 90 pg/ml) in 31 males with chronic prostatitis with leukocytospermia. Investigating at a cohort of infertile males, Ulcova-Gallova et al. (2009) reported a concentration of 2.6 pg/ml (range: 0.1 – 24.7 pg/ml) in 20 males with normospermia, 1.7 pg/ml (range: 0.1 – 26.7 pg/ml) in 17 males with asthenospermia and 3.4 pg/ml (range: 0.1 – 19.2 pg/ml) in 103 males with oligoasthenospermia. Interestingly, in a cohort of males from subfertile couples, divided into normospermic (n=14), asthenospermic (n=8), oligospermic (n=13), oligoasthenospermic (n=19), obstructive azoospermic (n=10) and non-obstructive azoospermic groups (n=9), Seshadri et al. (2009) reported undetectable levels in most samples, although a range across all groups was 0 – 73 pg/ml. Similarly, Naz & Kaplan (1994) did not detect TNF α in 20 semen samples.

Both serum and seminal TNF α negatively correlated with sperm concentration and total sperm count, but not ejaculation volume, in the cohort. There was also negative correlation with total motility, vitality and morphology for both serum and seminal TNF α . This is somewhat supported in the literature. Koçak et al. (2002) reported that seminal TNF α was negatively correlated with sperm motility in fertile and infertile males. Other authors have also reported negative correlations between seminal TNF α and sperm concentration and motility (Gruschwitz et al., 1996; Ulcova-Gallova et al., 2009). However, others have reported no such correlation in different male cohorts (Eggert-Kruse et al., 2007; Seshadri et al., 2009). There was no association between serum TNF α and any sperm parameter, except leukocytes, in seminal samples of asymptomatic males from subfertile couples as reported by Eggert-Kruse and colleagues (2007), and also in a report published by Camejo et al. (2001). Although Gruschwitz et al. (1996) found a correlation with sperm concentration and sperm motility, this study did not find a correlation with total sperm counts, viability, morphological alterations or testosterone in a very small cohort. Within this study, both serum and seminal TNF α correlated negatively with sperm DF. This was also observed in the MetS group, but not the CG. This may suggest that the cytokine may mediate damage to the DNA of spermatozoa, negatively influencing fertility.

As with other cytokines, TNF α associated with increased oxidative stress and pathological processes in semen (Sanocka et al., 2003). Martinez and colleagues (2007) showed that TNF α may negatively influence sperm peroxidation in ejaculated spermatozoa, concluding that higher concentrations in semen may negatively influence fertilisation.

4.6.4. Interleukin 1-beta and male reproduction

The interleukin 1 family, and particularly IL1 β , are classic proinflammatory polypeptides synthesised by a range of immunological and non-immunological cells, and known to promote the inflammatory response, typically inducing production of other proinflammatory cytokines (Tanaka et al., 1999; Jager et al., 2007). Leydig cells (Wang et al., 1991) and testicular macrophages (Kern et al., 1995) have been found to synthesis and secrete IL1 β . Human ejaculated spermatozoa have also been shown to synthesis and secrete an IL1-like molecule under *in vitro* conditions

(Huleihel et al., 1997). Increased IL1 has also been associated with chronic pelvic pain syndrome, irrespective of leukocytes (Alexander et al., 1998)

Within the CG, a range of 8.0 – 164.0 pg/ml was found for serum IL1 β , with 10 samples undetectable. A range of 15.6 – 181.0 pg/ml was found for serum IL1 β in the MetS group, with 8 samples undetectable. Although previous studies assessing serum IL1 β in health and disease has shown a variation in concentrations, the medians (reported) and means (unreported) in addition to the ranges appear to be generally lower than those reported in with previous studies assessing serum IL1 β concentrations. Gruschwitz et al. (1996) reported a concentration of 4.9 pg/ml in 8 normospermic males, 59.3 pg/ml in 8 subfertile males with positive sperm culture and 7.0 pg/ml in 9 subfertile males with negative sperm culture. Dousset et al. (1997) reported a mean of 5.0 pg/ml in 21 fertile males compared to 11.9 pg/ml in 119 males with androgenic disease undergoing routine infertility assessments. In 11 males with normal spermiograms, a concentration of 3.0 pg/ml was reported by Papadimas et al. (2002), in contrast to a concentration of 9.0 pg/ml in 42 males diagnosed with mild oligoasthenoteratospermia, 7.0 pg/ml in 10 males diagnosed with severe oligoasthenoteratospermia and 6.0 in 8 males diagnosed with azoospermia. These results are substantially lower than those reported in this study. In a cohort of 83 healthy and fertile men, Politch et al. (2007) reported a mean concentration of 2.3 pg/ml (range: 0 – 118 pg/ml). Ulcova-Gallova et al. (2009) reported a mean of 0.4 pg/ml (range: 0.1 - 3.2 pg/ml) in 20 males with normospermia, 0.8 pg/ml (range: 0.1 - 45.4 pg/ml) in 17 males with asthenospermia and 0.4 pg/ml (range: 0.1 - 2.1 pg/ml) in 103 males with oligoasthenospermia. Naz & Kaplan (1994) did not detect IL β in 20 semen samples. In contrast, Eggert-Kruse et al. (2005) reported a concentration of 37.8 pg/ml (range: 3 - 361.7 pg/ml) in 139 asymptomatic men from subfertile couples, generally a higher concentration than previous studies. Penna et al. (2007) reported similar concentrations as found in the in study, with a concentration of 17 pg/ml (interquartile range: 10 – 41 pg/ml) in 20 healthy males, 20 pg/ml (interquartile range: 10 – 32 pg/ml) in 23 men with symptomatic benign prostatic hyperplasia, 13 pg/ml (interquartile range: 9 – 19 pg/ml) in 9 males with chronic prostatitis without leukocytospermia, and 61 pg/ml (interquartile range: 22 – 108 pg/ml) in 31 males with chronic prostatitis with leukocytospermia.

Within the cohort of this study, seminal IL1 β did not correlate with any semen. Serum IL1 β did correlate negatively, yet weakly, with sperm concentration however. As with the other cytokines, the literature has offered inconclusive evidence of the role of IL1 β in sperm function. Gruschwitz et al. (1996) reported IL1 β to negatively correlate with sperm motility, but not with total sperm counts, viability, morphological alterations or testosterone. However, this study had low numbers (8 healthy and 14 infertile). IL1 β has also been associated with increased oxidative stress and pathological processes in semen (Sanocka et al., 2003).

4.6.5. Interleukin 6 and male reproduction

IL6 is produced by many cell types, including monocytes and macrophages, fibroblasts and endothelial cells (Bastard et al., 2006), with much of it estimated to be derived from adipose tissue in the absence of inflammation (Mohamed-Ali et al., 1997). This cytokine appears to play a very prominent role in the link between obesity, MetS and coronary heart disease (Yudkin et al., 2000). TNF α is also a strong inducer of IL6 from adipocytes themselves (Rotter et al., 2003). IL6 is a multifunctional cytokine involved in numerous processes in human spermatozoa, as well as autocrine and paracrine activity (Matalliotakis et al., 1998). The prostate appears to be a major source of IL6 in seminal plasma (Naz & Kaplan, 1994), with additional IL6 possibly originating from Sertoli cells or seminal vesicles (Seshadri et al., 2009). However, the role of IL6 in reproduction requires further investigation (Seshadri et al., 2009).

Within the CG, a range of 4.2 – 203.1 pg/ml was found for serum IL6, with 12 samples undetectable. A range of 5.3 – 255.0 pg/ml was found for serum IL6 in the MetS group, with 1 sample undetectable. Although previous studies assessing serum IL6 in health and disease has shown a variation in concentrations, the medians (reported) and means (unreported) in addition to the ranges, appear to be generally lower than those reported in with previous studies assessing serum IL1 β concentrations. Naz & Kaplan (1994) found a concentration of 23.6 pg/ml (range: 15 – 41 pg/ml) in 10 fertile (not defined) males, and 46.0 pg/ml (range: 15 - 62 pg/ml) in 10 males with infertility due to antisperm antibodies. Gruschwitz et al. (1996)

reported a concentration of 21.5 pg/ml in 8 normospermic males, 272 pg/ml in 8 subfertile males with positive sperm culture and 63.3 pg/ml in 9 subfertile males with negative sperm culture. Dousset et al. (1997) reported a concentration of 19 pg/ml in 21 fertile males compared to 22.9 pg/ml in 119 males with androgenic disease undergoing routine infertility assessments. In 29 males with normal spermograms, Matalliotakis et al. (1998) reported a concentration of 19.1 pg/ml (range: 3 – 92 pg/ml), with a concentration of 24.4 pg/ml (range: 5 – 75 pg/ml) in 45 males with sterile but abnormal spermogram and 39.0 pg/ml (range: 7 – 102 pg/ml) in 18 males with male accessory gland infections. Eggert-Kruse et al. (2001) reported a concentration of 15.0 pg/ml (range: 3.3 - 520 pg/ml) in 137 subfertile males. Koçak et al. (2002) reported a concentration of 18.8 pg/ml (range: 5 - 51.8 pg/ml) in 24 fertile males, 21.8 pg/ml (range: 7.4-51 pg/ml) in 23 infertile males diagnosed with varicocele and 42.8 pg/ml (range: 10 - 152.7 pg/ml) in 10 infertile males with male accessory gland infection. In a cohort of 79 healthy and fertile men, Politch et al. (2007) reported a concentration of 6.4 pg/ml (range: 0 – 110 pg/ml). Penna et al. (2007) reported a concentration of 16 pg/ml (interquartile range: 10 – 26 pg/ml) in 20 healthy males, 74 pg/ml (interquartile range: 21 – 132 pg/ml) in 23 men with symptomatic benign prostatic hyperplasia, 20 pg/ml (interquartile range: 13 – 39 pg/ml) in 9 males with chronic prostatitis without leukocytospermia, and 99 pg/ml (interquartile range: 31 – 130 pg/ml) in 31 males with chronic prostatitis with leukocytospermia. Kokab et al. (2010) reported a concentration of 16.3 pg/ml (range: 1 – 150 pg/ml) in 239 males uninfected with *Chlamydia trachomatis*, and 22.8 (range: 1 – 120 pg/ml) in 16 males infected with *Chlamydia trachomatis*. In a cohort of males from subfertile couples, divided into normozoospermic (n=14), asthenozoospermic (n=8), oligozoospermic (n=13), oligoasthenozoospermic (n=19), obstructive azoospermic (n=10) and non-obstructive azoospermic groups (n=9), Seshadri et al. (2009) reported concentrations of 18 pg/ml, 69.5 pg/ml, 38 pg/ml, 31 pg/ml, 42 pg/ml and 37 pg/ml respectively. A range across all groups was 4 – 1006 pg/ml. Investigating a cohort of infertile males, Ulcova-Gallova et al. (2009) reported a concentration of 29.6 pg/ml (range: 3.1 – 98.3 pg/ml) in 20 males with normozoospermia, 35.2 pg/ml (range: 1.9 – 492.6 pg/ml) in 17 males with asthenozoospermia and 40.1 pg/ml (range: 6.1 – 158.2 pg/ml) in 103 males with oligoasthenozoospermia.

Camejo et al. (2001) found no correlation between seminal IL6 and sperm parameters in males of infertile couples. This is supported by numerous additional studies (Dousset et al., 1997; Matalliotakis et al., 1998; Eggert-Kruse et al., 2001; Friebe et al., 2003). However, seminal IL6 correlated negatively with sperm concentration and morphology, and positively with DF, in the cohort, with some support from the literature. In a small study, IL6 has been associated previously with poor sperm motility, but not sperm concentration nor motility (Gruschwitz et al., 1996). IL6 has been negatively correlated with sperm concentrations in ejaculated semen, with increased IL6 associated with oligozoospermic and asthenozoospermic men (Seshadri et al., 2009). Further lines of evidence negatively correlate seminal IL6 with motility and oocyte penetration rates (Naz & Kaplan, 1994; Seshadri et al., 2009) Seminal IL6 has also been associated with increased oxidative stress and pathological processes in semen (Sanocka et al., 2003). Camejo et al., (2001) also reported that Increasing IL6 levels in semen of infertile men associated with detrimental effects on sperm lipid peroxidation.

4.6.6. Interleukin 8 and male reproduction

IL8, a well established proinflammatory and chemotactic cytokine, is an active component in acute inflammation as well as angiogenesis and endothelial cell proliferation (Baggiolini & Clark-Lewis, 1992). It is produced by immune cells (such as macrophages, neutrophils and T-lymphocytes) and non-immune cells (such as endothelial cells, fibroblasts and adipocytes) (Baggiolini & Clark-Lewis, 1992; Kim et al., 2006b). IL8 generally exerts its function in association with other cytokines and chemokines, and is crucially involved in numerous inflammatory conditions, including atherosclerosis (Lotti & Maggi, 2013b). Being classed as part of the chemokine family, it is suggested that IL8 has these functions within the reproductive tract (Politch et al., 2007).

Increased serum IL8 associated with increased waist-to-hip ratio and fat mass (Strackowski et al., 2002; Lotti et al., 2011). Furthermore, IL8 correlates positively with other proinflammatory cytokines, such as TNF α , IL1 β and IL6 (Strackowski et al., 2002; Lotti & Maggi, 2013b). Origins of seminal IL8 has not been fully elicited, but IL8 appears to be significantly associated with male accessory gland infections

suggesting a seminal vesicle and/or prostate origin (Seshadri et al., 2009). Seminal IL8 is considered an important and reliable predicting marker in the diagnostics of prostate inflammatory diseases, such as chronic pelvic pain syndrome and benign prostatic hyperplasia (Penna et al., 2007; Lotti et al., 2011; Lotti et al., 2013b).

Within the CG, a range of 10.3 – 4503 pg/ml was found for serum IL8, with 11 samples undetectable (ULOQ). A range of 420.9 – 4290.3 pg/ml was found for serum IL8 in the MetS group, with 14 samples undetectable (ULOQ). Seminal IL8 appears to be highly concentrated in human semen as compared to serum. As discussed below, this is in agreement with other studies. Based on medians obtained in this study, the CG had IL8 concentrated 116.1 times in semen, and the MetS group had IL8 concentrated 107.6 times in semen. However, there does not appear to be a physiological explanation for this cytokine to be concentrated.

Koumantakis et al. (1998) found a concentration of 5948.4 pg/ml (range: 2000 – 13500 pg/ml) in 29 males with a normal spermiogram, and 5670.4 pg/ml (range: 1550 – 17000 pg/ml) in 48 males with abnormal spermiograms. Eggert-Kruse et al. (2001) reported a concentration of 1257.0 pg/ml (range: 251 – 7854 pg/ml) in 137 subfertile males. In a cohort of 82 healthy and fertile men, Politch et al. (2007) reported a concentration of 1583.3 pg/ml (range: 384 – 14712 pg/ml). Penna et al. (2007) reported a concentration of 1984 pg/ml (interquartile range: 1164 – 2444 pg/ml) in 20 healthy males, 5044 pg/ml (interquartile range: 3063 – 11795 pg/ml) in 23 men with symptomatic benign prostatic hyperplasia, 2983 pg/ml (interquartile range: 2033 – 5287 pg/ml) in 9 males with chronic prostatitis without leukocytospermia, and 15240 pg/ml (interquartile range: 10630 – 19501 pg/ml) in 31 males with chronic prostatitis with leukocytospermia. Kokab et al. (2010) reported a concentration of 727.1 pg/ml (range: 100 – 7000 pg/ml) in 239 males uninfected with *Chlamydia trachomatis*, and 1457.8 (range: 70 – 12000 pg/ml) in 16 males infected with *Chlamydia trachomatis*. In a cohort of males from subfertile couples, divided into normozoospermic (n=14), asthenozoospermic (n=8), oligozoospermic (n=13), oligoasthenozoospermic (n=19), obstructive azoospermic (n=10) and non-obstructive azoospermic groups (n=9), Seshadri et al. (2009) reported concentrations of 1300 pg/ml, 2850 pg/ml, 1450 pg/ml, 1750 pg/ml 2050 pg/ml and 2100 pg/ml respectively. A range across all groups was 70 – 49500 pg/ml. Investigating a cohort of infertile

males, Ulcova-Gallova et al. (2009) reported a concentration of 693.5 pg/ml (range: 128.9 – 1532.3 pg/ml) in 20 males with normozoospermia, 836.8 pg/ml (range: 149.7 – 2000 pg/ml) in 17 males with asthenozoospermia and 919.2 pg/ml (range: 235.6 – 1925.7 pg/ml) in 103 males with oligoasthenozoospermia.

Within the cohort, CG and MetS groups, IL8 did not correlate with any sperm parameter. However, Seshadri et al. (2009) reported that serum IL8 has been associated with decreased sperm concentrations, as did Eggert-Kruse et al. (2001). Martinez and colleagues (2007) showed that IL8 may negatively influence sperm lipid peroxidation in ejaculated spermatozoa at physiological (50 pg/ml) and pathological (100 pg/ml) concentrations over 2 hours, concluding that higher concentrations in semen may negatively influence fertilisation.

4.6.7. Possible contribution from reactive oxygen species

In addition to a systemic low grade inflammatory state, there is an increase in systemic oxidative stress (OS) in patients with MetS (Furukawa et al., 2004; Holvoet, 2008). OS is the result of an imbalance between the production of reactive oxygen species (ROS) and total antioxidant concentrations (TAC), and has been implicated in the pathogenesis of numerous conditions, including MetS and related complications such as CVD, T2DM and cancer (Furukawa et al., 2004; Amaral et al., 2008; Holvoet, 2008; Makker et al., 2009; La Vignera et al., 2012a). Although ROS was initially thought to be exclusively toxic to human spermatozoa, recent studies have highlighted the physiological importance of these unstable molecules on fertilisation (Agarwal et al., 2009; Henkel, 2011a).

Low ROS concentrations play a fundamental role in triggering capacitation, hyperactivation, acrosome reaction, sperm zona binding and oocyte fusion (Henkel, 2005; Agarwal et al., 2009; Henkel, 2011a). Conversely, increased ROS concentrations (ROS-TAC mismatch) in seminal fluid have numerous detrimental effects on various parameters of sperm function (Henkel 2005; Henkel 2011a). Thus, the state between ROS and antioxidants (AO) needs to be finely balanced and maintained by various enzymatic and non-enzymatic processes (Henkel, 2005; Henkel, 2011a).

ROS are generated as a byproduct of spermatozoan oxidative phosphorylation, and are therefore intrinsic to the reproductive tract (Henkel, 2005; Henkel, 2011a). Furthermore, ROS in the male reproductive tract and ejaculate are also derived from seminal leukocytes, and play significant defensive and destructive roles in infections, inflammation and cellular defence (Henkel, 2005; Henkel, 2011a). Proinflammatory cytokines, particularly TNF α , IL1 β , IL6 and IL8, are known to modulate both OS and antioxidant status, with increased concentrations correlating positively with ROS and OS in seminal fluid (Sonocka et al., 2003). Additional ROS are derived from lifestyle and other exogenous sources, such as smoking, alcohol consumption, heavy metals and pesticides, and pathological states such as varicocele and spinal cord injuries, and all these factors have been linked to impairment of fertility status via OS (Henkel, 2005; Henkel, 2011a).

High levels of OS in the male reproductive tract and seminal fluid, associated with sperm lipid peroxidation of the polyunsaturated fatty acids in the spermatozoa membrane and DNA damage, has been extensively implicated in male factor infertility (Henkel, 2005; Pasqualotto et al., 2008; Agarwal et al., 2009). Excessive OS has been correlated with reduced sperm concentration and motility, morphological derangements and damage to both cellular and mitochondrial DNA (Henkel, 2005; Aitken et al., 2006; Amaral et al., 2008; Pasqualotto et al., 2008; Agarwal et al., 2009; Desai et al., 2009; Kefer et al., 2009; Makker et al., 2009; Henkel, 2011a; La Vignera et al., 2012a).

4.6.8. Inflammation and steroid hormones

Interestingly, free testosterone in saliva was negatively correlated with serum TNF α , IL1 β , IL6 in the cohort (as well as seminal IL8). This illustrates a relationship between MetS, inflammation and hypogonadism in males (Kasturi et al., 2008). Testosterone is associated with a down-regulation of cytokine-mediated inflammation in males (Cutolo et al., 2004; Malkin et al., 2004). It therefore appears that a decrease in testosterone may in part promote inflammation in MetS, and conversely, inflammation may in part down-regulate testosterone synthesis, as discussed below.

Furthermore, free progesterone negatively correlated with serum and seminal TNF α in the cohort, but not with any other cytokines. Progestins can stimulate monocyte production of inflammatory cytokines in woman (Jain et al., 2004). In woman, however, progesterone suppresses immune function during pregnancy (Oettel & Mukhopadhyay, 2004).

Positive correlations have been suggested between endogenous progesterone and CRP, IL6, and leptin in healthy, non-smoking males. In addition, a significant increase in IL6 and decrease in IL10 (inflammatory regulatory role) was found in males receiving testosterone and progestin treatment for hypogonadism as compared to males receiving testosterone plus placebo (Zitzmann et al., 2005). In a study of Chinese men and woman, progesterone concentration in males was found to be independently associated with atherosclerosis via assessment of carotid artery thickness (with no such correlation found in females) (Ma et al., 2009). As inflammation is closely associated with CVD and atherosclerosis, changes in progesterone may be an important mediator. However, with scanty information related to the impact of progesterone on immune function in males, this requires further exploration. Evidence indicates that endogenous progesterone beneficially regulates coronary artery reactivity in humans (Ma et al., 2009). Therefore, decreased progesterone in MetS may independently contribute to atherosclerosis and CVD in males. However, if this is mediated via the immune system remains to be studied. This is further discussed in the effect of cytokines on progesterone in TM3 Leydig cells below.

4.7. Steroid hormone synthesis in TM3 Leydig cells in the setting of insulin and inflammation

MetS is closely associated with the poorly understood phenomenon of hyperinsulinaemia (insulin resistance) and a low grade chronic and asymptomatic inflammatory state (Eckel et al., 2005; Kasturi et al., 2008; Huang, 2009; Monteiro & Azevedo, 2010). The results in the case-controlled arm of the study, as expected, have agreed with the extensive literature describing these associations. A novel finding is the increase in insulin, TNF α , IL1 β , IL6 and IL8 within the seminal fluid of

males diagnosed with MetS. In addition, hypogonadism (Pasquali, 2006; Guay, 2009; Saad & Gooren, 2009) is a feature of MetS in males.

The results of this study found both free testosterone and free progesterone to be decreased in the saliva of males with MetS compared to the control counterparts. Insulin (Lampiao et al., 2009) and inflammatory cytokines (Hales et al., 1999; Bornstein et al., 2004) are thought to directly and indirectly modulate the HPT axis. This is achieved by actions centrally, via the hypothalamus (modulating GnRH) and anterior pituitary gland (modulating LH), and peripherally (via action on Leydig cells and Sertoli cells) (Hales et al., 1999; Bornstein et al., 2004; Lampiao et al., 2009). However, the role of insulin and cytokines on modulation of male reproduction has not been fully elicited and requires further extensive research. Furthermore, this relationship has not been studied in light of known underlying phenomenon associated with MetS in males.

In order to further investigate the impact of insulin and inflammatory cytokines on steroidogenesis, a TM3 mouse Leydig cell line was used. Leydig cells stimulated with hCG were exposed to various concentrations of insulin, TNF α , IL1 β , IL6 and IL8. Cell viability, protein concentration, testosterone and progesterone concentrations were assayed.

4.7.1. Insulin

Results showed TM3 cell viability to be increased for all concentrations of insulin. This was most marked at 0.001 pg/ml concentrations, and generally less marked with increasing concentrations. At 10 pg/ml, the maximum concentration the cells were exposed to, this was no longer significant. At relatively lower concentrations of insulin, the increase in cell viability may indicate cellular stress. Higher concentrations of insulin (≥ 10 pg/ml) may indicate cellular death in a dose-dependent manner. Protein concentrations generally increased in a dose-dependant manner, with significant increases at 0.1, 1 and 10 pg/ml. Testosterone synthesis increased for all concentrations compared to the controls. This was most pronounced at the 0.01 and 0.1 pg/ml concentrations, and then appeared to be less pronounced at the higher concentrations, although still being a dose-dependent

increase. Similarly, testosterone-to-protein ratio was markedly and significantly increased at 0.01 pg/ml, further indicating cellular stress. There was a dose-dependant decline in this ratio for the higher concentrations, each one not statistically different from the control group. In stark contrast to testosterone concentrations, progesterone was significantly decreased for all concentrations assessed, in a dose-dependent manner. As a result, the progesterone-to-protein ratios were also significantly decreased in a dose-dependent manner.

The results of these experiments indicate that insulin appears to induce some cellular stress at the lower concentrations used. However, as testosterone and protein both increases, and testosterone-to-protein ratio, this may indicate a stimulation of metabolic activity.

Insulin resistance is closely associated with low testosterone in males. However, the mechanisms for this relationship remain unclear (Kasturi et al., 2008). There is a close relationship between insulin sensitivity and testosterone concentrations in men across a wide range of glucose intolerance, including those with T2DM, and independent of SHBG concentrations (Pitteloud et al., 2005b). This indicates a direct relationship between insulin and testosterone in males. Furthermore, males with hypogonadism are twice as insulin-resistant as eugonadal counterparts (Pitteloud et al., 2005b). Pitteloud and colleagues also published a paper (2005a) indicating that insulin resistance is associated with decreased secretion of testosterone from Leydig cells in a small male cohort using a novel model to systematically assess every level of the HPT axis. The results of the cell culture experiments in this study indicate that insulin increases testosterone secretion within the Leydig cells. This would agree with Pitteloud et al. (2005a) conclusions that insulin sensitivity is directly related to testosterone synthesis.

Insulin independently stimulates testosterone production and simultaneously inhibits SHBG in normal weight and obese males, and that this can be suppressed with diazoxide (inhibits secretion of insulin from the pancreas) treatments (Pasquali et al., 1995). Further experiments on insulin resistance in normal weight and obese males have been consistent with this hypothesis (Pasquali et al., 1997). Exposure of a crude preparation of primary mouse Leydig cell culture to insulin showed that

exposing the cells to 1 $\mu\text{g/ml}$ insulin (equivalent to 10^3 pg/ml , therefore higher concentrations than this study) for one hour prior to addition of LH for 3 hours increased testosterone production compared to no insulin pre-treatment (Bebakar et al., 1990). Lin et al. (1986) also demonstrated insulin stimulation of testosterone synthesis in a primary rat Leydig cell culture model, as well as potentiating hCG-induced cAMP formation, and this was blocked with administration of a protein synthesis inhibitor, cycloheximide.

In contrast, Benitez and Perez Diaz (1985) showed that destruction of pancreatic β -cells using streptozotocin, thus inducing type 1 diabetes mellitus, caused a dramatic decrease in serum testosterone in male rats. This was reversed with insulin treatments. More recently, 24-hour exposure of primary catfish Leydig cells to 1 ng/ml insulin stimulated testosterone synthesis (Dubey & Lal, 2009). Interestingly, these authors found a direct influence of testicular macrophages on Leydig cell steroidogenesis mediated by nitric oxide in catfish, which augmented insulin stimulation of testosterone synthesis (Dubey & Lal, 2009).

These series of publications indicate that both hypoinsulinaemia and hyperinsulinaemia (associated with insulin resistance) result in decreased testosterone synthesis. At optimal concentrations, insulin appears to therefore directly stimulate Leydig cells steroidogenesis, as indicated by the TM3 cell exposures to insulin concentrations. However, there are no studies identified that investigate the effect of insulin on progesterone synthesis. In the experiments with TM3 cells, results indicate that progesterone concentrations are significantly reduced, in the setting of an increasing testosterone synthesis. As progesterone is an essential precursor of testosterone, these results suggest enzymatic activity downstream of progesterone may be upregulated, and not those upstream. This hypothetical scenario would draw from the pool of progesterone for testosterone synthesis, without replacing the metabolised progesterone.

Hypothetically, insulin may upregulate one or more of the enzymes associated with progesterone metabolism into testosterone via the Δ^4 -steroid pathway, particularly cytochrome P450 17 α -hydroxylase (CYP17), cytochrome P450 17,20-lyase (CYP17,20) or 17 β -hydroxysteroid dehydrogenase (17 β -HSD) (Sherbet et al., 2003).

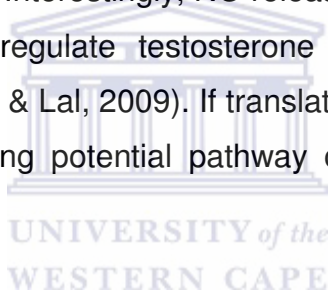
Insulin may therefore stimulate an upregulation of testosterone synthesis via the Δ^5 -steroid pathway, acting on one or more of these enzymes such as CYP17, CYP17,20, 3β -hydroxysteroid dehydrogenase (3β -HSD) or 17β -HSD. Of these, CYP17, CYP17,20 and 17β -HSD are downstream of progesterone on the Δ^4 -steroid pathway and are used in the Δ^5 -steroid pathway (Sherbet et al., 2003), and therefore more likely to explain these results. 3β -HSD, on the other hand, also metabolises pregnenolone into progesterone as the gateway to the Δ^4 -steroid pathway (Sherbet et al., 2003), and an up-regulation of this enzyme should be associated with an increase in progesterone. Alternatively, insulin may down-regulate 3β -HSD, leading to a decrease in the Δ^4 -steroid pathway, in the setting of an up-regulation of one or more of CYP17, CYP17,20 and 17β -HSD.

This evidence in this study and the literature indicates that insulin, at least acutely, increases testosterone synthesis in males, and may decrease progesterone synthesis. However, in animals and humans, increasing insulin medium- and long-term, such as hyperinsulinaemia and insulin resistance, is closely associated with decreased testosterone. This may imply that insulin resistance affects the Leydig cells insulin receptors and/or intracellular mediators to negatively affect steroidogenic cascades. This would explain both a decrease in progesterone and testosterone in the MetS group of this study. It was demonstrated by Pasquali et al. (1997) that increasing insulin resistance induced in obese males, as well as a subset of normal weight males, was associated with decreasing testosterone synthesis. However, acute hyperinsulinaemia stimulated testosterone synthesis.

A very recent study published in April 2013, Ahn and colleagues studied the potential effect of insulin on steroidogenesis in light of insulin resistance (Ahn et al., 2013). Insulin (20 & 40 nM) treated MA-10 Leydig cells demonstrated a dose-dependent decrease in cAMP mediated steroidogenesis, via the induction of DAX-1. This appears to disagree with most other cell culture, animal- and human-based studies, including the results in this study, in which insulin increases testosterone synthesis in the absence of insulin resistance (Benetiz & Perez Diaz, 1985; Lin et al., 1986; Bebakar et al., 1990; Benetiz & Perez Diaz, 1985; Pasquali et al., 1995; Pasquali et al., 1997; Pitteloud et al., 2005).

Furthermore, the Ahn et al. (2013) study found that injecting 1 unit insulin per Kg body weight into rats fed a high fat diet decreased testosterone synthesis (which also appears to disagree with results found by Pasquali et al. (1997) in human obese males). This was mediated by upregulation of intratesticular DAX-1. In addition, steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side chain cleavage (P450_{scc}) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) were all decreased in the rats (Ahn et al. 2013). If correct, this would explain a decrease in both progesterone and testosterone in the MetS group of this study.

Ahn and colleagues (2013) further reported that insulin activates the insulin signalling pathway in Leydig cells via the Akt kinase pathway. It is known that this intracellular pathway breaks down with insulin resistance (Huang, 2009). A down-regulation of this pathway is also associated with decreased nitric oxide (NO) production in endothelial cells. Interestingly, NO release from testicular macrophages has been suggested to up-regulate testosterone synthesis in Leydig cells in experiments on catfish (Dubey & Lal, 2009). If translated into mammals and humans, this may be another interesting potential pathway of IR induced steroidogenesis collapse in Leydig cells.



The impact of insulin on steroidogenesis is complex in insulin sensitivity, and appears to be augmented when insulin resistance is present. As MetS is associated with hyperinsulinaemia and hypogonadism in males, the fact that insulin exposure to Leydig cells resulting in increased testosterone indicated that insulin resistance is a key phenomenon in any downregulation of steroidogenesis in MetS. This can be mediated by downregulating StAR, P450_{scc} and 3 β -HSD transcriptions. However, the cell culture arm of this study indicated that in insulin sensitive TM3 Leydig cells, progesterone is decreased with increased testosterone. This may be due to up-regulation of CYP17, CYP17,20 and 17 β -HSD on both steroid pathways as an important mediator in insulin induced testosterone synthesis. These too would likely be compromised in the setting of insulin resistance. Further studies investigating these potential effects are required in order to fully elicit the effect of insulin (and insulin resistance), and the associated mechanisms, on steroidogenesis and hence testosterone synthesis.

4.7.2. Tumour necrosis factor-alpha

TNF α exerted a highly significant and detrimental dose-dependent effect on cell viability, protein concentration, testosterone and progesterone concentrations. The testosterone-to-protein ratio was initially increased compared to controls, before decreasing, as was the progesterone-to-protein ratio. These results indicate that at all TNF α exerts a significant toxic effect on the Leydig cells in ascending concentrations, and leads to a collapse of the steroidogenesis cascade, reflected in dose-dependent declines in all variables.

TNF α has been suggested to negatively affect the HPT axis. In animal and human experiments, administration of TNF α is associated with a decrease in serum testosterone (van der Poll et al., 1993; Bornstein et al., 2004). These results agree with previous publications that have assessed the impact of TNF α on Leydig cell function *in vitro* and *in vivo*. TNF α at a dose of 10 ng/ml reduced testosterone production in cAMP-stimulated primary mouse cultures of Leydig cells, but not basal stimulation. There was also a decrease in P450_{SCC} and CYP17 mRNA by 1.5% compared to stimulated controls (Xiong & Hales, 1993). The same authors further reported a negative effect of TNF α at similar concentrations on 3 β -HSD in addition to P450_{SCC}, CYP17 in cAMP both cAMP stimulated and basal primary cultures of mouse Leydig cells. TNF α was therefore shown to inhibit testosterone production in both stimulated and basal cultures (Xiong & Hales, 1997). Li and colleagues (1995) also showed that TNF α decreased testosterone production in cAMP stimulated MA-10 mouse Leydig cells in a dose-dependent manner from 0.1 – 10 ng/ml, with no more negative effect at 100 ng/ml. This was associated with a decrease in CYP17 mRNA.

In a primary culture of Leydig cells produced from immature porcine testes (2-3 weeks old), 0.02 pg/ml TNF α decreased hCG induced testosterone concentrations (but not basal testosterone concentrations) in a time dependent manner. Between 0.5 and 6 hours, no effect on testosterone was observed. A decrease was only observed after 24 hours, and peaked at 48 hours. This was associated with a decrease in StAR mRNA (Mauduit et al., 1998). Intratesticular delivery of TNF α has been shown to reduce StAR and testosterone secretion in both a basal and hCG stimulated rat model (Morales et al., 2003).

R2C Leydig cells exposed to 10 ng/ml TNF α (basal) decreased testosterone synthesis compared to control after 2 hours, with a corresponding decrease in StAR, P450_{scc}, CYP17 and 3 β HSD. Interestingly, after 12 hours, testosterone had recovered to approximately 50% to that of controls, and enzyme levels completely recovered, before decreasing at longer intervals (Hong et al., 2004). In the same study, 10 week-old male mice were injected intraperitoneally with TNF α at 50 μ g/Kg body weight. Both testosterone and progesterone levels reduced by 10 – 25% after 6 hours with a decrease in CYP17 in the testis (Hong et al., 2004). Wu and colleagues (2012) reported a significant decrease in cell viability in TM3 Leydig cells exposed to 0.01 pg/ml TNF α , which was prevented by co-stimulation of Sirt1 (associated with cellular protection from inflammatory stress) activation via resveratrol.

These results indicate that enzymes mediating cholesterol uptake into the mitochondria (regulated by StAR), conversion to pregnenolone (mediated by P450_{scc}), pregnenolone conversion to progesterone (mediated by 3 β HSD) and progesterone conversion to 17-OH-P (mediated by CYP17) are all negatively impacted by increasing TNF α concentrations. There are few previous studies measuring progesterone concentrations in the cell culture models, with Hong et al. (2004) reporting a decrease in progesterone in a mouse model. In the mouse tumour cell line mLTC-1, basal exposure of TNF α at 50 ng/ml was associated with an increase in StAR after 6 hours. Phosphorylation of StAR (p-StAR), an important requirement for steroid synthesis, was not seen in either the control or TNF α exposed cells, but only with exposure to hCG (50 ng/ml). No significant effect was found for any of these factors for P450_{scc} or 3 β -HSD. TNF α exposure was associated with a decrease in progesterone levels, whereas hCG increased progesterone levels (Manna et al., 2006). The increase in StAR found in this study, with generally no effect on P450_{scc} or 3 β -HSD does not generally agree with other studies reported.

The results of this study agree with much of the literature, as TNF α is associated with a decrease in progesterone and testosterone concentrations and collapse of steroidogenesis at concentrations similar to those associated with human serum concentrations. A down-regulation of StAR, P450_{scc} and 3 β -HSD would translate into

a decrease in progesterone synthesis, agreeing with the decrease in FP observed in the MetS group. Hypothetically, a decrease in these enzyme transcriptions would also be associated with a decline in 17-hydroxy-pregnenolone, DHEA and androstenedione via the Δ^5 -steroid pathway. The negative impact on cell viability and protein synthesis further indicates that TNF α is cytotoxic to Leydig cells. This may indicate that a down-regulation of steroidogenic enzymes may be related to a downregulation of cellular function. TNF α appears to cause a collapse in steroidogenesis in concentrations as low as 0.01 pg/ml (Wu et al., 2012). Increasing serum concentrations of TNF α associated with MetS is an important mechanism associated with male hypogonadism that required detailed investigations.

4.7.3. Interleukin 1-beta

IL1 β showed a more subtle decline in cell viability, but this was dose-dependent across all concentrations, as was protein and testosterone. However, the testosterone-to-protein ratio actually increased in a dose-dependent manner, with significant increases only at the 10 and 100 pg/ml concentrations. This reflects a more prominent negative effect on protein concentrations as compared to the testosterone decline, causing the ratio to increase. The effect on progesterone was marked, with a very large impact at all concentrations, as therefore the progesterone-to-protein ratio declined. These results suggest that IL1 β is associated with increased Leydig cell damage, with a decline in metabolic activity. A negative effect on progesterone is more marked than testosterone decline, suggesting direct effects on enzymes associated with cholesterol metabolism to progesterone, and less direct effect on progesterone metabolism to testosterone.

Previous studies on the effect of IL1 on Leydig cell steroid regulation have provided conflicting results. This appears to be due to a variety of different stages of puberty in which primary Leydig cells were obtained from animals. Also, both IL1 α and IL1 β have been reported to have a role in Leydig cell function, and results from experiments depend on which isoform is used (Svechnikov et al., 2001). Calkins et al. (1988) showed a decrease in hCG-stimulated testosterone synthesis by 1 U/ml IL1 β in a primary culture of immature rat Leydig cells after 8 hours of exposure. This was associated with a decrease in hCG stimulated cAMP production. Lin et al.,

(1991) indicated that IL1 β decreases testosterone and P450_{scc} mRNA in an hCG stimulated culture of highly purified Leydig cells prepared from adult male rats (55-65 days old). Concentrations of 1 – 100 ng/ml IL1 β were cultured over 24 hours, and a dose-dependent effect was observed, and these inhibitory effects could be reversed by the concomitant addition of IL1 receptor antagonist.

In contrast, IL1 β (0.5 – 10 U/ml) did not significantly alter basal or LH stimulated Leydig cell testosterone synthesis at 24, 48 or 72 hours of culture, neither did IL1 α (0.5 – 10 U/ml) significantly alter testosterone synthesis over the same exposure times (Sun et al., 1993). IL1 β (1 U/ml) markedly increased basal and low concentrations of LH stimulated C19 (testosterone and androstenedione) and C21 (progesterone, 17 α -OH-P and 20 α -hydroxypregnenolone) steroid hormone production in a primary culture of immature rat Leydig cells. Higher concentrations of LH, IL1 β inhibits C19 steroid production after 6 hours of exposure at 1 U/ml concentrations (Verhoeven et al., 1998). Leydig cell culture from 80 day old rats showed a dose-dependent decrease in hCG stimulated testosterone synthesis when exposed to 0.1 – 10 ng/ml (100 – 10000 pg/ml) IL1 β . Interestingly, IL1 β stimulated testosterone synthesis in cells obtained from 40 day old rats.

This study showed that this inhibitory effect was abolished by addition of androstenedione, suggesting that IL1 (both alpha and beta) suppresses CYP17 (Svechnikov et al., 2001). In the mouse tumour cell line mLTC-1, basal exposure of IL1 (unknown which isoform) at 20 ng/ml (20 000 pg/ml) was associated with an increase in StAR after 6 hours. Phosphorylation of StAR (p-StAR), an important requirement for steroid synthesis, was not seen in either the control or IL1 exposed cells, but only with exposure to hCG (50 ng/ml). No significant effect was found for any of these factors for P450_{scc} or 3 β -HSD. IL1 exposure was associated with a decrease in progesterone levels (which agrees with the results from this study), whereas hCG increased progesterone levels (Manna et al., 2006).

In 21 day old hemicastrated rats, a local bilateral injection or unilateral testicular administration of IL1 β (0.1 μ g/testis) resulted in a significant increase in basal testosterone secretion *in vitro* and serum testosterone concentration after 24 hours. Six days after treatment, the cytokine induced opposite effect in animals with two

testes *in situ*, i.e., it suppressed steroidogenesis. No effect was seen on LH or FSH. In adult animals subjected to bilateral treatment or to unilateral injection followed by hemicastration, IL1 β (1.5 & 15 μ g/testis) did not influence steroidogenesis and serum testosterone concentration. No change in serum LH and FSH concentration could be observed in any experimental group (Gerendai et al., 2005).

In a similar study design, intratesticular administration of IL1 β in immature hemicastrated rats induced a significant rise in testosterone secretion after 24 hours that could be prevented by vasectomy (that also means transection of the inferior spermatic nerve) in a study indicating an interaction between testicular nerves, IL1 β action and testicular serotonin (5-HT₂) receptors and local effect of IL1 β on testosterone secretion (Gerendai et al., 2007). In human NCI-H295R adrenocortical cells, IL1 β showed an increase in cortisol, androstenedione and DHEA synthesis in a dose-dependent relationship from 0.001 – 10 ng/ml (1 – 10000 pg/ml) at intervals of 24 and 48 hours. This was associated with increased mRNA's for StAR, CYP17 and 3 β -HSD (Tkachenko et al., 2011). More recently, Wu and colleagues (2012) reported that a significant decrease in cell viability in TM3 Leydig cells exposed to 0.02 pg/ml IL1 β . Interestingly, this was prevented by co-stimulation of Sirt1 (associated with cellular protection from inflammatory stress) activation via resveratrol (Wu et al., 2012).

4.7.4. Interleukin 6

Similarly to IL1 β , IL6 showed a more subtle decline in cell viability as opposed to that observed in TNF α . A dose-dependent decline in cell viability was observed. Protein was significantly decreased only at 100 pg/ml IL6 concentration. Again, very similar results to IL1 β for both testosterone and progesterone were found. Testosterone decline was observed in a dose-dependent manner for all concentrations. A more marked effect was observed for progesterone, with a very dramatic decline in progesterone concentrations at all IL6 concentrations. This more marked effect also indicates some specific action on the cholesterol conversions to progesterone pathways. As there was a generally small negative effect on testosterone and protein at all concentrations, the testosterone-to-protein ratio was not significantly different compared to the control at all concentrations. In contrast, there was a significant and

dose-dependent decline in progesterone-to-protein ratio at all IL6 concentrations, reflecting the specific negative action on progesterone synthesis.

Although the effects of TNF α and IL1 (to a lesser degree IL1 β) on Leydig cell steroidogenesis have been investigated, but not fully elicited, the effect of IL6 has been less studied in controlled experiments (Hales et al., 1999). It is indicated that IL6 compromised cAMP induced expression of CYP17 and, interestingly, 17 β -HSD, at concentrations of 100 ng/ml in primary mouse Leydig cells (Hales et al., 1999). Tsigos and colleagues (1999) injected increasing single doses of IL6 (0.1, 0.3, 1.0, 3.0, and 10.0 μ g/kg body weight) subcutaneously into 15 healthy male volunteers. The three higher IL6 doses caused significant decreases in testosterone levels at 24 and 48 hours, returning to baseline by 7 days, with maximal effect at 3 μ g/kg. This was associated with a small but significant increase in LH at these doses, but no change for FSH and SHBG.

IL6 may induce resistance to LH mediated steroidogenesis in Leydig cells (Tsigos et al., 1999). Hales (2002) has reported that IL6 decreases CYP17 and 3 β HSD. Wu and colleagues (2012) reported a significant decrease in cell viability in TM3 Leydig cells exposed to 0.02 pg/ml IL6 was prevented by co-stimulation of Sirt1 (associated with cellular protection from inflammatory stress) activation via resveratrol. IL6 is suggested to increase mineralocorticoids (aldosterone), glucocorticoids (cortisol) and androgens (DHEA) synthesis in a dose- and time- dependant manner. At concentrations of 10^{-8} – 10^{-12} mol/L, IL6 stimulation of adrenal hormones reached a peak after 48 hours in adrenal cells obtained from male patients 50 - 61 years of age following nephrectomy with unilateral adrenalectomy (Päth et al., 1997). IL6 appears to up-regulate adrenal steroidogenesis. However, this appears to be focused on glucocorticoids predominantly (Bornstein et al., 2004). Yet, it is not certain if the effects on androgens in testes would be similar however.

4.7.5. Interleukin 8

IL8 was associated with a dose-dependent and significant increase in cell viability. This would reflect either cellular stress or cellular stimulation, which is unclear with this data. Protein concentrations were not significantly different from the control at

0.1 or 1 pg/ml concentrations. There was a significant decrease in protein at 10 pg/ml. In contrast, there was a significant increase in protein at 100 pg/ml. The ANOVA repeated measures analysis of variance was not statistically significant, however, the ANOVA one-way analysis of variance did reach statistical significance.

There was no specific or significant effect on testosterone concentration, with no difference between the various IL8 concentrations and testosterone. The testosterone-to-protein ratio showed various results at the 0.1 and 10 pg/ml concentrations, with no significant change at the 1 and 100 pg/ml concentrations. At 0.1 pg/ml, this was significantly ($P < 0.0001$) increased, owing to a non-significant decline in protein and increase in testosterone at this concentration. As both protein concentration and testosterone changes at 0.1 pg/ml IL8 were not significant, the significance of the testosterone-to-protein ratio is questionable. In contrast, at a concentration of 100 pg/ml concentration, there was a significant decline in this ratio. This was mostly due to a significant increase in the protein concentration at 100 pg/ml IL8 (testosterone had a non-significant decrease at 100 pg/ml). This is associated with an increase in cell viability, indicating increased metabolic activity. Progesterone was decreased at all concentrations. Although this decrease was not as marked as the negative association with IL1 β and IL6, it indicates a more specific action on progesterone synthesis from cholesterol as opposed to testosterone synthesis from the progesterone pool. Based on the decline in progesterone, the progesterone-to-protein ratio showed a significant and dose-dependent decline at all concentrations of IL8.

Unlike TNF α , IL1 β and IL6, there appear to be no published investigations into the possible effects of IL8 on steroidogenesis in Leydig cells. In this study, testosterone is maintained, but progesterone decreased. It therefore appears that IL8 may influence progesterone via CYP17 or P450_{scc} or StAR. As testosterone is not different from controls over 48 hours, this would imply that the Δ^5 -steroid pathway is at least maintained, for which CYP17 or P450_{scc} and StAR are required. Progesterone is also a precursor hormone for mineralocorticoids and glucocorticoids. Therefore, it may be plausible that production of these hormones typically associated with adrenal glands is up-regulated by IL8, further drawing from the progesterone

pool, but leaving pregnenolone and the Δ^5 -steroid pathway intact for normal testosterone synthesis.

4.7.6. Potential mechanisms of Leydig cell dysfunction in metabolic syndrome

Adequate Leydig cell function with the central purpose of testosterone production via steroidogenesis cascades is critical not only for male reproduction, but in general for male health and well being (Walker et al., 2001; Midzak et al., 2009; Saad & Gooren, 2011). MetS is closely associated with hypogonadism in males, and a decline in testosterone is a significant risk and aetiological factor in the pathogenesis of MetS (Kasturi et al., 2008; Saad & Gooren, 2011).

It has been demonstrated that Leydig cell MMP, ATP synthesis and mitochondrial calcium concentrations are all required for steroidogenesis, and that this is a key control point for steroidogenesis (Hales et al., 2005). These mechanisms may be disrupted by oxidative stress (OS), and reactive oxygen species (ROS) are known to inhibit both ovarian and testicular steroidogenesis, most notably the initial step of cholesterol transfer into Leydig cell mitochondria (Diemer et al., 2003; Hales et al., 2005; Midzak et al., 2009; Midzak et al., 2011).

Typically, inflammation and OS occur in a close relationship together (in both infectious and non-infectious inflammation), and both of these phenomena have been well established in MetS patients (excessive OS in the setting of chronic inflammation) (Kasturi et al., 2008). As indicated by the results of the cell culture experiments and previous studies, inflammatory cytokines are able to down-regulate steroidogenesis at various stages. Furthermore, ROS disrupts various stages of steroidogenesis, including mitochondrial function and MMP, ATP synthesis by the mitochondria and StAR transcription (Diemer et al., 2003; Hales et al., 2005). Therefore, in the setting of chronic inflammation and OS associated with MetS, these mechanisms provide an important role in both the aetiology and propagation of MetS in males, negatively influencing reproductive potential and overall health and well being.

Ageing is associated with a significant decline in Leydig cell ability to produce testosterone in response to LH stimulation, with various mechanisms proposed (Midzak et al., 2009). For example, there is a reduction on cAMP production, StAR mRNA and the activity of P450_{SCC}, as are the activities of CYP17 and 3 β HSD (Zirkin and Chen, 2000; Midzak et al., 2009). This is associated with a gradual decline in testosterone in ageing males (Midzak et al., 2009). Age is also a significant risk factor closely associated with the prevalence of MetS (Cameron et al., 2004; Pais et al., 2009; Potenza & Meckanick, 2009; Razzouk & Muntner, 2009). There is likely a close relationship between these phenomena that requires further understanding, both in terms of age related non-communicable chronic disease (e.g. MetS, T2DM, CVD, cancer) and male reproductive health.

It is proposed that mitochondrial dysfunction has a key role in the development of insulin resistance (Kim et al., 2008). Furthermore, based on results from this study and previous studies (Lin et al., 1986; Bebakar et al., 1990; Pasquali et al., 1995; Pasquali et al., 1997; Pitteloud et al., 2005a; Pitteloud et al., 2005b), insulin appears to have an independent role in steroidogenesis. Inflammatory and ROS-induced damage on mitochondria may create insulin resistance in the Leydig cells, further negatively impacting testosterone synthesis. The relationship between the role of the mitochondrial, ROS, cytokines, insulin and testosterone require further research. Detailed understanding of these pathways may open the possibility of novel treatments to improve steroidogenesis in males with MetS, thereby potentially reversing the consequences of MetS and improving reproductive potential and sexual function.

A further point of consideration is the potential change in role of testicular macrophages in MetS. A detailed review by Hales (2002) outlines the key regulatory roles macrophages have in Leydig cell function (and hence steroidogenesis) and Sertoli cell function (and hence spermatogenesis). These macrophages are distinct from macrophages elsewhere in the body, and secrete a variety of proteins, including cytokines, in a distinct manner. It is plausible that there may be a change in testicular macrophage function with MetS, negatively influencing steroidogenesis in the Leydig cells. This potentially critical relationship will require further investigation.

4.8. Conclusions

The results of this study suggest that males diagnosed with MetS have reduced fertility potential as defined by standard fertility testing, MMP and DF. This has been observed in the absence of leukocytospermia and clinical detection of varicocele. Reduced peripheral free testosterone associated with males with MetS patients has been further confirmed. In addition, free peripheral progesterone concentrations have also been found to be lower in MetS patients compared to control, indicating a collapse in steroidogenesis. Closely associated with hyperinsulinaemia/insulin resistance, hyperleptinaemia and a proinflammatory state, seminal fluid analysis indicated an increase in seminal TNF α , IL1 β , IL6, IL8, leptin and insulin in males with MetS, which can have detrimental consequences on ejaculated sperm function. The association of MetS with decreased male fertility parameters and reproductive tract inflammation in the absence of leukocytospermia is a novel, and warrant further investigations into these mechanisms. Furthermore, insulin and IL8 are highly concentrated in human semen in both healthy and MetS males, for unknown reasons. This requires further investigation. Numerous correlations between parameters have been identified, however, these results do not investigate causation of pathological changes associated with MetS and male reproductive health, and many are likely indirectly associated via the poorly understood MetS pathophysiology.

TM3 cell culture data directly implicated the inflammatory cytokines TNF α , IL1 β and IL6 in a dose dependent decline in steroidogenesis, and IL8 may stimulate TM3 Leydig cell growth. Insulin is associated with a dose-dependent increase in testosterone synthesis, with a significant decline in progesterone synthesis. Insulin resistance may be associated with an impaired ability for insulin to stimulate testosterone synthesis. Although indications of these mechanisms have been previously published, the importance of inflammation and insulin resistance in male reproduction, spermatogenesis and steroidogenesis in the setting of MetS has not been previously reported, and is a novel and exciting area for further research in the fields of andrology, immunology and endocrinology.

Taken together, important underlying metabolic, immune and endocrine phenomenon associated with MetS likely negatively influence steroidogenesis and Leydig cell function, resulting in impaired reproductive potential and overall health and well being in males.

This study provides novel insights into the impact and potential mechanisms between metabolic syndrome and male reproductive health. These areas may have important implications not only in the field of andrology and, but also for other disciplines such as immunology and endocrinology.



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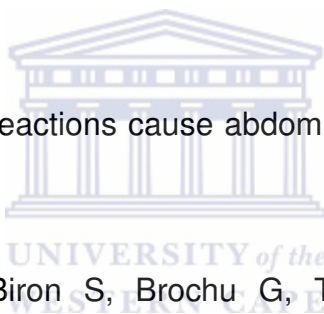
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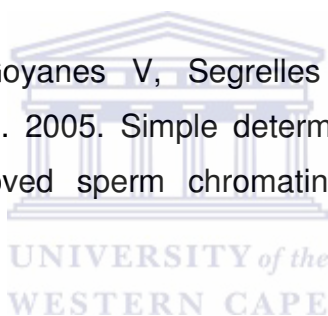
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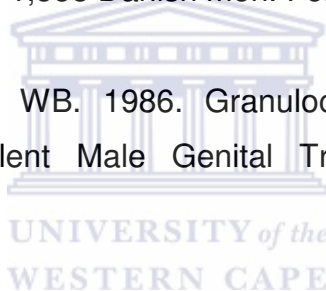
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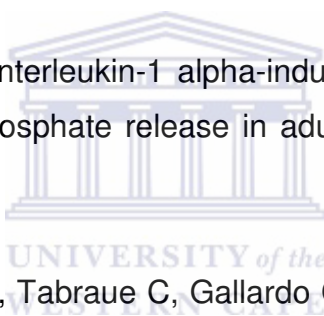
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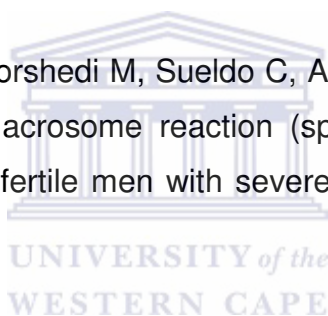
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