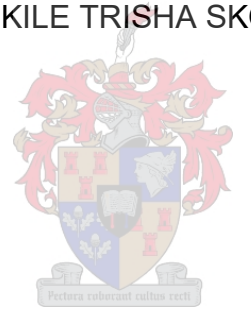
# AN INVESTIGATION OF OBESITY AS AN ETIOLOGY OF MALE INFERTILITY IN A RAT MODEL

BONGEKILE TRISHA SKOSANA




*Dissertation presented for the Degree of Doctor of Philosophy (Medical Physiology) in the Faculty of Medicine and Health Sciences,*

*Stellenbosch University*

Supervisor: Prof. Stefan S. du Plessis
Co-supervisor: Dr Guillaume Aboua
Co-supervisor: Prof. Gerhard van der Horst
Co-supervisor: Dr Erna Marais

March 2021

## Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously, in its entirety or in part, submitted it for obtaining any qualification.

The development and writing of this dissertation were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

Date: ……March 2021……..

## Abstract

Until recent decades, the inability to produce offspring has been seen as a female-related issue, but better understanding has made it evident that male fertility is likewise an essential determinant of couple fertility. Infertility in males has numerous causative factors, including lifestyle factors and obesity. Obesity has been shown to compromise fertility through changes in several aspects of reproductive function, including dysregulation of hormones (the HPG axis) and changes in the structure of the reproductive organs. There is, however, a great deal that is still unknown about how obesity and infertility interchangeably affect each other. New molecular techniques such as proteomics have been shown to provide insights into disease-causing mechanisms. These techniques may provide an avenue to discover more intrinsic changes that obesity may give rise to; it can also aid in the discovery of mechanisms through which obesity can act to bring about changes in male fertility.

The overall aims of this study were: (1) To examine the effects of obesity on male fertility by observing specific macroscopic (anthropometric), microscopic (sperm parameters, histology of the testis and epididymis) and molecular (antioxidant enzyme, reproductive hormones) changes in a diet-induced obesity animal model; (2) To examine changes in protein expression within the reproductive tissues of obese animals, quantify these changes, and identify the affected molecular pathways.

This study made use of an animal model of diet-induced obesity (DIO) to assess the effects of obesity on male reproductive organs and sperm parameters. Male Wistar rats (n=40) were randomly and equally divided into control (age-matched) and DIO groups and received standard rat chow or a high caloric diet *ad libitum* for 54 weeks, respectively. A long-term diet was chosen to mimic the gradual and chronic onset of obesity. At 60 weeks of age, rats were sacrificed by euthanasia. Each animal had their body weight measured and immediately examined post-mortem to determine visceral fat weight, testis weight and non-fasting blood glucose. Blood was collected from the thoracic cavity and used for plasma extraction and haematocrit analysis. Testis and epididymides were excised, weighed and preserved appropriately for subsequent sperm parameter evaluations (morphology and viability), histological analysis (H & E staining), protein determination, antioxidant evaluation (catalase, superoxide dismutase, glutathione, lipid peroxidation), and proteomics analysis (Liquid Chromatography Mass Spectrometry (LC-MS/MS)).

The chronic diet elevated body and visceral fat weights significantly in the DIO group compared to controls. Sperm morphology and viability, as well as estradiol production were negatively altered in the DIO group. These changes were associated with alterations in several macroscopic, microscopic and molecular changes including changes in relative testicular weight, histological aberrations, and a reduction in antioxidant enzymes within the testis and epididymis respectively.

Interestingly, testosterone was not significantly reduced, as seen in experiments with a shorter DIO feeding duration. This points towards a compensatory mechanism to counteract chronically increased testosterone concentrations. Protein expression profiles of the DIO and control groups suggest that the predominant molecular pathways affected by th treatment were related to metabolism. These seem to be the possible driver of changes in other proteins including those involved in the production of reactive oxygen species (ROS). Some lesser researched antioxidant proteins were increased in expression to counteract ROS. The negative histological changes observed in the DIO group were linked to the underexpression of structural proteins involved in cell-to-cell adhesion. Reproductive proteins including those involved in sperm production, fertilization and the early stages of embryonic development were reduced in expression in the DIO group. These negative changes were possibly instigated by the observed increases in stress proteins, redox and inflammatory proteins.

It is therefore evident that long-term obesity can impair male reproductive parameters and could be a contributing factor to the decline in male fertility by affecting sperm and reproductive parameters. Proteomic analysis of the epididymis and sperm showed that proteins essential in metabolism, ROS production, stress, inflammation and in the regulation of reproductive function as well as sperm and epididymis structure were negatively affected. In addition, long-term obesity can mask detrimental changes in physiology due to compensatory mechanisms, making changes in reproductive parameters difficult to explain.

## Opsomming

Tot ‘n paar dekades gelede was die onvermoë om 'n nageslag te produseer, gesien as 'n vroulike probleem, maar beter begrip het dit duidelik gemaak dat manlike vrugbaarheid ook 'n noodsaaklike bepalende faktor vir egpaarvrugbaarheid is. Onvrugbaarheid by mans het talle oorsaaklike faktore, insluitend lewenstylfaktore en vetsug. Daar is getoon dat vetsug vrugbaarheid in gevaar stel deur veranderinge in verskeie aspekte van voortplantingsfunksie, insluitend wanregulering van hormones (die HPG-as) en veranderinge in die struktuur van die voortplantingsorgane. Daar is egter nog baie onbekend oor hoe vetsug en onvrugbaarheid mekaar onderling beïnvloed. Nuwe molekulêre tegnieke, soos proteomika, het getoon dat dit insigte kan bied in meganismes wat siektes veroorsaak. Hierdie tegnieke kan 'n manier wees om meer intrinsieke veranderinge te ontdek waartoe vetsug kan lei; dit kan ook help met die ontdekking van meganismes waardeur vetsug kan optree om veranderinge in manlike vrugbaarheid teweeg te bring.

Die algemene doelstellings van hierdie studie was: (1) Om die effekte van vetsug op manlike vrugbaarheid te ondersoek deur spesifieke makroskopiese (antropometriese), mikroskopiese (spermparameters, histologie van die testis en epididymis) en molekulêre (antioksidant ensieme, voortplantingshormone) se veranderinge waar te neem in 'n dieet-geïnduseerde vetsugtige dieremodel; (2) Om veranderinge in proteïenuitdrukking binne die voortplantingsweefsel van vetsugtige diere te ondersoek, hierdie veranderinge te kwantifiseer en die aangetaste molekulêre paaie te identifiseer.

In hierdie studie is 'n diermodel van dieet-geïnduseerde vetsug (DIO) gebruik om die gevolge van vetsug op manlike geslagsorgane en spermparameters te bepaal. Manlike Wistar-rotte (n = 40) is ewekansig en gelykop verdeel in kontrole- (ouderdom-ooreenstemmende) en DIO-groepe en het onderskeidelik standard rotkos of 'n hoë kalorie-dieet ad libitum vir 54 weke lank ontvang. 'n Langtermyndieet is gekies om die geleidelike en chroniese aanvang van vetsug na te boots. Op 60 weke ouderdom is rotte deur genadedood geoffer. Elke dier se liggaamsmassa is gemeet en onmiddellik nadoods ondersoek om die viserale vetmassa, die testisgewig en die nie-vaste bloedglukose te bepaal. Bloed is uit die borsholte versamel en vir plasma-ekstraksie gebruik en hematokritanalise. Testis en epididimis is uitgesny, geweeg en toepaslik bewaar vir daaropvolgende spermparameter-evaluasies (morfologie en lewensvatbaarheid), histologiese analise (H & E-kleuring), proteïenbepaling, anti-oksidant-evaluering (katalase, superoksied-dismutase, glutathion, lipiedperoksidasie) en proteomika-analise (Vloeistofchromatografie massaspektrometrie (LC-MS / MS)).

Die chroniese dieet het liggaams- en viserale vet-massa aansienlik verhoog in die DIO-groep vergeleke die kontroles. Verskeie voortplantingsparameters was in die DIO-diere geaffekteer.

Spermmorfologie en lewensvatbaarheid, sowel as oestradiolproduksie, was negatief verander. Dit was geassosieer met veranderinge in verskeie makroskopiese, mikroskopiese en molekulêre veranderinge, insluitend 'n verandering in die relatiewe testikulêre gewig, histologiese afwykings en 'n vermindering in antioksidant ensieme binne die testis en epididimis. Interessant genoeg was testosteroon nie betekenisvol verminder, soos gesien in eksperimente met 'n korter DIO-voedingstydperk, nie. Dit dui op 'n kompenserende meganisme om chroniese verhoogde testosteroonkonsentrasies teë te werk. In hierdie geval kan dit toegeskryf word aan moontlike aanpassings aan die setpunt van die HPG-as om tekorte in die testikelfunksie of defekte in die testosteroon terugvoermeganisme teë te werk. Proteïen-uitdrukkingsprofiele van die DIO en kontrolegroepe dui op talle onderliggende veranderinge. Die oorwegende paaie wat geraak was, hou verband met metabolisme. Dit blyk die moontlike oorsaak van veranderinge in ander proteïene te wees, insluitend proteïene wat betrokke is by die produksie van reaktiewe suurstofspesies (ROS). Sommige anti-oksidant proteïene, wat minder ondersoek is, was egter verhoog in uitdrukking in 'n poging om ROS teë te werk. Die negatiewe histologiese veranderinge wat in die DIO-groep waargeneem is, kan gekoppel word aan die onderuitdrukking van strukturele proteïene wat betrokke is by sel-tot-sel-adhesie. Proteïene wat betrokke is by selmotiliteit, sel siklus, DNA herstel en replikasie was negatief beïnvloed; dit is noodsaaklik vir lewensvatbaarheid en die produksie van funksionele proteïene. Reproduksieproteïene, insluitend proteïene wat betrokke is by spermproduksie, bevrugting en die vroeë stadiums van embrionale ontwikkeling, was verminder in die DIO-groep. Die negatiewe veranderinge wat gelys is, is moontlik aangewakker deur die waargenome toename in stresproteïene, redoks en inflammatoriese proteïene.

Dit is dus duidelik dat vetsug op die lang termyn manlike reproduksieparameters kan benadeel en 'n bydraende faktor kan wees tot die afname in manlike vrugbaarheid deur sperm- en reproduksieparameters te beïnvloed. Proteomiese analise van die epididimis en sperm het getoon dat proteïene wat noodsaaklik is vir metabolisme, ROS-produksie, stres, inflammasie en die regulering van die voortplantingsfunksie en die sperm- en epididimis-struktuur negatief beïnvloed was. Daarbenewens kan vetsug op die lang termyn skadelike veranderinge in fisiologie as gevolg van kompenserende meganismes verberg, wat veranderinge in reproduktiewe parameters moeilik verklaarbaar kan maak.

## Acknowledgements

I would like to express my deepest gratitude to my supervisors for their support during my research project and the writing of this dissertation. Prof. van der Horst, Dr Marais and Dr Aboua, thank you for your valuable insights and contributions to this research and the writing of this dissertation. A special thank you to Prof. Stefan du Plessis for seeing me through this degree and for being both my mentor and my supervisor throughout my postgraduate studies. Being your student was an incredibly enriching experience and I will forever be grateful for all the skills that you have imparted upon me.

I would like to thank my friends and family for their unwavering support throughout my research, and for being my strength throughout the writing process. Thank you to Judith and Fanyana Skosana for your love and encouragement. To my thesis writing partners, Terri-Ann de Jager and Rozanne Adams, thank you for your support and friendship. Thank you for keeping me accountable and for being the best support group – I will always cherish the shared hours and meals together while we prepared our dissertations. The experience would not have been the same without you.

Thank you to my partner Peter Tumureebire for your constant support and your words of encouragement. You were with me throughout the entire journey. Thank you for being my shoulder to cry on when I felt overwhelmed, and for sharing in my happiness when all of the work was finally completed.

To my mentor Prof. Samantha Sampson, thank you for your advice and encouragement. Thank you for keeping me accountable and for giving of your time to council and guide me through this process.

I would like to acknowledge my funders, the National Research Foundation (NRF), for funding my research and the students that worked together with us on this project though the NRF Thuthuka Grant (Grant number 107095). I would also like to acknowledge the Subcommitee C of the Faculty of Medicine and Health Sciences for matching the funding contribution awarded to us by the NRF.

To my collaborators Prof. Amanda Lochner, Dr Ingrid Webster and Dr Suzel Hattingh, Prof. David Tabb, Dr. Xia Wang, thank you very much for your contributions to this study and for giving of your time and resources to allow this research to be completed successfully.

The work from this research was presented at the following conferences:

44th Annual conference of the Physiology Society of Southern Africa, 28-31 August 2016, The River Club, Cape Town, SA. B Skosana, S Hattingh, I Webster, A Lochner, G van der Horst, G

Aboua, SS Du Plessis. A proteomic search for biomarkers of obesity induced-male infertility. (Poster presentation)

Conference of the Polish Society of Andrology – 18th Andrology Day. 30 September - 1 October 2016. Novotel, Gdansk, Poland. B Skosana, SM Hattingh, I Webster, A Lochner, G van der Horst, YG ABoua, SS du Plessis. (2016) Obesity-related testicular histological changes explained by proteomics. (Oral presentation)

10th European Congress of Andrology, 11-13 October 2018. Budapest, Hungary. Bongekile Skosana, Suzel Hattingh, Ingrid Webster, Amanda Lochner, Stefan S Du Plessis. Sperm proteins implicated in obesity-induced male infertility (Poster presentation)

47th Annual conference of the Physiology Society of Southern Africa, 18-21 August 2019, East London, SA. Skosana B.T., Tabb D., Wang, X., Webster, I., Lochner, A., van der Horst, G., Aboua, Y.G., du Plessis, S.S. Oxidative stress in male reproductive organs: a proteomic investigation of a chronic diet-induced obesity model in male Wistar rats. (Oral presentation)

I acknowledge that opinions, findings and conclusions or recommendations expressed in any publication generated by this NRF supported research is that of the author(s), and that the NRF accepts no liability whatsoever in this regard.

This dissertation is dedicated to my Mother:

**Judith Nonhlanhla Skosana**

Without you, none of this would have been possible

# Table of contents

## List of abbreviations

| | |
|---|---|
| 1-DE | 1-dimensional electrophoresis |
| 2-DE | 2-dimensional electrophoresis |
| AgRP | Agouti-related peptide |
| AI | Adiposity index |
| AR | Androgen Receptors |
| ARC | Arcuate nucleus |
| ART | Assisted Reproduction Techniques |
| ART | Assisted reproductive treatment |
| BAT | Brown adipose tissue |
| BCA | Bicinchoninic acid |
| BMI | Body Mass Index |
| BMR | Basal metabolic rate |
| BSA | Bovine serum albumin |
| CART | Cocaine- and amphetamine-regulated transcript |
| CASA | Computer-aided sperm analysis |
| CAT | Catalase |
| CDC | Centers for Disease Control and Prevention of the United States |
| CNS | Central nervous system |
| CT | Computerized tomography |
| DDT | Dichlorodiphenyltrichloroethane |
| $dH_2O$ | Distilled water |

| | |
|---|---|
| DHT | Dihydrotestosterone |
| DIO | Diet-induced obesity |
| ED | Erectile dysfunction |
| ELISA | Enzyme-linked immunosorbent assay |
| ESI | Electrospray ionization |
| FFA | Free fatty acid |
| FSH | Follicle Stimulating Hormone |
| GHS | Reduced glutathione |
| GnRH | Gonadotropin Releasing Hormone |
| $H_2O_2$ | Hydrogen peroxide |
| HPA axis | Hypothalamic-pituitary-adrenal axis |
| HPG | Hypothalamic-Pituitary-Gonadal axis |
| HPLC | High-pressure liquid chromatography |
| ICSI | Intracytoplasmic sperm injection |
| IL1β | Interleukin-1 beta |
| IL-6 | Interleukin-6 |
| IVF | *In vitro* fertilization |
| LC | Liquid chromatography |
| LC-MS/MS | Liquid chromatography tandem mass spectrophotometry |
| LH | Luteinizing Hormone |
| LPO | Lipid peroxidation |
| m/z | Charge-to-mass |
| MALDI | Matrix-assisted laser desorption/ionization |

| | |
|---|---|
| MC4R | Melanocortin-4 receptor |
| MCH | Melanin-concentrating hormone |
| MDA | Malondialdehyde |
| MESA | Microscopic epididymal sperm aspiration |
| MRI | Magnetic resonance imagining |
| mRNA | Messenger RNA |
| MS | Mass spectrophotometer |
| MS/MS | Tandem mass spectrophotometry |
| NCDs | Non-communicable Diseases |
| NF-κB | Nuclear Factor Kappa B |
| NO | Nitric oxide |
| NPY | Neuropeptide Y |
| $O_2^-$ | Superoxide anions |
| OS | Oxidative stress |
| PESA | Percutaneous epididymal sperm aspiration |
| PDHA | Pyruvate dehydrogenase |
| POMC | Pro-opiomelanocortin |
| POPs | Persistent organic pollutants |
| PUFA | Polyunsaturated Fatty Acid |
| PVH | Paraventricular hypothalamus |
| RNA | Ribonucleic acid |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |

| | |
|---|---|
| SCA | Sperm class analyser |
| SDS | Sodium dodecyl sulphate |
| SHBG | Sex hormone binding globulin |
| SOD | Superoxide dismutase |
| TBARS | Thiobarbituric Acid Reactive Substances |
| TCA | Trichloroacetic acid |
| TESA | Testicular sperm aspiration |
| TESE | Testicular sperm extraction |
| TNF-α | Tumour necrosis factor |
| TOF | Time-of-flight |
| VMH | Ventromedial hypothalamus |
| WHO | World Health Organization |
| WHR | Waist-to-hip ratio |
| α-MSH | Alpha-melanocyte-stimulating hormone |

# List of Tables

# List of Figures

# Chapter 1: Introduction

## 1.1. Background

Until recently, the inability to produce offspring has been seen as a female-related issue, but better understanding of the human reproductive system has made it evident that male fertility is likewise an essential determinant of couple fertility (Hull *et al*., 1985; Hammadeh, Filippos & Hamma, 2009; Mehta *et al*., 2016; Rumbold, *et al.*, 2019; Murshidi, Choy & Eisenberg, 2020). Male factor infertility encompasses abnormalities within the male reproductive system that can impair structural and functional sperm parameters. It can contribute up to 50% to couple infertility (World Health Organization, 2014) and has many causative factors including hormonal disturbances, infections, genetic, endocrinological and reproductive anomalies (Pellicer *et al*., 1998). Importantly, however, male infertility can also be the result of modifiable risk factors that are the result of lifestyle. These include non-communicable diseases and obesity. Obesity, by definition, is an excess of body fat that poses a risk to health. The worldwide prevalence of obesity among children and adults doubled between 1980 and 2015, with approximately one third of the world's population now classified as overweight or obese (Afshin, *et al*., 2017; Chooi, Ding & Magkos, 2019). The prevalence is generally higher in women than in men, although the highest incidence was recorded in males aged 25-29 years residing in low-middle income countries.

Obesity is an etiological factor of male infertility and reductions in fertility have been shown to correspond with increased rates of obesity in countries with high obesity prevalence. Previous studies have shown obesity to result in poor sperm quality due to changes in sperm motility, morphology, viability, DNA integrity, and concentration (Du Plessis et al., 2010). Postulated mechanisms include increased adipokine concentrations, oxidative stress, inflammation (Fraczek and Kurpisz, 2007; Furukawa et al., 2004), hormone changes and hyperinsulinaemia (Teerds et al., 2011).

As many complexities regarding obesity-induced infertility and its effects on male fertility are still unanswered, molecular analyses are required to allow further insights. Proteomics is an analysis of proteins, which analyses the proteins present in an organism (Wilkins et al., 1995; Wilkins et al., 1997; Plebani et al., 2005). It produces protein profiles which allow the comparison between diseased tissues and healthy controls in order to identify affected proteins and disease-causing mechanisms (Anderson et al., 2000). This can then provide a great avenue to aid in the discovery of future diagnostic and treatment avenues.

## 1.2. Problem Statement

The principle question of the current study is whether obesity causes any changes in protein expression within the reproductive organs and spermatozoa of obese males, specifically in a rat model. If any changes are present, which molecular pathways are affected and could these alterations explain some of the phenotypic observations that have previously been described within obese, infertile males or similar animal models. In addition, can these protein changes describe the observations that are made in the current study.

## 1.3. Rationale of the Study

Whilst the effects of obesity on male fertility continues to receive attention, most of the published data focusses on observing changes to fertility parameters such as hormone changes and sperm parameters that are brought on by obesity. In many instances, the mechanisms leading to the observed phenotypic changes are not explored or have not been fully elucidated. As more technologies and molecular techniques become available to assess biological changes, more information can be gathered about how certain disease characteristics, e.g. obesity-related changes in fertility, are able to manifest.

## 1.4. Aims of the study

The overall aims of this study are:

- To examine the effects of obesity on male fertility by observing specific macroscopic (anthropometric), microscopic (sperm parameters, histology of the testis and epididymis) and molecular (antioxidant enzyme, reproductive hormones) changes in a diet-induced obesity animal model
- To examine changes in protein expression within the reproductive tissues of obese subjects, quantify these changes, and identify the affected molecular pathways

## 1.5. Hypothesis

We hypothesise that the chronic diet-induced obesity model will have a negative effect on sperm parameters, and on reproductive proteins that are responsible for normal reproductive function(s).

## 1.6. Outline of the Study

A summary of the literature will be provided in Chapter 2. This chapter will introduce male infertility and its causative factors, after which obesity will be defined and its effects on the male

reproductive system outlined. The chapter will end with a focus on proteomics and describe how it came into practice. Chapter 3 will provide the study design as well as describe the analyses performed within this study. The results will be presented in Chapter 4, along with the statistical analysis that were performed. These will be presented alongside the corresponding tables and graphs. Chapter 5 will discuss the findings of this study and provide an interpretation of the results. This chapter will also compare the findings of this study with those found in literature. The findings of this study will be concluded in Chapter 6, after which additional data will be provided in the Appendix.

## 1.7. Conclusion

Several reproductive parameters have been affected by the diet and the subsequent weight gain observed in the DIO animals. Changes in sperm morphology and viability, as well as estradiol production were seen to be affected by a culmination of several macroscopic, microscopic and molecular changes within the reproductive system. Among these include increased visceral fat weights which correlated to changes in sperm morphology, changes in epididymal catalse levels which related to alterations in sperm morphology and estradiol concentrations, as well as changes in testicular weight which were related to abberations in sperm viability. At a more molecular level, protein expression points towards numerous underlying abnormalities, with several protein pathways being affected by diet-induced obesity. The predominant pathways affected were related to metabolism, specifically influencing proteins involved in glycolysis, oxidative phosphorylation and β-oxidation. In addition, structural proteins involved in cell-to-cell communication, signal transduction and cell motility were negatively affected. These are essential for cell viability and the propagation of sperm towards to oocyte. Other proteins involved in cell cycle as well as DNA repair and replication proteins, which are also essential for cell viability as well as the production of functional proteins, were affected by the diet. Regulatory pathways essential for cell transport, signal transduction, the immune system, and the removal of aberrant cellular structures were dysregulated. These changes were possibly brought on by increases in stress proteins including redox, inflammatory and heat shock proteins. This is in agreement with by numerous studies which have shown obesity to increase biochemical stressors, which can lead to a cascade of biological changes and changes in bodily funtions. In addition to the listed changes, proteins responsible for proper reproductive function were differentially expressed between the study groups, including those responsible for sperm production, fertilization as well as the early stages of embryonic development. It is therefore evident from this study that long term obesity can impair male reproductive parameters and could be a contributing factor to the decline in male infertility by affecting sperm and reproductive parameters. Obesity in males is therefore a matter that needs

continuing intervention as it may lead to changes in fertilizing capacity. Lifestyle changes are thus encouraged to reduce its incidence and prevalence.

# Chapter 2: Literature Review

## 2.1. Introduction

The continued existence of a society is determined by the fertility of its people (Jauniaux, 2001). The desire to procreate is identified as one of our most primal needs, and is appropriately acknowledged in psychological ideology. In the works of psychologist Abraham Maslow (Maslow, 1943), his theory, the “Hierarchy of Needs”, places the need to procreate as one of the Immediate Physiological Needs, together with eating, breathing and maintaining homeostasis (Cherry, 2018). The inclusion of sexual reproduction at this level of the hierarchy highlights its importance to our existence and the propagation of the species.

In ancient societies, the primary occupation of a couple after establishing a home was to bear their own children (Morice, 1995). The inability to do so brought anguish and judgement, and in many cultures, this was just cause to allow a couple to separate (Robins, 1993). Furthermore, infertility has been recorded since biblical times and in the writings of ancient civilizations (Jones and Sills, 2013). Lack of reproduction had cultural, economic and political repercussions and was so essential to humankind that special consideration has been reserved for it in all existing belief systems (Morice *et al*, 1995).

The angsts of reproduction were not only reserved for peoples of the past, but are still evident in the present day. Until recently, most blamed infertility on the female, but better understanding of the human reproductive system in recent decades has made it evident that male fertility is likewise an essential determinant of couple fertility (Hull *et al.*, 1985; Hammadeh, Filippos and Hamma, 2009; Mehta *et al.*, 2016; Rumbold *et al.*, 2019; Murshidi, Choy and Eisenberg, 2020). A multicentre study by the World Health Organization (WHO) performed in the 1980’s observed that 10% of couples sought fertility treatment and amongst them, 20% had a male-related problem; 27% of the causes were related to both partners and 15% had an unknown cause (Comhaire *et al*., 1987). More recently, it was determined that male factor infertility can contribute up to 50% to couple infertility (World Health Organization, 2014). Male factor infertility encompasses abnormalities within the male reproductive system that can impair structural and functional sperm parameters, including genetic, anatomical, endocrine and immunological anomalies. Furthermore, chronic illnesses and sexual conditions impairing the male from inseminating his partner are included (Zegers-Hochschild *et al*., 2017).

The knowledge that men are contributing to couple infertility more than previously believed has encouraged the undertaking of more studies in order to understand how male infertility comes about. Human and animal studies have thus been employed, comparing fertile and infertile men to

uncover the derangements that can occur. Laboratory studies aiming to understand the underlying mechanisms of various pathologies, including infertility, are carried out in animal models. Laboratory rats have the same basic physiology, organ structure and similar body plans to humans. Moreover, almost all the disease-linked genes in humans have rat homologues, with rats sharing 90 percent of their genome with humans (Twigger, 2004; Iannaccone and Jacob, 2009; Jacob, 2010).

The physiological mechanisms governing male fertility will be reviewed, including the sperm parameters measured to determine the fertility of a male. This review will also analyse the causative pathologies of male fertility by looking at both human and animal studies and highlight the effect of non-communicable diseases on male factor infertility, with special attention to the role of obesity. As this study will make the case for why more molecular assessments (specifically proteomics) are needed in order for male infertility to be better understood, the sections dealing with genetics and genetic causes of infertility will be discussed in detail. This will establish the basis for the assessment of fixed versus dynamic molecular changes. Finally, a brief overview of the treatments of infertility will be outlined, followed by an analysis of new avenues that are available for future enquiry into the possible treatments of male infertility.

## 2.2. Overview of the Male Reproductive System

The reproductive system is one of the more complex organ systems of the body. From the Hypothalamus to the gonads, it encompasses a co-ordinated interplay between several organs and glands interacting together to determine sexual function. The organs, glands, essential hormones and processes necessary for the proper functioning of this system will be summarized below.

The male reproductive system (Figure 2.1) comprises most notably of the external genitalia and the testes, accompanied by accessory glands and ducts known as the internal genitalia (Silverthorn *et al*, 2014). The external genitalia, the penis and the scrotum, are the external protuberances of the male reproductive system. The testes are housed within the scrotum and are responsible for the production of spermatozoa and certain androgens. Internal genitalia, consisting of the prostate, seminal vesicles and bulbourethral (Cowper's) glands, produce secretions which contribute to the production of seminal fluid. This fluid is expelled together with spermatozoa as semen through the urethra, a shared passageway for both urine and semen. The urethra spans the length of the penile shaft and is surrounded by erectile tissue (Mawhinney and Angelo, 2013; Silverthorn *et al.*, 2014).

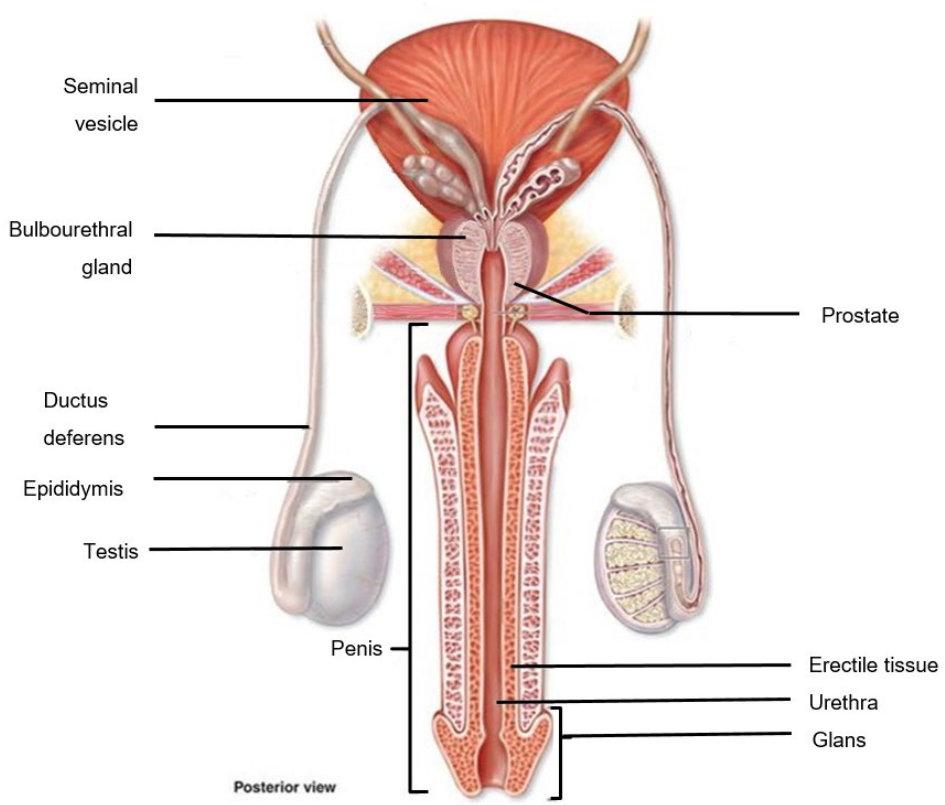



**Figure 2.1:** Posterior view of the human male reproductive system showing the eternal genitalia, the testes, internal genitalia and accompanying ducts. Figure adapted from McGraw Hill Companies, Inc**.**

The rat has the same reproductive organs as humans, differing mostly in size and proportion (Figure 2.2). The major difference is the protuberance of the penis, which is stored internally until it is erect. The rat penis also has a bone inside of it called a baculum (not shown).

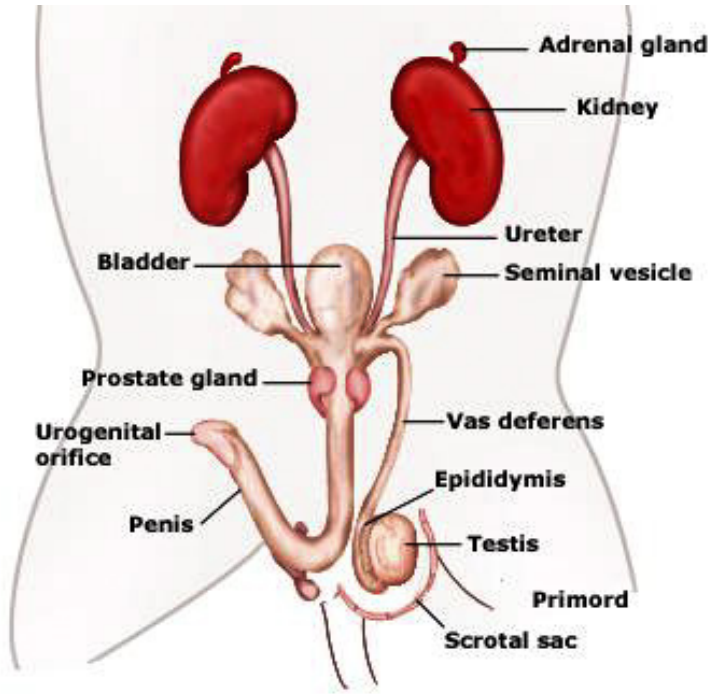



**Figure 2.2:** The male reproductive system of the rat showing the eternal genitalia, the testes, internal genitalia and accompanying ducts (Libretexts Biology, 2019)

### 2.2.1. Testes

As the histology of the testes and epididymis will be analysed in this study, it is worth briefly reviewing their structures. The testes (Figure 2) are present in both human and rat males. Testes are paired ovoid organs (Heffner and Schust, 2010) located together with the epididymides inside an external saclike structure known as the scrotum. The testes develop within the abdominal cavity during the foetal period in both humans and rats, and descend into the scrotum before and, in some instances, directly after birth. They are housed here due to the temperature requirements of spermatogenesis, which is optimally 1 - 2°C below normal body temperature (Silverthorn *et al.*, 2014). A patent inguinal canal (passageway in the anterior abdominal wall used by testes to descend into the scrotum) can be observed in rats, whereas the human equivalent is closed once the testes have descended into the scrotum (Werdelin and Nilsonne, 1999). The testes can thus communicate with the abdominal cavity in rats, and the inguinal canals are occupied by epididymal fat when the testes are in the scrotum. The ability to retract testes allows rats the ability to protect their testes and control testicular temperature (Okhura, Tsukamura and Maeda, 2000).

The primary functions of the testes include spermatogenesis and hormone production. Approximately 80% of the testis is occupied by seminiferous tubules, which are coiled tubes clustering into several hundred compartments (lobules) within the organ (Figure 2.3). Seminiferous tubules are enveloped by an external fibrous capsule called the tunica albuginea, which sends septa (fibrous connective tissue partitions) into the testes, dividing clusters of seminiferous tubules into lobules (Aitken *et al.*, 1997). The testicular interstitium is populated by interstitial cells including epithelial cells, macrophages, and most importantly, Leydig cells (Roberts and Pryor, 1997). Leydig cells are spherical cells containing large amounts of mitochondria and smooth endoplasmic reticulum. They are essential to the male reproductive system as they produce steroid hormones including the main male androgen, testosterone (Jambor, *et* al. 2015). During foetal development, testosterone production is required for the development and differentiation of male external genitalia and the onset of spermatogenesis. Adult Leydig cells are responsible for the androgen production necessary for masculinization at puberty and continued sperm production throughout the adult male life (Jambor, Tvrda and Lukac, 2015).

Within the seminiferous tubules are stratified (multiple) layers of male germinal (stem) cells, called the seminiferous epithelium. This epithelium is arranged in concentric cell layers progressing from primitive to differentiated germinal cells, under considerable support from surrounding somatic cells and structures. The most prominent supportive structure, the Sertoli cell, nurtures these cells as they divide and differentiate. It maintains the germ cells' cellular associations to the surrounding structures and other germ cells as they progress through spermatogenesis. Sertoli cells extend through the height of the seminiferous epithelium, penetrating through the differentiating layers to send arms to all germ cells (Hess and de Franca, 2005). These somatic cells are non-dividing, and are linked to one another via tight junctions. The junctions prevent passage of proteins and macromolecules from the interstitium into the lumen of the seminiferous tubules, where the developing spermatids are located (Roberts and Pryor, 1997). Sertoli cell secretions contain nutrients necessary for the development of germ cells, and are therefore called the "nurse cells" of the seminiferous tubules. Furthermore, they offer physical support for the developing germ cells by providing a scaffold onto which these cells can attach as they differentiate and move towards the lumen (Roberts and Pryor, 1997). Lastly, Sertoli cells create the blood-testis barrier via their tight junctions.

The seminiferous tubules are continuous with (and empty into) the rete testis, a network of tubules at the "exit" of the testes , which is called the hilum. The rete testis are continuous with the efferent ducts (ductuli efferentes), the collecting ducts which transport sperm into the epididymis. The seminiferous tubules are surrounded by a layer of muscle-like cells whose contractions assist in

the propulsion and expulsion of newly synthesized spermatozoa into ductuli efferentes and, therefore, into the epididymis. (Aitken *et al.*, 1997)

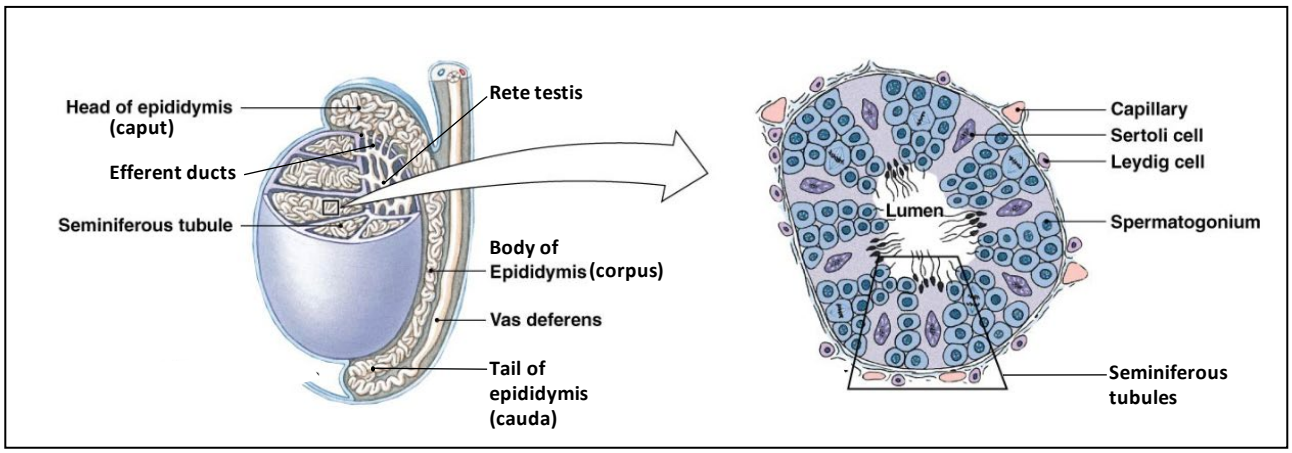



**Figure 2.3:** Schematic diagram depicting the human testis. Seminiferous tubules, the site of spermatogenesis, are illustrated here along with their internal cellular structures. Adapted from Silverthorn *et al*. (2014).

### 2.2.2. Epididymis

The epididymis carries out the same function in both human and rat reproductive systems; it is the storage organ of spermatozoa and the site of sperm maturation. The epididymis is an elongated crescent shaped organ tethered to the surface of each testis (Roberts and Pryor, 1997). The epididymis is essentially a single, convoluted duct (the ductus epididymis) lined by pseudostratified epithelium that is resting on a basement membrane (De Grava Kempinas and Klinefelter, 2015). Underlying the basement membrane is a connective tissue lining containing smooth muscle cells.

The epididymis is functionally divided into 3 regions; the caput (head), corpus (body) and the cauda (tail) (Figure 2.3). The caput lies atop the testis on its upper pole and contains the principal segment, and is the primary site of sperm maturation (Rogers, 2011). In this region, excess fluid from the testes is reabsorbed. Maturation continues in the corpus, where lipid containing secretions facilitate modification of the lipid content of the sperm cell membranes. The cauda is the major storage region of the epididymis. The cytoplasmic droplets expelled by maturing spermatozoa are absorbed. The sperm entering the proximal cauda are fully mature and capable of fertilization. Immobilin is then secreted to keep the sperm dormant during storage (De Grava Kempinas and Klinefelter, 2015).

There are slight variations in the structure of the human and rat epididymis. Humans have less differentiation between the caput, corpus and cauda; histological assessment shows no presence of a distinct initial segment (caput) and the caput does not exhibit the characteristic bulbous shape

that is seen in the rat and other mammals (Sullivan and Mieusset, 2016). Furthermore, the cauda in humans is poorly distinguishable from the vas deferens and it has a lower capacity to store sperm The human epididymis has a smaller lumen diameter, which could be related to the more rapid transit of sperm within the human epididymis (Bedford, 1994). Human sperm only takes 2-4 days to transit through the epididymis, whereas rat sperm take 10-12 days. This small reserve capacity in humans does not exceed the number of male gametes necessary to produce 2-3 normal semen samples (Bedford, 1990).

### 2.2.3. Hormonal regulation

The current study analyses the concentrations of two essential reproductive hormones, which necessitates a summary of the reproductive hormones within the male. The control of reproduction begins in the brain. The release of tropic hormones from the hypothalamus and the anterior pituitary set off physiological pathways which lead to the regulation of gonadal function (Harris, 1955; Silverthorn *et al.*, 2014) (Figure 3). The cascade begins with the release of a peptide hormone, Gonadotropin Releasing Hormone (GnRH), from the hypothalamus. This stimulates the production and release of two hormones from the anterior pituitary gland, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). FSH and LH are hormones which act directly on the gonads to initiate spermatogenesis and hormone production (Meethal and Atwood, 2005).

The release of GnRH is described as pulsatile (Hotchkiss and Knobil, 1996). Periodic secretion of this hormone by the hypothalamus every 1-3 hours ensures stimulation of GnRH receptors without their desensitization (Heffner and Schust, 2010). GnRH receptors are located on secretory cells of the anterior pituitary and their stimulation promotes the production and secretion of FSH and LH, the two main endocrine regulators of testicular function (Mutsumoto and Bremner, 2016), into the bloodstream (Meethal and Atwood, 2005). The receptors for FSH are located on Sertoli cells and activation of these, through a cascade of events, leads to an increase in spermatogenesis. LH stimulates Leydig cells in the testicular interstitium to produce estrogen and testosterone. (Gartner & Hiatt, 2000; Schulster *et al*, 2006). Estrogen production in the male is under the control of Leydig cells, Sertoli cells and germ cells. Sertoli cells contribute via the secretion of aromatase, an enzyme responsible for the conversion of testosterone to estradiol (Schulster, Bernie and Ramasamy, 2006). Germ cells also produce estradiol through this mechanism, providing majority contribution to the production of this hormone (Hess, 2003). Furthermore, estradiol is the dominant form of oestrogen (Schulster, Bernie and Ramasamy, 2006).

The system encompassing the 3 major organs involved in reproductive hormone production is appropriately termed the Hypothalamic-Pituitary-Gonadal (HPG) axis. The HPG axis is regulated by both positive and negative feedback systems. The release of hormones by the brain stimulates

gonadal hormone production which in turn, alter the secretion of their tropic hormones, GnRH, LH and FSH (Huhtaniemi, 1999). LH and FSH themselves inhibit the secretion of GnRH from the hypothalamus. The Sertoli cells assist in the regulation of hormone production via the secretion of paracrine factors (locally produced molecules which stimulate cell-to-cell communication) (Heffner and Schust, 2010). The purpose of these feedback systems is the precise control of hormone concentrations. Gonadal hormones always propagate a negative feedback of their stimulatory hormones and as their levels increase, their stimulatory hormones decrease. The regulation of the HPG axis by feedback mechanisms prevents the continued secretion of hormones by the gonads and possible dysregulation of the HPG axis. (Burger, 1988; Skinner *et al*., 1989). The HPG axis is well conserved among vertebrates, differing only by the duration of lifecycles, reproductive cycles and behaviours (Sower, Freamat and Kavanaugh, 2009). Experiments using animal models, such as the rat, to elucidate changes in HPG axis function during an intervention or disorder can therefore be extrapolated to humans with great accuracy.

Testosterone is the principal hormone of the male reproductive system. This steroid hormone is key to the development of the male foetus and in the growth and maturation of male internal and external genitalia (Sajjad, 2010; Creasy & Chapin, 2013) . Its release during puberty promotes the development of secondary sexual characteristics including facial hair, deeper voices, skull and bone virilisation and an enlarged larynx (voice box) (Herman-Giddens *et al.*, 2001). Testosterone is produced from cholesterol which undergoes enzymatic reactions and oxidation to become testosterone (Lyetjens and Weinbauer, 2012). The actions of testosterone are brought about directly by testosterone itself, or through its potent metabolite dihydrotestosterone (DHT). Testosterone is converted to DHT by the enzyme 5α-reductase which is present in the testes, epididymis, prostate, seminal vesicles, the liver, hair follicles, skin and the brain (Norman and Henry, 2015). The conversion to DHT occurs at these target tissues, where DHT has a regulatory effect. Both testosterone and DHT bind to androgen receptors (AR) within target tissues to control protein expression of essential regulatory proteins, with DHT having a more pronounced effect due to its higher potency (Lyetjens and Weinbauer, 2012). Testosterone regulates libido, contributes to the determination of bone mass, helps to regulate fat distribution, has an anabolic effect on muscle production, stimulates the production of red blood cells through its effect on erythropoietin and regulates spermatogenesis (Harrison, 2013).

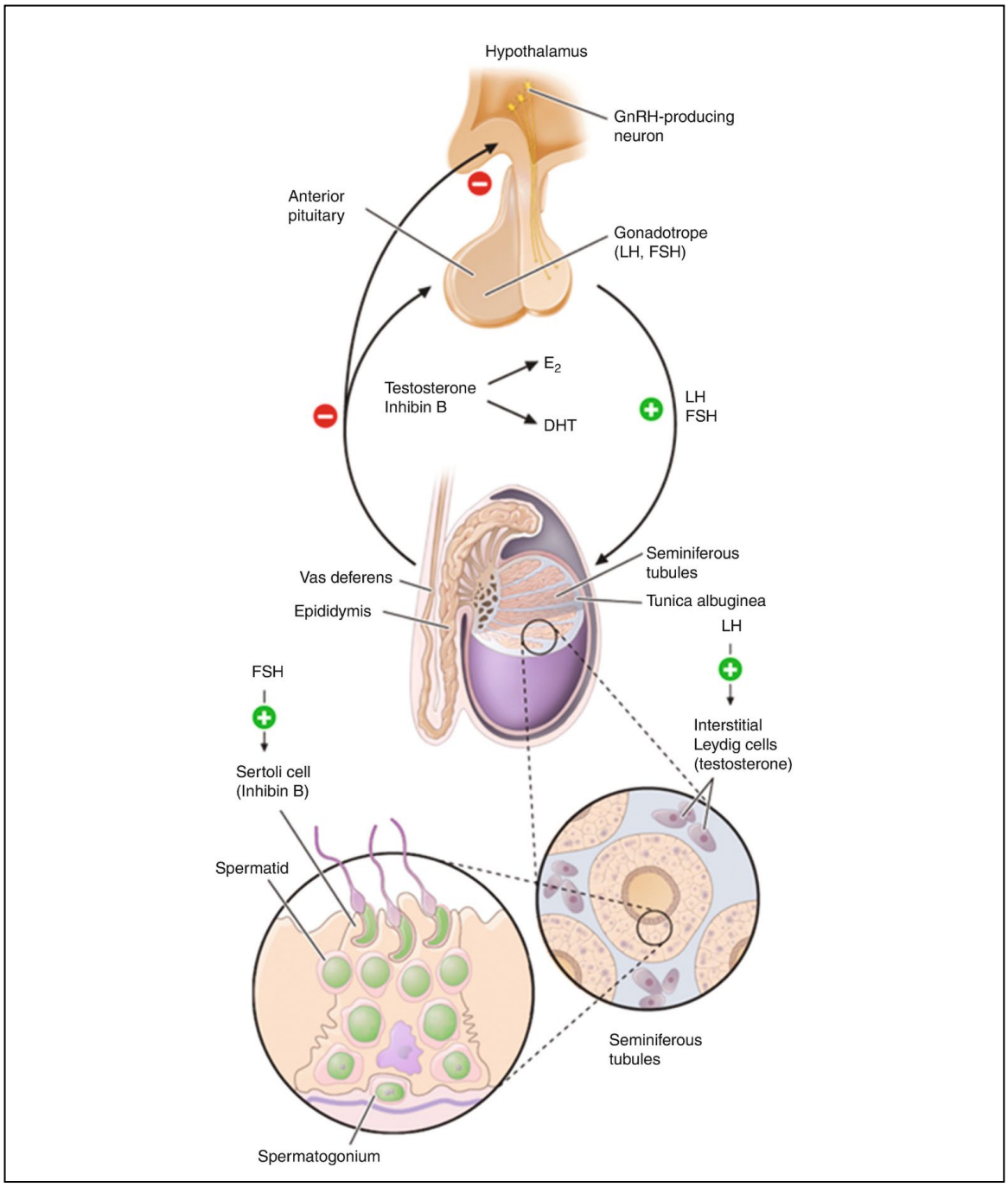



**Figure 2.4** The Hypothalamus-Pituitary-Gonadal (HPG) axis showing the glands, hormones and organs involved in the control of gonadal function. The hypothalamus and pituitary in the brain release gonadotropic hormones which stimulate the production of androgens from testicular tissue. Feedback systems then intricately control the amounts of gonadotropes released from the brain to regulate the production of testosterone, dihydrotestosterone (DHT) and estradiol (Dwyer and Quinton, 2019)

### 2.2.4. Spermatogenesis

Spermatogenesis describes the cellular transformation of male diploid spermatogonial stem cells into haploid spermatozoa through a complex process occurring within the male gonads (Figure 2.5 and 2.6). This transition occurs via carefully orchestrated cyclic cell ‘stages’ and ‘phases’ controlled by intrinsic and extrinsic testicular factors, culminating in continuous sperm production (Hess & de Franca, 2008).

The development of male germ cells is almost identical in all mammals, including humans and rats (Adler, 1996). Germ cells develop into spermatozoa within the walls of the seminiferous tubules (Roberts and Pryor, 1997). Each germ cell differentiates in close association with Sertoli cells for nutritive support during the development process. Development and differentiation occur from the basement membrane to the lumen, with spermatogonia, the immature germ cells, resting along the basement membrane (Creasy, 2002) (Figure 2.5). These cells undergo several mitotic divisions to colonize the seminiferous tubules with an adequate stem cell population and for promotion to primary spermatocytes (Gartner and Hiatt, 2000). Meiosis is thereafter the predominating mechanism through which germ cells differentiate. Meiotic cell divisions result in the halving of genetic material which permit male germ cells to become haploid cells (Mutsumoto and Bremner, 2016). Meiosis includes two divisions. Prophase of the first division is a lengthy process giving rise to several germ cell forms, which are different stages of the primary spermatocytes. These are leptotene, zygotene, pachytene and diplotene spermatocytes. This is followed by a diakinesis stage in which the chromosomes condense (Heffner and Schust, 2010; Mutsumoto and Bremner, 2016). The cells then undergo metaphase and anaphase, where the chromosomes line up at the equatorial plate and chromatids move to opposite poles of the cell, respectively. In the final stage, telophase, cytokinesis results in two secondary spermatocytes, which concludes the first meiotic division (Gartner and Hiatt, 2000). The second meiotic division allows sister chromatids to separate, giving rise to haploid spermatids with half the genetic material of the somatic stem cells. This culminates in 1 primary spermatocyte undergoing two divisions to give rise to 4 spermatids (Roberts and Pryor, 1997).

Spermatids undergo a subsequent maturation process termed spermiogenesis, which results in elongated sperm tails (Hess and de Franca, 2008). These endow the sperm with motile capabilities, allowing them to traverse the female reproductive system. Furthermore, spermiogenesis permits development of the acrosome, extrusion of excess cytoplasm and condensation of nuclear material (Creasy and Chapin, 2013). This packages the nuclear chromatin tightly to reduce nuclear volume and to protect the nuclear material from damage. The progression of germ cells through spermiogenesis allows them to move towards the lumen of the seminiferous tubules (Gartner and Hiatt, 2000). Spermatozoa are the final germ cell form, which are released

into the lumen by a process known as spermiation (the release of spermatozoa from Sertoli cells) (Mutsumoto and Bremner, 2016). The basement membrane is supported by fibroblasts and myoid cells, whose contractions create peristaltic movements that transport the spermatozoa out of the seminiferous tubules and into the epididymis (Roberts and Pryor, 1997).

Similarities and differences in spermatogenesis exists between mammals. Spermatogonial stem cells are always present and the process involves slow cycling through the different phases of division (Sharpe, 1944). The duration of the spermatogenic cycle, however, differs between humans and other mammals. The stages of differentiating spermatogonia last for 10.5 days in the rat compared to 16 days in human males. The human cells also only go through two rounds of mitotic division whereas the rat goes through six (Adler, 1996). The total process of spermatocyte development, including meiosis, takes 19 days in the rat (Figure 2.6) and 25 days in the human (Figure 2.5). Finally, spermatids develop for 12 days in the rat and 16 days in humans. Pathological insults cause different changes in the development of the spermatogenic series (Ehling, Doherty and Malling, 1972).

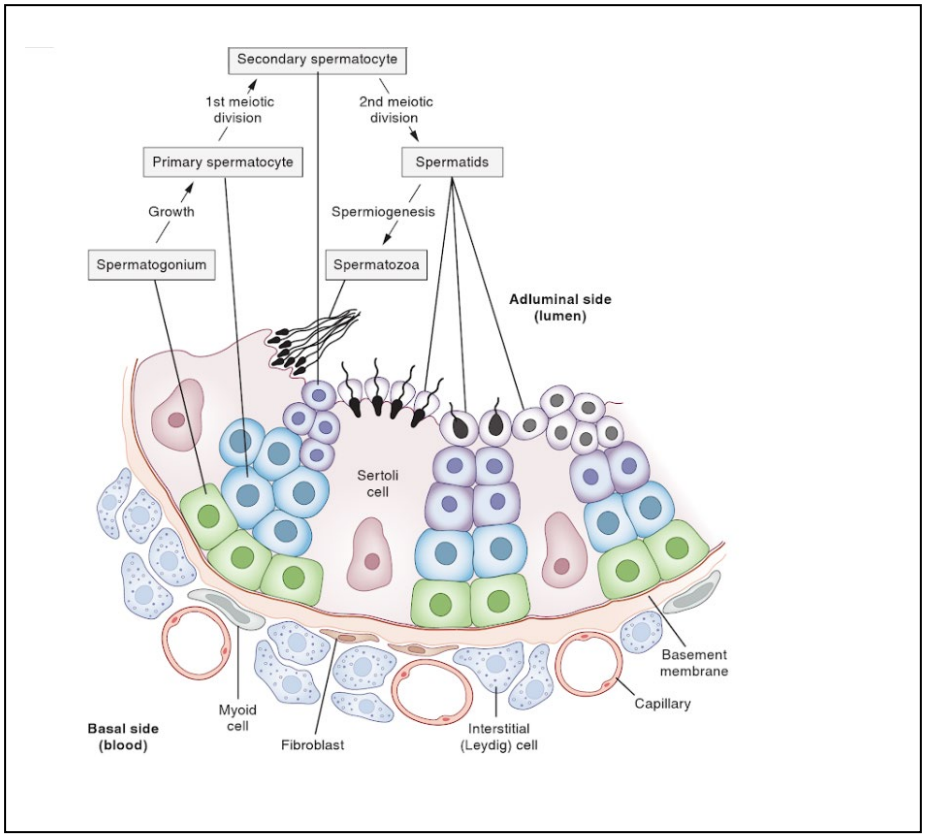



**Figure 2.5** Schematic diagram depicting spermatogenesis and the structure of the seminiferous tubules. This image shows the testicular tissue of a normal fertile male, detailing the seminiferous epithelium and the surrounding interstitial tissue. Progression of the spermatogenic series from spermatogonia on the basement membrane to spermatozoa in the adluminal portion of the seminiferous tubule is demonstrated. (Norman and Henry, 2015)

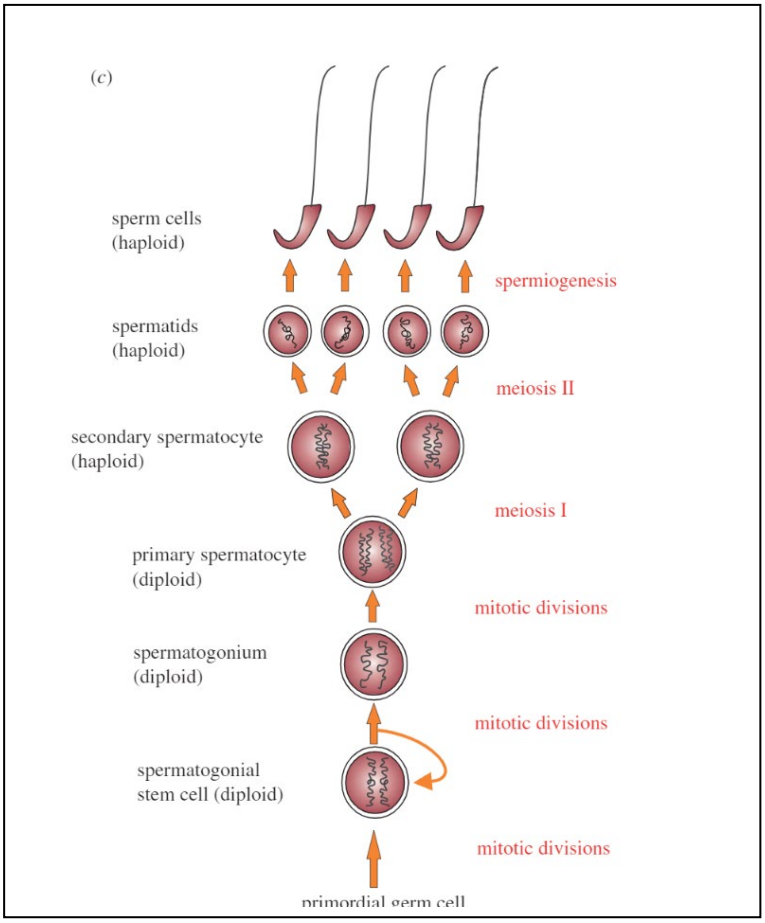



**Figure 2.6** Schematic diagram of spermatogenesis in the rat. Progression of the spermatogenic series from spermatogonia on the basement membrane to spermatozoa in the adluminal portion of the seminiferous tubule is demonstrated (Cheng & Mruk, 2010).

### 2.2.5. Structure of spermatozoa

Morphological analysis of spermatozoa is one of the reproductive parameters which was carried out in this study, so the structure of spermatozoa will be reviewed. Spermatozoa carry male genetic material to the female oocyte. Likewise, their structure is designed to optimize this process (Anderson and Personne, 1975). Its purpose is to travel towards the oocyte, fuse with its cortex and introduce its genetic material to the interior of the oocyte to create a viable embryo.

Mammalian sperm have a typical morphology, comprising of four separate regions: a head, neck, midpiece and tail (Auger, 2018) (Figure 2.7). Spermatozoa are enclosed by a plasma membrane largely containing polyunsaturated fatty acids (PUFAs) and glycoproteins. Morphologically normal human spermatozoa have an ovoid or piriform head measuring 3.7-4.7µm lengthwise and 2.5-3.2µm crosswise, with an acrosomal cap covering 40-70% of the head surface and a cytoplasmic region encompassing 30% of the head area (World Health Organization, 2010). The acrosome

covers the anterior part of the sperm head and encloses the nuclear material (Fléchon, 2016). The small residual cytoplasm of the sperm is a hydrodynamic advantage, but results in a lack of cytoplasmic material to cope with external insults. Thus, a condensed nucleus is a protective mechanism, compensating for a deficiency of DNA repair proteins, allowing the male nuclear material to journey safely through the male and female reproductive tract (Auger, 2018).

The acrosomal cap is a lysosome-like bag, containing digestive enzymes such as hyaluronidase necessary for sperm-egg fusion. It is surrounded by inner and outer acrosomal membranes, which meet at the equator of the sperm head. The outer membrane closely opposes the nuclear membrane while the inner membrane affixes to the sperm plasma membrane. The granules within the acrosome are released upon arrival of the sperm at the oocyte membrane (oolemma), a process called the acrosome reaction, allowing digestion of the oolemma to facilitate sperm-oocyte fusion. (Fléchon, 2016)

The nucleus contains paternal genetic information densely packaged within chromatin. DNA material is wrapped around histones, helically arranged proteins around which DNA can coil like thread around spools, allowing tight packaging of sperm DNA (McGhee and Felsenfeld, 1980). Further condensation of the male genome occurs during the late spermatid phase of spermatogenesis through an exchange of histones with protamines (sperm specific nuclear compaction proteins) within the nuclear material. This results in sperm DNA that is tightly packaged by protamines, making the chromatin seven times denser than that of a somatic cell (Ward and Coffey, 1991). Approximately 10-15% of human spermatozoa remain packaged by histones. This small percentage resembles the nuclear organization of somatic cells and is hypothesized to be due to the order in which genes need to be expressed upon fertilization (Rousseaux *et al*., 2008), where some nuclear material must remain uncondensed for easier and timeous translation. The nuclear cell membrane is closely applied to the nucleus and is devoid of any openings on its surface (Auger, 2018).

The neck of the sperm, occasionally referred to as the connecting piece, is the shortest segment of the spermatozoa. Its shape resembles that of a truncated cone, with a large base at its attachment to the head, tapering towards its attachment to the tail (Auger, 2018). Its role as the connecting piece is reinforced by the filaments found traversing its interior, linking the sperm head to the flagellum (tail). These perpendicular fibres make up the axoneme, the core of the connecting piece and the flagellum (Jequier, 2008). The axoneme is a collection of microtubules arranged in a cylindrical pattern, with 2 microtubules in the centre of the cylinder surrounded by 9 microtubule pairs creating its circumference (Figure 5). This, therefore, creates a “9 + 2” pattern, forming the core cytoskeleton of the neck and the flagellum. The axoneme is the motor apparatus of the flagellum (Fawcett, 1975). Importantly, the sperm neck contains two centrioles, distal and proximal.

The axoneme grows out of the distal centriole, which has the same microtubular structure. The proximal centriole is orientated 90 degrees from the distal, and is located at the base of the neck, at its attachment to the sperm head (Fawcett, 1970). The proximal centriole is key to fertilization as it is one of the materials deposited into the oocyte. After deposition, oocyte centrisomal proteins combine with this structure to form the centrosome, which is responsible for setting up the polarity of the oocyte and the assembly of the first mitotic spindle of the newly formed zygote (Manandhar *et al*., 2005).

The midpiece continues from the neck and contains the axoneme, encircled by mitochondria (Oates, 1997). These mitochondria are arranged in a helical pattern, end-to-end from one another, throughout the midpiece and are termed the mitochondrial sheath. They provide spermatozoa with the energy required for motility in the form of adenosine triphosphate (ATP) (Lindemann and Lesich, 2016).

The sperm tail, the longest segment of spermatozoa, is further subdivided into the principal piece and the end piece (Fawcett, 1975). The sperm ultrastructure includes a central axoneme surrounded by a fibrous sheath. The axoneme contains numerous protein fibres throughout its length, from the sperm neck to the tail, with as many as 200 being proposed (Ostrowski *et al*., 2002). The “9 + 2” microtubular structure is composed of tubulin proteins, with an additional 9 circumferential proteins termed outer dense fibres (ODF) found in the tail. This creates a new “9 + 9 + 2” microtubular pattern. ODFs extend two dynein arms each, which are structural proteins containing ATPases that catalyse the use of ATP for flagella movement (Gibbons, 1996). Dynein proteins facilitate the movement of flagella by attaching and detaching the axoneme microtubules, creating a sliding motion that propagates sperm forward. Each of the 9 outer microtubules attach to the central pair by protein fibres, giving the axoneme a wheel-spoke pattern (Afzelius, 1959; Oiwa *et al*., 2009).

Knowledge of the normal structure and function of the male reproductive system is essential to understanding any resulting pathologies from deviations of normality. Internal and external changes resulting in aberrations in the normal anatomy and physiology of the male reproductive system can reduce a male’s fertilizing potential, leading to infertility.

Sperm structure maintains the same components (head, midpiece, tail) but varies structurally between species. Rat spermatozoa differ most distinctly in head shape compared to human sperm. Rat sperm heads, as with most murine rodents, have a falciform, head with an apical hook (Immler *et al.*, 2007) (Figure 2.7 and Figure 2.8). This hook varies in size (sperm head length (ARC), sperm head width) and curvature (sperm head angle, sperm midpiece-head angle) (Figure 2.8) due to the variations in size and organization of the nucleus and acrosome (Gu *et al.*, 2019).

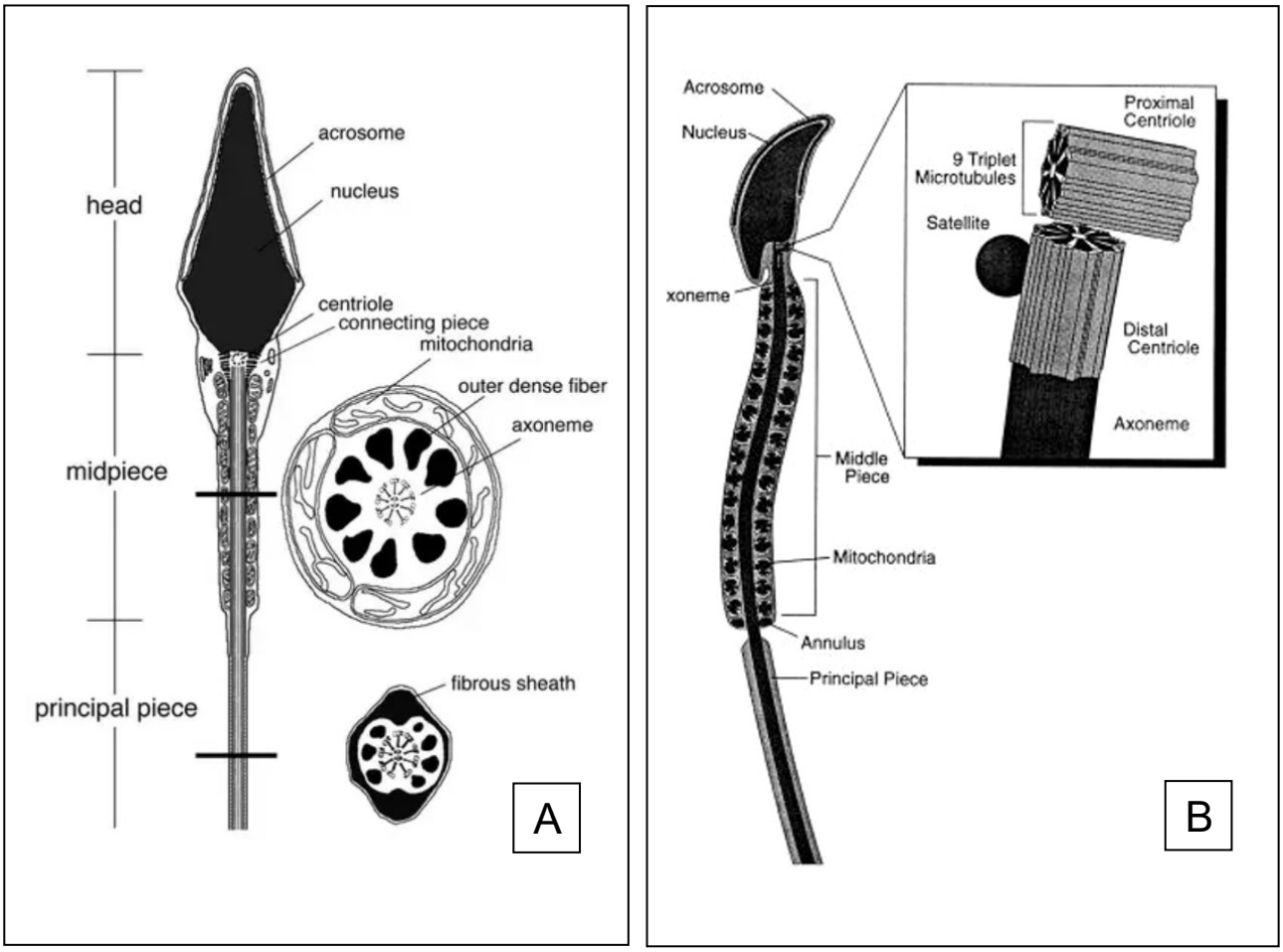



**Figure 2.7: Ultrastructure of spermatozoa. Image A depicts the structure of the human spermatozoa (Inaba, 2011); image B depicts the structure of the rat spermatozoa**

Van der Horst and colleagues (2018) recently characterised the size and curvature of rat spermatozoa in order to determine normal rat sperm morphometry measurements in Wistar and Sprague Dawley (SD) rats, particularly the sperm head and the anterior part of the tail, using CASA. This allowed the determination of upper and lower values of normal sperm morphometry parameters in order to allow for the differentiation between rat spermatozoa with normal and abnormal morphology. Table 2.1 below shows the minimum and maximum values obtained for normal sperm morphometry parameters. Spermatozoa with measurements outside of these ranges can be classified as morphologically abnormal. Using these cut off values, Van Der Horst *et al*. (2018) ascertained that, on average, 68-71% of spermatozoa in Wistar and SD rats have a normal morphology. Previous studies also determined head, midpiece and tail length of mammalian sperm including several wild-life rodents (Cummins, 1983; Cummins and Woodall, 1985; Vashishat, Dhanju and Cheema, 2012). Using manual measurements of mammalian spermatozoa, Cummins (1983) and Cummins and Woodall (1985) were able to provide insight on these sperm dimensions, which were corroborated by Vashishat and colleagues (2012) by means of semi-automated image

analysis. These authors found sperm head length, on average, to be > 25 µm. Van der Horst *et al.* (2018) found an average head length of > 21µm, which they attribute to their use of an isotonic/isosmotic stain. The use of staining methods which are not isotonic to that of the sperm intracellular fluid alters sperm morphometry measurements as the use of hypertonic or hypotonic staining solutions increase or decrease the size of the sperm due to osmosis. Several other sperm measurement parameters can be assessed to determine whether rat sperm possess a normal morphology, as listed in Table 2.1. The chord refers to the distance between the anterior tip of the acrosome and the posterior part of the sperm head; head linearity (LIN) and roughness are calculated using the equations outlined in Figure 2.8.

Sperm viability, motility and morphology are sperm parameters related to the fertility of a semen sample and are used to ascertain the fertility potential of an animal. Sperm morphology has been argued to be a strong indicator of fertility (Menkveld, Holleboom and Rhemrev, 2011) but it has been debated that male fertility cannot depend on the absolute number of a single parameter, nor be judged solely on the aforementioned sperm parameters (Bhattacharyya and Kanjilal, 2003; Petrunkina *et al.*, 2007; Vashishat, Dhanju and Cheema, 2012). The functional competence of sperm, which includes cervical mucous penetration, hypo-osmotic swelling, the hemi-zona assay and other *in vitro* tests, are also essential (Bhattacharyya and Kanjilal, 2003). Thus sperm should be subjected to multiple parameter analyses in order to assess fertility and fertilizing potential.

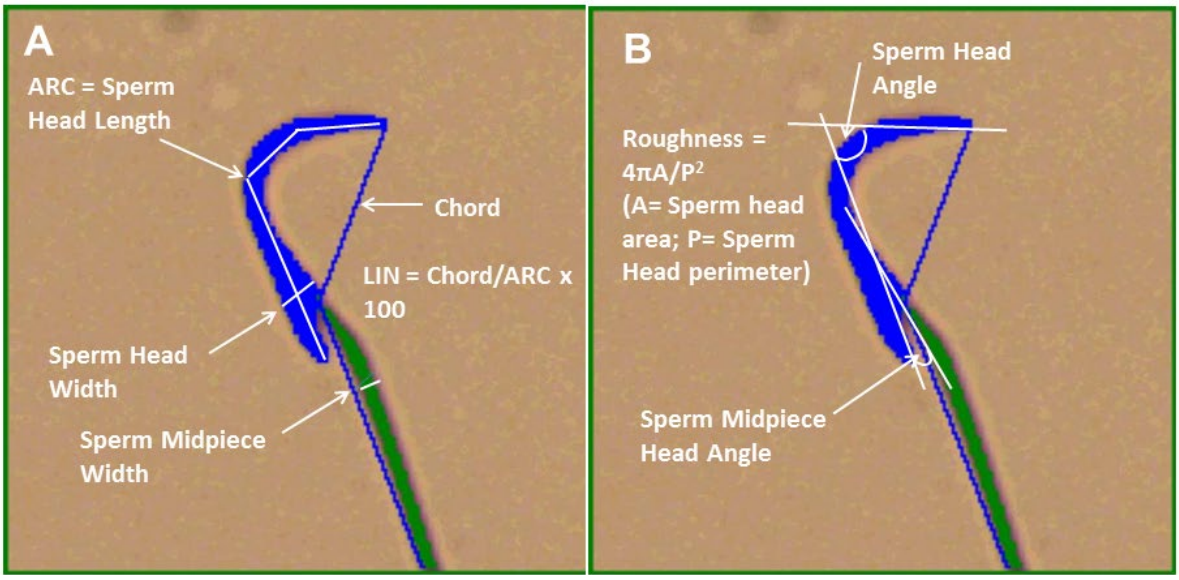



**Figure 2.8:** Morphology images obtained via computer-aided sperm analysis (CASA) showing normal sperm. The Head (blue) and midpiece (green), demarcated using the Sperm Class Analyser (SCA) system, can be assessed to determine normal sperm dimensions via the morphometry measurements depicted in the image.

**Table 2.1: Measurement values of rat sperm and mid-piece morphometry parameters as determined by Van Der Horst *et al.* (2018). Deviations from these morphometry values in Wistar and Sprague Dawley rats can be classified as having an abnormal sperm morphology.**

| Sperm head and mid-piece (MP) parameters | Minimum value | Maximum value |
|---|---|---|
| **Head** | | |
| Length (Arc) (µm) | 191 | 23 |
| Width (µm) | 0.9 | 1.90 |
| Perimeter (µm) | 40 | 50 |
| Area (µm) | 15 | 23 |
| Chord (µm) | 8 | 14 |
| Angle (degrees) | 50 | 87 |
| Linearity (%) | 40 | 65 |
| Roughness | 0.08 | 0.30 |
| **Mid-piece** | | |
| Width (µm) | 0.40 | 1 |
| Angle (degrees) | 0 | 60 |

## 2.3. Infertility

Defining infertility is essential for the clinical diagnosis and epidemiological investigation of infertility. The many definitions of infertility, stemming from the diverse criteria used to define a couple's fertility status and the many types of studies available to define its prevalence, can complicate this. Demographers, in measuring the human population, define infertility as the "absence of a live birth in a sexually active, non-contracepting woman" (Pressat and Wilson, 1985). The World Health Organization (WHO), however, defines it as "the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (World Health Organization, 2009). Demographers tend to estimate infertility over 5 years whereas clinical definitions prefer shorter periods. This is because clinicians prefer treatment to be initiated promptly while epidemiological studies seek to avoid false positives within their data. Furthermore, epidemiological research relies on Demographic and Health Surveys which contain complete birth histories but lack information regarding stillbirths, miscarriages, spontaneous and induced abortions. The WHO definition is preferred as it is clinically beneficial and can be applied in research across different disciplines (Larsen, 2005).

A combined meeting between the WHO and the International Committee for Monitoring Assisted Reproductive Technologies (ICMART) convened in 2017 to reassess the definition of infertility. The WHO-ICMART meeting expanded the WHO definition to include a wider array of conditions which could alter the ability of individuals and couples to reproduce. Furthermore, fertility interventions can now be performed prior to the 12 months benchmark, depending on patient age, diagnosis and physical findings noted during fertility assessments (Zegers-Hochschild *et al*., 2017).

Infertility affects 15% of couples of reproductive age globally (Gerrits *et al.*, 2017). A systematic analysis which assessed infertility in 195 countries between 1990 and 2017 found the age-standardized prevalence of male infertility to increase by 8.22% between 1990 and 2017, while female infertility increased by 14.96% (Sun *et al.*, 2019). This represents a 0.293% per year increase in infertility in males and a 0.396% increase in females. The Centers for Disease Control and Prevention (CDC) of the United States stated that infertility affects quality of life and carries a substantial number of public health consequences (Practice Committee of the American Society for Reproductive Medicine, 2013; Zegers-Hochschild *et al.*, 2017). These include psychological distress, social stigmatization, economic strain, and marital conflict. Furthermore, infertility has been shown to be a risk factor for chronic health conditions later in life, including cardiovascular disease (Kurabayashi *et al.*, 2016).

### 2.3.1. Causes of male infertility

The complex control of the male reproductive function lends itself to dysfunction, leading to a myriad of possible pathological changes. The causes of male infertility may be subdivided into 3 categories depending on the origin of the dysfunction, namely: pre-testicular, testicular and post-testicular.

**Pretesticular**

Pre-testicular causes of male infertility arise from extragonadal conditions that impede on testicular function by dysregulating the HPG axis, resulting in poor hormonal support to the testes (Wong, 1974).

Primary GnRH deficiency manifests in idiopathic hypogonadotropic hypogonadism (IHH) (a congenital deficiency of GnRH), Kallman Syndrome (results from a genetic mutation leading to the defective migration of GnRH neurons) and Prader-Labhart-Willi syndrome (hypothalamic deficiency manifesting in genital hypoplasia, scrotal hypoplasia and a small penis) (Kallman, Schoenfeld and Barrera, 1944; Prader, 1956; Hoefnagel, Costello and Hatoum, 1967; Schwanzel-Fukuda, Bick and Pfaff, 1989; Chowdhary and Tiwari, 2016). Diseases affecting the hypothalamic function present with reduced GnRH, LH, FSH and testosterone concentrations (Chowdhary and Tiwari, 2016).

Acquired causes of GnRH deficiency result from the destruction of hypothalamic tissue by diseases such as tuberculosis, metastatic tumours, hemochromatosis (excessive iron absorption) and sarcoidosis (inflammatory disease resulting in connective tissue deposition in the form of nodules in certain body organs) (Behre *et al*., 2010). Other instigators, such as obesity, may result in reduced sensitivity of GnRH or hypothalamic receptors, affecting the release of hypothalamic hormones and peptides (Teerds *et al*., 2011).

**Testicular**

Testicular causes of male infertility include primary defects of the testes (Wong *et al*., 1978) resulting in abnormal testicular ultrastructure and possible testicular failure (inability to produce sperm or hormones). Causes are predominantly genetic and acquired conditions, but some origins are unknown. Genetic causes of testicular abnormalities include chromosomal defects, namely Klinefelter syndrome (the inheritance of an extra X chromosome by genetic males) and the loss of genetic material from the Y chromosome (Y chromosome microdeletions) (Chowdhary and Tiwari, 2016). The Y chromosome contains genetic information vital to male development (O'Flynn O'Brien, Varghese and Agarwal, 2010), and gene deletions in this region have been correlated to

spermatogenic failure. Microdeletions in the long arm of the Y chromosome can affect several genes and are always inherited by the male offspring (de Vries *et al.*, 2001; Schlegel, 2004).

The acquired causes of testicular abnormalities include many well-known aetiologies of male infertility. Inflammatory conditions such as bacterial and viral infections resulting in orchitis (testicular inflammation), immunological disorders, environmental and lifestyle factors, gonadotoxins including drugs and chemotherapeutic agents, testicular cancers, undescended testes, systemic diseases such as renal failure, some endocrine and hormone disorders, testicular trauma, as well as structural anomalies including testicular torsion (rotation of the testes which twists the spermatic cord and the blood vessels within it, impeding blood supply), hydrocele (collection of fluid around the testes causing testicular swelling), varicocele (swollen testicular veins) and damage or changes brought on by surgery (Wong *et al.*, 1978; Nieschlag *et al*., 2010).

Testicular pathologies bring about improper sperm production, resulting in abnormal sperm morphology (teratozoospermia), immature sperm, oligozoospermia and azoospermia *(Testicular Causes of Male Infertilit>*, 2018).

**Post-testicular**

Post-testicular causes of male infertility include obstruction of the ducts exiting the testes. These succeed adequate sperm production by the male reproductive system and involve complications with the ductal system and ejaculation (Wong *et al.*, 1978).

Genetic aberrations giving rise to post-testicular changes include cystic fibrosis (resulting from a mutation of the CFTR gene) which leads to an absence of the vas deferens, and Young Syndrome (abnormal mucus secretions leading to bronchiectasis, chronic bronchitis and obstructive azoospermia) resulting in inspissated (thick) secretions into the epididymis that block the epididymal duct (van Ahlen and Hertle, 2001).

Acquired post-testicular changes result from trauma, infections and iatrogenic (medical treatment-related) causes (Chowdhary and Tiwari, 2016), with genital tract infections, hernia repair, vasectomy being prominent examples. Ejaculatory disorders include premature ejaculation, retrograde ejaculation and anejaculation (the inability to produce and ejaculate (Natarajan and Khan, 2019).

The common causes of male infertility mentioned above are categorised and further elaborated on in the sections below. As there are many causes of infertility, these will be briefly reviewed with an emphasis on lifestyle factors, and more especially on obesity.

### 2.3.2. Genetic

Genetic causes of male infertility account for approximately 15% of male infertility cases (Ferlin *et al*., 2007) and are alarming due to their ability to affect multiple physiological processes, including hormone production and spermatogenesis (O'Flynn O'Brien *et al*., 2010). Genetic errors may be inherited or occur sporadically throughout a male's life, affecting fertilization, embryogenesis and sexual differentiation of the conceptus (Carrell *et al*., 2006). Furthermore, genetic anomalies can impair male fertility, but may further be harmful to a foetus should they be passed on through assisted reproduction techniques (ART).

Approximately 5% of male infertility cases are attributable to chromosomal abnormalities (Ferlin *et al*., 2007). The most common of these are aneuploidies, also referred to as numerical chromosomal abnormalities, giving patients an incorrect number of chromosomes. These errors result in spermatozoa with an incorrect amount of genetic material and have been demonstrated to result in azoospermia (no sperm within the ejaculate) and oligozoospermia (sperm counts of less than 20 million spermatozoa/ml) (Carrell *et al.*, 2006; Emery & Carrell, 2006; Ferlin *et al*., 2007). Aneuploid sperm can also successfully fertilize an oocyte, passing on an incorrect number of chromosomes to the resulting offspring (Carrell, 2008).

Translocations (the exchange of genetic material from two chromosomes that are not from the same chromosome pair (non-homologous) can cause a gain or loss of genetic material which may alter the genetic code (Carrell, 2008). These are an additional source of aneuploidies. The consequence can be impaired gametogenesis as gametes can inherit an unbalanced combination of parental chromosomes (Ferlin *et al.*, 2007). Many of those with translocations are carriers and show no pathologies. Some can result in trisomies (inheritance of the 3 pairs of the same chromosome), which are incompatible with life, resulting in natural fetal abortion. Others can lead to common birth defects such as Down syndrome (trisomy 21). They can thus lead to a variety of phenotypes with varying changes to fertility (Georgiou *et al.*, 2006).

Genetic variation, referred to as polymorphisms if they occur in >1% of the population, in several genes can lead to male infertility. The resultant phenotypes from these variants are modified by other genetic factors as well as the environment; this influences the severity to which these variations are phenotypically expressed (O'Flynn O'Brien, Varghese and Agarwal, 2010). This means that some genetic variants will only be expressed if the correct interaction with other genes and the environment exists.

### 2.3.3. Hormonal

Hormonal causes of male infertility arise from any disorder which affects the HPG axis and its resultant gonadotropins and androgens. These could be initiated by primary deficiencies of the hypothalamus, the pituitary or gonads, or arise secondary to disease processes which affect organ structure, function and sensitivity.

GnRH deficiencies are the basis of a number of hormonal disturbances. In an animal model using an induced mutant mouse strain of GnRH deficiency, researchers observed a decline in pituitary secretion of both FSH and LH (Cattanach *et al.*, 1977). Congenital GnRH deficiencies bring about hypogonadism, and sufferers have an abnormal pubertal development as well as low plasma levels of LH and FSH (Kottler *et al*., 1999). Excess GnRH, such as GnRH administration used to treat GnRH deficiencies, leads to reduced LH and FSH secretion, due to the reduced sensitivity of GnRH receptors from constant stimulation. Therefore, excessive GnRH results in the reduction of pituitary gonadotropins (Hotchkiss and Knobil, 1996).

Hypopituitarism is the interruption of hormone secretion by the anterior pituitary gland (Simmonds, 1914) due to a primary inability of the pituitary to produce adequate hormones, or as a result of insufficient stimulation by hypothalamic hormones. It may also arise from several causes including organ damage (radiation ablation, infarction), pituitary neoplasms and tumours, inflammatory conditions and surgical intervention (Lamberts *et al*., 1998; Regal *et al*., 2002). Genetic causes of hypopituitarism are caused by alterations in the transcription factors responsible for the development of the pituitary. Aberrant production of these result in hypopituitarism and pituitary malformation (Dattani, 2005). GnRH deficiencies can also diminish the secretion of hypothalamic hormones.

Androgen deficiency is the failure of the testes to secrete physiological concentrations of testosterone (Bhasin *et al*., 2010). A diagnosis of androgen deficiency can be made in males with consistently low serum total testosterone levels, and in the case of low normal levels, the measurement of free testosterone. This must be accompanied by consistent symptoms of low serum testosterone (Bhasin *et al.*, 2010). Clinical symptoms of low testosterone have been listed as: erectile dysfunction (ED), low libido and hot flushes; non-specific symptoms include fatigue, poor concentration, depressed mood, decreased physical performance, irritability, sleeplessness and loss of vigour (Zitzmann, Faber and Nieschlag, 2006; Araujo *et al.*, 2007; Hall *et al.*, 2008). Clinically, testicular failure can be reversed with adequate hormone therapy, but is potentially irreversible with pre-testicular cases.

Therefore, a functional HPG axis and gonads are essential for the hormonal control of the male reproductive system. Furthermore, functional ARs are a requirement for the functions of androgens to manifest, thereby adequately controlling male reproductive physiology.

### 2.3.4. Infections

Micro-organisms have the ability to colonize the male genital tract and cause infections (Gimenes *et al.*, 2014). These micro-organisms have several origins including endogenous pathogens of the urogenital tract, transmission through sexual contact or travel via the bloodstream during a systemic infection (Schuppe *et al.*, 2017). They can initiate inflammatory processes within segments of the male reproductive tract, i.e. the urethra (urethritis), prostate (prostatitis), the epididymis (epididymitis) and testis (orchitis) (Brookings, Goldmeier and Sadeghi-Nejad, 2013), and subsequently affect sperm at different levels of their development, maturation and transport (Pellati *et al.*, 2008). Colonizing micro-organisms can be bacterial, viral or protozoal in nature (Gimenes *et al.*, 2014) and have several sequelae including genital cancer, hepatitis, reduced immunocompetence, vasculitis and infertility (Center for Disease Control, 2003). Reproductive tract infections can directly and indirectly lead to male infertility. This occurs via the recruitment of leukocytes into seminal fluid (Sarkar *et al.*, 2011), potentially resulting in infiltrations of >1 million cells per millilitre (M/ml) of semen, a state classified as leukocytospermia (World Health Organization, 2009). Leukocytes produce reactive oxygen species (ROS), unstable and highly oxidizing by-products of aerobic metabolism which directly damage neighbouring tissues via oxidative processes (Sikka, 2001). Spermatozoa are especially susceptible to this damage due to the composition of their cell membranes, which predominantly comprise of polyunsaturated fatty acids (Zalata *et al.*, 1998).These are damaged via lipid peroxidation, leading to reduced sperm viability, abnormal sperm morphology, and impaired spermatogenesis, sperm maturation and function (Pilatz *et al.*, 2016). Furthermore, leukocytes indirectly damage spermatozoa through the release of intracellular cytokines, thereby producing more ROS (Smith *et al.*, 2014). Inflammation additionally contributes to infertility by instigating accessory gland dysfunction and the blockage of seminal ducts (Rusz *et al.*, 2012), thereby hampering seminal fluid production and the release of spermatozoa via ejaculation.

### 2.3.5. Environment

Industrialization, urbanization and economic development have led to drastic changes in lifestyle, but also to the environment. Many hazardous chemicals from industries and agriculture have been released into the environment, exposing both humans and animals to their deleterious effects (Mathur and D'Cruz, 2011). Industrialized countries have consequently observed reduced reproductive health in both humans and their wildlife, and a 50% decline in sperm concentrations

observed from 1940 to 1990 has raised concern regarding the contribution of synthetic chemicals to this decline (Carlsen *et al.*, 1992; Colborn, Vom Saal and Soto, 1993; Sinawat, 2000).

Reproductive malfunction is a sensitive indicator of environmental hazards due to the reactiveness of testicular tissue to external stressors (Mattison and Thomford, 1987). The testis is appreciably more sensitive to radiant heat and radiation than any other bodily organ. Numerous environmental agents that humans are exposed to, have been shown to be hazardous to their reproductive potential. This was observed from the 1970's when male reproductive function was seen to be reduced in men employed within industrial and agricultural sectors making use of the compound 1,2-dibromo-3-chloropropane (DBCP) (Whorton *et al.*, 1977; Slutsky, Levin and Levy, 1999). Whorton *et al*. (1977) observed azoospermia, oligozoospermia, and increased LH and FSH within pesticide workers exposed to DBCP. After this revelation, subsequent studies interrogated different classes of pesticides and solvents in animal models to investigate any effects on reproductive parameters (Sundaram and Witorsch, 1995). Rats exposed to the insecticide methoxychlor (MXC) have shown numerous effects including cellular apoptosis and reductions in Sertoli cell number nuclear volume, implying impairment of spermatogenesis by MXC (Johnson *et al.*, 2002). Certain chemicals, though, cause undetectable changes in the testes due to the ability of testicular tissue and germ cells to continuously produce spermatozoa (Sharpe, 1944). Thus, reproductive parameters could be impaired without noticeable changes in sperm concentrations.

Several mechanisms have been put forward as to how these chemicals can affect fertility. Reproductive function can be impaired via disruptions to hormone secretion and damage to testicular microanatomy. By targeting specific cell types including Sertoli cells and germ cells, environmental hazards can disrupt the microanatomy of the testes and disturb male fertility. In addition, mutations to germ cells may lead to genetic diseases, malformations and miscarriage of the fetus. Neurotoxic compounds can disturb hormone production resulting in abnormal erection, emission as well as ejaculatory disorders (Telöken, Juncal and Graziottin, 2012).

Environmental pollutants have been implicated in the disturbance of the antioxidant/pro-oxidant system within cells by stimulating the production of ROS (Bagchi and Stohs, 1993). This promotes the oxidation of cellular membranes, particularly those of spermatozoa, reducing sperm motility and viability (Aitken and Clarkson, 1987; Mathur, Saradha and Vaithinathan, 2008). Endocrine disruptors are external sources or pollutants with estrogenic properties that mimic the effects of endogenous hormones, thereby disrupting normal endocrine function (Saradha and Mathur, 2006). They can to bind estrogen receptors and antagonize or stimulate their natural actions within the body (Zava, Blen and Duwe, 1997). Bisphenol A (BPA), an endocrine disruptor used to make hard, clear plastics and in regularly used substances such as dental sealants and food cans, has been found to: reduce sperm concentrations within the epididymis (Chitra, Latchoumycandane and

Mathur, 2003); decrease motility and viability (Lukacova *et al.*, 2015); reduce sperm acrosome and plasma membrane intactness, and reduce sperm mitochondrial integrity and morphology (Wisniewski *et al.*, 2015).

### 2.3.6. Lifestyle factors

There are many well-established health risks that many individuals are exposed to on a regular basis that may affect their fertility.

#### Smoking

The burning of a tobacco cigarette releases approximately 4000 compounds including vaporized liquids, gases and particles (Hruska *et al.*, 2000). Cigarette smoke contains high levels of ROS, including hydrogen peroxide and hydroxyl anions and radicals (Saleh *et al.*, 2002) as well as carcinogens responsible for numerous cancers. Virtually all semen parameters (concentration motility, viability, morphology) are negatively affected by cigarette smoke, with some studies reporting no “safe” amount of cigarette smoking to be recommended for the maintenance of normal semen quality (Gaur, Talekar and Pathak, 2007; Oyeyipo, Maartens and du Plessis, 2014). Since human spermatozoa have nicotine receptors, the presence of high amounts of nicotine found in male smokers causes activation of these receptors, eliciting deleterious effects which reduce normal sperm morphology and concentration (Görnig and Schirren, 1996; Oyeyipo *et al.*, 2011). Smoking additionally affects the accessory sex glands, with Pakrashi and co-workers (1995) observing significant reductions in the ejaculate contents of seminal vesicles and the prostate in smokers. The compounds found within cigarette have been shown to increase ROS and reduce antioxidants (Saleh *et al.*, 2002), cause DNA damage within sperm nuclei (Zhang *et al.*, 2000; Elshal *et al.*, 2009) and varicocoels (Klaiber *et al.*, 1987). In addition, compounds within cigarettes have been shown to reduce sperm proteins such as axonemes and acrosin (a proteolytic enzyme which degrades the acrosome membrane during the acrosome reaction) which are responsible for normal sperm motility and function (Gerhard *et al.*, 1989; Zavos Ed.S., Ph.D. *et al.*, 1998; Yeung *et al.*, 2009).

#### Physical exercise

Physical activity includes any form of movement which relies on muscle contraction (Vaamonde Martin *et al.*, 2014). This includes everyday activities such as walking, gardening and housework. Exercise is a purposeful and planned activity performed to increase physical fitness which includes cycling, running, weight training and swimming. Physical activities and exercise have widespread positive effects on the body including the cardiorespiratory, immune and endocrine system, bringing about protection from several diseases and disorders including obesity, diabetes and

osteoporosis (Vaamonde Martin *et al.*, 2014). Resistance training has an anabolic response as it increases testosterone production within the body thereby stimulating protein synthesis and muscle growth, while also enhancing the production of growth hormone (McArdle, Katch and Katch, 2006). This increase in testosterone release is both an increase in frequency and amplitude, which decreases during post-exercise rest (Kraemer and Ratamess, 2005). Excessive exercise, though, has been shown to reduce testosterone secretion (Hackney, 2005; Vaamonde *et al.*, 2006). In endurance and ultra-endurance exercise, hypothalamic and testicular endocrine functions can be negatively affected via the suppression of GnRH release (Kujala, Alen and Huhtaniemi, 1990). This, however, is related to training volume: runners performing low-to-medium amount of training showed either no significant changes in free and total testosterone (Mathur, Toriola and Dada, 1986; Bagatell and Bremner, 1990; De Souza *et al.*, 1994), or a decrease in free and/or total testosterone (Ayers *et al.*, 1985; Griffith *et al.*, 1990; Wheeler *et al.*, 1991); ultra-endurance runners training 10-20 hours a week have been shown to be hypogonadal and possess low resting testosterone levels (Hackney, Moore and Brownlee, 2005). Regular, moderate exercise has been shown to be beneficial to overall health and not detrimental to semen parameters (Vaamonde *et al.*, 2006). Moreover, regular exercise is more anabolic than strenuous resistance exercise as it increases testosterone levels to a better extent (Viru and Viru, 2003; Grandys *et al.*, 2009). According to Rivier and Rivest (1991) and Borer (2003), strenuous exercise stimulates catabolic and stress-related hormones including cortisol, β-endorphins, corticotropin-releasing hormone and ACTH, while also decreasing testosterone. Physically active individuals partaking in moderate regular exercise were shown by Vaamonde and her colleagues (2012) to have higher testosterone and lower cortisol levels than sedentary individuals, which better regulated homeostasis and spermatogenesis. They also observed high levels of LH and FSH, as well as higher proportions of morphologically normal and progressively motile sperm in these individuals. Conversely, individuals taking part in strenuous exercise have lower sperm concentrations, lower motility and lower percentages of normal sperm (De Souza *et al.*, 1994; Vaamonde *et al.*, 2006).

**Alcohol consumption**

Binge-drinking (≥ 5 drinks/units at a time) and heavy alcohol consumption (≥ 2 drinks per day) are generally considered to be harmful drinking patterns (Stewart *et al.*, 2019). Alcoholic men and women as well as laboratory animal models receiving high concentrations of ethanol have evident reductions in reproductive function, with high alcohol volumes in males shown to induce hypogonadism, testicular and prostatic atrophy, decreased libido, impotence and gynaecomastia (Sundaram and Witorsch, 1995). Alcohol-induced testicular dysfunction has been shown to result from dysregulation of the HPG axis. Human and animal studies have shown the testes, in response to high alcohol consumption, to have a reduced testicular response to exogenous human

chorionic gonadotropin (hCG) as subsequent testosterone release is reduced relative to controls (Sundaram and Witorsch, 1995). Moderate alcohol consumption (1-2 drinks per day) has not, however, been established as a deleterious factor for sperm concentrations (Marinelli *et al.*, 2004; Martini *et al.*, 2004; Choi Yoon-Jung Myung Seung-Kwon, 2018), but chronic alcoholism (habitual, heavy alcohol consumption for a minimum of 5 days per week for ≥ 1 year) has been observed to impair spermatogenesis and reduce testosterone levels (Villalta *et al.*, 1997; Muthusami and Chinnaswamy, 2005). Using time-to-pregnancy (the number of contraceptive-free menstrual cycles it takes for a couple to conceive) as an outcome, a European study assessed the alcohol consumption of 4000 couples and determined time-to-pregnancy to be similar among all alcohol intake groups, implying that alcohol had no significant effect on how long a couple will take to conceive a child (Olsen *et al.*, 1997). If any detrimental results were observed, however, it was in the female partners who conceived ≥ 8 drinks per week. In chronic alcoholics, however, changes in sperm count, morphology and viability were observed (Muthusami and Chinnaswamy, 2005). The mechanisms of alcohol toxicity within the reproductive system have been investigated: the oxidative metabolism of ethanol increases the testicular NADH/$NAD^+$ ratio, thereby causing a redox readjustment which reduces the availability of this co-factor to take part in steroid production within the testis; long-term administration of ethanol to rats elicits biochemical changes in testicular membranes including a decreased glutathione and unsaturated fatty acid content but increased saturated fatty acid and malonaldehyde concentration, which predisposes membranes to damage via lipid peroxidation; isolated Sertoli cell cultures showed reduced ATP and $O_2$ when exposed to alcohol, thereby reducing cell viability, which corroborates reports that ethanol is permeable to the blood-testis barrier of Sertoli cells (Sundaram and Witorsch, 1995). Other factors found to affect reproductive function in alcoholic men includes the effect of the phytoestrogens identified in the non-alcoholic portion of alcoholic beverages which have been found to cause a feminizing effect on males. Furthermore, increases in the production of prolactin have been identified as the causative factor in gynecomastia in alcoholic males, but this is a controversial finding (Sundaram and Witorsch, 1995).

**Psychological stress**

Psychological stress can result from, but also cause, infertility. It has been shown to increase serum levels of glucocorticoids and suppress testosterone production within the testes (Whirledge and Cidlowski, 2010). Moreover, stress has also been observed to induce both structural and meiotic alterations to sperm, thereby making the sperm less capable of locating an oocyte (Collodel *et al.*, 2008). On the other hand, couples struggling with fertility can become psychologically stressed, which can further exacerbate their infertility (Nasim, Bilal and Qureshi, 2019).

### Non-communicable diseases

Non-communicable diseases (NCDs) are non-transmissible, non-infectious chronic diseases, including cardiovascular diseases, diabetes, cancers and chronic lung diseases (Kim and Oh, 2013; World Health Organization, 2019), These accounted for 71% (41 million) of global deaths in 2016, with approximately 31.5 million (over three quarters) disproportionately occurring in low- and middle-income countries (World Health Organization, 2018a). NCD mortality has a greater probability in Africa, accounting for 22% of NCD deaths worldwide in 2016.

NCDs largely occur as a result of modifiable lifestyle factors including poor diet, lack of physical activity, alcohol abuse and tobacco smoking, which largely result in four physiological changes including increased blood glucose, blood pressure, blood lipids and obesity (World Health Organization, 2018b). Obesity is both a sequelae of adverse lifestyle factors and a risk factor for several NCDs including hypertension, diabetes, stroke, coronary heart disease, cancers and obstructive sleep apnea (World Health Organization, 2016a).

## 2.4. Obesity

Obesity, by definition, is an excess of body fat that poses a risk to health. It is assessed clinically via the expression of body weight as a function of height – the body mass index (BMI). This is calculated by dividing an individual's weight (in kilograms) by the square of their height (in meters) ($kg/m^2$) (World Health Organization, 2018c). BMI is used in adults to delineate overall body fatness where a BMI of 18.5 – 24.9 $kg/m^2$ is considered to be normal. Abnormal BMI's are sub-categorized according to severity, where a BMI ≥ 25 $kg/m^2$ is considered to be within the overweight range, BMIs ≥ 30 $kg/m^2$ are considered as obesity and BMI ranges ≥ 40 $kg/m^2$ are classified as severe/morbid obesity.

Obesity was largely ignored in the previous millennium (W. P. T. James, 2008). The WHO recognised obesity as a disease in 1948 when it was first established but did not recognise it as a health problem for a substantial amount of time. When escalations in the number of obese patients were noticed in the 1970's by physicians in the United States and the United Kingdom (UK), attempts were made to alert the WHO of this impending public health problem. Obesity was, however, regarded as a disease of opulence, easily remedied by the reduction of food intake and considered to be irrelevant elsewhere. Furthermore, obesity was not a major concern for the WHO as their priority was to deal with malnutrition and the problems of the Third World. By the mid-1990's, obesity had become a prominent problem for obesity specialists but was still not being taken seriously by most national governments. The International Obesity Task Force (IOTF) was subsequently launched by a group of physicians who were exasperated by the issue to try to

establish a new approach on how to deal with the rise in prevalence and to raise awareness. After meeting with the WHO, the Organization accepted that more attention was warranted, and it later tasked the IOTF with assessing the risk that obesity and other risk factors imposed on premature death and disability. Excess adult BMI's were revealed as part of the top ten risk factors of the burden of disease in high, middle- and low-income countries. The WHO and the rest of the world have since accepted the role played by obesity in the amplification of many diseases.

### 2.4.1. Epidemiology

The worldwide prevalence of obesity among children and adults doubled between 1980 and 2015, with approximately one third of the world's population now classified as overweight or obese (Afshin *et al.*, 2017; Chooi, Ding and Magkos, 2019). The prevalence is generally higher in women than in men, although the highest incidence was recorded in males aged 25-29 years residing in low-middle income countries. In children, the obesity prevalence was highest in high-income countries, with only a low prevalence amongst boys and girls aged 10-14. In middle and high-middle income countries, however, this prevalence was highest in boys, but the trend reversed in late adolescence. The steepest increase in childhood obesity in the aforementioned time period was observed in both girls and boys residing in middle income countries (Afshin *et al.*, 2017). Thus, the worldwide prevalence of obesity continues to increase in most countries; although childhood obesity was lower than adult obesity over the measured 25-year period, the rate of increase in childhood obesity was greater than that of adult obesity.

Many clinical and epidemiological studies on obesity have been conducted and anthropometric measurements are always the basis for the establishment of obesity. Several measurements are employed to assess body fat composition, such as height, weight, skinfold thickness, as well as chest, waist and hip circumferences. From these measurements, several indices are created to establish obesity and body fat content including BMI and the waist-to-hip ratio (WHR) (Bray, 1989). The convenient and low-cost nature of these measurements have encouraged their wide use, although other, more reproducible measurements may be applied. Magnetic resonance imaging (MRI), computerized tomography (CT), total body electrical conductivity, bioelectrical impedance, ultrasound and the measurement of body density are some of the more precise measurements that may be employed, with the "gold standard" being the assessment of body density by means of hydrostatic weighing, from which fat and fat-free mass may be determined (Bray, 1989). Body density is determined using Archimedes' principle of water displacement, where an individual's weight is determined in and out of water; measurements using electrical conductance and impedance quantify lean and fat tissue through the ability of electromagnetic waves to pass through them, where fat is more resistant to electrical charge (Bray, 1976; Segal *et al.*, 1985; Garrow, 1988; Khalil, Mohktar and Ibrahim, 2014). MRI and CT scans allow the ability to visualize

and thus quantify regional fat distribution through the ability of fat, muscle and other tissues to reflect ultrasonic waves; these permit the measurement of fat thickness in regional locations and thus an estimation of intra- and extra-abdominal fat. The availability of these instruments as well as the expense of their use, however, is a deterrent and widely limits their use.

Measuring the WHR provides an indication of regional body fat distribution; the accumulation of fat around the waist/abdomen, which indicates a visceral deposition of fat, is referred to as central obesity and colloquially as the “apple-shape”, whilst the deposition of fat around the hips/buttocks indicates a subcutaneous (beneath the skin) accumulation of fat and is commonly referred to as the “pear-shape” (Segula, 2014). Central obesity is classified clinically as a WHR ≥ 0.95 in females and ≥ 0.85 in males (Castro *et al.*, 2003).

Body fatness is a useful predictor of health and provides a guide of an individual’s health risk (Bray, 1989). The accumulation of fat, especially in the abdominal region, carries a high risk for morbidity and mortality as this increases and individual’s odds of suffering from obesity-related diseases including diabetes, cardiovascular disease, strokes and cancer (Vague, 1947). Furthermore, the risk of developing these chronic ailments progressively increases at BMIs above 21 kg/m$^2$ (Wilding, 2001).

Animal studies which measure the effects of obesity also employ anthropometric measurements to assess body fatness within animal models. Many previous studies of obesity in animals generally accepted any significant increase in energy content or body weight relative to the control animals as obesity (Rothwell and Stock, 1984). More recently, obesity in animals is predominantly measured using 2 approaches. The Lee index, a similar measure to BMI, assesses the dimensions of the animal; it is defined by the cube root of the animal’s body weight in grams divided by the naso-anal length of the animal (cm) multiplied by a thousand [(body weight$^3$/length) x 1000]. The second measurement of obesity in animals is the assessment of body fat content, which can be quantified by comparing the body weight (or fat) of the obesity group with that of the controls (Hariri and Thibault, 2010), or by measuring the adiposity index (Leopoldo *et al.*, 2016). The adiposity index (AI) is defined by the total body weight divided by total body fat (epididymal + retroperitoneal + visceral/omental fat) multiplied by a hundred [(total body weight/total body fat) x 100] (Truett *et al.*, 1991; Leopoldo *et al.*, 2016). Using the Lee index, obesity can be classified in animals with values above 310, whereas the AI and percentage increase in body weight/fat compared to controls are less defined (Hariri and Thibault, 2010). Studies using the percentage increase in body weight/fat compared to the control group define 10-25% increases in body weight/fat as moderate obesity, and >40% increases as severe obesity (Harrold, Williams and Widdowson, 2000; Levin and Dunn-Meynell, 2002; Woods *et al.*, 2003; Hariri and Thibault, 2010). The measurement of body fat and adiposity has been shown to be a more sensitive measurement to assess obesity in

animals (Woods *et al.*, 2003). A universally adopted categorization of animal obesity according to the AI has not been established although a study by Leopoldo *et al*. (2016) has attempted to do so by means of a cluster analysis in an experiment using animals fed control or high fat diets. At the end of the feeding protocol, 84 animals were stratified according to their AI (mean ± SD) into normal (≤ 4.36 ± 0.49), overweight (≤ 5.96 ± 0.60) and obese (≤ 8.78 ± 1.62) categories, with the obese group showing significantly higher total body fat and AIs compared to the overweight group. The animals on the high fat diet showed obesity indicators that resemble human obesity and its comorbidities including hyperinsulinaemia, hyperlipidaemia and high levels of total cholesterol, triglycerides and glucose. The classification of animal obesity by Leopoldo can be seen as a potential means of characterising degrees of adiposity in animals that represents obesity in humans. Furthermore, experiments presently stratify experimental animals, including rats, into obesity prone or resistant categories based on their body weight/fat gain at the end of the experimental period (Hariri and Thibault, 2010; Leopoldo *et al.*, 2016). In the exposure of study animals to obesogenic diets *ad libitum*, the sensitive (obesity prone) animals become obese while others grow normally and resist the obesogenic effects of the diet (obesity resistant) (Levin and Dunn-Meynell, 2002).

### 2.4.2. Control of body weight

To understand how the body controls food intake and how the dysregulation of this system can lead to obesity in humans and animals, the control of body weight will be reviewed. This literature also provides a background for experimental models used to induce obesity.

The brain is central to the regulation of food intake and energy balance. Peptides released by several tissues and organisms within the body such as adipose tissue (Schuster, 2009), gut microbiota (Tehrani *et al.*, 2012) and the gastrointestinal tract (Griffen *et al.*, 1981) stimulate specific regions of the brain, especially the hypothalamus, which have an abundance of receptors responsible for the central pathways which regulate the energy balance. Approximately 40 neurotransmitters have been implicated in the control of body weight, which can be categorized as those involved in stimulating the intake of food while decreasing energy expenditure (orexigenic) and those which decrease food intake while increasing energy expenditure (anorexigenic) (Wilding, Widdowson and Williams, 1997). The control of body weight is thus a highly precise endeavour which makes use of several homeostatic mechanisms. The combined goal of these mechanisms is to control the energy balance, thereby minimizing excessive loss or gain in body weight (Martinez, 2000).

Adipose tissue, or fat, is the principle storage site for energy. It has the ability to buffer energy imbalances by storing excess energy and releasing it during energy deficiency (Schuster, 2009).

Fat mass is dependent on the number of adipocytes present in the body and the independent size of each adipocyte. It can increase through the differentiation of preadipocytes into adipocytes and via increased lipid deposition into existing adipocytes (Knittle *et al.*, 1979; Ailhaud, 1982). Beyond its function of storing excess energy, adipose tissue plays a pivotal role in numerous homeostatic processes such as appetite regulation, energy expenditure and glucose regulation. It is also involved in regulating the immune response, thyroid gland function, reproductive processes and blood clotting via the secretion of numerous hormones. Furthermore, it is an active endocrine organ responsible for the secretion of a variety of adipokines (cytokines secreted by adipose tissue) including leptin, adiponectin, free fatty acids, angiotensinogen, complement factor 3, adipsin and the important inflammatory mediators tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (Kissebah *et al.*, 1982). The dysregulation of these processes is linked to many obesity-related disorders such as cardiovascular disease, stroke and lipid disorders (Schuster, 2009).

Fat is distributed and stored in different regions within the body. The two major storage depots are beneath the skin (subcutaneous – superficial and deep) and surrounding intra-abdominal organs (visceral), with the superficial subcutaneous layer believed to be the primary adipose tissue storage compartment (Sniderman *et al.*, 2007). The superficial and deep subcutaneous layers are separated by facia and differ in the organization of their fat lobules, where the superficial subcutaneous compartment displays well organized, tightly packed lobules compared to the larger, irregular and less organized lobules observed in the deep subcutaneous compartment (Goodpaster *et al.*, 1997; Misra, 1997; Kelley *et al.*, 2000; Smith *et al.*, 2001). Visceral adipose tissue has more blood supply (more vascularized), with less defined lobules; it is the smallest of the 3 compartments but is substantially more metabolically active. The expansion of these fat depots brings about different disease risks. Expansion of the deep subcutaneous and visceral adipose tissue has been shown to have adverse metabolic outcomes as this has been linked to dyslipidaemia (high mounts of blood triglycerides and low-density lipoproteins, and reduced levels of high-density lipoproteins) and vascular disease (Abate *et al.*, 1995; Tchernof *et al.*, 1996).

Energy expenditure comprises of 3 essential elements: the basal metabolic rate (BMR), dietary thermogenesis and energy utilization during physical activity (Bray, 1987). BMR is the energy required to maintain normal metabolism in all cells during the resting state; it accounts for 70% of total body energy expenditure and is influenced by age, gender, weight, climate and genetics (Bray, 1976). BMR is related most accurately to fat-free body mass; it is also closely related to total body weight and skin surface area as heat is primarily lost via dissipation through the skin. Higher metabolic rates are thus correlated with increased body weight and higher percentages of lean body mass, as observed in obese and lean males (Bray, 1989). On the other hand, low resting metabolic rates in relation to fat-free body mass is a possible predictor of increased body fat,

reduced physical activity and a higher likelihood of becoming obese (Ravussin *et al.*, 1988). Dietary thermogenesis is the energy required to digest, absorb and store a meal. The metabolic rate increases after food consumption and remains elevated for several hours thereafter. During this process, 10-15% of the total caloric value of the meal is expensed in order to digest and absorb the meal and store its nutrients. This increased in metabolic rate has been attributed to the increased activity of the sympathetic nervous system (Glick, Teague and Bray, 1981). Physical activity accounts for variable amounts of energy expenditure. Experiments on energy expenditure during physical activity, for instance in cycling, assess the effectiveness of energy usage by exercising muscle and have found that approximately 30% of total body energy is required by muscle to turn a flywheel on an exercise bike during cycling (Bray, 1983). Obese individuals have, however, been observed to expend more energy than leaner individuals while performing the same task, i.e. walking the same distance (Wilding, 2006). A positive energy balance where energy intake is greater than expenditure, results in increased energy storage in the form of fat. A continued positive balance is associated with an increase in body weight and thus and increased tendency towards obesity (Ravussin, 2005).

Leptin is a hormone predominantly secreted by adipocytes (Zhang *et al.*, 1994; Halaas *et al.*, 1995). The serum concentration of this hormone is proportional to an individual's adipose tissue mass; its secretion results in the activation of hypothalamic receptors responsible for reduced food intake and increased energy expenditure (Considine *et al.*, 1996; Bray and York, 1997). It is therefore viewed as the "switch" which signals a change from the fed to the starved state (Ahlma *et al.*, 1996). It is produced by the *Ob* (obesity) gene, which has a well-conserved sequence between different species including human, mice and rats (Friedman *et al.*, 1991; Wang *et al.*, 1996). Leptin secretion is controlled by hormones such as insulin (Houseknecht *et al.*, 1998) where it stimulates expression of the leptin gene in adipocytes, thereby increasing leptin levels in blood. The release of leptin results in its binding to leptin receptors predominantly concentrated within the central nervous system, where the main receptor isoform is highly expressed within the hypothalamus in a region central to appetite control (Houseknecht and Portocarrero, 1998). Other tissues where shorter isoforms of the leptin receptor have been located include the liver, kidney, pancreatic β-cells, the lung, muscle (Löllmann *et al.*, 1997) and almost all tissues responsible for the regulation of energy expenditure (Houseknecht and Portocarrero, 1998). This can explain the direct effect that the release of this hormone can have on the respective organ functions (Cohen, Novick and Rubinstein, 1996; Muoio *et al.*, 1997) including the reproductive system. Leptin reduces food intake, stimulates thermogenesis (Collins *et al.*, 1996) and reduces the size of adipocyte tissue due to the mobilization of lipid stores and apoptosis of adipocytes; obese people have been shown to have high expression levels of leptin in their adipocytes as well as increased circulating

concentrations of this hormone, but they have a lack of leptin receptor activation, indicating leptin resistance. Their energy intake and expenditure are thus, in many cases, dysregulated.

The binding of leptin to its hypothalamic receptors affects the release of several neuropeptides; anorexigenic neuropeptides such as pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) are synthesized whilst orexigenic neuropeptides agouti-related peptide (AgRP) and neuropeptide Y (NPY) are inhibited, thereby decreasing food intake and increasing energy expenditure (Anand and Brobeck, 1951). Leptin also inhibits neuropeptides melanin-concentrating hormone (MCH) and orexin, both released from the lateral hypothalamus, resulting in reduced food intake (Kishi and Elmquist, 2005). The leptin-induced synthesis of POMC results in the production of peptides classified as melanocortins (Pantel and Cone, 2013), which are formed by the cleavage of POMC. A prominent and important melanocortin is alpha-melanocyte-stimulating hormone (α-MSH), an important regulator of body weight, food intake, glucose homeostasis, and an essential mediator of numerous biological actions of leptin (Cone, 2006). It acts by stimulating the melanocortin-4 receptor (MC4R) which is widely distributed with the central nervous system (CNS) (Mountjoy *et al.*, 1994) and the peripheral nervous system (Gautron *et al.*, 2010). Stimulation of this receptor results in activation of the sympathetic nervous system, activating sympathetic tone in brown adipose tissue (BAT), muscle, and kidney, for instance, (Haynes *et al.*, 1999) thereby stimulating thermogenic metabolism and thus energy expenditure (Egawa, Yoshimatsu and Bray, 1991). The MC4R is inhibited by AgRP where overexpression of AgRP has been shown to lead to a drastic increase in hyperphagia and thus obesity (Ollmann *et al.*, 1997). During the fasting state, leptin levels are reduced, thereby stimulating AgRP and NPY, resulting in increased food intake and reduced energy usage, which is further illustrated by increases insulin and glucocorticoids (Spiegelman and Flier, 1996; Flier, 1998; Friedman and Halaas, 1998; Ahima *et al.*, 1999; Cowley *et al.*, 2001). Ghrelin, a hormone released from the gut and also synthesized in the hypothalamus, works in concert with leptin (Kojima *et al.*, 1999; Pusztai *et al.*, 2008). Its levels increase during starvation and thus acts as a signal for hunger, with its levels being highest just prior to meals (Cummings *et al.*, 2001, 2002). Ghrelin is secreted to increase appetite and reduce energy usage, while leptin is simultaneously reduced so as to not antagonize the actions of ghrelin (Saper, Chou and Elmquist, 2002). The stimulation of ghrelin receptors in the hypothalamus stimulates NPY production (Luckman, Rosenzweig and Dickson, 1999; Willesen, Kristensen and Rømer, 1999). The stimulation or inhibition of anorexigenic and orexigenic neuropeptides from the hypothalamus thereafter affects the release of numerous hormones from the pituitary, and influence the adrenal, thyroid and reproductive axes, thereby affecting energy use and production (Swerdloff, Batt and Bray, 1976; Ahlma *et al.*, 1996; Légràdi *et al.*, 1997; Guo *et al.*, 2004). It is therefore essential that energy is regulated and food intake is controlled so that body weight is managed.

### 2.4.3. Causes of obesity

Obesity is an unintended consequence of modernization (Martinez, 2000) where economic, social and technological advances (Pi-Sunyer, 2002) have resulted in urbanisation and reduced physical activity (Walker, Adam and Walker, 2001). Diets have transitioned from natural, organic foods to refined, high fat and high sugar alternatives, leading to a nutrition transition parallel to the observed economic growth (Alaba and Chola, 2014). These changes have led to a rapid increase in the prevalence of obesity (Martinez, 2000).

As a historically Western disease, obesity was largely localized to developed countries until recent decades where middle- and low income-countries began to experience rates comparable to those of high income countries. The rapid economic expansion and modernization experienced by middle and low income countries, as well as changing diets, are the greatest contributors to this epidemic (Alaba and Chola, 2014) as more and more of these countries adopt Western diets and lifestyles. Close to 30% of the populations within middle income countries are classified as overweight or obese, with South Africa (SA) having the highest prevalence in sub-Saharan Africa, where the prevalence was reported to be 27% in adults over the age of 18 years in 2016 (World Health Organization, 2018b).

While the predominant causes of the energy imbalance seen in obesity are inadequate physical activity and unhealthy diets, not all those who are exposed to these unfavourable behaviours will develop the disease. Obesity is a multifactorial disease and many underlying factors disproportionately predispose subsects of the population to its development, and several of these are non-modifiable factors including age, gender, race, and genetics. These will be discussed below.

#### 2.4.3.1. Genetics

Family studies together with twin and adoption studies have revealed a high heritability of obesity, where the offspring of obese individuals have a 40-70% probability of inheriting the condition (Apalasamy and Mohamed, 2015). The BMI of adoptees has been shown to correlate with that of their birth parents and not with that of their adoptive parents. Obesity amongst twins has been shown to range between 0.6 and 0.9, with only slightly reduced levels observed in twins raised apart versus those raised together (Stunkard *et al.*, 1986). Thus, an individual's risk of being obese are amplified if any of their relatives are obese. The mechanisms of this heritability have been examined using *in vivo* (animal) and *in vitro* (primary cell culture, three-dimensional cell culture and co-culture) models, which have revealed genes, growth factors and hormones that are instrumental in the control of body weight and in the feeding behaviours that underlie obesity development

(Apalasamy and Mohamed, 2015). Monogenetic obesity (obesity caused by a mutation in a single gene), as well as polygenic obesity (the interaction of environmental and genetic factors) investigated using both *in vivo* and *in vitro* models have revealed genes which are mutated in obese mice. These studies have also assessed the role of environmental factors in the body's responses to high fat or high sugar diets, and the epigenetic (heritable non-genetic changes in DNA which can affect gene expression/activity) changes elicited by obesogenic environments (Speakman *et al.*, 2008; National Institutes of Health National Library of Medicine, 2019). Several genes found to partake in the control of central and peripheral pathways of energy intake and expenditure in the aforementioned models have subsequently been analysed in humans, with the most recent version of the Gene obesity map (2006) implicating over 600 genetic contributions (genes, chromosomal regions and genetic markers) to the development of obesity and body weight regulation.

The mutations resulting in monogenetic obesity have a large effect size; they result in severe obesity of early onset, with hyperphagia as a principal feature accompanied by numerous endocrine abnormalities (Clement, 2006). There are several predominant genes whose mutations give rise to monogenetic obesity, namely the leptin (*LEP*), leptin receptor (*LEPR*), proprotein convertase 1 (*PSCK1*), *POMC*, *MC4R*, single-minded homology 1 (*SIM1*), brain-derived neurotrophic factor (*BDNF*) and the neurotrophic tyrosine receptor kinase 2 (*NTRK2*) gene (Montague *et al.*, 1997; Holder, Butte and Zinn, 2000; Yeo *et al.*, 2004; Farooqi *et al.*, 2006, 2007; Gray *et al.*, 2006; Dubern *et al.*, 2007; Benzinou *et al.*, 2008). The discovery and understanding of these gene mutations were facilitated by animal models, barring SIM1 which was discovered by means of *in vitro* cell lines (Holder, Butte and Zinn, 2000). The majority of the aforementioned genes produce proteins which are critical to the leptin-melanocortin pathway, which positions this pathway as an essential regulator of energy homeostasis (Mutch and Clément, 2006). The knowledge of these gene mutations has enabled a better understanding of the biological pathways of the disease. Mutations in the LEP gene lead to obesity as abnormal versions of this protein are produced, or its production is impaired. For instance, a frameshift mutation in the leptin gene of two Pakistani cousins resulted in leptin deficiency as this mutation led to the production of a truncated leptin protein sequence and therefore a lack of leptin secretion (Farooqi, 2005). This deficiency resulted in the typical characteristics of monogenetic obesity including hyperphagia and increased insulin levels, which stimulate obesity. Furthermore, in a study assessing patients with mutations in the leptin receptor gene, the phenotypic expression of obesity was similar to those with leptin deficiency, exhibiting rapid weight gain in early life, severe hyperphagia, normal resting metabolic rates, basal temperature, cortisol and glucose levels, but high plasma leptin and insulin levels (Zurbano *et al.*, 2004; Farooqi, 2005). Mutations in the LEP gene in mice has been shown to result in obesity, overfeeding, insulin resistance, infertility and numerous other endocrine anomalies

(Houseknecht and Portocarrero, 1998). While familial transmission of obesity is high and clear genetic contributions at the level of overall obesity have been established through such studies, these mutations, however, only account for about 6-11% of obesity cases within a population (Tchernof and Després, 2013). Thus DNA alone cannot explain the high heritability rates of obesity, which has necessitated the observance of gene-gene and gene-environment interactions in order for a broader understanding of obesity genetics to be acquired.

Polygenic obesity is a more common form of obesity, with the obese phenotype stemming from numerous genes making minor contributions towards its development (Glazier, Nadeau and Aitman, 2002). These genetic contributions are common in the general population and make an individual susceptible to obesity; it is the interplay between this genetic susceptibility and the individual's lifestyle and environment which determine whether obesity will develop (Hinney and Hebebrand, 2008). Obesogenic environmental factors are those which stimulate energy consumption above energy expenditure such as lack of physical activity and food abundance (Mutch and Clément, 2006). These factors together with individual genetic susceptibility lead to the typical form of obesity that is rampant in society today. Genetic variances in humans determine an individual's energy storage and expenditure, lipid and glucose metabolism and the development of their adipose tissue (Rankinen *et al.*, 2006); these contribute to whether individuals exposed to an identical obesogenic environment will develop obesity (Martínez-Hernández *et al.*, 2007). Gene-environment interactions arise when environmental changes can give rise to a particular phenotype, i.e. obesity, depending on the genotype or genetic susceptibility of an individual (Martinez, 2000). Two established genetic variants which predispose individuals to obesity include the MC4R and FTO (fat mass and obesity associated) genes (Herbert *et al.*, 2006; Frayling *et al.*, 2007). Initially observed in rodent studies showing increased body weight and obesity in homozygous and heterozygous knockout mice respectively, human studies of the MC4R gene were initially carried out where population screening for mutations yielded a small number of different mutations, which were found in obese individuals only (Vaisse *et al.*, 1998; Hinney *et al.*, 1999; Yeo *et al.*, 2003). Further analyses uncovered numerous but infrequent mutations of the MC4R gene in humans, with the majority of the mutations observed in individuals with extreme obesity (Hinney *et al.*, 2006). Large population screenings for mutations of the MC4R gene and subsequent meta-analyses uncovered a polygenic mode of inheritance for the gene due to its small effect size and the environmental dependence of the phenotypic expression of this mutation (Geller *et al.*, 2004). Most cases of MC4R mutations have been shown to either significantly predispose individuals to obesity or provide them with a minor protective effect against it (Hinney and Hebebrand, 2008). Thus, different individuals have a genetic predisposition towards obesity (Farooqi, 2005) but such genetic differences only infer the risk of developing obesity and not necessarily an absolute acquisition of this phenotype (Hinney and Hebebrand, 2008).

A number of clinical syndromes arising from genetic defects and chromosomal abnormalities have obesity as a prominent feature. Following the definition of a syndrome, which is a “collection of signs and symptoms which frequently occur together” (Kaur *et al.*, 2017), syndromic obesity was coined to represent the presence of obesity along with other frequently accompanying clinical symptoms which frequently includes developmental abnormalities, dysmorphic features and mental retardation (Bell, Walley and Froguel, 2005). Approximately 30 such syndromes exist and most of these arise from the mutation of a single gene, where at least 4 are known to elicit hyperphagia in addition to hypothalamic dysfunction (Lunsky and Meyre, 2019). Of the known obesity syndromes, Prader-Willi, Bardet–Biedl and Alström syndrome are the most frequently occurring (Chung, 2012). Prader-Willi syndrome (PWS) is characterised by hyperphagia, endocrine abnormalities, early-onset obesity, mental retardation, short stature and hypogonadotropic hypogonadism (Prader, 1956; Muscogiuri *et al.*, 2019). It occurs in one out of 10,000 to 30,000 individuals and is the result of the loss of expression of a paternally inherited gene on chromosome 15 (specifically 15q11-q13) (Angulo, Butler and Cataletto, 2015). PWS has also been shown to affect fertility. Although the mechanisms of this disease are not fully understood, patience with PWS show changes in food intake and energy expenditure which are ascribed to dysfunctions within the satiety centre of the hypothalamus, as well as the hormonal pathways that it is linked to, resulting in hormone deficiencies (Burman, Ritzén and Lindgren, 2001; Butler *et al.*, 2007). Syndromic obesity data seems to suggest specific neuroanatomical and functional aberrations, specifically of the hypothalamus, which occur as a result of genetic abnormalities that lead to changes in food intake as the predominant mechanism of obesity development (Chung, 2012).

#### 2.4.3.2. Ethnicity

The distribution of adipose tissue within the body varies between different ethnic groups (Tchernof and Després, 2013). Some ethnic groups are predisposed to store excess adipose tissue subcutaneously while others are more prone to a visceral accumulation of fat (Lovejoy *et al.*, 1996). An American study assessing ethnic differences in obesity by oversampling minority groups such as African-Americans and Hispanics found these groups to have a higher risk of obesity compared to white people of European decent (McTigue, Garrett and Popkin, 2002), where the onset of obesity occurred 2 times earlier for African-Americans and 1.5 times earlier for Hispanics compared to Whites. A wider meta-analysis of ethnic differences in body fatness conducted by Deurenberg *et al*. (1998) revealed significant differences in BMI among African-Americans, Whites, Ethiopians, Polynesians, Chinese, Thais and Indonesians of the same age, gender and body fat content. Asian populations have been shown to have lower BMI values at a higher body fat content in comparison to Whites, which could be attributed to ethnic differences in body fat composition between the two ethnicities (Deurenberg, Deurenberg-Yap and Guricci, 2002; Lear *et al.*, 2009).

These differences in body fatness among ethnicities necessitated a revision of the WHO BMI threshold applied in the classification of obesity for different age groups (children and adults), especially for Chinese, South Asians and Aboriginals as lower BMI values were shown to already exhibit high levels of blood glucose, hypertension and dyslipidaemia in these ethnic groups compared to those of European descent (Razak *et al.*, 2007).

Ethnic groups can also be stratified according to their tendency to store fat in visceral fat depots. White people have significantly more visceral adipose tissue than African-Americans at comparable levels of total adiposity, while Asians and Indian-Asians are even more susceptible to visceral obesity than other ethnic groups despite having lower total adiposity levels (Kadowaki *et al.*, 2006; Lear *et al.*, 2009; Misra and Khurana, 2009). Attempts to explain the physiological mechanisms behind these differences have led to genetic and epigenetic differences being proposed as the major determinants of body fat deposition (Albu *et al.*, 1997; Després *et al.*, 2000; Misra and Khurana, 2009), although these mechanisms need further clarification. Sniderman *et al*. (2007) have proposed ethnic differences in the development of the superficial adipose tissue compartment to be an underlying mechanism for the propensity towards visceral obesity. These authors have hypothesized the primary adipose tissue compartment of South Asians to be less developed compared to Whites and have thus a reduced capacity to store adipose tissue. South Asians make earlier use of their secondary storage compartments, the deep subcutaneous and visceral adipose tissue, making them more prone to the development of visceral obesity. This hypothesis, which Sniderman and colleagues (2007) have termed “the adipose tissue overflow hypothesis” is also proposed by Sniderman to possibly explain why Whites are less prone to fat storage in visceral fat depots and are thus more protected against the complications of visceral obesity, such as metabolic syndrome and diabetes, compared to other ethnic groups including South Asians, native Americans, Africans and populations of the Middle East (Forouhi, 2006). A multi-ethnic cohort study assessing the propensity for intra-abdominal and hepatic adiposity in 5 different ethnic groups showed, however, that African-Americans had the lowest relative amounts of visceral fat, Japanese-Americans had the highest, and the other races comprising of European (white), Latino and Native Hawaiian ancestry were intermediate (Lim *et al.*, 2019). This is supported by several other studies (Gillum, 1999; Carroll *et al.*, 2008; Kelly, Wilson and Heymsfield, 2009; Heo *et al.*, 2013; Barreira *et al.*, 2014; Eastwood *et al.*, 2015). Therefore, in most studies, South Asians have more visceral adipose tissue than whites, whereas those of African descent have less; the amount of visceral adiposity is thus unlikely to be an explanation for the amount of metabolic syndrome and diabetes observed in Africans (Eastwood *et al.*, 2015).

#### 2.4.3.3. Gender

Body fat distribution varies amongst human males and females (Pond, 1992). Females have a higher body fat percentage and a different body distribution pattern to males (Karastergiou *et al.*, 2012). Males have a tendency to store excess fat in the upper body region including the trunk/abdomen, whereas women have a propensity to store fat in the lower body such as the hips and thighs (gluteofemoral); these are classified as android (male) and gynoid (female) fat distributions, respectively (Vague, 1947; Krotkiewski *et al.*, 1983; Pond, 1992). Krotkiewski *et al*. (1983) hypothesized sex hormones to be the regulating factors behind these gender differences in fat deposition. They further added that males have possibly more fat cells in the trunk region while females have more cells in the gluteofemoral region. The hormone hypothesis has been shown to have some clout in body fat distribution due to observation of males and females undergoing hormone therapy for the purpose of gender reassignment. In female-to-male transsexuals administering intramuscular testosterone injections, a shift in fat deposition from the gynoid to the android distribution was observed. Similarly, male-to-female reassignment via estrogen injections significantly increased subcutaneous fat deposition in the thigh and hip region in transitioning males (Elbers *et al.*, 1997, 1999, 2003). An android fat distribution and high WHR has been observed in females with high testosterone levels (Evans, Barth and Burke, 1988; Wild, 1995). Studies have observed visceral adipose tissue to accumulate in males as total body fat increases, whereas the gynoid pattern of deposition is less dependent on total body fat. Even correcting for body fat mass has shown a lower visceral adipose tissue accumulation in females and, for any given increase in waist circumference, males displayed a higher accumulation of visceral fat tissue than females (Seidell *et al.*, 1988; Ross *et al.*, 1992; Pouliot *et al.*, 1994; Kuk *et al.*, 2005). Furthermore, for the same BMI, women generally present with a ± 10% higher body fat content than males (Womersley and Durnin, 1977; Jackson *et al.*, 2002).

#### 2.4.3.4. Age

Adults gain weight as they age (Heymsfield *et al.*, 2016). In young men and women, and women of middle age, excess energy is predominantly stored in the subcutaneous adipose tissue (Enzi *et al.*, 1986). This preferential storage changes with age where fat storage begins to accumulate within visceral adipose tissue, with increased visceral fat accumulation observed in both older men and postmenopausal women (Kotani *et al.*, 1994). The Baltimore Longitudinal Study of Aging also reported an increase in the WHR with age in both men and women (Shimokata *et al.*, 1989), which can be attributed to an abdominal adipose tissue accumulation, as fat is more likely to amass in the abdominal region with age as opposed to the gluteofemoral region (Lanska *et al.*, 1985). Premenopausal women are generally more protected from the visceral deposition of fat due to their

endogenous estrogens, a protection that is diminished once menopause begins (Godsland *et al.*, 1987; Scheffer *et al.*, 1997).

## 2.5. Effect of Obesity on the Male Reproductive System

Urbanization and increased economic activity have redefined the occupations that men partake in, with professional, managerial and administrative careers now dominating over more physical employment (Alaba and Chola, 2014). This has encouraged sedentary behaviour, which is contributing significantly to the rise in obesity amongst males. In low- and middle-income countries such as South Africa, obesity is more prevalent in higher socioeconomic groups (Steyn and Damasceno, 2006), where wealthier individuals are more likely to be obese, and the obesity risk is more pronounced in men. However, as countries transition from middle- to high-income, the higher prevalence in obesity is observed in poorer socioeconomic groups (Sobal and Stunkard, 1989; Steyn and Damasceno, 2006). Cultural perceptions are also implicated in the prevalence of obesity as a larger body size, particularly in black South Africans, is seen as a sign of health, prosperity and wealth which has spurred on the obesity prevalence, especially in wealthy men (Mvo, 1999; Puoane *et al.*, 2002; Alaba and Chola, 2014).

Obesity has been reported to cause male infertility, as reductions in reproductive potential have been observed in countries with high obesity prevalence (Carls*en et al*, 1992; Du Plessis, Cabler, McAlister, Sabanegh & Agarwal, 2010). The female contribution to infertility has long been researched as couple infertility is largely viewed as a female issue. It is only in the recent decades that the contribution of males has been explored, with obesity being researched as one of its more modern etiological factors (Du Plessis *et al.*, 2010). One study published in 2005 studied couples presenting themselves for fertility treatment and observed the obese male patients to have a three-fold higher likelihood of having poor semen quality (Magnusdottir *et al.*, 2005), and higher BMIs further correlated with reduced sperm concentration. Other studies assessing couples undergoing assisted reproductive treatment (ART) have reported obese male partners to contribute to reduced pregnancy rates, loss of pregnancy and an increased time to pregnancy (Magnusdottir *et al.*, 2005; Ramlau-Hansen *et al.*, 2007; Keltz *et al.*, 2010; Sermondade *et al.*, 2013).

Perhaps the most basic measurement of infertility is the analysis of microscopic sperm parameters including sperm motility, viability, concentration (count) and sperm morphology (World Health Organization, 2010). Studies on the effect of obesity on male fertility have yielded variable results regarding the extent to which these parameters are altered. An analysis of semen quality in relation to BMI amongst a cohort of young males in a general Denmark population showed reduced total sperm count (TSC) and sperm concentration in males with a BMI > 25 kg/m$^2$ compared to those

with normal BMIs (Jensen *et al.*, 2004). In addition, these authors observed no changes in the percentage of motile sperm between normal and obese individuals.

In a Chinese study of fertile males from their general population, Qin *et al*. (2007) noted reduced sperm motility in overweight and obese males, but the association lost statistical significance when the study adjusted for age, existing reproductive disorders, lifestyle factors, etc., thus finding no change in sperm motility. They also found no association between semen volume and changes in BMI. A Hungarian assessment of normozoospermic (semen samples with normal sperm concentration, motility and morphology) males attending fertility treatment at a clinic reported reduced sperm concentrations in the obese patients compared to men with a normal BMI, a change observed to worsen with age (Koloszár *et al.*, 2005). In contrast, an assessment of subfertile men with oligozoospermia (low sperm concentration) presenting for ART within the same country observed males with BMIs > 25 kg/m$^2$ to have reduced sperm concentrations compared to men with lower BMIs (Fejes *et al.*, 2006).

Hammoud *et al*. (2008) performed a retrospective analysis of American couples undergoing fertility treatment and observed higher incidences of oligozoospermia with increasing BMI as well as a low progressive sperm concentration in males with higher BMIs. A recent study performed in India assessing sperm quality in obese males found obesity to be related to lower sperm volume, concentration, motility and morphology, as well as a higher likelihood of having oligozoospermia and asthenozoospermia (low number of progressively motile sperm) compared to males with lower BMIs

Animal studies have also been performed to assess the relationship between obesity and male fertility, with equally variable results. In a study of diet-induced obesity by Bakos *et al*. (2011a), six-week-old male mice were placed on a high fat diet (HFD) in order for their sperm parameters and other molecular parameters to be assessed. These authors noted no changes in the sperm concentrations in the obese mice compared to lean controls, although the percentage of motile sperm declined in the HFD group. Similarly, Ghanayem and his co-workers (2010) corroborated this finding by observing a reduced motility in their obese mice. Rat studies have also observed similar results, where motility in the obese rats examined by Jia and colleagues (2018) was reduced compared to controls, along with higher amounts of abnormal sperm. The change was supported in another rat study analysing the alteration in sperm function within obese male rats where a higher proportion of sperm head defects were observed in obese rats compared to their leaner controls (Vigueras-Villaseñor *et al.*, 2011). A longer term intervention where rats were given a HFD for 15, 30 and 45 weeks showed normal sperm counts but decreases in progressively motile sperm (C. D. B. Fernandez *et al.*, 2011).

Several mechanisms have been implicated in the reduction of fertility within obese subjects including perturbations of the HPG axis, disruptions in endocrine regulation, oxidative stress, genetic inheritance and alterations, the additive effects of environmental toxins and the physical changes and strain brought on by increases in body weight, amongst others. These will be discussed in brief below.

### 2.5.1. Dysregulation of the HPG axis

The most important cause of infertility is the failure of spermatogenesis. This is a process that is tightly controlled by numerous proteins, most essentially the reproductive hormones. Spermatogenesis is largely controlled by the HPG axis, which early studies by Glass *et al*. (1977), Amatruda *et al*. (1978) and Strain *et al*. (1982) observed to be affected by obesity. These studies showed obese males to have normal LH, but reduced total testosterone and sex hormone binding globulin (SHBG) levels and thus compromising spermatogenesis. Later studies (Zumoff *et al.*, 1990; Vermeulen, Kaufman and Giagulli, 1996) were further able to show correlations between BMI, testosterone and SHBG where men with higher BMIs exhibited lower testosterone and SHBG levels. Data from studies analysing free testosterone levels in obese males has been discordant as the use of BMI as a variable differed in the respective studies. Using different categories of BMI namely obese (BMI ≥ 30-40) and morbid obesity (BMI ≥ 40), Giagulli and co-workers (1994) observed obese males to have normal levels of free testosterone within their plasma in contrast to morbidly obese males who had reduced free testosterone. Furthermore, LH levels remained normal in the obese males whereas LH secretion and LH amplitude in the morbidly obese were reduced. This decrease in LH amplitude was also observed by Vermeulen (1996). Levels of testosterone are affected by the increased adipose tissue observed within obese males. Adipose tissue releases an enzyme called aromatase which converts testosterone into estradiol in a process called aromatization (Yoshio *et al.*, 1987); this subsequently results in a negative feedback loop where the HPG axis is inhibited (Schneider *et al.*, 1979; Kley *et al.*, 1980). The Massachusetts Male Aging Study, a longitudinal study of 942 males aged 40-70, corroborated earlier findings of reduced SHBG as well as total and free testosterone, adding that these reductions become more sever with age (Derby *et al.*, 2006).

Leptin released from white adipose tissue has been shown by numerous studies to affect the HPG axis. It stimulates GnRH release from the hypothalamus and LH and FSH from the anterior pituitary (Yu *et al.*, 1997). It was also observed to be a direct regulator of testosterone production in the testes via the stimulation of leptin receptors on the Leydig cells (Ishikawa *et al.*, 2007). In obesity, leptin resistance predominates, resulting in high plasma levels of leptin. This, in turn, causes reduced testosterone production, which was observed by Isidori *et al*. (1999) to show a negative correlation between adiposity and testosterone levels.

### 2.5.2. Disruptions in the Endocrine System

In line with the findings by Vague (1947), adipose tissue is not only a storage site for excess fat but an active endocrine organ which affects lipid and glucose metabolism. In addition to its release of endocrine factors such as leptin and adiponectin, and inflammatory mediators such as TNFα and interleukins, adipose tissue is the site of production for numerous autocrine, paracrine and neuroendocrine molecules responsible for energy regulation, the oxidation of lipids, immune functions and vascular function (La Merrill *et al.*, 2013).

Excess adipose tissue is associated with numerous perturbations of the endocrine system, such as changes in hormone and protein concentrations via alterations in their secretion, metabolism, transport and their ability to act on their target tissues (Kahn and Flier, 2000; Pinkney and Kopelman, 2004). Whether the changes in these hormones and proteins precede or result from excess adipose accumulation is still under debate. It was previously believed that these changes were brought on by obesity and alleviated through weight loss, but further evidence has implicated these endocrine changes as part of the underlying mechanism of obesity development, with these preceding the onset of obesity and its accompanying metabolic disorders (Wajchenberg, 2000).

Obesity affects lipid and glucose metabolism differentially based on the amount and distribution of adipose tissue within the body. The “android” pattern of fat distribution is correlated with increased mortality as well as numerous disorders including diabetes, hypertension atherosclerosis and stroke to a higher extent than the “gynoid” pattern (Lapidus *et al.*, 1984; Larsson *et al.*, 1984; Ducimetiere, Richard and Cambien, 1986; Donahue *et al.*, 1987). Subsequent studies have more clearly ascribed these detrimental changes to accumulations of fat within the intra-abdominal fat depots (omental, mesenteric and retroperitoneal fat) with the aid of CT scans; fat accumulation in this region has been correlated with hyperinsulinemia, glucose intolerance, alterations in plasma lipoprotein lipid (dyslipidaemia) and cholesterol concentrations due to its high metabolic activity and easier drainage of secreted factors into systemic circulation (Despres *et al.*, 1989; Park *et al.*, 1991; Pouliot *et al.*, 1992). Further to this, the endocrine abnormalities observed in obesity involving growth hormone, steroid hormones and insulin was already conceptualized more than a decade ago to stimulate abdominal fat accumulation (Björntorp, 1991, 1995). The concept of visceral fat being more stimulatory of insulin insensitivity than subcutaneous fat has, however, been challenged. Using MRI and CT, Abate *et al*. (1995) and Goodpaster *et al*. (1997) were able to show that subcutaneous fat correlated at least as strongly to insulin insensitivity as visceral fat. Furthermore, a review of 23 published manuscripts on weight loss interventions by Smith and Zachwieja (1999) highlighted that individuals with larger visceral fat depots lose more total body fat during weight loss, with the greatest loss seen from visceral fat depots. This is because visceral fat is lost more easily due to its highly lipolytic nature and increased metabolic activity. Taking all

these studies into consideration, it appears that both visceral and subcutaneous tissue lead to disruptions in glucose metabolism and thus insulin insensitivity, but visceral fat is likely to be more deleterious due to its increased metabolic activity and expansive activation of cardiovascular disease risk factors such as dyslipidaemia and hyperinsulinemia.

Lipid mobilization, the “net release of free fatty acids (FFAs) from triglycerides stored within adipose tissue to other organs”, is controlled by numerous hormones as well as the nervous system (Carlson, Boberg and HÖgstedt, 2011). It is stimulated by the sympathetic nervous system as well as increased levels of norepinephrine and epinephrine, growth hormone and glucagon. Similarly, obesity increases sympathetic nervous system activation and causes activation of the hypothalamic-pituitary-adrenal (HPA) axis, thus increasing cortisol (Björntorp, 1995). It does, however, also lead to reduced growth hormone and sex hormones, as well as an increase in insulin production. It would appear then, that obesity favours lipid accumulation over mobilization, as insulin, growth hormone, testosterone and cortisol stimulate lipid accumulation within adipocytes and thus an increase in fat mass (Bjorntorp *et al.*, 1990). Visceral adipose tissue, however, is more sensitive to lipolytic stimuli (Björntorp, 1990), thus the combination of increased visceral adipose tissue and increased sympathetic tone can more greatly increase lipid mobilization within obese patients, especially those with central obesity. Large adipose tissue masses lead to increased FFAs making their way into blood circulation due to lipid mobilization which occurs within adipose tissue; visceral fat, specifically, drains into the portal vein, which pumps directly into the liver circulation (Carlson, Boberg and HÖgstedt, 2011).

High levels of FFAs in the portal vein have been shown to inhibit adequate insulin uptake by the liver, thus resulting in hyperinsulinemia (Svedberg *et al.*, 1991). Elevated portal FFAs have been postulated to lead to elevated liver triglycerides, as excess FFAs are transferred into existing triglycerides within the liver. In an experiment comparing insulin clearance between obese and lean rats, a strong negative correlation was observed by Strömblad and Björntorp (1986) between insulin uptake and liver triglyceride levels within study animals, suggesting this hypothesis to be valid (Svedberg *et al.*, 1991). Increased triglycerides and FFAs within the liver do not only lead to the storage of these molecules. FFAs in the liver are incorporated into lipoproteins, especially into very low density lipoproteins (VLDL), and are oxidised to form ketone bodies (Borgström and Carlson, 1957; Langdon, 1957; Carlson, 1960; Engel and Amatruda, 1963). Ketosis may result if the production of ketone bodies exceeds the rate at which they are utilized within the peripheral circulation, which can lead to metabolic acidosis (Laurell, 1956; Bressler, 1963; Engel and Amatruda, 1963). Furthermore, the mobilization of FFAs and lipid into plasma influences glucose metabolism. Increased FFAs within blood reduces glucose uptake into the liver and its metabolism by muscles, and chronic activation of this system can lead to hyperglycaemia (Randle, 1963).

Increased FFAs in peripheral circulation causes hyperlipidaemia and can also stimulate FFA deposition within other organs including blood vessel walls and the heart myocardium itself (Maling and Highman, 1958). This can lead to vascular complications including atherosclerosis, hypertension and cardiovascular disease. Taken together, obese individuals, especially those with central obesity, are prone to increased peripheral FFAs which stimulate hyperlipidaemia, hyperinsulinaemia and thus insulin resistance, as well as cardiovascular disease and diabetes, all of which are hallmarks of metabolic syndrome.

Metabolic syndrome itself has been observed to lead to male infertility (Kasturi, Tannir and Brannigan, 2008). High levels of visceral obesity and insulin insensitivity affect the production of GnRH and SHBG, dysregulating the HPG axis and thereby affecting spermatogenesis (Pasquali, 2006). Insulin insensitivity has also been found by authors such as Pitteloud and colleagues (2005) to directly reduce testosterone production at the level of the testis, which can lead to hypogonadotropic hypogonadism and a reduction in fertility. Cardiovascular disease, in particular hypertension, is a risk factor for erectile dysfunction which affects the ability to copulate and thus affects the formation of a conceptus (Esposito *et al.*, 2005). End-organ damage as a result of hypertension can also target the testes, subsequently affecting spermatogenesis, but the mechanism in which this occurs needs more clarity (Kasturi, Tannir and Brannigan, 2008). Hypertension has been shown by several studies to be correlated with reduced total plasma testosterone, free testosterone and SHBG (Khaw and Barrett-Connor, 1988; Phillips *et al.*, 1993; Fogari, Preti, *et al.*, 2002; Fogari, Zoppi, *et al.*, 2002). Some authors have suggested that the resultant arterial stiffness caused by hypertension is the underlying instigator of this androgen deficiency (Dockery *et al.*, 2003).

### 2.5.3. Oxidative Stress

Free radicals are highly reactive molecules formed during regular oxygen metabolism within cells (Griveau and Lannou, 1997). Due to this origin, they are termed reactive oxygen species (ROS); other forms of free radicals are derived from the chemical alteration of nitric oxide (NO) and are termed reactive nitrogen species (RNS) (Aitken and Clarkson, 1987; Sikka, 2001). Free radicals have physiological functions within the male reproductive system and have now been established as prominent signalling molecules involved in the regulation of spermatogenesis, capacitation, hyperactivation, the acrosome reaction and sperm-oocyte fusion (Kothari *et al.*, 2010). The quantity of free radicals within the male reproductive system is kept at homeostatic levels by endogenous antioxidants (Sikka, 1996). Antioxidants stabilize free radicals by donating electrons to their atomic structure and by scavenging them from a system (Aitken, 1994). Should there be an increase in free radicals due to their heightened production or an inability of antioxidants to maintain their concentrations at physiological levels, oxidative stress (OS) will ensue (Aitken, 1999). Oxidative

stress results in damage to cellular components of all forms, including lipids, proteins and DNA (Schafer and Buettner, 2001). Spermatozoa are susceptible to damage by free radicals as their membranes are predominantly composed of lipids, specifically PUFAs, which are prone to peroxidation by ROS (Aitken and Clarkson, 1987).

Together with their susceptibility to peroxidative damage, spermatozoa have a limited capacity to protect themselves from an attack by free radicals as they have small cytoplasms and a limited capacity to repair their membranes (Jones, Mann and Sherins, 1979). Sperm are also active producers of free radicals and were interestingly the first cells discovered to produce ROS (Thompson, Agarwal and Plessis, 2013). Mounting evidence points towards OS as a central mechanism in the etiology of sperm damage and thus infertility (Aitken, 1994). Sperm under stress from a variety of disorders can produce ROS and be additionally attacked by increased free radicals from numerous endogenous and exogenous sources. Furthermore, the peroxidative damage incurred by sperm during OS damages their membranes and makes them non-viable, and their membranes lose the fluidity conferred to them by PUFAs, which is necessary for sperm-oocyte fusion and the acrosome reaction (Aitken and Clarkson, 1987).

Obesity is said to both cause and result from OS. Adipose tissue secretes pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, as well as adipokines; these induce the production of ROS within the body, thereby attacking nearby tissues at sites of ROS accumulation (Fonseca-Alaniz *et al.*, 2007; Fernández-Sánchez *et al.*, 2011a). Moreover, the attack of adipocytes by ROS begets the production of more ROS, exacerbating OS. TNF-α stimulates the immune system to instigate an inflammatory response, adipocyte apoptosis, lipid metabolism, insulin signalling and ROS production; the latter is activated via the binding of TNF-α to receptors that promote Nuclear Factor Kappa B (NF-κB) signalling (Chandel, Schumacker and Arch, 2001; Wang and Trayhurn, 2006). TNF-α is heightened in obesity and is also found to increase the interaction between electrons and oxygen, thereby generating superoxide anions ($O_2^-$) which are the most predominant ROS species in the male reproductive system (Wang and Trayhurn, 2006). IL-6 regulates energy homeostasis as well as inflammation and influences the transition from acute to chronic inflammation, therefore stimulating chronic inflammation in obesity as well as insulin resistance (Naugler and Karin, 2008). Arguments for ROS preceding obesity state that ROS stimulate insulin insensitivity within adipose cells and peripheral tissue, which is a hallmark of obesity and metabolic syndrome (Sowers, Whaley-Connell and Hayden, 2011). Furthermore, Youn *et al*. (2014) used the premise that obesity is associated with vascular dysfunction and vascular OS to establish that vascular-derived ROS is generated via NADPH oxidase activation of vascular smooth muscle cells. This predisposes individuals to obesity. After genetic alteration of genes responsible for NADPH oxidase production in order to increase its function, Youn and colleagues fed mice with a high fat

diet and found that the mice with increased NADPH function acquired exaggerated obesity, gaining 50% more weight than the control mice.

OS hampers male fertility by affecting basic sperm parameters as it reduces sperm motility (Jones, Mann and Sherins, 1979; Aitken and Clarkson, 1987; De Lamirande and Gagnon, 1992), viability (Jones, Mann and Sherins, 1979; Mahfouz *et al.*, 2010), normal sperm morphology (Gil-Guzman *et al.*, 2001) as well as sperm function including capacitation (Rivlin *et al.*, 2004), hyperactivation (S Oehninger *et al.*, 1995), sperm-oocyte fusion (Aitken, Buckingham and Harkiss, 1993; Aitken, 1999; Das *et al.*, 2008) and causes spontaneous acrosome reactions (S Oehninger *et al.*, 1995).

### 2.5.4. Environmental toxins

Adipose tissue is an important site for the bioaccumulation of toxins (Jackson *et al.*, 2017). Organic pollutants are lipophilic compounds that are considerably stable and resistant to environmental degradation, more especially persistent organic pollutants (POPs) such as dichlorodiphenyltrichloroethane (DDT), polychlorinated dibenzo-p-dioxins (dioxins) and polycyclic aromatic hydrocarbons (US EPA, no date). The principal human exposure to POPs is via diet, through the consumption of contaminated fatty foods of animal origin such as meat, fish and dairy, exposing individuals to many classes of POPs (Jackson *et al.*, 2017). A few studies have suggested that adipose tissue plays an important role in the storage and toxicokinetics of hydrophobic xenobiotic POPs (Kim *et al.*, 2011, 2012). Xenobiotic chemicals are well metabolised by the body via an extensive metabolizing system including receptors, transporters and metabolizing enzymes which assist in their elimination from the body (Barouki, 2010). POPs are, however, resistant to metabolism. Instead, they distribute to lipophilic compartments within the body, especially adipose tissue (Jackson *et al.*, 2017). They are absorbed into adipocytes and can be localized within lipid droplets. The storage of POPs within adipose cells has both beneficial and detrimental consequences. Their deposition within lipophilic tissues removes them from blood circulation and reduces their bioavailability, thereby protecting other tissues from their toxic effects. This was shown by Geyer *et al*. (1993) where the 30 day exposure of 20 terrestrial animals to a POP showed reduced toxic effect in the species with the higher fat masses. Conversely, higher amounts of POPs can accumulate in individuals with higher adiposity, and their fat will become a long term reservoir (La Merrill *et al.*, 2013). When these individuals lose weight, however, these POPs are released into circulation, exposing other body cells to their toxic effects (De Roos *et al.*, 2012; Dirinck *et al.*, 2015). This was shown in both human and animal studies after weight loss via lifestyle changes or bariatric surgery in humans (Ohmiya and Nakai, 1977; Jandacek *et al.*, 2005; Hue *et al.*, 2006; Kim *et al.*, 2011) or periods of starvation in animals. The pre-treatment of mice with DDT by Ohmiya and Nakai (1977) showed increased blood concentrations of DDT after weight loss, as well as its increase in several other lipid-rich tissues including heart, lung, spleen,

kidney, liver and brain. Furthermore, the newly released POPs in blood will circle back into the remaining adipose tissue, thereby becoming more concentrated within the adipocytes (Dewailly *et al.*, 2000; Kim *et al.*, 2011). As mentioned previously, environmental pollutants such as POPs have a negative effect on hormone concentrations and fertility parameters. They further exacerbate the effects of obesity on male fertility by affecting the normal functions of adipose tissues. POPs have been shown to increase the release of adipokines (Taxvig *et al.*, 2012) and inflammatory mediators from adipose tissue (Kern *et al.*, 2002; Li, Vogel and Matsumura, 2007; Arsenescu *et al.*, 2008; Nishiumi *et al.*, 2010), thereby promoting inflammation and affecting insulin signalling and fertility.

### 2.5.5. Physical changes

The increased fat deposition observed in obesity can result in fat being deposited directly above the pubic area, the upper thigh region and the scrotum (Shafik and Olfat, 1981). This increased amount of fat can raise the temperature around the scrotum, which is usually 2°C below core body temperature (Chowdhury and Steinberger, 1970; Mieusset and Bujan, 1995). All tissues and organs are susceptible to the heat damage associated with increased body temperature, but the testes are unique in that they are damaged by normal core body temperature (Setchell, 1998). Increased fat around the scrotum as well as sedentary behaviour such as extended periods of sitting, exposes the scrotum (and thus the testes) to normal body temperature, causing heat stress and damage within the genitalia (Durairajanayagam, Agarwal and Ong, 2015).

Spermatogenesis requires lower temperatures for optimal functioning and increases in scrotal temperature have been shown to negatively affect sperm quality (Hjollund *et al.*, 2000). Even small increases of 1°C in scrotal temperature have a negative effect on sperm production, reducing spermatogenesis by approximately 14% (Durairajanayagam, Agarwal and Ong, 2015). Studies have shown genital heat stress to result in reduced sperm counts, spermatogenic arrest and atrophy of the germinal epithelia (Chowdhury and Steinberger, 1970), but Hjollund *et al*. (2002) observed no changes in sperm motility and morphology as well an semen pH. A study on young male rats exposed to 33-35°C for 3-5 weeks showed decreased levels of testosterone (Bedrak, Chap and Fried, 1980) which can explain the observation by other studies which observed marked decrease in testis size due to heat stress (Setchell, 1998).

Erectile dysfunction (ED), the consistent inability to achieve and/or maintain penile erection over a 3 month period, is the sequelae of hyperglycaemia, hyperlipidemia and cardiovascular disease, all of which are conditions that can be instigated by obesity (Seftel, Sun and Swindle, 2004; Fisher *et al.*, 2009; Montorsi *et al.*, 2010; Dong, Zhang and Qin, 2011; Dursun *et al.*, 2018). Other causes of ED include smoking, low level of testosterone, anemia, peripheral vascular disease, drug and alcohol abuse, and surgery or trauma to the pelvic area or the spine (Johannes *et al.*, 2000; Dong,

Zhang and Qin, 2011). ED is an obvious cause of infertility due to its hindrance of sexual activity within couples (Fisher *et al.*, 2009) and is a commonly reported symptom in obese and diabetic males. A study by Corona *et al*. (2006) reported 96,5% of their study participants with metabolic syndrome to have ED. A Health Professionals Follow-up Study observed that men with a BMI < 25 had a 30% reduction in their risk of developing ED (Bacon *et al.*, 2003), while a study by Andersen *et al*. (2008) observed higher BMIs in young and elderly men to correlate with increased frequency of ED. The co-morbidities of obesity impair the release of NO from vascular endothelia to cause changes to endothelial function and reduce plasma testosterone levels, which promote ED (Feeley and Traish, 2009). In addition, adipose tissue also secretes numerous cytokines and adipokines which stimulate inflammation, endothelial dysfunction and insulin resistance, all contributing towards the pathophysiology of ED (Guzik, Mangalat and Korbut, 2006). The testosterone deficiency observed in obesity and ED have been linked to further alterations in fertility. Insulin resistance has been shown to reduce testosterone levels as insulin is a known stimulant of testosterone production (Pasquali *et al.*, 1995). Further to that, leptin receptors are found in the testes and the binding of leptin to these can reduce androgen production, as observed by Caprio *et al*. (1999). This mechanism explains the hypogonadism observed in obese men and those with ED, which can further affect fertilizing potential via the reduction of basic sperm parameters.

Sleep apnea, which is shallow or infrequent breathing during sleep, is common among obese individuals (Katib, 2015). It is more common in males with central obesity (Luboshitzky *et al.*, 2005). Sleep apnea commonly leads to disrupted sleep which has been shown to disrupt the nocturnal rhythm of testosterone production, with studies showing this to lead to reduced functioning of the HPG axis and a reduction in the morning levels of testosterone (Luboshitzky *et al.*, 2001).

## 2.6. Animal models of obesity

Having observed the similarities between laboratory animals and humans, it is evident that these mammals can interchangeably be assessed in order for disruptions in normal physiology to be understood.

Obesity is assessed in both rats and mice in order for the observed clinical changes in humans to be better assessed in a laboratory setting and for therapies to be devised. These models induce obesity in a manner that would normally occur in humans, including alterations to genetics, diet, physical activity, hormone dysregulations, endocrine changes, aging and via exposure to environmental toxins and medications that predispose laboratory animals to obesity. The most widely used animal models of obesity will be briefly highlighted below.

### 2.6.1. Genetic

Genetically induced obesity animal models are classified according to the mode of inheritance, e.g. monogenic (single-gene dominant, single gene recessive) and polygenic obesity. Monogenic models of obesity have been widely used, with dominant models being obese (ob/ob), diabetic (db/db) and Zucker or fatty rats (fa/fa) (Sclafani, 1984). It has been argued, though, that polygenic obesity is more representative of human obesity as animals with these mutations become spontaneously obese or acquire obesity via additional exposure to cafeteria, high fat or high carbohydrate diets (Festing, 1979; N. J. Rothwell, Saville and Stock, 1982). Mutations of the leptin (ob) gene create the genetically obese ob/ob model by preventing the production leptin (Orahilly, 2009). This results in early-onset obesity, hyperphagia, reduced energy expenditure, insulin resistance, hyperglycaemia, and hyperinsulinaemia. Mutating the leptin receptor (db) gene creates the diabetic db/db animal model, which is phenotypically similar to the ob/ob model, but the animals develop leptin resistance and marked hyperleptinaemia (Lutz and Woods, 2012). Zucker rats are leptin receptor deficient rats with mutations in the extracellular domain of this receptor. This is an autosomal recessive form of obesity, and also exhibits hyperphagia and reduced energy expenditure (Bray, 1977). They do, however, have normal blood glucose levels. POMC, AgRP, and MC4R mutations are also utilized as monogenic models of obesity.

### 2.6.2. Diet-induced

Although genetics plays an essential role in the regulation of body weight, body composition, body size and the metabolic response to feeding, an exponential increase in weight gain in humans and animals over a short period of time cannot be explained by genetics (Hariri and Thibault, 2010). Individual susceptibility to obesity that is conferred by genetics is influenced by environmental factors including diet (Bouchard and Tremblay, 1997; Jéquier, 2002; W. James, 2008). Diets are crucial determinants of health and disease, and habits that cause an unbalanced diet are a significant risk factor in the development of obesity and its related metabolic disorders (Preguiça *et al.*, 2020).

Maintaining laboratory rats on a modified *ad libitum* diet is a widely applied approach to inducing weight gain in laboratory animals. Additionally, overfeeding is employed as a direct approach where tube feeding (gavaging) animals at intervals throughout the day has shown vast increases in body weight (Hansen, 1979). Altering the palatability and/or calories of food seems to be the easiest method to increase weight gain, where high fat and/or high sugar diets are provided *ad libitum* to elicit diet-induced obesity models (Sclafani, 1978). Standard rat chow is most often in the form of pellets that contain carbohydrates derived from wheat, corn and barley. These are whole grains which are high in fibre and contain phytochemicals, and are thus protective against insulin

resistance and obesity (Isken *et al.*, 2010). The addition of sugars (fructose, sucrose, glucose), polysaccharides and highly palatable calorie-rich foods such as sweetened milk and processed meats can increase the palatability of the standard chow, causing hyperphagia (Sclafani and Springer, 1976; Rolls, Rowe and Turner, 1980; N. J. Rothwell, Saville and Stock, 1982). Some studies place experimental animals on normal rat chow after obesogenic feeding; in these experiments, some of the animals in the treatment/diet group are observed to lose weight whereas others remain obese (Rolls, Rowe and Turner, 1980). This is anticipated to be the result of changes in adipocyte cellularity, the length of diet duration and differences in rat or mouse strains (I M Faust *et al.*, 1978; Sclafani, 1978; Mandenoff, Lenoir and Apfelbaum, 1982). Diet-induced obesity (DIO) animals exhibit reduced insulin and leptin sensitivity, and seem to directly affect intracellular signalling pathways of the hypothalamus which control body energy. The standard animals used in dietary obesity studies include Wistar, Sprague-Dawley and C57BL/6C rats (Hariri and Thibault, 2010).

There are several types of animal diets used to induce obesity, namely tube feeding, high-fat, high-carbohydrate, low-protein and varied diets.

#### 2.6.2.1. Tube feeding

Force feeding of animals involves the delivery of liquid diets orally or via gastric intubation, which is used to administer several large meals per day. This can lead to obesity as it reduces dietary thermogenesis, thereby increasing fat deposition (Rothwell and Stock, 1984). These meals are usually provided during the day-time, which differs from the normal nocturnal feeding pattern observed in rodents. Rats ingest 70-80% of their meals at night (during the dark phase), with the two peaks in meal frequency occurring at the beginning of the night (dusk) and towards the end (dawn) (Le Magnen and Devos, 1970).Tube-fed rats have been found to gain significantly more weight than their controls, and their percentage weight gain is in proportion to the intake provided via intubation (Rothwell and Stock, 1984).

#### 2.6.2.2. High fat diets

Epidemiological studies in humans have shown that as the fat content of a diet increases, the incidence of obesity also increases (Saris *et al.*, 2000; Hariri and Thibault, 2010; Piché *et al.*, 2018). High-fat diets have been proposed to lead to obesity as fat is an energy dense macronutrient. It is also less satiating than either carbohydrates and proteins, leading to passive overconsumption (Moussavi, Gavino and Receveur, 2008). High fat diets are diets with 40-60% of the macronutrient composition derived from lipids (Fernandes *et al.*, 2016). The lipid sources in these diets include lard, vegetable oils and soybean. Diets with a high fat content usually result in

excessive weight gain. They are more palatable than regular chow/pellets alone and are more energy dense. These diets also require less energy for fat storage as dietary energy is derived directly from lipids, which have a lower energy cost than *de novo* lipogenesis from carbohydrate sources (6 vs 14 kJ/g) (Rothwell and Stock, 1984). This results in more efficient weight gain. Some researchers have reported increased weight gain in the high fat diet groups despite there not being a significant increase in food intake compared to controls (Lemonnier, 1972; Herberg *et al.*, 1974). In a review of animal models of obesity using experiments published 2010-2016, Fernandes *et al*., (2016) showed that diets containing 60% lipid gained weight in all high fat groups regardless of strain. In addition, these animals gained epididymal fat and exhibited hyperglycaemia and insulin resistance. Obesity has, however, been shown to be induced using diets with as low as 13% of total energy derived from fat, but these were high-energy diets (Harrold, Williams and Widdowson, 2000). This is because a rat's daily fat requirement is only 5%. Furthermore, high-fat diets have been shown to derive up to 85% of their macronutrient composition from lipids (Mickelsen, Takahashi and Craig, 1955). The type of fat used in high fat diets is important. This is because the metabolic behaviours (i.e. oxidation and deposition rate) of fatty acids differ. The characteristics of dietary fatty acids, such as structure, chain length, degree of saturation, and the configuration and position of double bonds, can affect this metabolic behaviour (DeLany *et al.*, 2000; Madsen, Petersen and Kristiansen, 2005). Metabolic studies in animals and humans have shown that short- and medium-chain fatty acid are preferentially oxidised compared to long-chain fatty acids (Leyton, Drury and Crawford, 1987; DeLany *et al.*, 2000). Diets high in medium-chain fatty acids (C6-C12) have been shown to gain less weight than diets using lipids with longer chain fatty acids such as corn oil or lard (Gurr, Rothwell and Stock, 1979). This is because the fatty acid composition of fat sources can affect the cellularity (i.e. the fat cell number and volume) of adipose tissue (Moussavi, Gavino and Receveur, 2008).

#### 2.6.2.3. High carbohydrate diets

Diets with a 60% mean proportion of macronutrients derived from carbohydrates constitute a high carbohydrate diet. These carbohydrates are derived from corn starch, sucrose and fructose and other starches and sugars. Animals on high carbohydrate diets show increases in body weight and body fat, as well as increased triglycerides, hypertension and hyperglycaemia (Fernandes *et al.*, 2016). Many animals show a preference for foods with carbohydrates, especially those with a high sugar content. These also increase the palatability of foods and can cause hyperphagia. In a study observing the difference in dietary thermogenesis of sucrose and glucose solutions administered via gastric intubation over several weeks, the two sugars were shown to have the same acute effect on thermogenesis. Sucrose, however, was shown to be higher in energy (which increases

the balance towards a higher energy intake) and a higher oxygen consumption (N J Rothwell, Saville and Stock, 1982).

#### 2.6.2.4. Low protein diets

Protein-deficient diets in rats have a range of 4-13% of the macronutrient composition derived from protein (Langley-Evans, 2000; Lieberman, Yeghiayan and Maher, 2005; Malta *et al.*, 2014; Pezeshki *et al.*, 2016; Ajuogu *et al.*, 2020). Normal daily intake of protein in rodent diets is approximately 22% and reductions in this macronutrient results in malnutrition, increased food consumption and increased body weight and fat mass (Pezeshki *et al.*, 2016). Furthermore, moderate protein restriction in rodents has been shown to be associated with changes in body composition such as reduced lean muscle mass, increased fat weight and a fatty liver (Du, Higginbotham and White, 2000; White, Porter and Martin, 2000; Huang *et al.*, 2013; Solon-Biet *et al.*, 2015). These changes are the result of alterations in food consumption and dietary thermogenesis following a low protein diet. Reduced dietary protein concentrations lead to the overconsumption of fats and carbohydrates in order to compensate for insufficient protein intake, leading to an increase in total energy consumption (White, Porter and Martin, 2000; Pezeshki *et al.*, 2016). The thermogenesis required to break down protein is 20-35% of dietary energy consumed, in comparison to the 5-15% required for carbohydrates (Westerterp, Wilson and Rolland, 1999). The lower thermic effect of low protein diets, especially those supplemented by higher carbohydrate and fat consumption, result in reduced energy expenditure and increased energy (fat) storage (Dauncey and Bingham, 1983; Karst *et al.*, 1984; Crovetti *et al.*, 1998; Halton and Hu, 2004).

#### 2.6.2.5. Varied

Some diets do not fit the characteristics of the aforementioned high-fat, high-carbohydrate, low-protein diets, and cannot be classified. The key is to make sure that the diet exhibits higher or lower macromolecule proportions compared to the control diet, and that the high-energy macromolecule percentages are above those that are required daily by the experimental animals. In addition, the macromolecule proportions, e.g. percentage of fat, included in a diet intervention can be based on quantities previously shown by other DIO experiments to be sufficient to elicit weight gain.

High fat, high-salt, low-fibre, energy-dense diets are used in animal experiments to induce obesity. An example of this includes the cafeteria diet. This diet includes highly palatable, energy dense foods consisting of human food items such as chocolate chip cookies, cheese, marshmallows, processed meats, cereals, bananas, milk chocolate and peanut butter (Sclafani and Springer,

1976; Sampey *et al.*, 2011; Johnson *et al.*, 2016). Cafeteria diets are designed to mimic the variety of energy dense foods consumed by humans on a Westernized diet, and it induces hyperphagia and rapid weight gain (Sampey *et al.*, 2011). Furthermore, energy-dense foods are known to alter normal appetite regulation as they disable the regular homeostatic mechanisms used by the body for normal energy balance, most possibly via the activation of neural reward systems (Erlanson-Albertsson, 2005; Rolls, 2009; Sampey *et al.*, 2011). In addition, high-sugar, high-fat foods upregulate the expression of hunger signals while suppressing satiety signals, leading to increased food consumption (Erlanson-Albertsson, 2005). Animals on cafeteria diets have been shown to develop prediabetic parameters including hyperinsulinaemia and glucose intolerance, and an increase in fat mass and obesity-related inflammation (Johnson *et al.*, 2016). Other highly palatable diets may also be termed as hyperphagic diets.

#### 2.6.2.6. Diet duration

The duration of exposure to obesogenic diets in animal models varies between studies. Previous research has shown that it is more effective to begin obesogenic diets in animal models at a young age (after weaning, ± 4-6 weeks of age) and for the period of feeding to continue for several weeks to months after its inception (Peckham, Entenman and Carroll, 1962; Quinn, 2005; Sengupta, 2013; Dutta and Sengupta, 2016). Increases in body weight can be observed from as few as 2 weeks after the commencement of obesogenic feeding, but the obese phenotype becomes more apparent after 4 weeks or more of feeding (Buettner, Schölmerich and Bollheimer, 2007). The majority of existing models are predominantly acute as animals are placed on varying diets for ± 4-28 weeks to bring about the obese phenotype (Woods *et al.*, 2003; Carroll, Zenebe and Strange, 2006; Parasuraman *et al.*, 2010, 2013; Puig *et al.*, 2012; Shao *et al.*, 2012). Although findings from these studies are relevant, they may not wholly mimic the true onset of obesity and its chronic nature in the adult human. Several studies using feeding periods between 4 – 28 weeks have reported increases in body weight/fat in the DIO animals, but studies including those from Harrold (2000), Chang (1990) and Leopoldo (2016) reported obesity to occur in only the obesity prone animals, with a large proportion of their rats unable to attain the obese phenotype. Furthermore, some studies report the length of their diets as insufficient, stating that a longer treatment duration would be beneficial in yielding results more representative of the of the obese condition (Sampey *et al.*, 2011; Watson, Bruggeman and Parent, 2012; Selfridge *et al.*, 2014; Cecconello *et al.*, 2015; Oosterman *et al.*, 2015; Aly and Polotsky, 2017). Prolonged periods of obesogenic feeding can produce 10 – 20% increases in body weight in the DIO group compared to controls (Buettner, Schölmerich and Bollheimer, 2007). A study by Peckham *et al*. (1962) established a 62 week hypercaloric diet and was able to attain a 37% increase in the body weights of DIO rats compared to age-matched controls, with the increase in weight being attributed to a higher fat weight in the

DIO rats. Other long-term diets have found significant increases in body weight after extended exposure of rats to hyperphagic diets or excessive amounts of chow. Oscai (1982) induced severe obesity in a rat model by providing unrestricted access to a high-fat, hyperphagic diet to rats immediately after weaning and continuing with the diet until the rats reached 29 – 58 weeks of age. Body weights stabilized at a sizable 1,213 ± 62g in the high fat diet group. Scotellaro *et al*. (1991) took a group of 100 rat pups and placed them in individual cages after weaning to remove food competition and created an excess supply of normal rat chow for 10 weeks. After this period, the heaviest and lightest rats were fed for another 40 weeks, and the obese rats were observed to consume significantly more food than the lean rats and attain 59.53% more body fat. Schemmel *et al*. (1969) fed male and female weanlings normal rat chow or a high fat diet for 64 weeks and managed to attain weights of ± 1,050 g in the obese animals compared to ± 600 g in the control group, a significant increase in body weight which correlated with a higher body fat mass. From this information it can be deduced that diet durations applied in obesity research using animal models vary. Calorie-dense diets can cause obesity in short term diets from 2 weeks of feeding, with long term diets being shown to be very effective in inducing obesity that is representative of the accumulative increase in body and fat weight observed in the obese condition.

### 2.6.3. Endocrine

Several methods are used as endocrine-related obesity models (Wade and Gray, 1979). Methods of induction include chronic administration of hormones including insulin, hormones and glucocorticoids (Hollifield, 1968; Lotter and Woods, 1977). The excessive administration of a glucocorticoid, such as cortisol and growth hormone, is a common example of obesity induced by known inciting agents. In an experiment where 11-dehydrocorticosterone was administered via a subcutaneously implanted pellet, increased fat was observed in the older mice (Hollifield and Parson, 1959). This was not as a result of increased food intake or weight gain but from increased gluconeogenesis and insulin production, which resulted in increased fat synthesis by adipose tissue and decreased protein synthesis. In addition, observances from glucocorticoid-induced obesity studies have revealed that excess glucocorticoids do not induce obesity in young animals and humans during their growth period (Blodgett *et al.*, 1956; Hollifield and Parson, 1959; Morris *et al.*, 1968). Fluctuations in circulating levels of hormones are able to alter body composition and body weight, including the behaviours which control body weight, e.g. food intake and exercise (Wade and Gray, 1979). Numerous gonadectomy and hormone replacement experiments have been performed over the past few decades to establish the effect of ovarian and testicular hormones on body weight and regulatory behaviours (Hausberger and Hausberger, 1966; Krotkiewski, Kral and Karlsson, 1980; Holmäng and Björntorp, 1992; Ouerghi, Rivest and Richard, 1992; Russell *et al.*, 1993; Chen *et al.*, 2002; Keto *et al.*, 2012; Kashiwagi *et al.*, 2020). In male

rats, different androgens have been administered to animal models and their effect on eating and body weight vary depending on the type and concentration of androgen used. Androgens regulate energy metabolism in males. One of the mechanisms in which this occurs is via direct stimulation of the androgen receptor or the indirect stimulation of oestrogen receptors after the aromatization of androgens (Movérare-Skrtic *et al.*, 2006). A heightened activation of the androgen receptor can result in obesity via the alteration of lipid metabolism and glucose oxidation (Movérare-Skrtic *et al.*, 2006). Testosterone, however, has been shown to supress adiposity over a range of concentrations (Wade and Gray, 1979). This is because testosterone is an anabolic hormone with influence on male body fat distribution. It has been shown to influence glucose and insulin concentrations, as well as the levels of circulating triglycerides and cholesterol (Christoffersen *et al.*, 2006). Furthermore, testosterone deficiency increases body fat, which can alter carbohydrate and fat metabolism (Seidell *et al.*, 1990; Mauras *et al.*, 1998). Castration (the surgical removal or artificial destruction of gonads) has been used as a model to study extreme testosterone deficiency in animals. Orchiectomy (the removal of one or both testes) increases fat content in male rats (Hoskins, 1925; Kemnitz, Goy and Keesey, 1977; Drago *et al.*, 1982; Christoffersen *et al.*, 2006; Movérare-Skrtic *et al.*, 2006). Furthermore, castration has been shown to reduce food intake, increase fat percentage and reduce lean body mass via the direct alteration of glucose and fat metabolism in rats, among other mechanisms (Gentry and Wade, 1976; Krotkiewski, Kral and Karlsson, 1980; Holmäng and Björntorp, 1992).

### 2.6.4. Neural

Hypothalamic obesity is the most well-known neural model of obesity. These models of obesity affect feeding behaviour as the “satiety centre” of the brain is targeted. The original induction of hypothalamic obesity was via an electrolytic lesion to the ventromedial hypothalamus (VMH) to induce overeating and obesity (Bray, 1977).This region of the hypothalamus contains a large proportion of neurons which are gluco-responsive, as well as neurons responding to histamine, dopamine and serotonin stimulation (King, 1991, 2006; Paasch *et al.*, 2010). VMH lesions most likely lead to the destruction of POMC neurones, resulting in these animals showing a decrease in sympathetic tone and an increase in parasympathetic tone. Furthermore, VMH-lesioned rats display elevated circulating insulin. Other methods include knife-cuts to the medial hypothalamus (MH), lesions to the paraventricular hypothalamus (PVH) and the injection of certain substances such as monosodium glutamate (MSG), bipieridyl mustard and gold thioglucose to destroy the arcuate nucleus (ARC) (Sclafani, 1984). ARC lesions result in hyperphagia, insulin resistence and hyperinsulinaemia (Nemeroff, Lipton and Kizer, 1978).

## 2.7. Treatments of male infertility

In the case of infertility caused by lifestyle factors, the simplest intervention is lifestyle modification (Anderson, Nisenblat and Norman, 2010). Weight loss in males has been shown to increase SHBG as well as total and free testosterone, with an increased LH being observed in more marked weight loss (Chambers and Anderson, 2015). Furthermore, serum concentrations of insulin and leptin are reduced after weight loss (Kaukua *et al.*, 2003; Niskanen *et al.*, 2004; Sharman and Volek, 2004). Regular, moderate exercise as well as low-to-moderate resistance training is further able to increase testosterone production (Kraemer and Ratamess, 2005; Vaamonde Martin *et al.*, 2014; Chambers and Anderson, 2015), thereby assisting to possibly restore function to the HPG axis of infertile men.

Medical treatment of an underlying illness or disorder responsible for the initiation of infertility may alleviate the cause of infertility. The treatment of infections, especially those of the reproductive tract, should be resolved in order to reduce inflammation and halt the generation of ROS. Leukocytospermia can occur as the result of an infection; antibiotic treatment and frequent ejaculation have been shown to resolve this, but no immediate changes in male fertility parameters were observed by some researchers (Branigan and Muller, 1994; Kamischke and Nieschlag, 1999) while others observed an improvement in semen quality and/or fertility (Aparicio *et al.*, 1980; Berger *et al.*, 1983; Giamarellou *et al.*, 1984).

Obesity can be treated with medication where lifestyle modifications have been deemed insufficient. Anti-obesity drugs reducing the absorption of fat via the intestine (Orlistat) and suppressing appetite via deactivation of norepinephrine, serotonin and dopamine (Sibutramine) have been approved by the Food and Drug Administration (FDA) for long-term use but weight loss with these drugs is modest. Aromatase inhibitors have been prescribed to obese males with elevated estradiol and low testosterone levels; these prevent the production of aromatase, thereby reducing the conversion of testosterone to estradiol. Aromatase inhibitors have been found to be effective in improving fertility, but with varying efficacy. A study comparing aromatase inhibitors Anastrozole and Testolactone on lean, overweight and obese patients, showed both drugs to improve patient testosterone levels, thereby improving their testosterone:estradiol ratio and increasing semen parameters with varying effectiveness (Raman and Schlegel, 2002). Morbidly obese, infertile patients treated with Anastrozole were seen to have increased LH, FSH and testosterone levels and an improvement in spermatogenesis (Roth, Amory and Page, 2008).

Testosterone replacement therapy can be prescribed to men with low testosterone concentrations and symptoms of hypogonadism. The use of these medicines is, however, controversial as it has been shown to have numerous benefits (increased libido, improvement of sexual function, muscle

mass, bone density, erythropoiesis and body composition) as well as detrimental side effects (liver toxicity, exacerbation of benign prostatic hypertrophy, dysregulation of the HPG axis, erythrocytosis and hyperviscosity, and sever heart failure) (Bassil, Alkaade and Morley, 2009). Antioxidants are used to help alleviate some of the sequelae of OS. Several studies have reported the use of antioxidants to treat male infertility. Glutathione has been shown to increase progressive motility and morphology (Lenzi, Gandini and Picardo, 1998) and reduce lipid peroxidation (LPO) of sperm membranes (Dabaja and Schlegel, 2014). Superoxide dismutase (SOD), which catalyses the conversation of superoxide anions to oxygen and water, has also been observed to prevent LPO, thereby increasing sperm motility (Naz and Kamal, 2017). Vitamin C and E supplementation in healthy men has been shown by Eskenazi *et al*. (2005) to increase sperm concentration and progressive motility respectively. Although the abovementioned antioxidants have been shown to have positive effects on male fertility, a single antioxidant is limited in its capacity to tackle OS in infertile men and a combination of them has been recommended as a better approach (Lanzafame *et al.*, 2009). As free radicals are necessary for normal sperm functions, caution should be taken not to reduce concentrations too drastically as this can be detrimental to sperm function.

Leptin has been found to affect the function of GnRH within the brain (Quennell *et al.*, 2009) and controlling the levels of this hormone can treat male infertility. Leptin deficiency as seen in obese individuals can be treated with leptin therapy. Leptin therapy given to leptin deficient mice due to a LEP mutation showed a correction in the infertility of the mice, with increases in testis weight, spermatogenesis and improved Leydig cell morphology (Mounzih, Lu and Chehab, 1997); leptin therapy has, however, been shown to have limited therapeutic action in polygenic/common obesity in humans, but impressive effects have been elicited in congenital leptin deficiency individuals (Yildiz and Haznedaroglu, 2006).

Perhaps the most well-known treatment for infertility is ART, which is performed in numerous fertility clinics the world over. The most widely used methods of ART include *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) which have shown considerable success in the treatment of couple infertility (MacDonald *et al.*, 2009). IVF is the placement of sperm and oocytes into a laboratory (petri) dish to allow for conception to occur while ICSI pertains to the direct insertion/injection of a sperm cell into an oocyte *in vitro* (Practice Committee of the American Society for Reproductive Medicine, 2017). ART does, however, bypass the underlying causes of infertility and in most cases, these issues are not adequately dealt with (Siddiq and Sigman, 2002). As long as some viable spermatozoa can be extracted from the male, the underlying cause of infertility is overlooked and curative measures are mostly not attempted. ART is also expensive and has variable success (Kushnir *et al.*, 2017).

Surgical methods are additionally used in the attempt to improve male fertility. These can be divided according to the intended purpose of the surgery, including diagnostic surgery, surgery for the improvement of sperm production, surgery for the improvement of sperm delivery, and surgery for the retrieval of spermatozoa (Lopushnyan and Walsh, 2012). Diagnostic methods including testicular biopsy, can be used to determine the presence of cancers as well as spermatozoa within the testes. In the case of non-obstructive azoospermia, a needle can be inserted into the testes to assess the presence of sperm to determine whether these cells can be extracted for ART (Saleh *et al.*, 2010). Sperm production can be facilitated through the relief of anomalies impeding normal spermatogenesis. Varicocele (the enlargement of the veins within the scrotum) results in reduced thickness of the germinal epithelium and disorganized spermatogenesis due to hypoxic damage to spermatozoa and oxidative stress, which can be reversed via varicocelectomy (Masson and Brannigan, 2014). Obstruction within the ducts of the male reproductive system can be alleviated through surgery in order to facilitate the delivery of sperm to the female genital tract. Ejaculatory duct obstruction, for example, results in low semen volume, low seminal pH and/or the absence of fructose in seminal plasma (Pryor and Hendry, 1991). This can be treated by a resection of the ejaculatory ducts (Farley and Barnes, 1973). Sperm retrieval can be performed on patients with azoospermia. These include testicular sperm aspiration (TESA), testicular sperm extraction (TESE), percutaneous epididymal sperm aspiration (PESA) and microscopic epididymal sperm aspiration (MESA) (Lopushnyan and Walsh, 2012). These allow for sperm to be directly extracted from the testes and epididymis, respectively.

These interventions have shown success. In the endeavour to develop more curative treatment strategies, additional knowledge needs to be gained on the mechanisms underlying infertility, especially those governing the interactions between infertility and the innumerable diseases and disorders which can alter fertility potential such as obesity. Furthermore at least 25% of men with infertility have an unidentifiable cause (Siddiq and Sigman, 2002). Their medical work up including history and examination are normal but their semen parameters are below WHO recommended ranges. New strategies need to be considered in order for the causative factors of such cases to be elucidated.

## 2.8. Novel approaches for treatment

Previous research, especially using *in vivo* and *in vitro* models, have only explained certain aspects of obesity and male infertility; animal and cell models need to be further examined through the use of new molecular techniques in order for a better understanding of the pathophysiology of obesity to be acquired, thus permitting the exploration of improved and novel therapeutic approaches (Apalasamy and Mohamed, 2015).

Genetics is believed to play a significant role in both obesity and infertility and several studies have endeavoured to reveal the intricacies of this aetiology, with the most recent using novel genetic methods to gain new insights. Gene analyses such as candidate gene association studies (associations between genetic variations and specific genes, phenotypes and diseases), linkage analyses (studies establishing links between genes) and genome-wide association studies (genome-wide assessments of genetic variations in multiple individuals to establish if a variant is associated with a trait) have been employed for the identification of novel susceptibility genes in an effort to further elucidate the genetic processes underlying function; these techniques have been used in obesity research to understand the regulation of body weight, but with limited success (Bouchard and Pérusse, 1996; Rankinen *et al.*, 2006; Goodarzi, 2018). Lack of knowledge regarding the numerous genetic variations that exist among humans prohibited the thorough elucidation of these processes, until the successful sequencing of the entire human genome by the Human Genome Project (HGP) in 2001 (Craig Venter *et al.*, 2001). This significantly expanded our knowledge of variations within the human genome that are responsible for diseases and allowed the expansion of the molecular techniques that are available for the further elucidation of disease mechanisms.

The major limitation of genetics research is that genetic mutations confer susceptibility to certain diseases, but unless the mutation is monogenic, these mutations merely predispose individuals to certain diseases without necessarily determining whether the phenotype will be expressed. Individuals can inherit a mutation but not develop a disease, and whether or not this mutation will be expressed can be determined by an environment. Furthermore, the environment can confer new mutations that predispose individuals to new diseases.

Another limitation which hampers genetic research is the dynamic nature of a cell. Gene sequences do not necessarily determine the day-to-day functioning of a cell and gene products can vary based on a cell's physiological state. According to the central dogma of molecular biology, DNA undergoes transcription into ribonucleic acid (RNA) which, by means of messenger RNA (mRNA), is translated into proteins (Crick, 1958). Due to the dynamic nature of a cell, not all encoded genes are transcribed, and not all transcribed genes are translated into proteins. Furthermore, a single gene can give rise to multiple gene products: RNA processing into mRNA can generate numerous RNA transcripts, mRNAs produced by a cell are dependent on the efficacy and stability of translation, and the numerous protein products that can be generated by a cell are determined by the RNA transcripts that have been produced. Furthermore, protein sequences can be altered by post-translational modifications, proteolysis and protein compartmentalization (Graves and Haystead, 2002). Therefore, it is now recognised that cellular information cannot be obtained or understood from the study of genetics alone. This has caused many to consider the

assessment of the numerous gene products that can inform the functioning of a cell as opposed to only genes themselves.

New molecular research has expanded to include genomics (the study of genes and their functions), transcriptomics (the translation of genetic information into transcripts and thus functional proteins), proteomics (the study of proteins and their functions) and metabolomics (study of the chemical processes concerning metabolites, the substrates, intermediates and products of metabolism) in order to understand the biology of diseases and to elucidate disease mechanisms (European Bioinformatics Institute, no date; Plebani, 2005; Shah, 2011; World Health Organization, 2016b; Lowe *et al.*, 2017; Manzoni *et al.*, 2018). All the “omics” biomolecular techniques have their appropriate applications as well as their unique advantages and disadvantages. Genomic material, unless altered by the environment, is quite static and eludes to the *possible* phenotypes that can occur; transcriptomics can show which genes and transcripts are active and exerting an influence on an organism, but does not elude to the concentrations of proteins produced by the mRNA transcripts; proteomics can provide information regarding the structure, function, abundance, interactions and properties of proteins but, at this stage, only known proteins can be identified and further work is necessary to trace their origins and functions (Plebani, 2005; Gupta and Kumar, 2014).

Even with the challenge that the novelty of the field of proteomics presents regarding the number of proteins (and their variants) that are yet to be discovered, it continues to be an attractive molecular technique as it is able to capture the dynamic state of a cell during different physiological and pathological states, thereby representing the link between genes, proteins and diseases (Petricoin *et al.*, 2002).

### 2.8.1. Proteomics

Proteins are the actual “factories” of a cell. They control cellular functions (in the form of receptors, enzymes, transporters, regulatory proteins and co-factors) and give structure to all cells. The production of proteins is greatly dependent on the physiological state of a cell, and it is therefore proteins that are responsible for cell phenotypes. (Graves and Haystead, 2002; Plebani, 2005; Wright *et al.*, 2012; Silverthorn *et al.*, 2014)

Proteomics can also be described as the characterization of the complete protein complement of an organism, cell or tissue (O’Farrell, 1975; Wasinger *et al.*, 1995; Wilkins *et al.*, 1996). Protein expression and function are altered in different physiological conditions and diseases, and the goal of proteomics is to assesses the signalling, metabolic and regulatory proteins (and thus networks) that become dysfunctional in these conditions in order to understand how and why these changes

occur (Anderson, Matheson and Steiner, 2000). Furthermore, the comparison of protein expression between different samples (e.g. normal vs. diseased tissue) that differ by some variable, known as expression proteomics, enables the identification of disease-specific proteins and markers, allowing for the elucidation of disease mechanisms.

A typical proteomics experiment has 3 phases: i) separation and isolation of proteins; ii) elucidation of protein structural information for the identification and characterization of proteins present; iii) searching of protein sequences in a database to identify proteins and their functions (Graves and Haystead, 2002).

#### 2.8.1.1. Protein separation and isolation

Proteins are complex and made of multiple amino acid sequences that are folded into different structures with differing complexities (Silverthorn *et al.*, 2014). These proteins therefore need to be broken down into their individual components so that they can be visualized and their sequences identified and characterized.

Two predominant methods are utilized for this purpose: gel electrophoresis and/or protein digestion. During the earlier years of proteomics, gel electrophoresis was a gold-standard method for the separation of proteins. In this method, proteins are solubilized using sodium dodecyl sulphate (SDS) and separated by electrophoresis through a polyacrylamide gel according to mass (1-dimensional electrophoresis, 1-DE) or mass and charge (2-dimensional electrophoresis, 2-DE) (Aebersold *et al.*, 1987; Anderson and Anderson, 1996). The more complex protein mixtures, such as protein lysates, are resolved using 2-DE. This method is also able to resolve proteins which have undergone post-translational modification as this modification results in a difference in protein charge and a difference in mass. 1-DE and 2-DE produce gels with spots which denote the presence of different proteins, and it allows the quantitative and qualitative comparison of gels from different samples (e.g. control and diseased); the appearance or disappearance of spots denotes the presence or absence of a protein compared to the other sample, while the intensity of the spot denotes the quantity of the protein present (its expression) (Plebani, 2005). Gel electrophoresis has advantages and disadvantages: it is reproducible and can resolve a wide range of protein masses, but it is time consuming. Additionally, large hydrophobic proteins do not enter the gel and extremely acidic or basic proteins are not well resolved. It also does not allow for proteins found in small concentrations to be analysed, even though the change in expression between samples could be significantly different (Celis and Gromov, 1999; Görg *et al.*, 2000).

Protein digestion is the conversion of proteins into peptides by means proteolytic enzymes, usually with the use of the enzyme trypsin (Switzar, Giera and Niessen, 2013). Proteins can then be

purified via several different methods such as liquid chromatography (LC) before being analysed (Opiteck *et al.*, 1997; Link *et al.*, 1999). This method bypasses gel electrophoresis and significantly more proteins can be represented in the peptide mixture.

#### 2.8.1.2. Elucidation of protein structure

Proteins that have been separated by gel electrophoresis can either be digested directly from the gel (in-gel digestion) via a protease, or be transferred electrophoretically (electroblotted) onto an inert membrane such as nitrocellulose in order for proteins to be visualized on the membrane surface. In the latter, the proteins are excised from the membrane and fragmented via cleavage of proteins at methionine or tryptophan residues into large peptide fragments. If in-gel digestion has been used, the proteins are digested into peptides directly from the gel and not placed on a membrane (Snevechenko *et al.*, 1996). The peptides then need to be purified to remove contaminants that are generated during an in-gel digest including detergents, salts and buffers which could interfere with the sequencing instrument (Yates, 1998); this can be done using methods such as high-pressure liquid chromatography (HPLC). The protein fragments are then placed into a sequencing instrument in order for amino acid sequences to be elucidated. This was previously performed on an Edman sequencer but more regularly a mass spectrophotometer (MS) (Aebersold *et al.*, 1987; Celis and Gromov, 1999; Graves and Haystead, 2002).

MS is an analytical method which allows structural information about proteins and peptides to be obtained, such as peptide masses and amino acid sequences. This information can then be used in protein identification and to determine the presence and location of protein modifications (LibreTexts Chemistry, no date; Graves and Haystead, 2002).

Sequencing via MS requires peptide molecules to be charged and dried, which occurs via ionization. The predominant forms of ionization includes electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). In ESI, peptides are allowed to flow into the microcapillary tubing of the MS instrument to make their way to the opening (inlet) of the MS, a process which creates a potential difference between the capillary tubing and the inlet, resulting in the peptides being dispersed into a fine mist of charged droplets as they enter the MS. The electrospray can additionally be connected to an LC system, which purifies the droplets as they enter into the MS. In MALDI, proteins are ionized by being incorporated into organic molecules which absorb UV radiation (called matrix molecules) and then irradiating the proteins and peptides with a laser (Karas and Hillenkamp, 1988; Yates, 1998).

The MS has a built-in mass analyser which is a component that takes ionized proteins and peptides and separates them based on their charge-to-mass (m/z) ratios and outputs them to a

detector. The detector then senses the proteins and creates a digital output (Yates, 1998). The masses of the individual peptides are measured and a mass spectrum is created. There are several mass analysers available, each with different applications:

Quadrupole mass analysers transmit ions through an electrical field created by four parallel metal rods, known as the quadrupole. It either filters ions according to their m/z ratio before they are transmitted or it transmits all ions through the electrical field. If multiple quadrupoles are combined, as seen in triple quadrupoles, they can effectively obtain information regarding the amino acid sequence of a peptide (Yates, 1998; Graves and Haystead, 2002).

Time-of-flight (TOF) mass analysers make use of a tube which is a vacuum containing two parallel electrodes and a detector at the end of the tube. Ionized proteins and peptides (ions) are energized by the electrodes (which create an electrostatic field at the beginning of the mass analyser) and this energy creates a beam of high energy ions that “shoots” the ions towards the detector. The beam of ions moves through a 1-2 meter portion of the tube with no electrical or magnetic field (field-free region) before striking the detector. The ions essentially “fly” through this field-free region due to their newly acquired energy – the field-free region is therefore named a “flight tube”. TOF measures the protein and peptide m/z ratios by measuring the time that it takes for ions to move through the length of the flight tube. The heavier ions will take longer to reach the sensor. (Karas and Hillenkamp, 1988; Guilhaus, Mlynski and Selby, 1997; Guilhaus, Selby and Mlynski, 2000)

Ion trap analysers make use of electric or magnetic fields to trap ions within the mass analyser (Clarke, 2016). This is similar to the quadrupole, but the parallel rods are replaced by two electrodes which face each other as well as a ring electrode halfway between the two electrodes. Ions are thus trapped in a circular flight path due to the electric field, but linear electric fields can also be generated by changing the configuration of the electrodes. An example of this type of analyser is the Orbitrap – this stores ions in a stable flight path (an orbit), and the frequency of an ion’s orbit around the electrode is related to its m/z ratio.

#### 2.8.1.3. Database search

Once the structural information of a protein has been acquired through MS, this information can be used for protein identification. Online databases can be searched using MS data to accurately identify large numbers of proteins (Quadroni and James, 1999). The most specific online databases use amino acid sequences of peptides to identify the proteins from which the peptides were derived. Numerous databases are available, and these allow the identification of proteins from raw MS data, examples of which include SEQUEST, Mascot and SONAR (Eng, McCormack and Yates, 1994; Perkins *et al.*, 1999; Fenyö, 2000).

#### 2.8.1.4. Choice of proteomics method for this study

The most commonly used MS to obtain amino acid sequences is the triple quadrupole (Graves and Haystead, 2002). This version of the quadrupole uses two stages of the analysis: i) Once all the proteins and peptides have been ionized, the instrument scans all ions above a certain m/z ratio and transmits these ions to its third quadrupole; ii) The instrument undergoes a second MS scan (therefore called MS/MS mode or Tandem mass spectrophotometry) where the selected ions are channelled into a collision chamber and strike one another, breaking into fragments. This fragmentation allows peptide bonds to be broken. The fragments are then determined based on their m/z ratio by the third quadrupole, and the amino acid sequence of each of the fragments can be determined (as each amino acid has a unique mass).

## 2.9. Purpose of research

The last decade has provided numerous studies investigating the link between obesity and male fertility. Numerous mechanisms have been elucidated and information has been provided about the various pathways which become dysfunctional.

Even with all of this knowledge, there is a great deal that is unknown about both obesity and infertility, and how these pathologies interchangeably affect each other. Moreover, non-consensus on some of the disease causing mechanisms and resulting pathological changes leaves a lot unanswered regarding the true consequences of both of these disorders. Could it be that the individuals that show pathological changes are merely the ones that have succumb to the onslaught of damage that ensues when an individual becomes obese? To what extent is fertility affected and could there be more molecular changes occurring that are undetectable at the gross level of functioning?

New molecular techniques such as genomics, transcriptomics, proteomics and metabolomics that have been made possible by the decoding of the human genome through the Human Genome Project have provided us with the ability to research biomolecular changes that could be occurring within a diseased individual. Moreover, they have been shown to provide insights into disease-causing mechanisms. Molecular techniques may provide a great avenue to discover the more intrinsic changes that obesity may give rise to and, furthermore, aid in the discovery of mechanisms through which obesity can act to bring about the observed changes in infertile men.

## 2.10. Aims and objectives

The overall aims of this study are:

- To examine the effects of obesity on male fertility by observing specific macroscopic, microscopic and molecular changes to structure and function in a diet-induced obese animal model
- To examine changes in protein expression within the reproductive tissues of the obese subjects, quantify these changes, and identify the affected molecular pathways

The aims are thus threefold:

**Aim 1**: To establish an animal model of obesity by means of an obesogenic diet

**Objectives**

- Measure anthropometric and macroscopic measurements including body weight, visceral fat weight and adiposity to ascertain the establishment of the obesity model
- Monitor other macroscopic changes including testicular weight and haematocrit

**Aim 2**: To assess microscopic and molecular changes related to obesity and potential male fertility

**Objectives**

- Measure sperm parameters (viability and morphology), histological sections (testis and epididymis), hormone levels (testosterone and estradiol), unfasted glucose and oxidative stress parameters
- Corroborate experimental data with data found in literature

**Aim 3**: Perform proteomic analysis on reproductive tissues (testis, epididymis, spermatozoa) in order to identify differences in protein expression between obese and control animals

**Objectives**

- Determine proteins over- and under-expressed in obesity
- Relate changes in protein expression to the observed changes in anthropometric, macroscopic, microscopic and molecular parameters
- Perform enrichment analysis to determine molecular pathways that are up- and down-regulated in obesity
- Perform functional network analysis and identify affected protein networks. Visualise the protein networks affected by changes in protein expression
- Correlate changes in protein expression to the macroscopic, microscopic and molecular parameters that will be measured in the control and obese subjects.

**Hypothesis**: We hypothesise that the chronic diet-induced obesity model will have a negative effect on sperm parameters, and on reproductive proteins that are responsible for normal reproductive function(s).

# Chapter 3: Materials and Methods

This chapter will provide a detailed account of the study procedures, the animal model and the materials and methods utilized during the course of the study.

## 3.1. Ethical Clearance

Project approval was obtained from the Research Ethics Committee: Animal Care and Use (REC: ACU) of the Faculty of Medicine and Health Sciences, Stellenbosch University after the study was deemed to comply with the guidelines of the revised South African National Standard for the Care and Use of Animals for Scientific Purposes.

Ethical approval was granted for the parent study titled, “High fat diet and obesity: effects of melatonin”. An amendment to the initial protocol to include the harvesting of reproductive organs and plasma for the purposes of this study from rat carcasses was approved.

Ethics Reference Number: SU-ACUM13-00035.

## 3.2. Study design

The figure below (Figure 3.1) provides an overview of the experimental groups used in the study, as well as the analyses performed under each type of interrogation that was carried out.

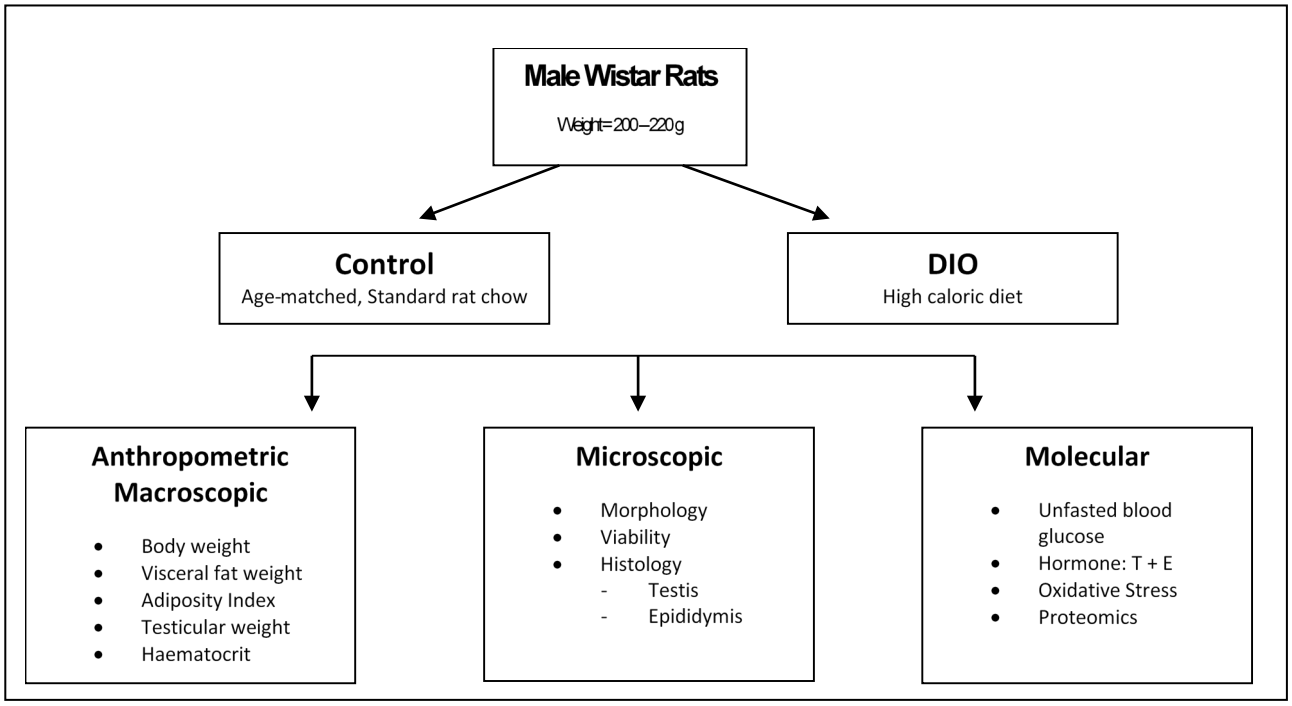



**Figure 3.1** Diagram outlining the experimental design followed, the individual study groups as well as the analyses carried out during the course of the study (T = testosterone, E = estradiol). The study used forty male Wistar rats on a 54 week chronic obesogenic diet to assess effects of obesity on male fertility.

Forty male Wistar rats were utilized for this study (breed originating from Charles River, United Kingdom, Ltd.). The animals were bred and maintained at the Stellenbosch University Animal Facility under conventional environments (22°C, ±40% relative humidity, 12 hour day/night cycle) with *ad libitum* access to food and water until ± 6 weeks of age or until adequate weights (200-220 g) were obtained. Animals were then randomly and equally divided into control (age-matched) and diet-induced obesity (DIO) groups where they received a high caloric diet or standard rat chow for 54 weeks, respectively.

The diet used for this model was based on a hyperphagia inducing diet adapted from Pickavance and coworkers (Pickavance et al., 1999), successfully implemented by previous studies (Huisamen et al., 2012; Salie et al., 2014), where standard commercially available rat chow was supplemented with both sucrose and Holsum cooking fat (68% saturated fat, 26% mono-unsaturated fat and 5% poly-unsaturated fat) (Sime Darby Plantation, Liberia). Diet compositions are summarized in Table 3.1 below:

**Table 3.1 Macronutrient composition of diets used in the rat feeding protocol. Analysis of the diets was performed by Microchem (Cape Town, South Africa). (DIO, Diet-induced obesity)**

| | **Control** | **DIO** |
|---|---|---|
| Carbohydrate (%) | 34.6 | 42 |
| Protein (%) | 17.1 | 8.3 |
| Total fat (g/100g) | 4.8 | 11.5 |
| Saturated fat (g/100g) | 0.9 | 7.6 |
| Cholesterol (mg/100g) | 2.2 | 13 |
| Sucrose (g/100g) | 5.3 | 25.4 |

At 60 weeks of age, rats were sacrificed by euthanasia using an overdose of sodium pentobarbital (intraperitoneal 100mg/kg). Each animal had their body weight measured and immediately examined post-mortem to determine visceral fat weight and non-fasting blood glucose. Blood was collected from the thoracic cavity and used for plasma extraction and haematocrit analysis. Testes and epididymides were excised, weighed and preserved appropriately for subsequent sperm

parameter evaluations, histological analysis, protein determination, antioxidant evaluation (catalase, superoxide dismutase, glutathione, lipid peroxidation), and proteomic analysis.

## 3.3. Anthropometric and macroscopic parameters

### 3.3.1. Body weights and body fat

The body weights of fully sedated male Wistar rats were recorded using a Mettler (Mettler K5, E.Mettler, Zurich) scale. At post-mortem, an incision was made across the abdomen to allow access to abdominal fat depots, where retroperitoneal, epididymal and omental fat were removed; the combined weight of the three fat deposits were recorded and expressed as the visceral fat weight. The Adiposity Index (AI) was determined by substituting the body weight and weights of each fat depot into the following equation:

$$AI = \frac{Total\ Body\ Fat}{Final\ Body\ Weight} x\ 100$$

where Total Body Fat = Epididymal fat + Retroperitoneal fat + Omental fat.

The AI was used as a measure of obesity. This index is a measure of fat storage, demonstrating the degree of adipose tissue accumulation in a study animal (Levin et al., 2003; Sinitskaya et al., 2007; Leopoldo et al., 2016). Animals with an AI of 10-25% above the mean body weight of the age-matched controls were considered to be moderately obese, while those with ≥40% increase were considered to be morbidly obese (Levin *et al.*, 2003; Woods *et al.*, 2003; Leopoldo *et al.*, 2016).

Immediately after collection, the testes were dissected free from associated tissue and weighed using a Mettler Toledo Ab204-5 digital scale (Mettler-Toledo, Columbus, Ohio, USA).

### 3.3.2. Haematocrit

Whole blood from the chest cavity was collected into two non-heparinized capillary tubes for haematocrit analysis. The tubes were then sealed using white sealing clay and centrifuged in a micro-haematocrit centrifuge (Hawksley, Lancing, UK) at 2500xg for 5 minutes to allow maximum packing of red cells. Haematocrit was subsequently determined with a haematocrit tube reader (Hawksley, Lancing, UK) and reported as a percentage.

## 3.4. Microscopic parameters

### 3.4.1. Semen analyses

Rat epididymides harvested from each rat were placed in a petri dish containing a 5ml solution of Hams F-12 nutrient media (Sigma Chemicals, St Louis, MO, USA) supplemented with 3% Bovine Serum Albumin (BSA) (Roche Diagnostics GmbH Mannheim, Germany) at 37°C. The caudal portion of each epididymis was resected by radial section using fine pointed scissors and placed in 2ml of 3% Hams-BSA solution for 5 minutes, with occasional agitation to facilitate the release of sperm into the media. Morphology and viability analyses were performed on sperm suspensions from both groups to determine sperm quality.

#### 3.4.1.1. Morphology and morphometry

For morphology analysis, 10µl of sperm was pipetted and smeared onto a glass slide. After air drying at room temperature, slides were immersed into a staining tray containing SpermBlue® (Microptic SL, Barcelona, Spain) fixative for 20 minutes, and stained thereafter using SpermBlue® stain for 15 minutes in the same manner. Stained slides were then gently immersed in distilled water for 3 seconds to remove excess stain, allowed to air dry, and mounted using DPX mountant (Merk Millipore, Modderfontein, Gauteng) and a coverslip. Sperm morphometry was performed by means of computer-aided sperm analysis (CASA) using the Sperm Class Analyser version 6 Rat Tox (SCA®; Microptic, Barcelona, Spain) software and a Nikon E50i, with bright field optics at 600 x magnification using a blue filter. Analyser settings were as follows: optics Ph+; contrast 166; brightness 465; scale 600x; capture 100 images per slide. The SCA software automatically analyses spermatozoa to detect acrosome, head and mid-piece defects, and accurately measures these and other variables to determine whether sperm are normal or abnormal.

#### 3.4.1.2. Viability

Viability determines the integrity of sperm plasma membranes by means of a dye exclusion technique. Sperm (10µl) was mixed with eosin (Sigma-Aldrich, South Africa) and nigrosin (Sigma-Aldrich, South Africa) dyes (1:1:1 ratio) in a 2ml Eppendorf; 10µl was pipetted out onto a glass slide and a thin smear was made. Smears were allowed to air dry then mounted using DPX mounting medium and a coverslip. 200 cells were counted using a Nikon Eclipse E200 light microscope (Tokyo, Japan) at 40x magnification. Live spermatozoa remained unstained as their membranes were impermeable to eosin while the dead sperm stained red, indicating permeability and a damaged membrane. The number of viable sperm was expressed as a percentage of the total number of sperm counted.

### 3.4.2. Histology

#### Tissue collection and fixation

Immediately after collection, testes and epipidymides were freed from associated tissue and placed in specimen containers containing 10% paraformaldehyde (formalin) solution, for a minimum of 48 hours to allow fixation. This fixation step stops tissue degradation and cytosis and ensures that structural integrity is maintained. Tissues were processed for embedding in paraffin wax. This was done in a 3-step process which includes dehydration in alcohol, clearing in xylene and infiltration with paraffin wax, all done automatically using a Duplex processer (Shandon Elliott). Tissues were placed in metal embedding moulds and filled with wax; cassettes were fixed to the moulds and placed onto an iced surface to set, creating tissue blocks. Tissue blocks were trimmed and cut into uniform 5µm thick sections using a microtome (Ultra- cut UCT, Leica, Vienna, Austria). Sections were then placed into a 40ºC water bath before being mounted onto glass slides and stained using haematoxylin and eosin (H&E) stains. Slides were then mounted using DPX mounting medium and glass coverslips to complete the histological preparation process.

#### Analysis

Microscopic evaluation of the morphological features of testicular and epididymal tissues were performed using a Leica photomicroscope (Leica Microsystems, Wetzlar, Germany), equipped with an Olympus C-5060 digital camera (Olympus, Tokyo, Japan) to capture photomicrographs at a constant magnification (x 10). Micromorphometric analyses of the tissue samples were performed using Quick Photo Micro Image software, version 4 (Olympus, Tokyo, Japan). Qualitative analysis of the microscopic structure of the testes and epididymis were assessed at first. Relative volumes (%) of the epithelium, lumen and interstitium were then subsequently evaluated, followed by the evaluation of metric parameters (µm) - the diameter of the seminiferous and epididymal tubules, epithelial height and lumen diameter.

## 3.5. Molecular analyses

### 3.5.1. Blood glucose

Non-fasting blood glucose was measured via collection of blood from a tail prick. A drop of blood was then placed onto the absorbent film of a slip that was immediately inserted into a glucometer (Gluco Plus™, Cipla MedPro, South Africa).

### 3.5.2. Hormone analyses

Hormone analyses were performed using plasma. Blood samples collected from the abdominal cavity were placed into EDTA blood tubes using a Pasteur pipette and placed on ice for 30 minutes before being centrifuged at 1000 x g (10min, 4ºC) to yield plasma.

#### 3.5.2.1. Testosterone

The plasma levels of this hormone were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA; Demiditec Diagnostics GmbH, Kiel, Germany) which makes use of competitive binding principles to determine unknown testosterone concentrations. Microplate wells were pre-coated with testosterone antiserum directed towards antigen sites of testosterone molecules. The testosterone present within experimental samples competes with a defined amount of testosterone-horseradish peroxidase conjugates to bind to antiserum binding sites. After incubation, the unbound conjugates are removed through multiple washing steps. The amount of peroxidase conjugates bound inversely corresponds to the testosterone concentration in each sample. After a substrate solution is added, the intensity of the colour reaction is inversely correlated to the optical density (OD) measured.

10 µl of each standard (0, 0.1, 0.4, 1.5, 6.0, 25.0 ng/ml), control and samples were pipetted into microplate wells in triplicate. 100 µl of incubation buffer was added into each well, followed by 50 µl of enzyme conjugate. These solutions were incubated at room temperature for 1 hour on a microplate mixer/shaker. The contents of the wells were then expelled and microplates thoroughly rinsed with a 4 step wash using 300 µl of diluted wash solution. Wash solution was then completely removed by vigorous beating of the microplate on absorbent paper to eliminate any residual droplets. 200 µl of substrate solution was then dispensed into each well and incubated in the dark for 30 minutes. The enzymatic reaction was then halted with the addition of 50 µl of stop solution. Absorbance was measured at 450nm within 15 minutes of the addition of stop solution.

A standard curve was created by plotting protein standards on the x-axis vs 450nm absorbance readings of the standards on the y-axis in Excel. A calibration curve was used to calculate sample protein concentrations (ng/ml) using the mean absorbance readings of each sample.

#### 3.5.2.2. Estradiol

The plasma levels of this hormone were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA; DRG Diagnostics GmbH, Marburg, Germany) which makes use of competitive binding principles to determine unknown estradiol concentrations. Microplate wells were pre-coated with estradiol antiserum directed towards antigen sites of estradiol molecules. The

estradiol present within experimental samples competes with a defined amount of estradiol-horseradish peroxidase conjugates to bind to antiserum binding sites. After incubation, the unbound conjugates are removed through multiple washing steps. The amount of peroxidase conjugates bound inversely corresponds to the estradiol concentration in each sample. After a substrate solution is added, the intensity of the colour reaction is inversely correlated to the optical OD measured.

Each standard (0, 25, 100, 250, 500, 1000, 2000 pg/ml), control and samples (25 µl) were pipetted into microplate wells in triplicate. Enzyme conjugate (200 µl) was inserted into each well using a multi-channel pipette and mixed rigorously for 10 seconds. These solutions were then incubated uncovered at room temperature for 2 hours. The contents of the wells were then discarded and microplates thoroughly rinsed with a 3 step wash using 400 µl of diluted wash solution. Wash solution was then completely removed by vigorous beating of the microplate on absorbent paper to eliminate any residual droplets. Substrate solution (100 µl) was then dispensed into each well and the microplate incubated at room temperature for 15 minutes. The enzymatic reaction was then halted with the addition of 50 µl of stop solution. Absorbance was measured at 450±10nm within 10 minutes of the addition of stop solution.

A standard curve was created by plotting protein standards on the x-axis vs 450nm absorbance readings of the standards on the y-axis in Excel. Sample protein concentrations (pg/ml) were read off the standard curve using the mean absorbance readings of each sample.

### 3.5.3. Protein determination

The amount of protein present in each tissue sample had to be quantified prior to antioxidant and proteomic analysis. This was in order to determine whether adequate protein concentrations were present in each sample (for proteomics) and as a variable used to calculate antioxidant concentrations (per milligram of protein for catalase and superoxide dismutase assays). Testis and epididymis tissue as well as sperm pellets (200mg) were thawed on ice and placed in Sorvall tubes after which 2mL of lysis buffer (Table 3.2) was added. The tissue was then homogenized on ice using a polytron. Homogenates were transferred to appropriately marked Eppendorf tubes and centrifuged at 10000 x g (15 minutes, 4ºC). Supernatants were pipetted out without agitating the pellet, transferred to new marked Eppendorf tubes and kept on ice. Two different protein determination procedures were used to determine protein concentrations of the collected tissues; bicinchoninic acid (BCA) and Bradford protein determination assays. This is due to the constituents of the respective lysis buffers used for the antioxidant and proteomic assays. The presence of SDS interrupts the bonding of the coomasie blue dye used in Bradford assays, providing an incorrect quantitation of protein. If SDS was present, BCA protein determination was used.

#### 3.5.3.1. BCA

The BCA protein determination assay is formulated based on bicinchoninic acid (BCA), utilizing colorimetry to detect and quantify total protein. It uses the reduction reaction of $Cu^{2+}$ to $Cu^{+}$ caused by proteins in alkaline media, a chemical reaction called the biuret reaction. A certain reagent containing BCA is used to sensitively and selectively detect the colorimetric change that occurs when the cuprous anion (Cu+) is emitted by proteins. A purple colour change occurs when two BCA molecules chelate with one cuprous anion, allowing a powerful absorbance at 562nm which increases with increasing protein concentrations in an almost linear fashion over a 20–2,000 µg/ml range.

Each standard and sample (25µL) were pipetted into microplate wells in triplicate. 200 µL of Working Reagent was then added to each well and placed on a plate shaker for 30 minutes to ensure thorough mixing. The plate was then covered to prevent dehydration and incubated for 30 minutes in a $CO_2$ incubator (Heal Force, Shanghai, China). The microplate was then cooled to room temperature before absorbance readings were taken. Absorbances were measured at ~562nm using a plate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany). A standard curve was the plotted using the known concentrations vs 562nm absorbance measurements of the standards. This was then used to determine the concentration of each sample.

#### 3.5.3.2. Bradford

The Bradford protein assay uses spectrophotometric analysis to measure the protein content of a solution. It includes several steps, which are outlined below:

##### 3.5.3.2.1. Setup

Bradford reagent was formulated by combining 20ml of Bradford stock solution (0.6mM (w/v) Coomassie Blue G-250, 95% (v/v) ethanol and 85% (v/v) phosphoric acid) with 80ml of dH2O in a 1:5 ratio, which was then filtered twice using two filter papers. Bradford standards of known concentration were prepared using serial dilutions of bovine serum albumin (BSA). These were used to create a standard curve by plotting 595nm absorbance readings of the standards vs their known protein concentrations (1-20µg range). Unknown sample protein concentrations were then determined using the resultant linear equation of this curve. Tubes were then set out on a rack in the following formation before running the assay:

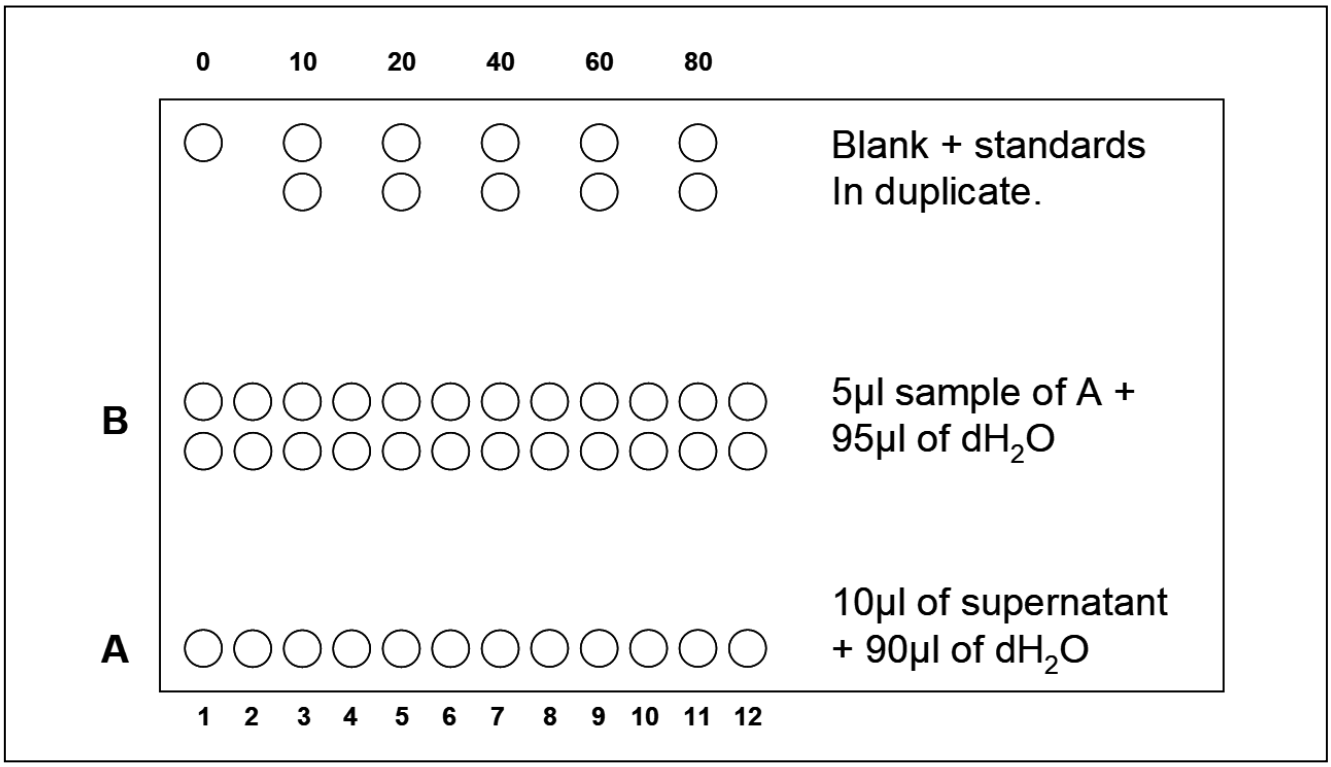



**Figure 3.2:** Diagram showing a setup example for a Bradford assay of 12 samples. Row A is the first dilution and contains a single file of tubes labelled 1-12, one for each sample. Row B is the second dilution step and contains tubes labelled 1-12 in duplicate. The final row contains tubes into which the specified serial dilutions of BSA will be prepared in duplicate.

#### 3.5.3.2.2. Sample dilution

Two dilution steps were used to prepare samples for analysis. An initial 1:10 dilution (10µl sample supernatant + 90µl $dH_2O$) was performed on the experimental samples (row A), after which a 1:20 dilution was performed where 5µl was taken from samples in row A, placed into corresponding tubes (row B) and diluted with 95µl $dH_2O$. This was done to ensure protein concentrations of the experimental samples do not exceed that of the highest standard (20µg), allowing the unknown protein concentrations of the samples to fit onto the standard curve.

**Table 3.2:** Composition of lysis buffer used to create testis, epididymis and sperm homogenates for antioxidant analyses

| Reagent | Stock solution | Concentration of working solution | Amount used for 30mL lysis buffer |
|---|---|---|---|
| **Tris-HCl EGTA (pH 7.5)** | 200mM | 20 mM | 3 mL |
| **EDTA** | 100mM | 1 mM | 300 µL |
| **NaCl** | 1M | 150 mM | 4.5 mL |
| **β-glycerolphosphate** | | 1 mM | 0.006 g |
| **Tetra-Na-Pyrophosphate** | | 2.5 mM | 0.03 g |
| **Sodium orthovanidate ($Na_3VO_4$)** | 0.018 mg/10mL | 1 mM | 3 mL |
| **Leupeptin and Aprotinin** | 10 µg/mL | 10 µg/mL | 30 µL |
| **Triton V-100** | 10% | 1 % | 3 mL |
| **Phenylmethylsulfonyl fluoride (PMSF)** | 50 µg/mL | 10 µg/mL | 90 µL |
| **$dH_2O$** | | | Fill up to 30 mL |

#### 3.5.3.2.3. Assay

The Bradford protein assay uses spectrophotometric analysis to measure the protein content of a solution. It includes several steps, which are outlined below:

Filtered Bradford reagent (900µl) was then added to all standards and samples in row B and vortexed. Solutions were allowed to stand for 15 minutes before measurement with a spectrophotometer at 595nm. Spectrophotometric readings were performed on the standards starting with the blank to set the “zero”, then read from 10 to 80; samples were read from 1 to 12.

The standard curve was plotted using the known protein concentrations/standards (x-axis) vs 595nm absorbance (OD) readings of the standards (y-axis) using Chart Wizard in Excel. A trendline is then added to the curve, and the resultant straight line equation used to calculate sample protein concentrations (µg protein per ml).

### 3.5.4. Oxidative stress parameters

Mammals have an innate enzymatic antioxidant system which regulates the amount of ROS within their bodies, protecting against oxidative stress. The reproductive system, sperm in particular, possesses this system, with the quantity of each antioxidant enzyme varying between species. Enzyme activity was quantified using enzymatic antioxidant assays, measuring enzyme activities between the control and experimental group

#### 3.5.4.1. Catalase

Catalase is an abundant antioxidant enzyme found in nearly all aerobic organisms. It is responsible for catalysing the breakdown of a powerful ROS molecule, hydrogen peroxide ($H_2O_2$), into oxygen ($O_2$) and water ($H_2O$). $H_2O_2$ is a by-product of normal aerobic metabolism and found to be toxic when present in large quantities, i.e. in conditions causing pathogenic ROS production. Catalase is responsible for the degradation of the majority of $H_2O_2$, thereby detoxifying a system.

#### 3.5.4.1.1. Tissue preparation

Tissue samples were thawed at room temperature then placed on ice to prevent enzymatic reactions from taking place. Each tissue (±200mg) was resected using fine pointed scissors, placed in 2ml cold phosphate buffer, and homogenized on ice using a polytron. Homogenates were then transferred to appropriately marked Eppendorf tubes and centrifuged at 10000 x g (15 minutes, 4ºC). Supernatants were pipetted out without agitating the pellet, transferred to new marked Eppendorf tubes and kept on ice.

BCA protein determination was performed on all tissue homogenates (testis and epididymis).

#### 3.5.4.1.2. Assay

Each standard and sample (20µL) were pipetted into UV microplate wells in triplicate. Catalase assay buffer (150µL) was dispensed into each well using a multi-channel pipette. Catalase's enzyme reaction was activated with the addition of 75 µL of H2O2 and immediately analysed on a plate reader (Multiskan spectrum) at 240nm using SkanIt RE for MSS 2.2 (ThermoScientific™ Inc.) software. The software recorded the linear absorbance decrease of catalase for 1 minute at 15 second intervals.

The average absorbance (ΔA/T) was calculated for each standard and sample. The absorbances of the standards were then corrected by subtracting the average absorbance of standard A, then plotted as a function of the catalase activity (U/ml). Catalase activity (CAT) of all samples was then calculated by substituting corrected absorbance values of all samples into the following equation, acquired from the linear regression of the plotted standard curve:

CAT (U) = [(sample absorbance – y-intercept)/slope x 0.25mL/0.005mL].

Catalase activity was expressed as U/mg protein concentration.

### 3.5.4.2. Superoxide dismutase

Superoxide dismutase (SOD) is an essential, widely distributed antioxidant enzyme within aerobic cells. SOD is responsible for dismutation of the superoxide anion ($O_2^-$), an abundant reactive oxygen specie (especially within the mammalian reproductive system), into $H_2O_2$ and $O_2$. Superoxide dismutase is increased in conditions causing pathogenic ROS production to prevent tissue damage by $O_2^-$ and its metabolites.

SOD was measured using a microplate SOD enzyme assay dependent on the spectrophotometric assessment of $O_2^-$. The assay uses two chemicals, 6 hydroxydopamine (6HD) and diethylenetriaminepentaacetic acid (DETAPAC), to generate $O_2^-$ anions, which are reduced in the presence of SOD. The reaction yields a colorimetric signal where samples with reduced amounts of SOD emit a lesser signal. SOD activity was calculated using a resultant linear regression curve, where activity was expressed as U/mg protein concentration.

#### 3.5.4.2.1. Tissue preparation

Tissues were prepared in the same procedure outlined in the Catalase tissue preparation protocol, as described previously.

Tissue homogenates contained both cytosolic and mitochondrial SOD. Centrifuging the supernatant at 1000 x g (15 minutes, 4ºC) separated the two enzymes, keeping cytosolic SOD in the supernatant and containing the mitochondrial SOD within the pellet. The supernatant was used to measure cytosolic SOD activity.

BCA protein determination was performed on all tissue homogenates (testis and epididymis).

#### 3.5.4.2.2. Assay

The SOD enzyme assay measures the auto-oxidation of 6-HD. One unit of SOD is defined as the amount of SOD required to exhibit 50% dismutation of the $O_2^-$ radical.

12µL of each standard and sample were pipetted into microplate wells in triplicate. 170 µL assay buffer was dispensed into each well using a multi-channel pipette. SOD's enzyme reaction was activated with the addition of 15 µL of 6-HD and immediately analysed on a plate reader (Multiskan spectrum) at 490nm using SkanIt RE for MSS 2.2 (ThermoScientific™ Inc.) software. The software recorded linear absorbance decreases of catalase for 4 minutes at 1 minute intervals.

The average absorbance (ΔA/T) was calculated for each standard and sample. Standard A's absorbance was divided by itself, and the absorbance of the remaining standards and samples divided by the average absorbance of standard A to produce a linearized rate (LR), then plotted as a function of the final SOD activity (U/ml). SOD activity of all samples was then calculated by substituting linearized absorbance values of all samples into the following equation, acquired from the linear regression of the plotted standard curve:

SOD (U/mL) = [(sample LR – y-intercept)/slope] x sample dilution

SOD activity was expressed as U/mg protein concentration

### 3.5.4.3. Glutathione

Widely distributed in both animals and plants, Glutathione (GSH) is a co-factor essential in the reduction of lipid hydroperoxides into their corresponding alcohols and $H_2O_2$ to $H_2O$, through electron donation to glutathione peroxidases (GPx). GSH bears the brunt of ROS attack by buffering high levels of $H_2O_2$ through its oxidation into a disulphide dimer, oxidised glutathione (GSSG).

This assay quantifies GSH via an enzymatic recycling method which uses glutathione reductase (GR) to recycle GSSG into GSH. Cells under oxidative stress accumulate high levels of GSSG,

reducing the GSH:GSSG ratio. Thus, the GSSG:GSH ratio and the enumeration of GSH are good indicators of the oxidative status of cells and tissues.

GSH's sulphhydryl group reacts with a colorimetric detector, 5,5,'-dithiobis-2-nitrobenzoic acid (DTNB), converting it to 5-thio-2-nitrobenzoic acid (TNB) to produce a yellow colour. The resultant mixture of TNB with GSH (GSTNB) is reduced by GR, catalysing the production of more TNB and the recycling of GSH. The rate at which TNB is produced directly correlates to this enzyme recycle reaction, which in turn, is directly proportional to the amount of GSH within a sample. The absorbance of TNB is measured at 405 or 414nm by a fluorometer, providing an accurate approximation of the GSH concentration. The use of GR allows both GSH and GSSG to be quantified, reflecting total glutathione.

#### 3.5.4.3.1. Tissue preparation

Tissues were prepared in the same procedure outlined in the Catalase tissue preparation protocol, as shown above.

#### 3.5.4.3.2. Assay

Each standard and sample (50µL) were pipetted into microplate wells in triplicate. Assay buffer (50µL) was dispensed into each well using a multi-channel pipette. DTNB (50µL) was then added to every well followed by 50µL GR. The solution was mixed and incubated for 5 minutes at room temperature. The reaction was activated with the addition of 50µL NADPH and immediately analysed on a plate reader (Multiskan spectrum) at 412nm using SkanIt RE for MSS 2.2 (ThermoScientific™ Inc.) software. The software recorded the linear absorbance for 5 minutes at 1-minute intervals.

The absorbance values for all standards and samples were plotted as a function of time, creating an *i*-slope. Plotting the *i*-slope of each standard as a function of the concentration of total GSH then created an *f*-slope.

Total GSH activity of all samples was then calculated by substituting the absorbance values of all standards and samples, calculated from the i- and f-slopes, into the following equation:

Total GSH or GSSG concentrations (µM GSH) = {(*i*-slope for sample) – (y-intercept)}/*f*-slope x dilution factor x sample dilution

### 3.5.4.4. Thiobarbituric Acid Reactive Substances (TBARS)

Oxidative stress results in cellular injury through several mechanisms, lipid peroxidation being a prominent one. Malondialdehyde (MDA) is a typical product of lipid peroxidation. Polyunsaturated fats yield lipid peroxides, which, due to their instability, disintegrate into several compounds including reactive carbonyl compounds, i.e. MDA.

Biological specimens under oxidative stress contain Thiobarbituric Acid Reactive Substances (TBARS) such as aldehydes and hydroperoxides, which increase with increasing oxidative stress. MDA forms adducts with TBARS, thiobarbituric acid in particular, with which it forms a 1:2 adduct. This MDA-TBA complex, forming under acidic, high temperature (90-100ºC) conditions, elicits a colorimetric reaction measured with a fluorometer at 530-540nm. This assay is therefore a direct measure of the amount of MDA present in a sample, indicating the amount of lipid peroxidation taking place.

#### 3.5.4.4.1. Tissue preparation

Tissues were prepared in the same procedure outlined in the Catalase tissue preparation protocol, as shown above, using 10% Trichloroacetic acid (TCA) as the homogenizing media.

#### 3.5.4.4.2. Assay

The appropriate number of Eppendorf tubes for all standards and samples were labelled. 100µL of each standard and sample were pipetted into their corresponding Eppendorf tubes. 2.5ml TBA/buffer solution was forcefully added down the side of each tube. Tubes were then sealed and placed in a 100ºC waterbath for 1 hour. The tubes were then cooled in an ice bath for 10 minutes and then allowed to reach room temperature on the bench. The samples and standards were then centrifuged at 3000rpm for 15 minutes, and their supernatants used for analysis.

Each standard and sample (300µL) were pipetted into microplate wells in triplicate and analysed on a plate reader (Multiskan spectrum) at with excitation at 530nm and emission at 550nm using SkanIt RE for MSS 2.2 (ThermoScientific™ Inc.) software. The software recorded the absorbance of TBA.

The average absorbance (ΔA/T) was calculated for each standard and sample. TBA activity was then calculated by plotting the corresponding mean MDA value for each standard on an x-axis versus their average absorbance on a y-axis to construct a standard curve. A straight line equation was obtained for the standard curve, and MDA concentration calculated by substituting mean MDA value for each sample into the equation.

TBARS are expressed in terms of MDA (nmol/ml).

### 3.5.5. Proteomics

Proteomic investigation of the testis, epididymis and sperm were carried out to assess changes in protein profiles between the control and obese samples. Samples were homogenized and whole cell lysates were created. Protein concentrations were measured by means of a Bradford assay, after which equal protein concentrations (10µg/µl) of 4 (or 8) samples were each pooled together. Eight pools (4 control, 4 obese) of testis and epididymis samples and two pools (8 control, 8 obese) of sperm samples, resulting in 16 samples of each organ being pooled together and analysed as shown in Table 3.3 below:

**Table 3.3:** Table outlining the epididymis, testis and sperm samples pooled for proteomic analysis

| Sample pools | | |
|---|---|---|
| **Tissue** | **Samples contained within each pool** | |
| | **Control** | **Diet** |
| **Epididymis** | | |
| Pool 1 | Control 1-4 | Diet 1-4 |
| Pool 2 | Control 5-8 | Diet 5-8 |
| Pool 3 | Control 9-12 | Diet 9-12 |
| Pool 4 | Control 13-16 | Diet 13-16 |
| **Testis** | | |
| Pool 1 | Control 1-4 | Diet 1-4 |
| Pool 2 | Control 5-8 | Diet 5-8 |
| Pool 3 | Control 9-12 | Diet 9-12 |
| Pool 4 | Control 13-16 | Diet 13-16 |
| **Sperm** | | |
| Pool 1 | Control 1-8 | Diet 1-8 |
| Pool 2 | Control 9-16 | Diet 9-16 |

#### 3.5.5.1. In-solution digest with trypsin

Protein digestion was carried out using Trypsin to break down proteins into their respective peptides. Briefly, 30µg of each protein pool was dried down within Eppendorf tubes and made up to a final volume of 10µl using Triethylammonium bicarbonate (TEAB; 50nM, Sigma 17902). Proteins were then reduced using tris (2-carboxyethyl) phosphine (TCEP; Sigma 646547) to a final concentration of 10mM TCEP. After incubation (60 minutes, 60°C) and subsequent cooling of

samples to room temperature, alkylation was performed using methylmethanethiosulphonate (MMTS; Sigma 208795) where after samples were brought to a final concentration of 10mM MMTS. Following incubation at room temperature (15 minutes), a 1µg/µl trypsin solution was added to each sample in a 1:20 (trypsin:protein) ratio. The addition of TEAB corrected samples to a 50mM concentration before an 18-hour trypsin digest at 37°C. Peptides then underwent vacuum centrifugation before resuspension in trifluoroacetic acid (TFA; 0.1%, Sigma T6508). C18 desalting was then carried out to conclude the digest.

#### 3.5.5.2. Analysis

Label-free protein quantitation of samples was performed using a Q-Exactive quadrupole-Orbitrap Liquid Chromatography Mass Spectrometer (LC-MS/MS) (Thermo Fischer Scientific, USA). The extracted peptides (1.5µg) were resuspended in sample loading buffer and introduced into the C18 trap column (300µm x 5mm x 5µm) of the Mass Spectrophotometer, followed by chromatographic separation by the C18 column (75µm x 20mm x 2µm). Peptide separation was then carried out via a multi-step gradient generated by a solvent system composed of solvent A (Water, 0.1% Formic Acid) and solvent B (Acetonitrile; 0.1% FA) at 300nl/min.

Four biological and four technical replicates were assessed to relatively quantify the protein expression of each sample pool by means of the Progenesis QI software (Nonlinear, UK). Acquired MS-MS spectra were converted from RAW data files into the mzML format using ProteoWizard 3.0 msConvert with vendor peaklisting before being run through MSGF+ software (July 16, 2014 version) (Kim et al., 2010) against the RefSeq Rat protein sequence database (updated February 8, 2016). IDPicker 3.1 software (Ma et al., 2009) was utilized and four-on-four comparisons were run to allow protein assembly and filtering. Peptide identification stringency was applied in the initial protein assembly to allow for accurate identification of proteins. A peptide-to-spectrum match (PSM) false discovery rate (FDR) of 2% was applied where a minimum requirement of 4 spectra was applied to remove erroneously identified protein spectra.

### 3.6. Statistical analysis

Biometric, macroscopic, microscopic and molecular data were analyzed using Graphpad Prism software (version 5). Normality was tested using the Kolmogorov-Smirnoff test after which unpaired t-tests or Mann-Whitney tests were respectively applied. Data is represented as mean ± Standard Deviation (SD), and a p value below 0.05 was considered significant.

Spearman correlation analyses were carried out to analyze the associations between all measured variables. Results are expressed as mean ± SD and statistical significance was set at $p < 0.05$.

Proteomics data was analyzed using Multiple Comparisons performed on R version 3.3.2 (2016-10-31). Spectral count data was preprocessed to remove sparse proteins with low spectral counts (< 12) (Slebos *et al*., 2014). Statistical analysis was performed on the remaining proteins. In each sample type, for a given protein (protein $i$), the following Poisson regression model was applied:

$$y_{ij} = offset_j + \beta_0 + \beta_1 \cdot Treatment$$

$y_{ij}$ represents the spectral count of the $i^{th}$ protein in the $j^{th}$ replicate, where , $i = 1, \dots, \#\ used\ proteins$, and $j = 1, \dots, 8,\ \ and\ Treatment = 1$ if the replicate is for diet-induced obesity and =0 if from control samples.

Differences in sensitivity (abundance) were adjusted, where total spectral counts in each replicate were used as the offset. The two-sided hypothesis tested was $H_0: \beta_1 = 0$ versus $H_1: \beta_1 \neq 0$.

Raw p values were obtained for each protein and adjusted to allow for multiple comparisons. “BH” (Benjamini & Hochberg (1995)) was used to control for the false discovery rate (FDR) at 0.05.

# Chapter 4: Results

The results obtained during this study are presented in this chapter, with data represented in tables and figures to visually depict and summarize the findings. Statistically significant results will be described herein and will inform the subsequent discussion.

## 4.1. Anthropometric and macroscopic parameters

Anthropometric measurement of the study animals (Figure 4.1) showed the diet-induced obesity (DIO) animals to exhibit a significantly higher body weight, visceral fat mass and a higher AI compared to the control group. Haematocrit was observed to be lower in the DIO group compared to controls. Using the mean body weight of the control group as a threshold, 3 (15%) of the control animals demonstrated a ≥ 10% increase in body weight compared to the mean control body weight and can be classified as moderately obese and/or obesity prone, while 13 (65%) of the DIO animals showed a similar increase and categorization. Two (10%) of the DIO animals showed a reduction in body weight compared to controls and were thus observed to have low (relative) body weights and classified as obesity resistant. All the animals were included in the statistical analysis and analysed within their study groups. No animals were excluded from analysis based on their proclivity towards obesity as their removal did not change the statistical significance of the measured parameters.

Testicular weight measurements did not show differences in absolute testis weight between DIO and control groups (Figure 4.1). However, when these weights were expressed as a ratio of body weight (Testis: Body Weight ratio; Testis:BW), (Bailey et al., 2004; Sellers et al., 2007), known as the relative testis weights, we observed the relative weights of the DIO testes to be significantly lower compared to controls (Figure 4.1).

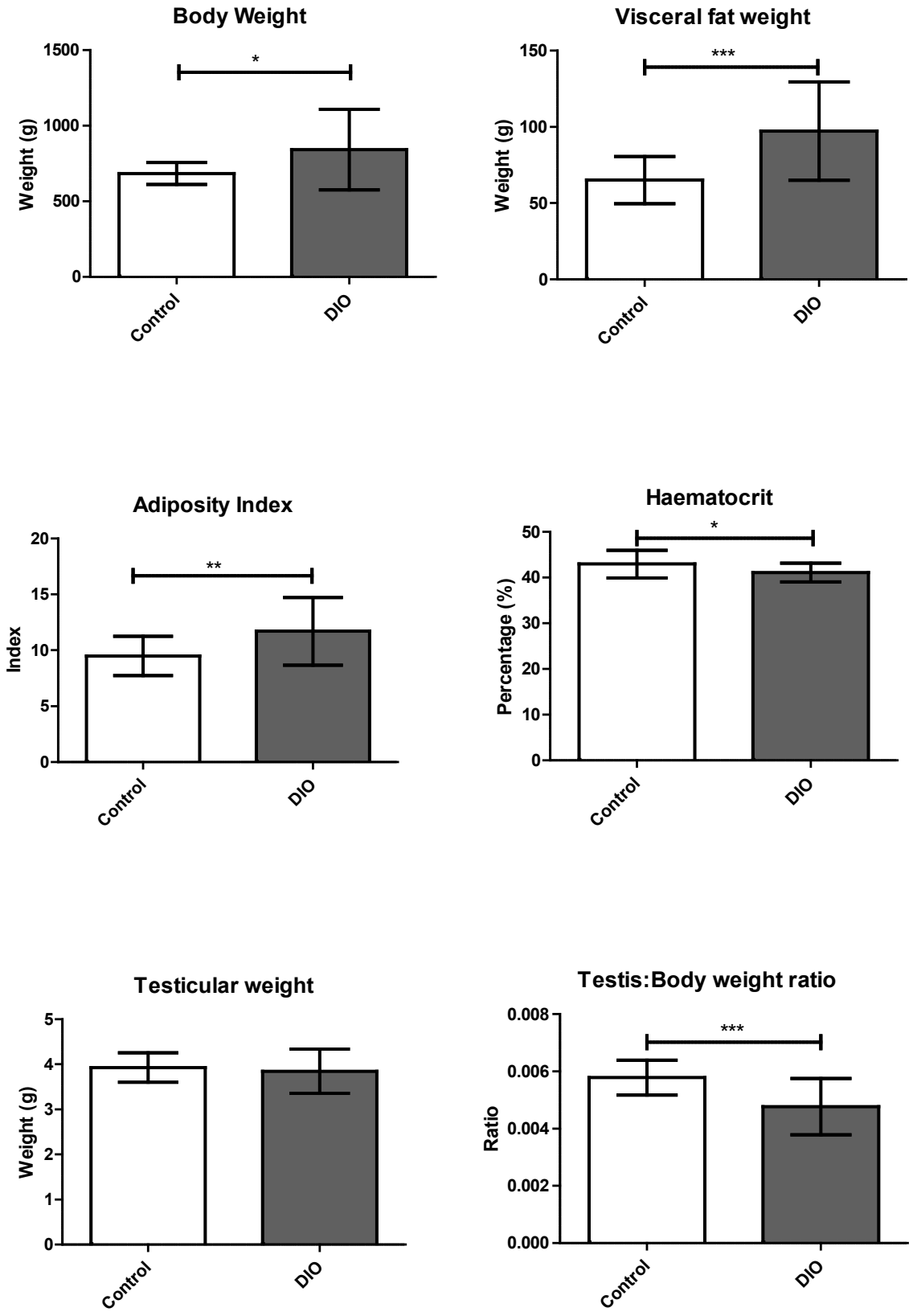



**Figure 4.1:** Histograms depicting anthropometric and macroscopic parameters of control (n = 20) and DIO (n = 20) groups.

## 4.2. Microscopic parameters

### 4.2.1. Sperm analyses

Semen analysis of the DIO and control groups revealed changes in several sperm parameters of the DIO animals as a result of the treatment. The results are summarized in Table 4.1, with examples of the observed changes in sperm morphology and viability illustrated in the accompanying figures, Figure 4.2 and 4.3, respectively. The percentage of morphologically normal sperm was significantly lower in DIO subjects compared to controls. Sperm from the DIO group exhibited a higher proportion of head defects, with a significant number observed to have a reduced head area when compared to controls. Sperm viability of the DIO animals were also significantly lower compared to controls.

**Table 4.1:** Summary of sperm viability, sperm morphology and morphometry data in the control and DIO groups. ($^{\#}$ = statistically significant result)

| | Groups | | |
|---|---|---|---|
| | **Control** | **DIO** | **p-value** |
| **Normal Sperm Morphology (%)** | 74.27 ± 2.2240 | 60.53 ± 2.8720 | 0.0008$^{\#}$ |
| - Head defects (%) | 23.93 ± 2.1550 | 37.47 ± 2.9050 | 0.0004$^{\#}$ |
| - Midpiece defects (%) | 2.80 ± 0.0470 | 3.07 ± 0.8016 | 0.8412 |
| **Sperm Morphometry** | | | |
| ***Head*** | | | |
| - Length = Arc (µm) | 21.32 ± 0.0901 | 21.11 ± 0.1564 | 0.2546 |
| - Width (µm) | 1.48 ± 0.0205 | 1.47 ± 0.0241 | 0.8674 |
| - Area (µm$^2$) | 19.06 ± 0.2498 | 18.13 ± 0.3091 | 0.0266$^{\#}$ |
| - Perimeter (µm) | 45.73 ± 0.2060 | 45.39 ± 0.3380 | 0.3968 |
| - Linearity (%) | 50.52 ± 0.3714 | 50.09 ± 0.5333 | 0.5181 |
| - Angle (degrees) | 59.37 ± 1.1510 | 57.82 ± 1.1880 | 0.3589 |
| - Roughness | 0.11 ± 0.0016 | 0.11 ± 0.0009 | 0.1646 |
| - Chord (µm) | 10.80 ± 0.0596 | 10.60 ± 0.1007 | 0.0964 |
| ***Midpiece*** | | | |
| - Width (µm) | 0.71 ± 0.0104 | 0.68 ± 0.0159 | 0.1032 |
| - Area (µm$^2$) | 2.23 ± 0.1648 | 2.05 ± 0.1490 | 0.4315 |
| - Angle (degrees) | 10.77 ± 0.3925 | 10.25 ± 0.6506 | 0.5027 |
| **Sperm viability (% live sperm)** | 58.80 ± 5.4120 | 43.20 ± 2.3470 | 0.0030$^{\#}$ |
| **Teratozoospermic Index (TZI)** | 1.02 ± 0.0110 | 1.02 ± 0.0113 | 0.6162 |
| **Sperm deformity index** | 0.26 ± 0.0244 | 0.41 ± 0.0317 | 0.0013$^{\#}$ |

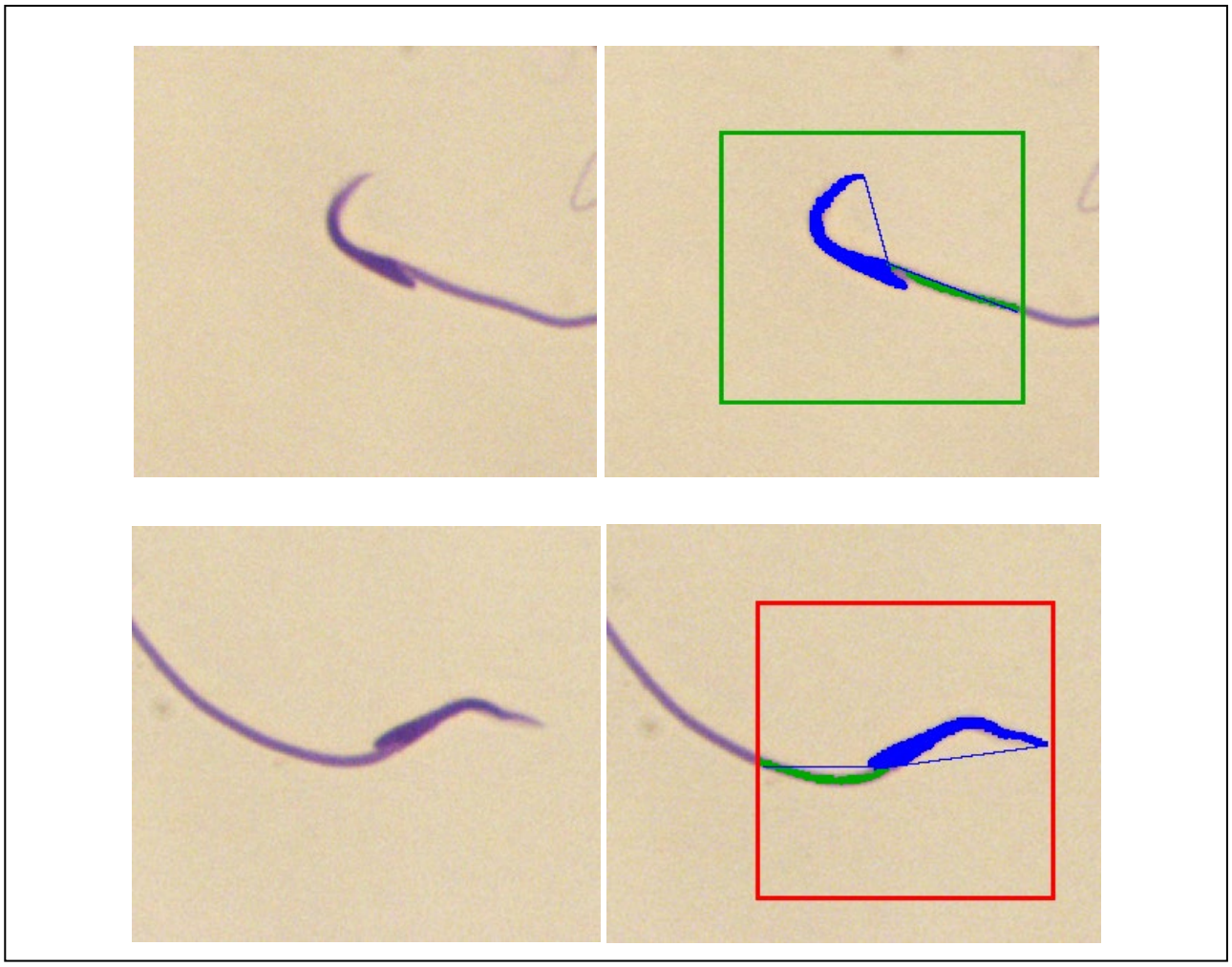


**Figure 4.2:** Representative images of morphometrically normal and abnormal spermatozoa. Analyses were performed using The Sperm Class Analyser (SCA) © Computer Assisted Sperm Analysis (CASA). Images are shown with and without thresholding (left vs. right), with green boxes around spermatozoa denoting normal sperm, and red boxes denoting abnormal sperm.

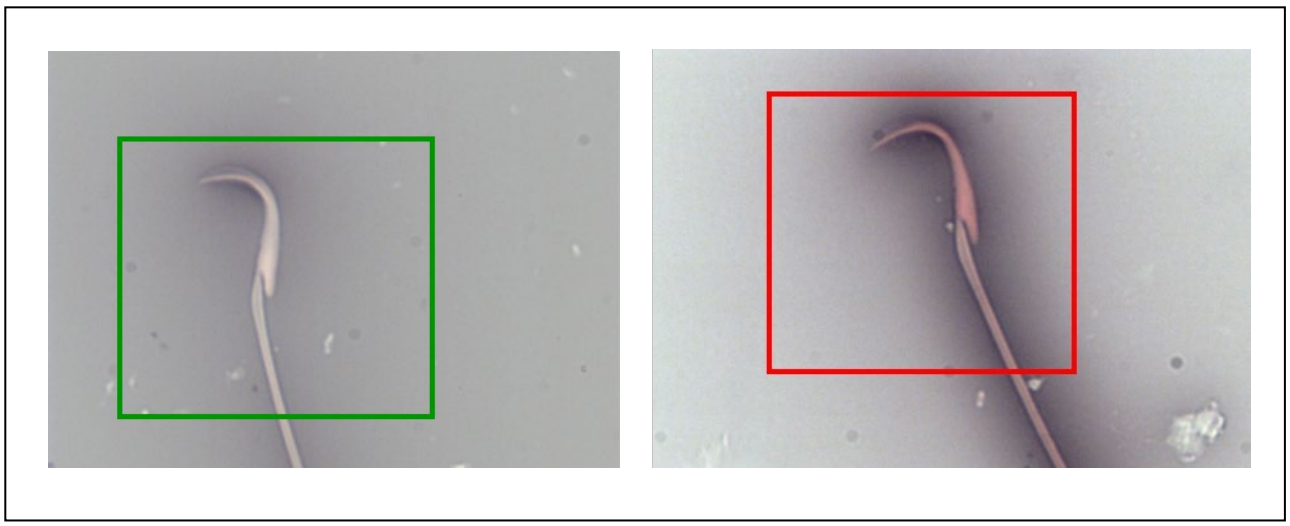


**Figure 4.3:** Images showing a viability analysis performed using the Cell Counter module of the SCA© CASA program, depicting viable (green boxes) and non-viable (red boxes) spermatozoa.

### 4.2.2. Histology

Analysis of the testicular and epididymal histology revealed several changes in reproductive organs between the two study groups. The results of these will be subdivided into quantitative and qualitative sections and presented accordingly.

#### 4.2.2.1. Quantitative analysis

Significant reductions in the epithelial height of the seminiferous tubules were observed in the DIO testes compared to controls (Table 4.2, Figure 4.4). Similarly, the relative volumes of the seminiferous tubules were also lower in the DIO testes compared to controls (Table 4.2, Figure 4.6). Luminal diameters and relative luminal volumes were significantly smaller in the DIO group, compared to controls. Seminiferous tubular diameters were significantly decreased in the obese animals. The relative interstitial volume was increased in the obese testes compared to controls (Table 4.2, Figure 4.6). The seminiferous epithelia of the DIO epididymis showed no changes in epithelial height and relative epithelial volume compared to controls (Table 4.3, Figure 4.5). Luminal diameters and relative luminal volumes were reduced in the DIO group compared to controls. Epididymal tubular diameters and relative tubular volumes were reduced in the obese epididymides while the relative interstitial volume significantly increased compared to the age-matched controls (Table 4.3, Figure 4.6).

**Table 4.2:** Summary of the observed differences in testicular histomorphometric parameters between the control and DIO groups. Illustrations of these measurements can be observed in Fig. 4.4 and 4.6.

| | Seminiferous tubules | | | | | | | | | | Interstitium | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Epithelium | | | | Lumen | | | | Tubular Diameter (µm) | | Relative volume (%) | |
| | Height (µm) | | Relative volume (%) | | Diameter (µm) | | Relative volume (%) | | | | | |
| | Control | DIO | Control | DIO | Control | DIO | Control | DIO | Control | DIO | Control | DIO |
| Mean | 48.51 | 38.66 | 72.32 | 61.16 | 157.9 | 137.3 | 29.15 | 22.73 | 206.40 | 176.00 | 4.49 | 10.29 |
| SD | 9.38 | 2.37 | 3.83 | 6.32 | 16.85 | 9.85 | 3.29 | 5.07 | 23.41 | 11.26 | 1.16 | 1.49 |
| CV | 19.35 | 6.14 | 5.29 | 10.42 | 10.67 | 7.18 | 14.45 | 17.39 | 11.34 | 6.40 | 28.80 | 14.49 |
| P value | 0.0317 | | <0.0001 | | 0.0459 | | 0.0003 | | 0.0306 | | <0.0001 | |

**Table 4.3:** Summary of the observed differences in epididymal histomorphometric parameters between the control and DIO groups. Illustrations of these measurements can be observed in Fig. 4.5 and 4.6.

| | Epidydimal ducts | | | | | | | | | | Interstitium | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Epithelium | | | | Lumen | | | | Tubular Diameter (µm) | | Relative volume (%) | |
| | Height (µm) | | Relative volume (%) | | Diameter (µm) | | Relative volume (%) | | | | | |
| | Control | DIO | Control | DIO | Control | DIO | Control | DIO | Control | DIO | Control | DIO |
| Mean | 23.27 | 22.74 | 9.56 | 9.09 | 356.40 | 241.50 | 80.91 | 73.65 | 382.70 | 264.20 | 9.53 | 17.32 |
| SD | 2.12 | 1.55 | 3.60 | 2.00 | 86.10 | 41.19 | 7.09 | 4.01 | 67.98 | 41.44 | 2.56 | 11.26 |
| CV | 9.15 | 6.85 | 28.50 | 22.02 | 23.96 | 17.06 | 8.76 | 5.45 | 15.99 | 15.69 | 11.34 | 6.40 |
| P value | 0.6686 | | 0.7240 | | 0.0246 | | 0.0018 | | 0.0261 | | 0.0007 | |

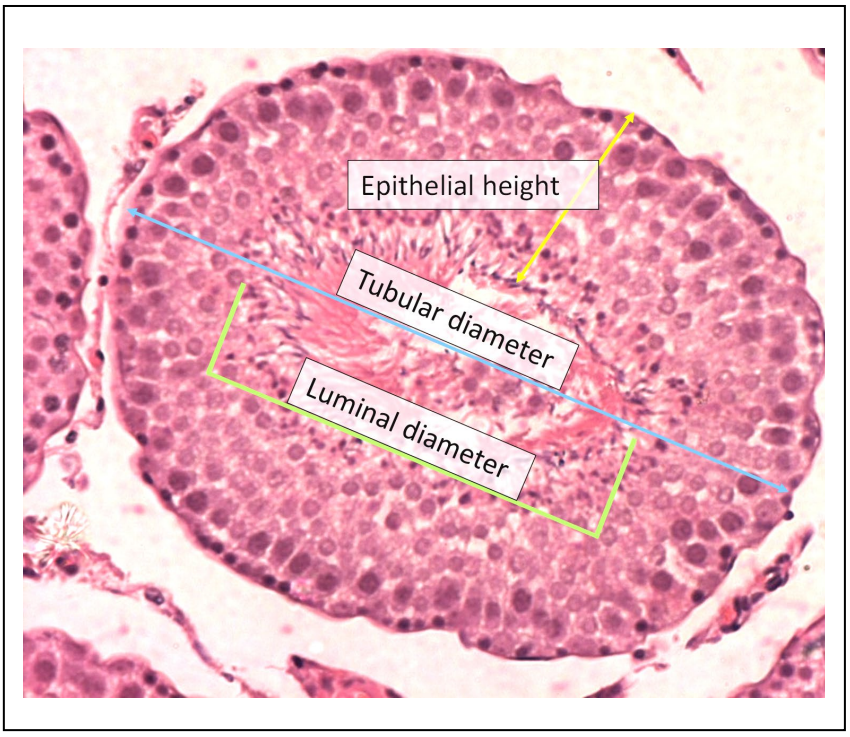



**Figure 4.2:** Photomicrograph of testicular tissue (H&E staining, 40x objective) illustrating the quantitative measurements performed on seminiferous tubules of the testes.

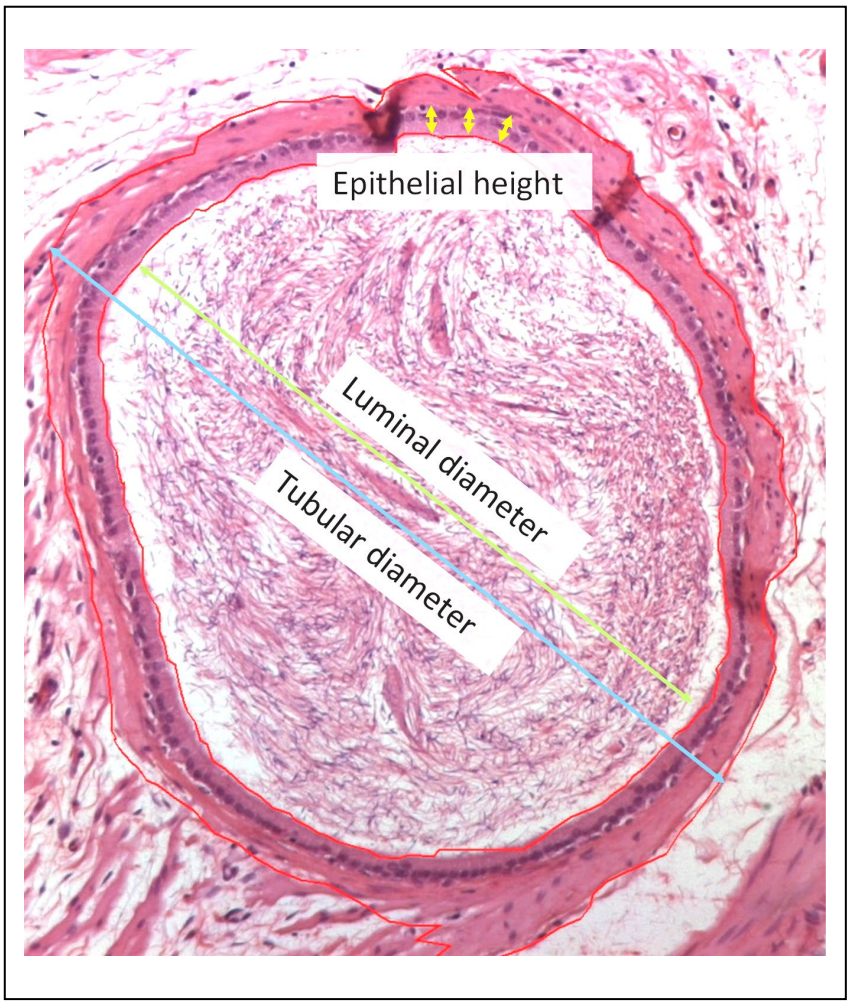



**Figure 4.3:** Photomicrograph of epididymal tissue (H&E staining, 40x objective) illustrating the quantitative measurements performed on epididymal ducts.

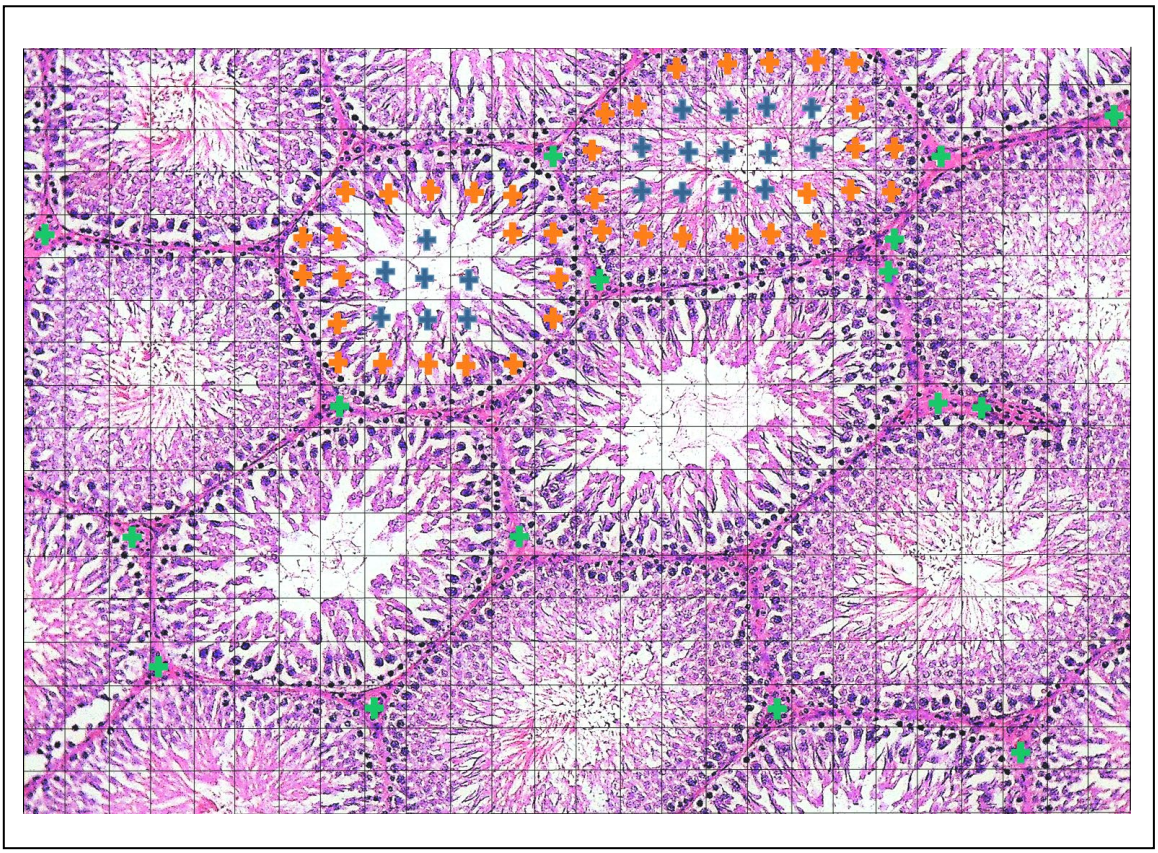


**Figure 4.4:** Photomicrograph (H&E staining, 10x objective) depicting the histological analysis of relative luminal volume (blue crosses), relative epithelial volume (orange crosses) and relative interstitial volume (green grosses) using testicular tissue as an example. The same analysis was carried out on epididymal tissue to assess relative volumes, which were expressed as a percentage (%) of the total testicular or epididymal volume. A grid is overlaid to appropriately divide the tissue into different tissue regions, after which crosses are respectively inserted to demarcate cubes of the grid which contain ≥60% of the relevant tissue. The number of cubes were then quantified and expressed as a % of the total number of cubes (per field). Two seminiferous tubules and all relevant cubes of interstitial tissue are shown as an example.

#### 4.2.2.2. Qualitative alaysis

The most frequently observed histological pattern in the DIO testes is a mosaic distribution of normal and affected tubules. This means that within the microscopic fields analysed, there is a “pattern” or distribution of damaged seminiferous tubules interspersed with normal, intact tubules. The normal tubules (Figure 4.6A) show intact seminiferous epithelium, with many groups of developing sperm attached to Sertoli cells and spermatids released into the lumen. The damaged tubules (Figure 4.6B) exhibit a shortening of the pseudostratified seminiferous epithelia (evidenced by significantly reduced epithelial heights (Table 4.2)), a degeneration of the epithelial layer and a disruption of the cell-to-cell connections between the epithelia. Large vacuoles may also be seen between the germ cells. Less sperm can be observed within the lumen. Spermatogonia and Sertoli cells are seen to detach from the basement membrane. In the epididymis (Figure 4.7), a similar mosaic distribution is observed. Control epididymides show columnar and basal epithelia that is tightly adherent to the basement membrane, with well-organized steriocilia. In the DIO group (Figure 4.7B), epithelia is seen breaking away from the basement membrane (with a portion of epithelia observed in the lumen, possibly from an “upstream” section of the duct) and steriocilia are not well-defined.

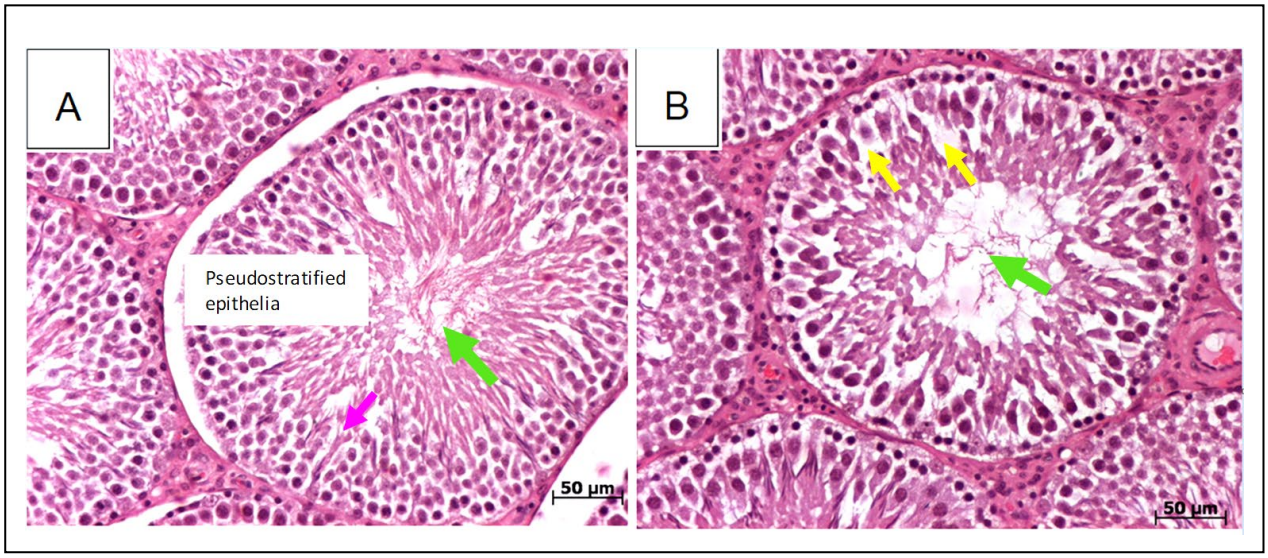



**Figure 4.5**: Photomicrographs of sections of testicular tissue (H&E staining, 60x objective) showing (A) control and (B) DIO testes. The pseudostratified (germinal) epithelium consisting of spermatogenic cells can be visualized, along with the supportive Sertoli cells (pink arrows). The lumens of control and DIO testes can also be observed (green arrows), with DIO tubules containing less sperm within the lumen. Numerous vacuoles (yellow arrows) can be seen in the between DIO pseudostratified epithelia.

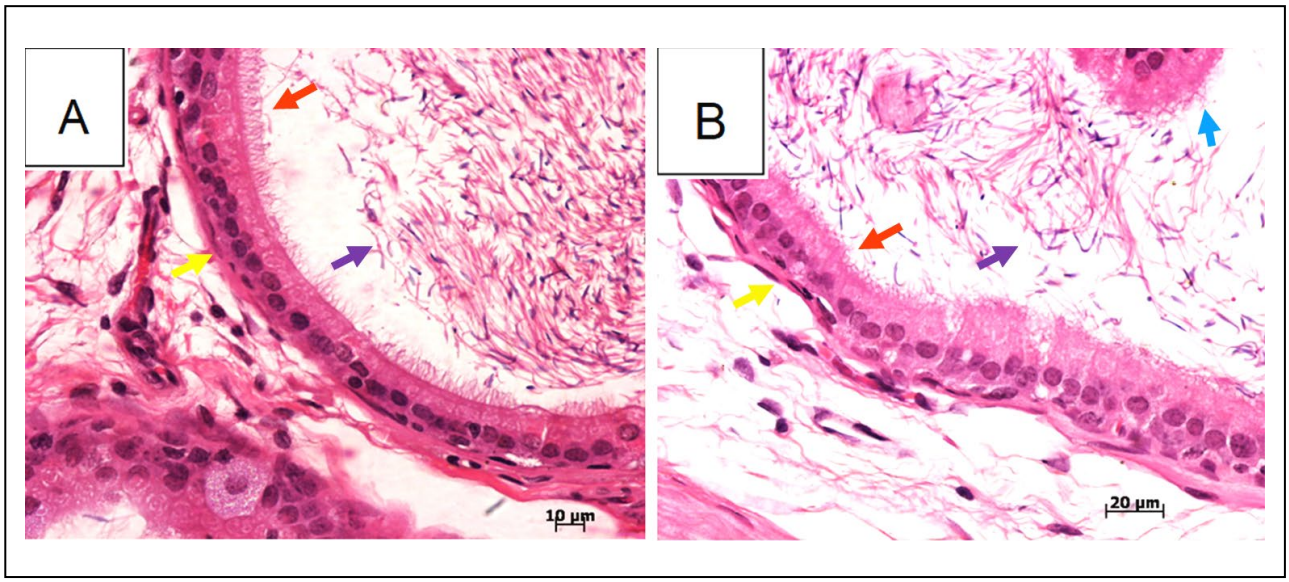



**Figure 4.6:** Photomicrographs of sections of epididymal tissue (H&E staining, 60x objective) showing control (A) and DIO (B) epididymides. The columnar and basal epithelia (red arrows) lie on basement membranes (yellow arrows) which separate the epididymal ducts from the interstitium below. The stereocilia of the control epididymis is well developed and pronounced, stretching into the lumen of the duct whereas the steriocilia in the DIO epididymis appear disrupted in places and less defined. The DIO epididymis is seen to have less sperm (purple arrows). Epididymal columnar epithelia is seen in the lumen of the DIO epididymis (blue arrow) which was likely to have broken off from an “upstream” section of the duct.

## 4.3. Molecular analyses

The following table summarizes the results obtained from the molecular analyses performed during experimentation: Each of the significant results will be elaborated in the text below.

**Table 4.4:** Table outlining the changes in molecular parameters between control and diet-induced obesity (DIO) animals. Results are presented as mean ± standard deviation (SD) with p values provided to show the significance of each parameter change

| | Control | DIO | p value |
|---|---|---|---|
| **Blood glucose (mmol/l)** | 6.57 ± 0.117 | 6.78 ± 0.1806 | 0.335 |
| **Haematocrit (%)** | 42.97 ± 0.711 | 41.11 ± 0.4792 | 0.037 |
| **Testosterone (ng/ml)** | 5.64 ± 1.125 | 5.36 ± 1.064 | 0.708 |
| **Estradiol (pg/ml)** | 34.51 ± 4.058 | 37.09 ± 4.732 | 0.207 |
| **Testosterone:Estradiol ratio** | 0.166 ± 0.033 | 0.148 ± 0.036 | 0.248 |
| **Testosterone:BW ratio** | 0.008 ± 0.002 | 0.007 ± 0.002 | 0.076 |
| **Catalase (U/mg)** | | | |
| Testis | 2373 ± 112.600 | 2109 ± 63.44 | 0.048 |
| Epididymis | 1550 ± 71.910 | 1532 ± 49.89 | 0.402 |
| **Superoxide dismutase (U/µg)** | | | |
| Testis | 26.60 ± 0.4854 | 24.78 ± 0.5123 | 0.014 |
| Epididymis | 22.78 ± 0.5030 | 22.92 ± 0.6916 | 0.871 |
| **GSH** | | | |
| Testis (total GSH; tGSH) | 338.00 ± 17.940 | 275.90 ± 13.87 | 0.009 |
| **TBARS (umol/g MDA)** | | | |
| Testis | 0.151 ± 0.0139 | 0.148 ± 0.0139 | 0.878 |
| Epididymis | 0.102 ± 0.0131 | 0.098 ± 0.0125 | 0.474 |

### 4.3.1. Blood glucose

Despite a slight elevation, unfasted blood glucose levels were not different between control and DIO groups and remained within normal ranges (3.3 – 7.2 millimoles/litre (mmol/L)) for both lean and DIO groups (Kohn and Clifford, 2002; Ringler and Dabich, 2013) (Table 4.4).

### 4.3.2. Hormone analyses

The obese phenotype showed no difference in the measured steroid hormones, as spectrophotometric quantification of total testosterone and estradiol yielded lower (-5%) and higher (7%) hormone concentrations respectively when compared to controls, but not to statistically significant levels (Table 4.4). Estradiol levels of DIO rats did, however, exceed the normal physiological range (10 – 36 nanograms/millilitre (ng/ml)) (DRG Diagnostics, 2014) (Table 4.4). A calculated Testosterone:Estradiol (T:E) ratio showed no differences in T:E ratios between control and DIO animals (Table 4.4).

### 4.3.3. Oxidative stress parameters

CAT activity (U) was lower in the testes of DIO animals (2109 ± 283.7 U/mg protein vs. 2373 ± 503.4 U/mg protein; p = 0.048) but unchanged in the epididymis (1532 ± 211.7 U/mg protein vs. 1550 ± 305.1 U/mg protein; p = 0.402) in comparison to control animals (Table 4.4). SOD activity was significantly lower in the DIO testes (24.78 ± 2.291 U/µg protein vs. 26.60 ± 2.171 U/µg protein; p = 0.014) but not altered in the epididymides (22.92 ± 3.093 U/µg protein vs. 22.78 ± 2.193 U/µg protein; p = 0.871) compared to controls. LPO was not significantly changed between the two groups in both the testes (0.148 ± 0.0624 umol/g MDA vs. 0.151 ± 0.0622umol/g MDA; p = 0.8778) and epididymides (0.098 ± 0.0545 umol/g MDA vs. 0.102 ± 0.0571 umol/g MDA; p = 0.4743). Total GSH concentration in the testes of DIO animals was lower than in the controls (275.9 ± 62.05 tGSH vs. 338.0 ± 80.24 tGSH; p = 0.009).

### 4.3.4. Correlations

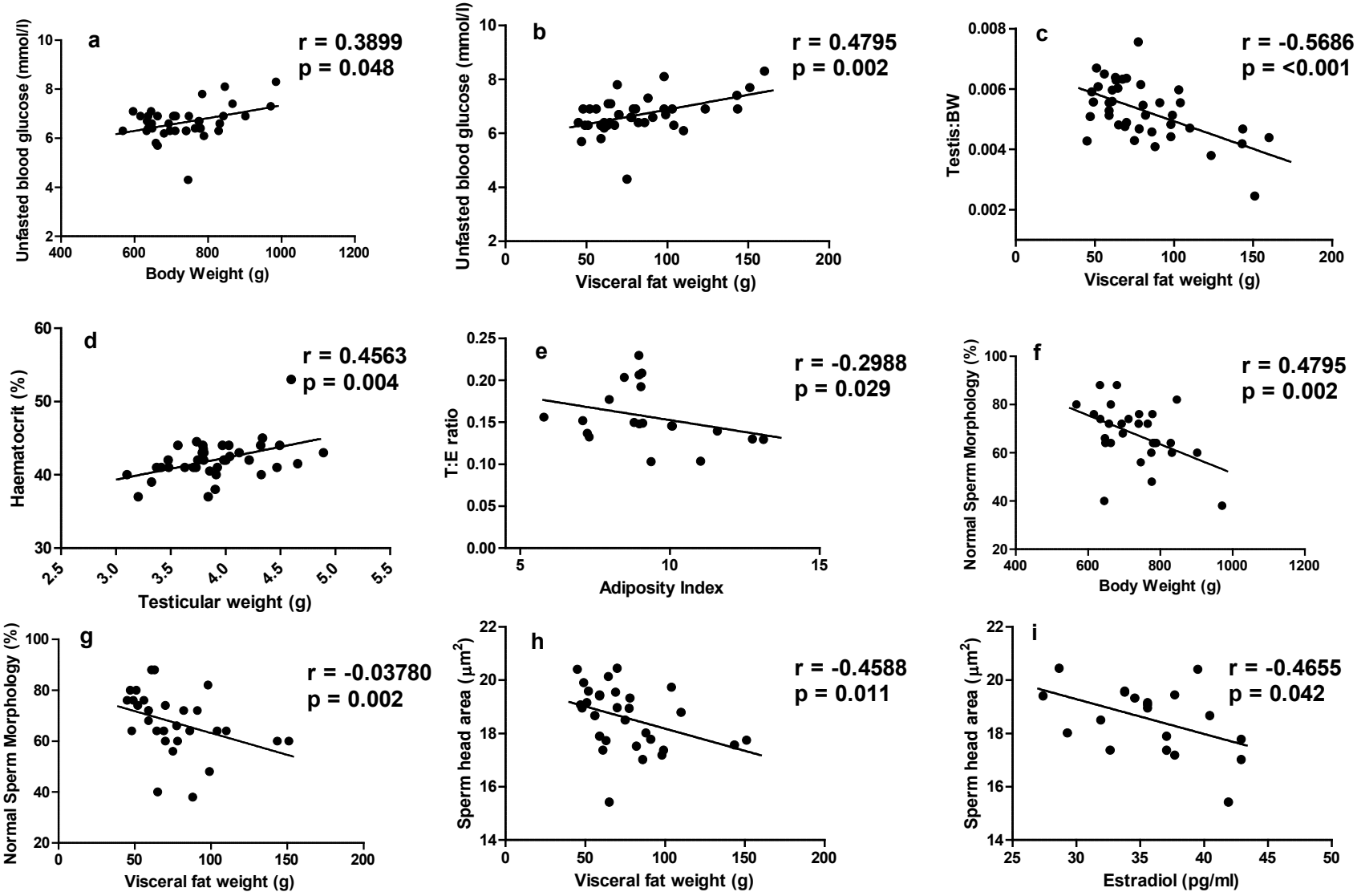
Correlations between biometric, haematocrit, sperm, histomorphological, blood glucose, hormone and antioxidant enzyme data revealed several relationships between the measured variables (Figure 4.9).

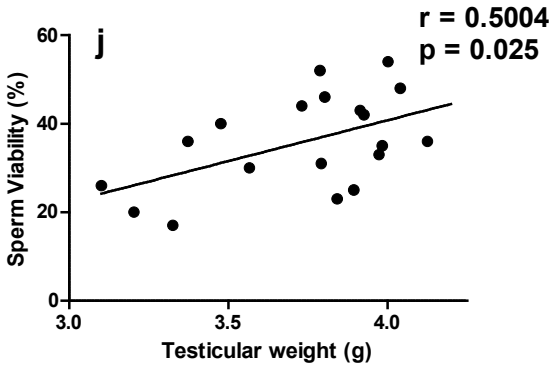
Animals with larger body weights showed higher blood glucose levels (r = 0.3899; p = 0.048). Similarly, greater visceral fat weights yielded higher blood glucose levels (r = 0.4795; p = 0.002) and were associated with lower Testis:BW ratios (r = -0.5686; p < 0.001). Haematocrit positively correlated with testicular weight (r = 0.4563, p = 0.004). Reduced T:E ratios were found in animals with greater adiposity indexes (r = -0.2988; p = 0.029). Lower percentages of morphologically normal sperm were found in animals with higher body weights (r = 0.4795; p = 0.002) as well as in those with higher visceral fat weights (r = -0.3780; p= 0.002). Furthermore, reduced sperm head area correlated with high visceral fat weight (r = -0.4588; p = 0.011) and high estradiol (r = -0.4655; p = 0.042). Animals with higher testicular weight had higher percentages of viable spermatozoa (r = 0.5004, p = 0.025).

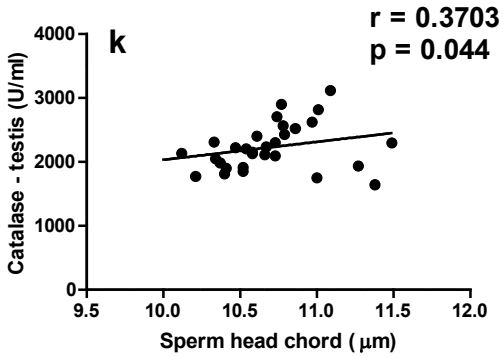
Antioxidant and peroxidation measurements showed significant relationships with molecular and microscopic parameters. Catalase activity in the testis correlated with changes in sperm morphometry through a positive correlation between catalase and sperm head chord (r = 0.3703, p = 0.044). Epididymal catalase activity was higher in animals with higher estradiol levels (r = 0.4208, p = 0.0456), and similarly correlated with sperm morphometry via correlations with sperm head width (r = -0.5018, p = 0.006), head linearity (r = 0.4074, p = 0.028), head chord (r = 0.3974, p = 0.033) and midpiece angle (r = 0.4821, p = 0.008). Epididymal catalase activity furthermore correlated with epididymal histomorphologcal changes, including tubular diameter (r = -0.7167, p =

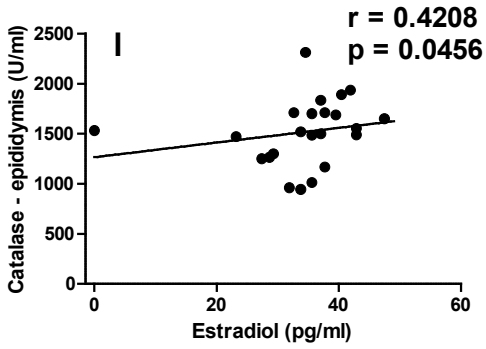
0.037), luminal diameter (r = -0.7167, p = 0.037), relative luminal volume (r = -0.9333, p < 0.001) and relative interstitial volume (r = 0.9167, p = 0.001). SOD activity in the testes showed a significant relationship to the changes observed in several sperm morphometry parameters, including head area (r = 0.4389, p = 0.015), head roughness (r = 0.5382, p = 0.002) and midpiece defects (r = 0.3949, p = 0.031). SOD activity further showed a negative relationship with the relative epithelial volume (r = -0.685, p = 0.035) and the relative luminal volume of the testes (r = -0.8061, p = 0.007). SOD activity in the epididymis similarly correlated positively with midpiece defects observed in the sperm (r = 0.4232, p = 0.022). Total GSH in the testes correlated negatively with all 3 body weight and fatness measurements, where lower tGSH levels were observed in animals with higher body weight (r = -0.3421, p = 0.031), visceral fat weight (r = -0.3371, p = 0.033) and higher AI (r = -0.3318, p = 0.0365). Lower tGSH levels were found in animals with higher percentages of morphologically normal sperm (r = 0.4882, p = 006), and lower sperm head defects (r = -0.5205, p = 0.003). Testicular histology measurements significantly correlated with tGSH, with reduced tGSH levels observed in testes with higher tubular diameters (r = 0.7212, p = 0.023) and luminal diameters (r = 0.7576, p = 0.015).

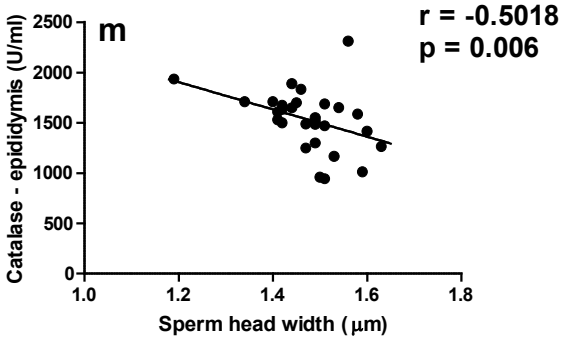
Histological measurements of the testes and epididymis showed several relationships with measured molecular and microscopic parameters. Testicular epithelial height was negatively associated with blood glucose (r = -0.6160, p = 0.031), as well as positively related to sperm head area (r = 0.7820, p = 0.011). Luminal diameters of the seminiferous tubules demonstrated a positive association with relative testicular weight (r = 0.6610; p =0.044), although a negative correlation was observed with testosterone (r = -0.7580; p = 0.015). Relative testicular interstitial volume exhibited a negative relationship with the Tesits:BW ratio (r = -0.7090; p = 0.027) and sperm head roughness (r = -0.6460, p = 0.049). Epididymal histomorphological changes also correlated with various measured parameters. Relative epithelial volume correlated positively with sperm head roughness (r = 0.7218; p = 0.023). Relative interstitial volume further correlated with sperm viability (r = -0.7939, p = 0.009).

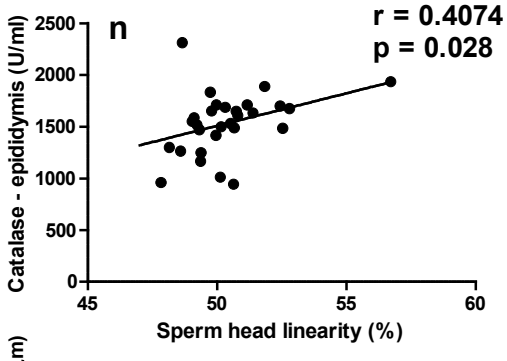


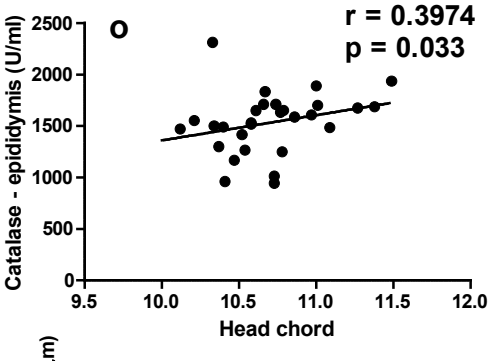


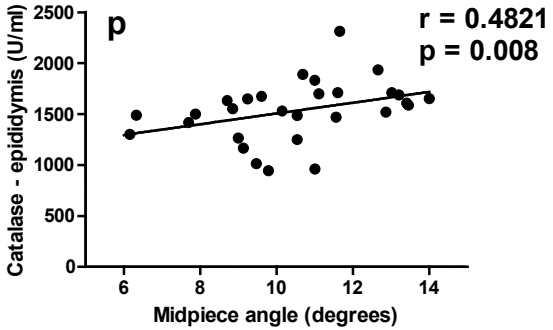



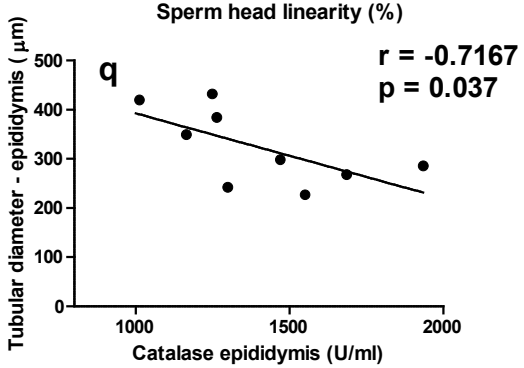



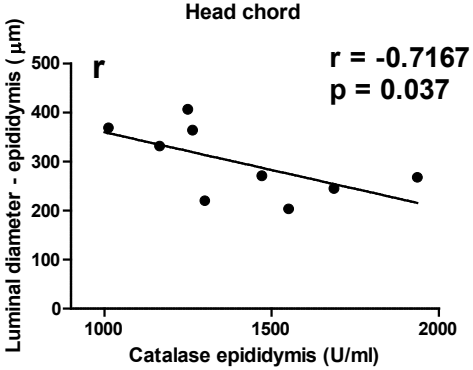



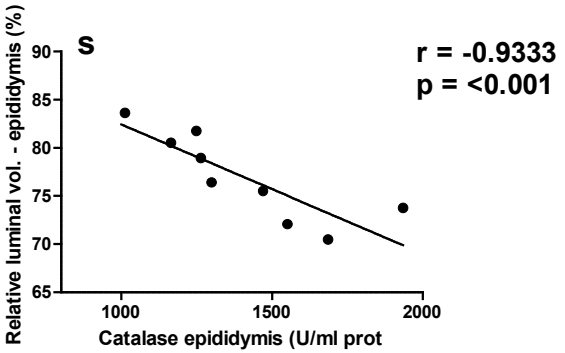



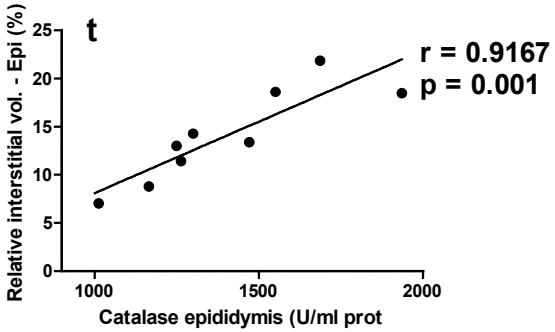



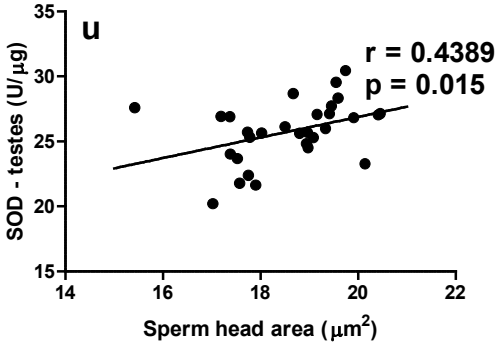



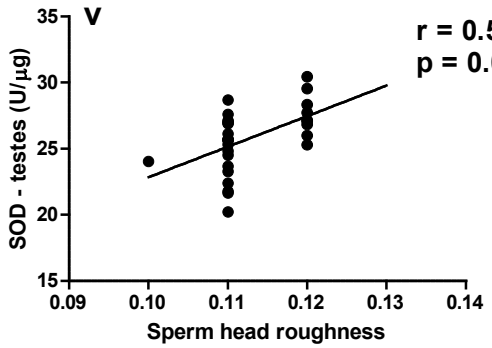



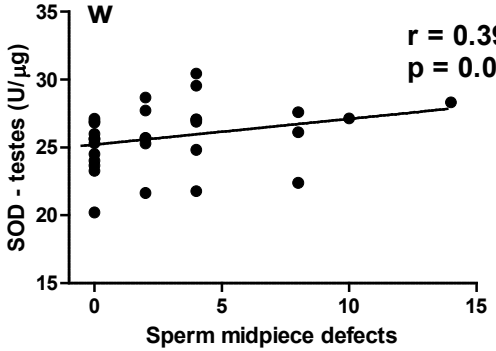



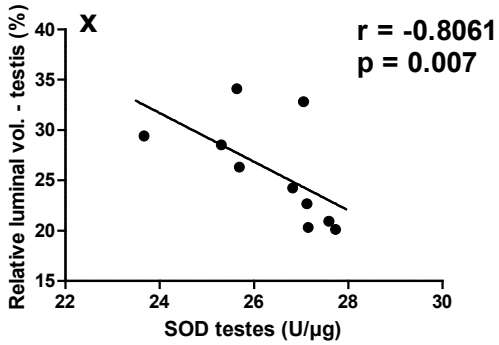


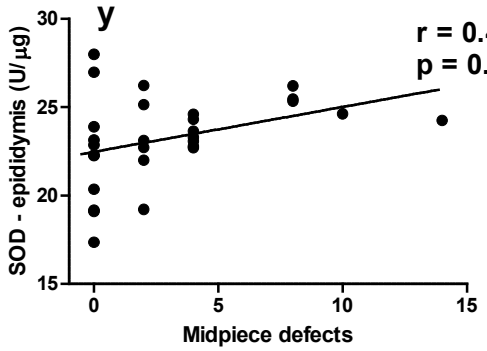



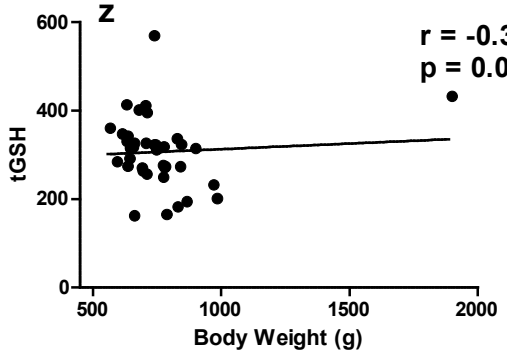



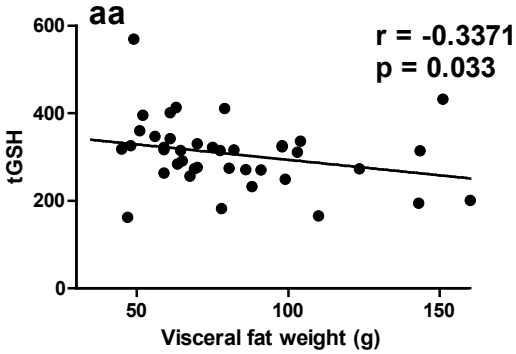



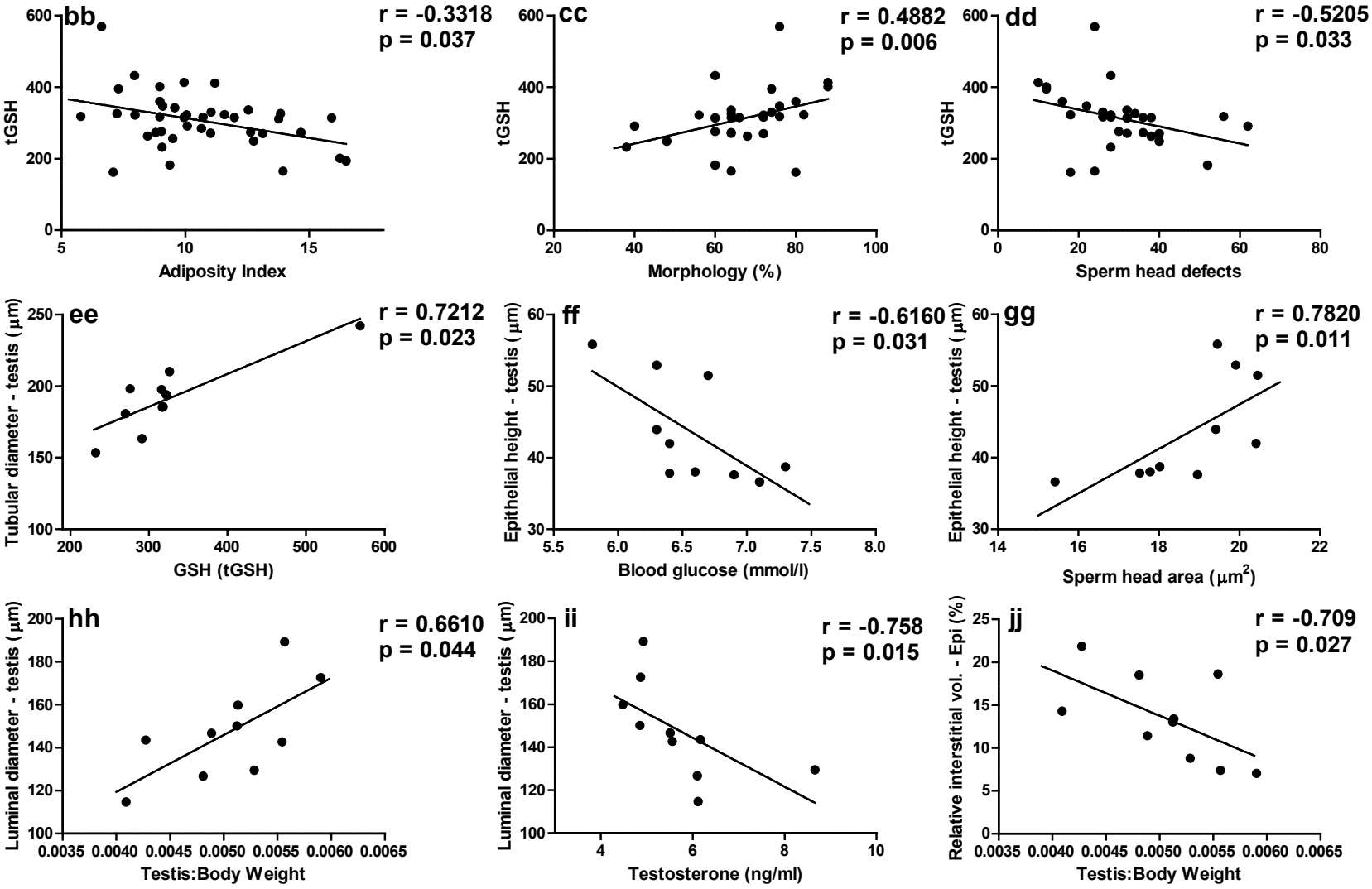





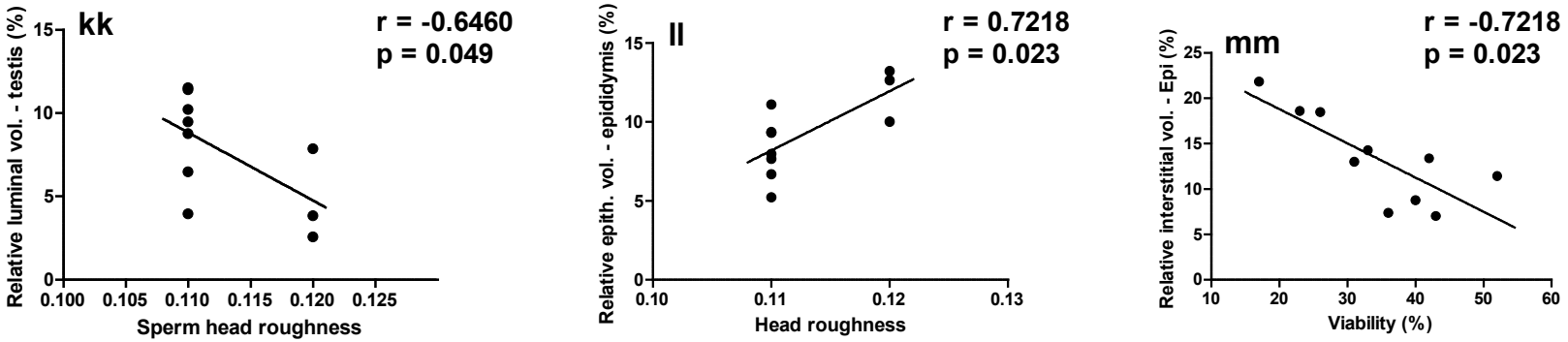



**Figure 4.7:** Correlation plots showing significant correlations between biometric, molecular, sperm, histomorphological, and antioxidant enzyme data.

### 4.3.5. Proteomics

A total of 3754 proteins were identified within the testes, epididymis and spermatozoa by proteomic analysis. After removal of sparse proteins and the application of identification stringency, 246 epididymal and 61 sperm proteins tested as significantly different between the control and DIO groups, whilst no testicular proteins were significantly expressed (change in expression reaching statistical significance).

Of the epididymal proteins, 199 were successfully mapped and assigned functions using the Uniprot database. Forty-four proteins had identified sequences with no known functions. The remainder of the epididymal proteins were unidentified. Of the sperm proteins that showed significance, 37 were successfully mapped and identified by the Uniprot database, while 20 were mapped but had no further information or assigned functions.

After ascertaining protein functions, all epididymal and sperm proteins were divided into 6 categories: metabolic, structural, reproduction-specific, antioxidant/pro-oxidant, regulatory and immune-related proteins. Several proteins had multiple functions and were classified as multifunctional. Due to the long list of significant proteins, only the top 6 (where appropriate) most abundant proteins in each category will be displayed in this section (Table 4.5 and Table 4.6), with the full list of proteins displayed in the addendum. Schematic diagrams showing protein-protein interactions of the abundantly expressed proteins grouped according to function are shown in Figure 4.12 to Figure 4.16 to summarize proteomics findings.

**Table 4.5: Annotation of the most abundant significantly expressed Epididymal proteins of each functional category performed using Uniprot**

| Accession number | Gene name | Protein name | Control Mean | Diet Mean | Adjusted p value | Function |
|---|---|---|---|---|---|---|
| **Metabolic proteins** | | | | | | |
| Q5XIV1 | Phosphoglycerate kinase | Pgk2 | 100 | 118 | 0.017 | Carbohydrate degradation; glycolysis; conversion of pyruvate from D-glyceraldehyde 3-phosphate. Flagellated sperm motility. |
| P04764 | Alpha-enolase (Enolase 1) | Eno1 Eno-1 | 97 | 113 | 0.023 | Multifunctional enzyme involved in glycolysis. Has a role in various processes including growth control, hypoxia tolerance and allergic responses. Serves as a receptor and activator of plasminogen on the cell surface of several cell-types. Stimulates immunoglobulin production. |
| P00507 | Aspartate aminotransferase mitochondrial (mAspAT) Glutamate oxaloacetate transaminase 2 (Got2) | Got2 Maat | 61 | 79 | 0.004 | Essential role in amino acid metabolism. Important for metabolite exchange between mitochondria and cytosol. A fatty acid-binding protein which facilitates cellular uptake of long-chain free fatty acids |
| P01026 | Complement C3 | C3 | 66 | 45 | 0.008 | Activation of the complement system. Activated to become C3b and C3a (an anaphylatoxin and mediator of local inflammation). Induces smooth muscle contraction, increases vascular permeability and causes histamine release from mast cells and basophilic leukocytes. In chronic inflammation, acts as a chemoattractant for neutrophils. Adipogenic hormone stimulating triglyceride (TG) synthesis and glucose transport within adipocytes, thus regulating fat storage and postprandial |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | TG removal. Appears to stimulate TG synthesis via activation of the PLC, MAPK and AKT signalling pathways |
| P16290 | Phosphoglycerate mutase 2 | Pgam2 | 32 | 52 | <0.001 | Gluconeogenesis, glycolytic process, regulation of pentose-phosphate shunt. Interconversion of 3- and 2-phosphoglycerate with 2,3-bisphosphoglycerate as the primer.<br><br>Spermatogenesis |
| P11030 | Acyl-CoA-binding protein (ACBP) (Diazepam-binding inhibitor) (DBI) | Dbi | 27 | 49 | <0.001 | Binds medium- and long-chain acyl-CoA esters with very high affinity and may function as an intracellular carrier of acyl-CoA esters. Able to displace diazepam from the benzodiazepine (BZD) recognition site located on the GABA type A receptor |
| **Structural proteins** | | | | | | |
| P63269 | Actin, gamma-enteric smooth muscle (Alpha-actin-3) (Gamma-2-actin) (Smooth muscle gamma-actin) | Actg2 Acta3 Actsg | 171 | 215 | <0.001 | Cell motility. Ubiquitously expressed in all eukaryotic cells. |
| P60711 | Actin, cytoplasmic 1 (Beta-actin) | Actb | 153 | 190 | <0.001 | Cell motility. Ubiquitously expressed in all eukaryotic cells. |
| P68136 | Alpha-actin-1 | Acta1 | 158 | 203 | <0.001 | Cell motility. Ubiquitously expressed in all eukaryotic cells. |
| Q6P9T8 | Tubulin beta-4B chain (Tubulin beta-2C chain) | Tubb4b Tubb2c | 77 | 93 | 0.020 | The major component of microtubules. |
| P31000 | Vimentin | Vim | 37 | 58 | <0.001 | Vimentins are class-III intermediate filaments found in numerous non-epithelial cells, more especially mesenchymal cells. Attached to the nucleus, endoplasmic reticulum, and mitochondria. Is involved in the stabilization of type I collagen mRNAs. |
| P58775 | Tropomyosin beta chain (Beta- | Tpm2 | 26 | 69 | <0.001 | Binds to actin filaments in muscle and non-muscle cells. Plays a central role in calcium dependent striated muscle contraction. Smooth muscle |

| | | | | | | |
|---|---|---|---|---|---|---|
| | tropomyosin) (Tropomyosin-2) | | | | | contraction is regulated by interaction with caldesmon. In non-muscle cells is implicated in stabilizing cytoskeleton actin filaments. The non-muscle isoform may have a role in agonist-mediated receptor internalization. |
| **Reproduction specific** | | | | | | |
| Q5XIV1 | Phosphoglycerate kinase | Pgk2 | 100 | 118 | 0.017 | Flagellated sperm motility, glycolytic process |
| P12020 | Cysteine-rich secretory protein 1 (32 kDa epididymal protein) (Acidic epididymal glycoprotein)) | Crisp1 Aeg | 100 | 151 | <0.001 | Supposedly helps spermatozoa undergo functional maturation while they move from the testis to the ductus deferens. |
| P0DP29 | Calmodulin-1 | Cam1, Cam2, Cam3 | 37 | 48 | 0.027 | Mediates the control of a large number of enzymes, ion channels, aquaporins and other proteins through calcium-binding. Stimulate protein kinases and phosphatases in a calcium-dependant mechanism. Together with centrin, is involved in a genetic pathway that regulates the centrosome cycle and progression through cytokinesis. Positively regulates calcium-activated potassium channel activity. |
| P04182 | Ornithine aminotransferase, mitochondrial (Ornithine--oxo-acid aminotransferase) | Oat | 32 | 44 | 0.012 | Expressed in the head and flagellum of epididymal sperm but not in testicular sperm (at protein level) .Amino-acid biosynthesis; L-proline biosynthesis; L-glutamate 5-semialdehyde from L-ornithine. |
| P14668 | Annexin A5 (Anchorin CII) (Endonexin II) (Lipocortin V) (Placental | Anxa5 Anx5 | 17 | 25 | 0.049 | An anticoagulant protein which acts as an indirect inhibitor of a thromboplastin-specific complex, which is involved in the blood coagulation cascade. |

| | | | | | | |
|---|---|---|---|---|---|---|
| | anticoagulant protein 4) (PP4)) | | | | | |
| P81155 | Voltage-dependent anion-selective channel protein 2 (VDAC-2) (Outer mitochondrial membrane protein porin 2) | Vdac2 | 14 | 6 | 0.020 | Expressed in the head region of epididymal sperm. Forms a channel through the mitochondrial outer membrane that allows diffusion of small hydrophilic molecules. |
| **Antioxidant/pro-oxidant** | | | | | | |
| P04904 | Glutathione S-transferase alpha-3 (Glutathione S-transferase Yc-1) (GST Yc1) | Gsta3 Gstyc1 | 60 | 83 | <0.001 | Conjugates reduced glutathione (GSH) to exogenous and endogenous hydrophobic electrophiles to detoxify the system. |
| O35244 | Peroxiredoxin-6 (Acidic calcium-independent phospholipase A2) (Antioxidant protein 2) | Prdx6 Aipla2 Aop2 Tsa | 36 | 50 | 0.006 | Plays a role in cell protection against oxidative stress by detoxifying peroxides and in phospholipid homeostasis. Catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively. Can reduce H2O2 and short chain organic, fatty acid, and phospholipid hydroperoxides. Also has phospholipase activity. |
| P07632 | Superoxide dismutase [Cu-Zn] | Sod1 | 13 | 29 | <0.001 | Destroys radicals which are normally produced within the cells and which are toxic to biological systems. |
| Q63716 | Peroxiredoxin-1 (HBP23) (Heme-binding 23 kDa protein) (Thioredoxin peroxidase 2) | Prdx1 Tdpx2 | 22 | 32 | 0.015 | Thiol-specific peroxidase that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively. Plays a role in cell protection against oxidative stress by detoxifying peroxides and as sensor of hydrogen peroxide-mediated signaling events. Might participate in the signaling cascades of growth factors and tumor |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | necrosis factor-alpha by regulating the intracellular concentrations of H2O2 |
| Q64057 | Alpha-aminoadipic semialdehyde dehydrogenase (Alpha-AASA dehydrogenase) (Aldehyde dehydrogenase family 7 member A1) | Aldh7a1 Ald7a1 | 22 | 12 | 0.019 | Protects cells from oxidative stress by metabolizing a number of lipid peroxidation-derived aldehydes. Involved in lysine catabolism Multifunctional enzyme mediating important protective effects. |
| Q6AXX6 | Redox-regulatory protein FAM213A (Peroxiredoxin-like 2 activated in M-CSF stimulated monocytes) (Protein PAMM) (Sperm head protein 1) | Fam213a Pamm Shp1 | 12 | 19 | 0.041 | Involved in redox regulation of the cell. Acts as an antioxidant. Inhibits macrophage production of inflammatory cytokines, probably through suppression of the MAPK signaling pathway. Expressed by the principal cells of the epididymis. Detected in the head region of epididymal sperm (at protein level) |
| **Regulatory** | | | | | | |
| P02770 | Serum albumin | Alb | 449 | 536 | <0.001 | A protein with a binding capacity for water, plams ions, fatty acids, hormones, bilirubin and drugs. A major zinc transporter (±80%) in plasma. |
| P31044 | Phosphatidylethanolamine-binding protein 1 (PEBP-1) | Pebp1 Pbp Pebp | 264 | 311 | <0.001 | Binds ATP, opioids and phosphatidylethanolamine. Serine protease inhibitor which inhibits thrombin, neuropsin and chymotrypsin. Inhibits the kinase activity of RAF1 by inhibiting its activation and dissociating the RAF1/MEK complex. Spermatid development; sperm capacitation. |

| | | | | | | |
|---|---|---|---|---|---|---|
| P12346 | Serotransferrin (Transferrin) (Beta-1 metal-binding globulin) | Tf | 113 | 132 | 0.013 | Iron binding transport proteins which bind two Fe(3+) ions in association with an anion, usually bicarbonate. Transports iron atoms from sites of absorption and heme degradation to sites of storage and utilization. |
| Q66HD0 | Endoplasmin (94 kDa glucose-regulated protein) (GRP-94) (Heat shock protein 90 kDa beta member 1) | Hsp90b1 Grp94 Tra1 | 72 | 89 | 0.012 | Molecular chaperone - processes and transports secreted proteins. Involved in endoplasmic reticulum associated degradation. Has ATPase activity.<br><br>Acute-phase response |
| P06761 | Endoplasmic reticulum chaperone BiP (78 kDa glucose-regulated protein) (GRP-78) (Binding-immunoglobulin protein) (BiP) (Heat shock protein family A member 5) | Hspa5 Grp78 | 84 | 111 | <0.001 | Endoplasmic reticulum chaperone. Necessary for correct folding of proteins and degradation of misfolded proteins.<br><br>Cellular response to glucose starvation, cellular response to interleukin-4 |
| F7FJQ3 | NPC intracellular cholesterol transporter 2 | Npc2 | 43 | 55 | 0.026 | Cholesterol efflux, cholesterol homeostasis, intracellular cholesterol transport |
| **Immune-mediated** | | | | | | |
| P14740 | Dipeptidyl peptidase 4 (GP110 glycoprotein) (T- | Dpp4 Cd26 | 9 | 3 | 0.004 | Cell surface glycoprotein receptor involved in costimulation signal essential for T-cell receptor (TCR)-mediated T-cell activation. Acts as a positive regulator of T-cell coactivation. Induces T-cell proliferation and NF-kappa-B activation in a T-cell receptor/CD3-dependent manner. |

| | | | | | | |
|---|---|---|---|---|---|---|
| | cell activation antigen CD26) | | | | | Regulates lymphocyte-epithelial cell adhesion. May be involved in the promotion of lymphatic endothelial cells adhesion, migration and tube formation. When overexpressed, enhances cell proliferation. Also regulates various physiological processes by cleaving peptides in the circulation, including many chemokines, mitogenic growth factors, neuropeptides and peptide hormones.<br><br>Detected in the serum and the seminal fluid |
| Q5PQW8 | Guanylate binding protein 2 (Guanylate nucleotide binding protein 2) (Guanylate-binding protein 1) | Gbp2 | 4 | 1 | 0.049 | Hydrolyzes GTP to GMP in 2 consecutive cleavage reactions, but the major reaction product is GDP. Promotes oxidative killing and deliver antimicrobial peptides to autophagolysosomes, providing broad host protection against different pathogen classes |
| Q63663 | Guanylate-binding protein 1 (GTP-binding protein 2) (GBP-2) (Interferon-induced guanylate-binding protein 2) (p67) | | | | | |
| B2RZC6 | Ilf2 protein (Interleukin enhancer-binding factor 2) | Ilf2 | 3 | 0 | 0.008 | Immune response |
| **Multi-functional** | | | | | | |
| P18418 | Calreticulin (CALBP) (CRP55) | Calr | 61 | 74 | 0.03557251 7069555 | Calcium-binding chaperone that promotes folding, oligomeric assembly and quality control in the endoplasmic reticulum (ER) via the |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (Calcium-binding protein 3) | | | | | calreticulin/calnexin cycle. This lectin interacts transiently with almost all of the monoglucosylated glycoproteins that are synthesized in the ER. |
| | | | | | | Involved in maternal gene expression regulation. May participate in oocyte maturation via the regulation of calcium homeostasis. |
| P46462 | Transitional endoplasmic reticulum ATPase (TER ATPase) (Valosin-containing protein) (VCP) | Vcp | 53 | 74 | <0.001 | Fragmentation and reassembly of Golgi stacks during and after mitosis. Is part of a complex (UFD1, VCP and NPLOC4) that binds ubiquitinated proteins and exports misfolded proteins from the ER to the cytoplasm to be degraded by the proteasome. The complex also regulates disassembly of spindles at the end of mitosis and is essential for the formation of a closed nuclear envelope. Involved in a stress-induced mechanism that selectively regulates the translocation of stressed/damaged mitochondrial outer-membrane proteins to be degraded by proteasomes. Essential for the clearance of ubiquitinated proteins via autophagy. May play a role in the ubiquitin-dependent sorting of membrane proteins to lysosomes where they undergo degradation. Key role in caveolin sorting within cells. |
| | | | | | | Involved in the DNA damage response and is recruitment to double-strand breaks |
| | | | | | | Controls the steady-state expression of the IGF1R receptor |
| P04785 | Protein disulfide-isomerase (PDI) (Cellular thyroid hormone-binding protein) (Prolyl 4-hydroxylase subunit beta) | P4hb Pdia1 | 37 | 72 | <0.001 | Catalyzes the formation, breakage and rearrangement of disulfide bonds. At the cell surface, seems to cleave disulfide bonds of proteins attached to the cell. May therefore cause structural modifications of proteins. Inside the cell, seems to form/rearrange disulfide bonds of new proteins. At high concentrations, functions as a chaperone that inhibits aggregation of misfolded proteins. At low concentrations, facilitates aggregation (anti-chaperone activity). Shows receptor activity, where activation increases disulfide reductase activity at the plasma membrane, altering the plasma membrane redox state and enhancing cell migration. |
| | | | | | | May be involved with other chaperones in the structural modification of the TG precursor in hormone biogenesis. |
| | | | | | | Also acts on structural subunits of various enzymes such as prolyl 4-hydroxylase and microsomal triacylglycerol transfer protein MTTP. |

| | | | | | | |
|---|---|---|---|---|---|---|
| O88767 | Protein/nucleic acid deglycase DJ-1 (Contraception-associated protein 1) (Protein CAP1) (Fertility protein SP22) | Park7 Cap1 | 31 | 57 | <0.001 | Functions as a protein deglycase that glycated proteins, releasing repaired proteins, lactate or glycolate, respectively. Deglycates cysteine, arginine and lysine residues in proteins, and thus reactivates these proteins by reversing glycation by glyoxals. Acts on early glycation intermediates (hemithioacetals and aminocarbinols), preventing the formation of advanced glycation endproducts (AGE) that cause irreversible damage. Also functions as a nucleotide deglycase able to repair glycated guanine in the free nucleotide pool (GTP, GDP, GMP, dGTP) and in DNA and RNA. Is thus involved in a major nucleotide repair system named guanine glycation repair (GG repair), dedicated to reversing methylglyoxal and glyoxal damage via nucleotide sanitization and direct nucleic acid repair. |
| | | | | | | Plays an important role in cell protection against oxidative stress and cell death acting as an oxidative stress sensor and redox-sensitive chaperone and protease |
| | | | | | | It is involved in neuroprotective mechanisms. Regulates astrocyte inflammatory responses |
| P38659 | Protein disulfide-isomerase A4 (Calcium-binding protein 2) (CaBP2) (Endoplasmic reticulum resident protein 70) (ERp70) | Pdia4 Cabp2 Erp70 | 22 | 32 | 0.01757223 12589536 | Part of a large chaperone multiprotein complex. Involved in cell redox homeostasis; chaperone-mediated protein folding; response to endoplasmic reticulum stress |

**Table 4.6: Annotation of the most abundant significantly expressed spermatozoal proteins of each functional category performed using Uniprot**

| Accession number | Gene name | Protein name | Control Mean | Diet Mean | Adjusted p value | Function |
|---|---|---|---|---|---|---|
| **Metabolic** | | | | | | |
| P18163 | Acyl CoA Synthase (Long-chain-fatty-acid--CoA ligase 1) | Acsl1 Acs1 Acsl2 Facl2 | 87 | 79 | 0.049 | Initiates fatty acid utilization by producing acyl CoA synthase from fatty acids. Results in the production of free fatty acids. Activation of long-chain fatty acids for both synthesis of cellular lipids, and degradation via beta-oxidation. Activated by Adiponectin. |
| Q68FY0 | Cytochrome b-c1 complex subunit 1, mitochondrial (Complex III subunit 1) (Core protein I) (Ubiquinol-cytochrome-c reductase complex core protein 1) | Uqcrc1 | 16 | 33 | 0.005 | This is a component of the ubiquinol-cytochrome c reductase complex, which is part of the mitochondrial respiratory chain. This protein may mediate formation of the complex between cytochromes c and c1. |
| Q66HF1 | NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial | Ndufs1 | 8 | 19 | 0.004 | Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed to belong to the minimal assembly required for catalysis. Complex I s in the transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone (By similarity). This is the largest subunit of complex I and it is a component of the iron-sulfur (IP) fragment of the enzyme. It may form part of the active site crevice where NADH is oxidized. |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q06437 | Pyruvate dehydrogenase E1 component subunit alpha, testis-specific form, mitochondrial (PDHE1-A type II) | Pdha2 | 6 | 18 | 0.001 | The pyruvate dehydrogenase complex catalyzes the overall conversion of pyruvate to acetyl-CoA and CO(2), and thereby links the glycolytic pathway to the tricarboxylic cycle.<br><br>Testis specific isoform |
| Q99NA5 | Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial (Isocitric dehydrogenase subunit alpha) (NAD(+)-specific ICDH subunit alpha) | Idh3a | 6 | 15 | 0.013 | Catalytic subunit of the enzyme which catalyzes the decarboxylation of isocitrate (ICT) into alpha-ketoglutarate. The heterodimer composed of the alpha (IDH3A) and beta (IDH3B) subunits and the heterodimer composed of the alpha (IDH3A) and gamma (IDH3G) subunits, have considerable basal activity but the full activity of the heterotetramer (containing two subunits of IDH3A, one of IDH3B and one of IDH3G) requires the assembly and cooperative of both heterodimers. |
| P29410 | Adenylate kinase 2, mitochondrial (AK 2) (ATP-AMP transphosphorylase 2) (ATP:AMP phosphotransferase) (Adenylate monophosphate kinase) | Ak2 | 5 | 12 | 0.037 | Catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP. Plays an important role in cellular energy homeostasis and in adenine nucleotide metabolism. Adenylate kinase activity is critical for regulation of the phosphate utilization and the AMP de novo biosynthesis pathways. Plays a key role in hematopoiesis. |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Structural** | | | | | | |
| M0R8B6 | Tubulin beta chain | Tubb1 | 38 | 26 | 0.003 | Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain. |
| P08733 | Myosin regulatory light chain 2 (MLC-2) (MLC-2v) (Myosin light chain 2) | Myl2 Mlc2 | 5 | 22 | <0.001 | Contractile protein that plays a role in heart development. Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to actin binding sites to sustain thin filament activation as a means to fine-tune myofilament calcium sensitivity to force. During cardiogenesis plays an early role in cardiac contractility by promoting cardiac myofibril assembly. |
| D4A0A0 | Dynein light chain roadblock | Dynlrb2 | 16 | 10 | 0.044 | Acts as one of several non-catalytic accessory components of the cytoplasmic dynein 1 complex that are thought to be involved in linking dynein to cargos and to adapter proteins that regulate dynein . Cytoplasmic dynein 1 acts as a motor for the intracellular retrograde motility of vesicles and organelles along microtubules. |
| Q6AXV2 | Tektin-4 | Tekt4 Tek4 | 4 | 12 | 0.020 | Structural component of ciliary and flagellar microtubules. Forms filamentous polymers in the walls of ciliary and flagellar microtubules. |
| **Reproduction specific** | | | | | | |
| Q6AYW7 | Capping protein (Actin filament) muscle Z-line, alpha 3 (F-actin-capping protein subunit alpha-3) | Capza3 | 1 | 5 | 0.011 | F-actin-capping proteins bind in a Ca(2+)-independent manner to the fast growing ends of actin filaments (barbed end) thereby blocking the exchange of subunits at these ends. Unlike other capping proteins (such as gelsolin and severin), these proteins do not sever actin filaments. |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Regulatory** | | | | | | |
| Q6AXN7 | 5'-nucleotidase, cytosolic IB | Nt5c1b | 170 | 157 | 0.003 | Adenosine metabolic process |
| P02091 | Hemoglobin subunit beta-1 (Beta-1-globin) (Hemoglobin beta chain, major-form) (Hemoglobin beta-1 chain) | Hbb | 70 | 62 | 0.049 | Involved in oxygen transport from the lung to the various peripheral tissues. |
| P63039 | 60 kDa heat shock protein, mitochondrial (60 kDa chaperonin) (Chaperonin 60) (Heat shock protein 60) | Hspd1 Hsp60 | 16 | 29 | 0.050 | Chaperonin implicated in mitochondrial protein import and macromolecular assembly. Together with Hsp10, facilitates the correct folding of imported proteins. May also prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix. |
| Q6PDU6 | Hbb-b1 MGC72973 | Beta-glo | 25 | 18 | 0.049 | Hemoglobin complex. Glutathione metabolic process, oxygen transport |
| Q68FS4 | Cytosol aminopeptidase (Leucine aminopeptidase 3) (LAP-3) | Lap3 | 3 | 14 | <0.001 | Presumably involved in the processing and regular turnover of intracellular proteins. Catalyzes the removal of unsubstituted N-terminal amino acids from various peptides |
| D4A9P2 | RGD1563714 | Armh1 | 4 | 11 | 0.046 | |

| **Multifunctional** | | | | | | |
|---|---|---|---|---|---|---|
| Q6IMF3 | Krt1 Kb1 | Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1) (K1) (Type-II keratin Kb1) | 6 | 2 | 0.008 | May regulate the activity of kinases such as PKC and SRC via binding to integrin beta-1 (ITB1) and the receptor of activated protein C kinase 1 (RACK1). In complex with C1QBP is a high affinity receptor for kininogen-1/HMWK. |

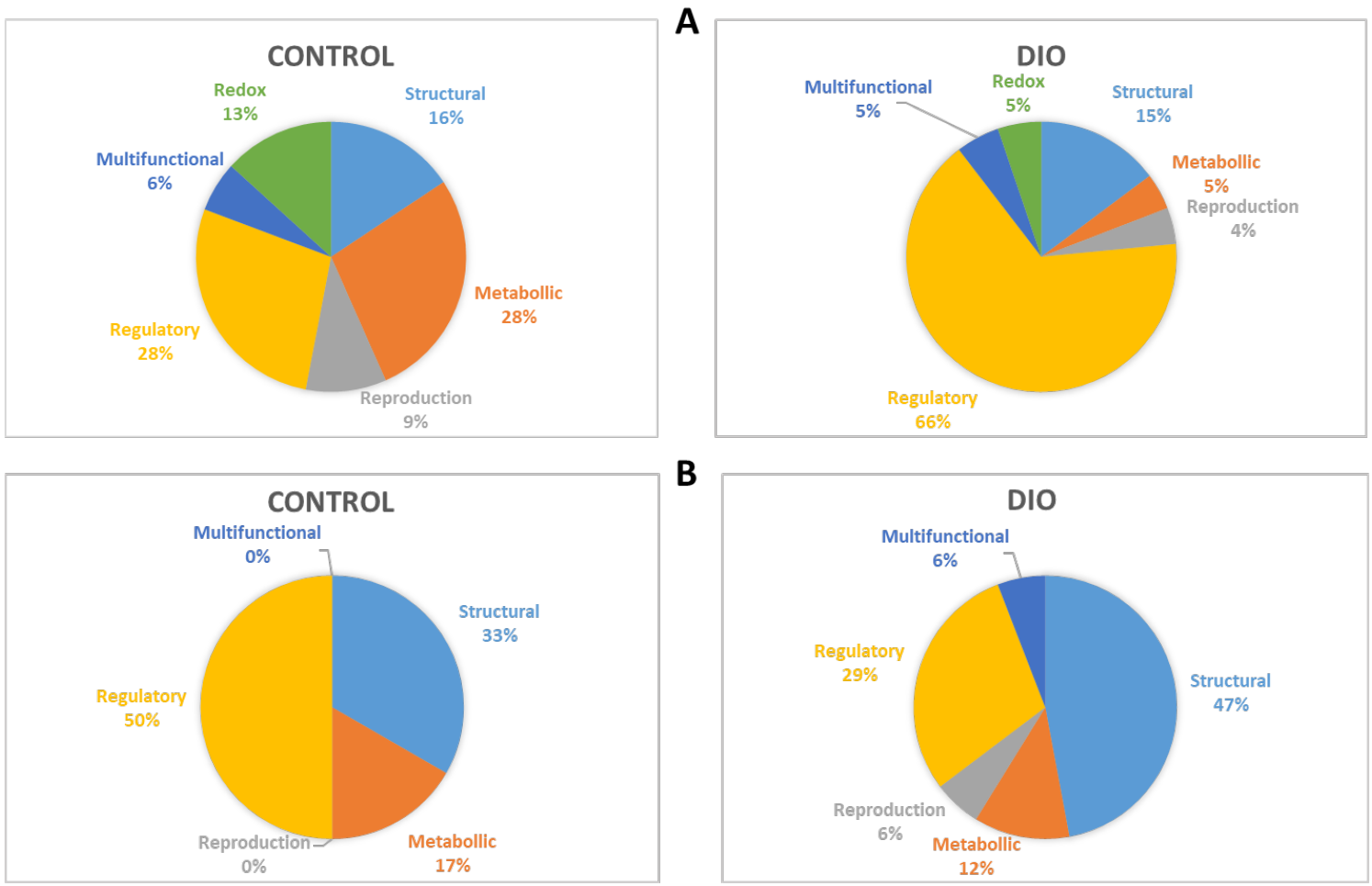



**Figure 4.8:** Pie charts representing the percentage of overexpression in each functional category for (A) epididymal and (B) sperm proteins. Regulatory proteins as a category are seen to have consistently high abundance in both the epididymis and sperm. Metabolic proteins are the most abudant in the control groups, with structural proteins being the highest percentage of proteins being affected in the DIO group.

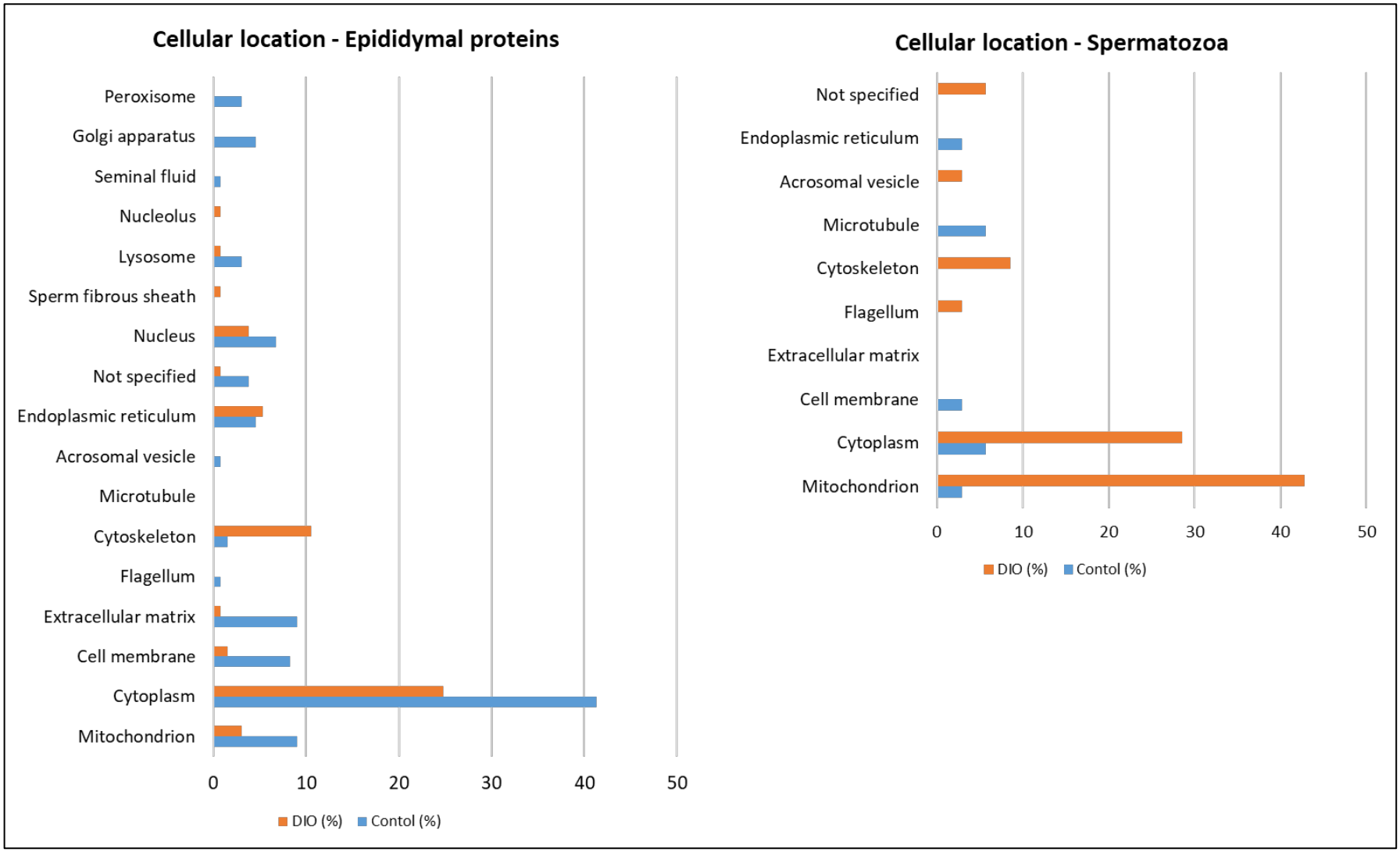



**Figure 4.9:** Bar charts visually illustrating and comparing the overexpression of proteins in the epididymis and sperm based on cellular location. Proteins from the cytoplasm of epididymal cells were the most overly expressed, while proteins from both the cytoplasm and mitochondria were most affected in the sperm.

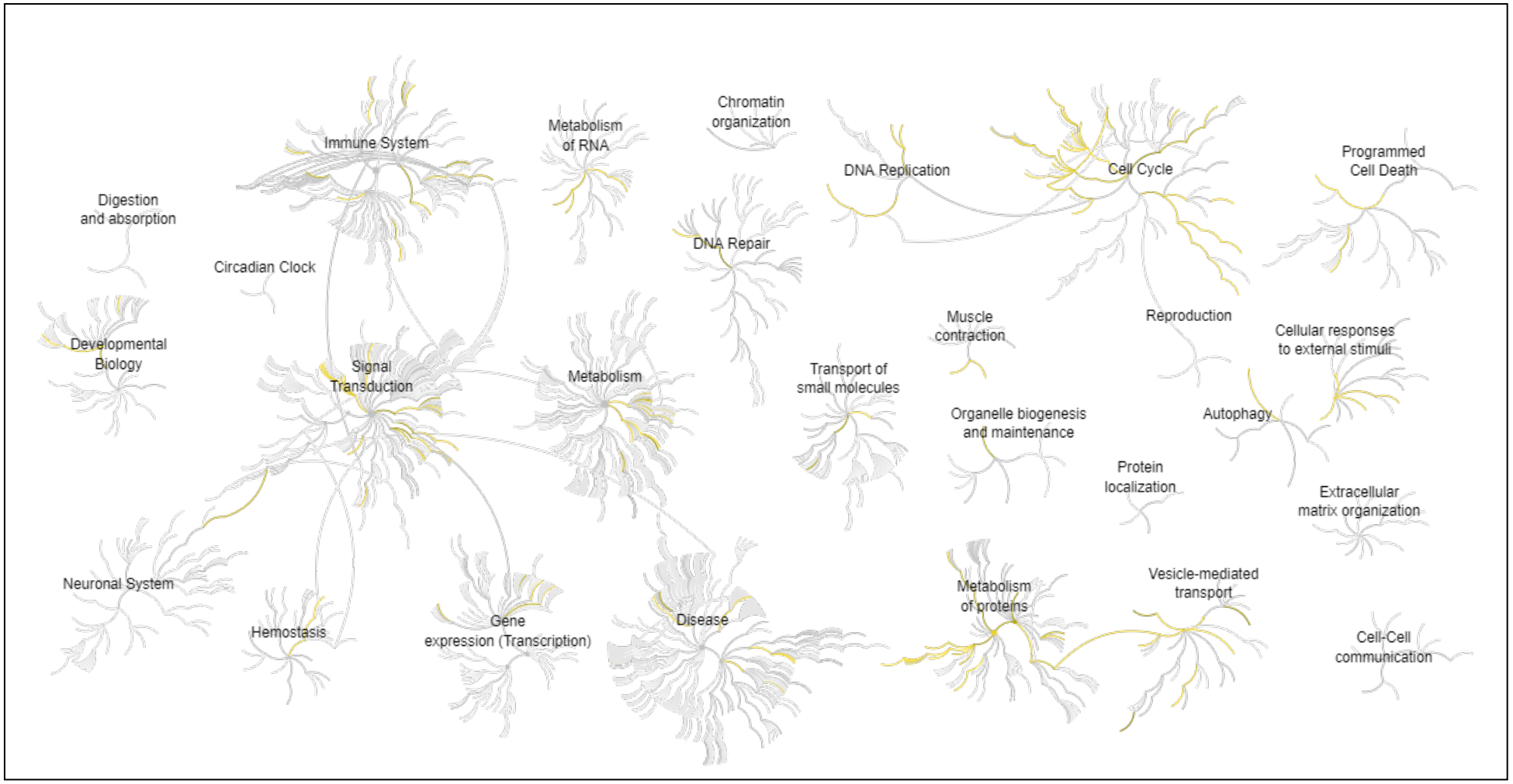



**Figure 4.10:** A schematic diagram showing protein-protein interactions of epididymal proteins grouped according to function. The pathways in yellow are the ones in which the abundantly expressed proteins are involved. Expression profile image created in Reactome (Jassal *et al*., 2019). Several pathways are affected including proteins from the immune system, signal transduction, metabolism and cell cycle.

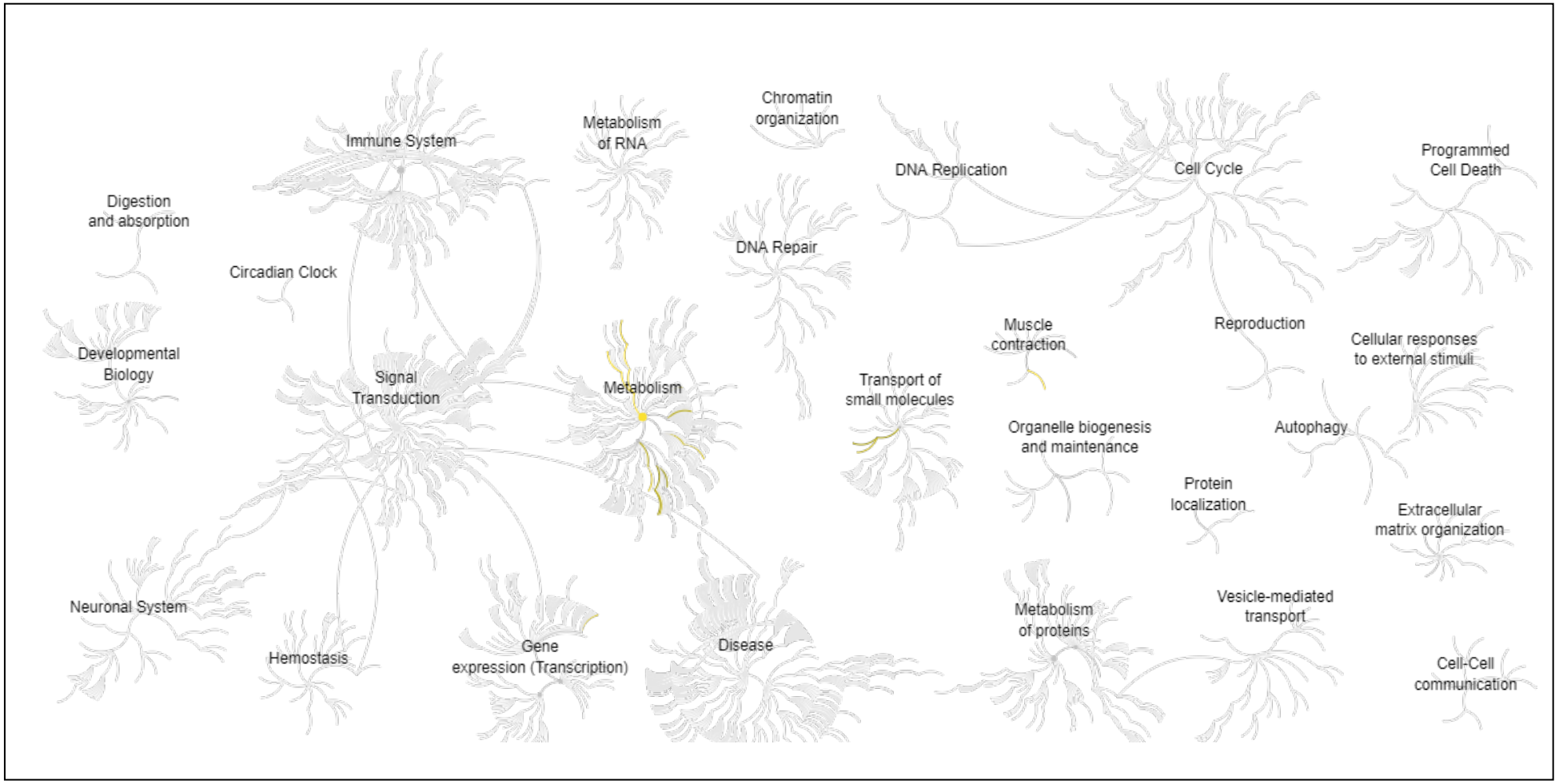



**Figure 4.12:** A schematic diagram showing protein-protein interactions of sperm proteins grouped according to function. The pathways in yellow are the ones in which the abundantly expressed proteins are involved. Expression profile image created in Reactome (Jassal *et al*., 2019). An emphasis on metabolic, transport and contractile protein pathways is evident, with proteins from these pathways showing the highest change in expression.

# Chapter 5: Discussion

This chapter is divided into three sections in order to discuss the various study objectives respectively. The first section describes the study model and the attainment of obesity by the study animals. In the second section, the results from study will be discussed. The implications of these results will be assessed and compared to findings and interactions obtained by other models of DIO. The results of the current study will thereafter be discussed in combination with the proteomics data obtained in order to assess the contribution of the significant proteins to the observed anthropometric, macroscopic, microscopic and molecular results. The third section will summarize the proteomics results and discuss the functional networks affected by the DIO model, especially in the context of male fertility. The associations and interactions between the study variables are summarised in Figures 5.1 and 5.5 – 5.9.

## 5.1. Achievement of obesity

Obesity is a multifaceted disorder with numerous factors contributing towards its manifestation. Despite this, its current rate of incidence suggests that behavioural and environmental factors have been significant contributors to the current epidemic, as genetic factors can only account for a limited number of cases (Marques *et al.*, 2016). Polygenetic, and not monogenetic, animal models of obesity are therefore preferred as DIO represents the predominant mode of obesity development in humans (Rosini, Ramos da Silva and de Moraes, 2012). The presence of multiple genetic mutations in individuals does not necessarily lead to the onset of obesity but confers a predisposition to the disorder which can be brought to the fore by lifestyle habits and the environment. The environment alone contributes to the inherent ability of an individual to develop obesity (de Castro, 2004; Karnehed *et al.*, 2006). Furthermore, the induction of obesity via diet and a sedentary lifestyle provides similar metabolic responses in animals as those observed in humans. Therefore, diet-induced obesity animal models are useful in mimicking the pathophysiology of vascular and metabolic complications, which have been shown to manifest during obesity (Rosini, Ramos da Silva and de Moraes, 2012).

Maintaining laboratory rats on a modified *ad libitum* diet is a widely applied approach to inducing weight gain in these animals. The consumption of large portions of high calorie-containing foods as well as frequent feeding due to its greater availability replicates the predominant mechanism of obesity development in humans. Keeping animals in cages with no exercise for the duration of the experiment promotes sedentary behaviour, and also mimics the two predominant causes of obesity in humans (Hill *et al.*, 1984). Altering the palatability and/or calories of food with the addition of sugars, polysaccharides, fats and calorie-rich foods causes hyperphagia and thus increases the likelihood of animals developing obesity (Sclafani and Springer, 1976; Rolls, Rowe and Turner,

1980; N. J. Rothwell, Saville and Stock, 1982). In the current study, supplementation of the DIO diet with condensed milk and lard (Holsum cooking fat) increased the palatability and caloric content of the food to further stimulate the induction of obesity. Furthermore, longer diets have been shown to be more representative of the long-term development of obesity and are more effective in inducing obesity in animals. A long-term diet was therefore used in the current study, where a control or hyperphagic (DIO) diet was provided to ± 6 week old rats for 54 weeks to obtain significant increases in body weight and a higher likelihood of obese animals in the DIO group.

The chronic diet elevated body and visceral fat weights for both control and DIO rats, with DIO rats having significantly higher body and visceral fat weights compared to controls. In comparison to shorter feeding protocols used to induce obesity (I. M. Faust *et al.*, 1978; Farley *et al.*, 2003; Nascimento *et al.*, 2008; Olivares *et al.*, 2010; Bakos *et al.*, 2011b; Saravanan *et al.*, 2014), the control rats in this study were considerably larger. This is due to the diet duration and the age of the animals at the end of the experimental protocol. Although not a measured variable in this study, age is associated with changes in adiposity as it leads to alterations in body composition. These include reductions in muscle mass, increased visceral fat and decreased fat free mass (Wannamethee *et al.*, 2007). This is a generally observed phenomenon in humans, as older males tend to have higher body weights compared to younger males. The resultant body weights are an advantage for the current study as they better represent the body composition of adult males. Nonetheless, the DIO rats had a significantly higher body weight and visceral fat weight compared to controls.

The AI was used in this study as a measure of obesity. Several studies have correlated the AI with the Lee index (Lee, 1929), a reliable measure of body fat content (Bernardis and Patterson, 1968; Bernardis, 1970; Kanarek and Marks-Kaufman, 1979; Rogers and Webb, 1980). In the current study, the AI increased to ranges well above normal (Leopoldo *et al.*, 2016), with the obese group exhibiting significantly higher adiposity compared to controls.

Unfasted blood glucose remained within normal ranges (3.3-7.2 mmol/l) (Kohn and Clifford, 2002; Ringler and Dabich, 2013) for both the control and obese animals, which is consistent with the hyperphagia inducing diet described by our colleagues (Salie, Huisamen and Lochner, 2014), where the diet caused a small, but insignificant increase in blood glucose concentrations. The rats were, similarly, only pre-diabetic and did not develop diabetes. The model used in this study is therefore a model of obesity and does not test the effects of hyperglycaemia. The current study found unfasted blood glucose to correlate with visceral fat weight, as animals with higher visceral fat weights possessed higher blood glucose levels, an observation corroborated by Du Toit *et al.* (2005) and Salie *et al.* (2014).

The glycaemic status of this study's animals is in agreement with (and therefore reinforces) the results from experiments carried out by Westermark and colleagues which showed that dietary intervention in rats does not lead to type II diabetes and therefore hyperglycaemia (Westermark *et al.*, 1986, 1987). This has been attributed to a lack of amyloid deposition in rat pancreatic β-cells by the amyloid producing peptide, islet amyloid polypeptide (IAAP), a deposition normally observed to occur in type II diabetes.

IAAP is a peptide located within β-cells, specifically within the islets of Langerhans (Westermark *et al.*, 1987). It is co-stored with insulin within the secretory granules of β-cells (Lukinius *et al.*, 1989) in humans and other mammals, including the rat (Asai *et al.*, 1990). This peptide is secreted together with insulin upon glucose stimulation (Mitsukawa *et al.*, 1990). IAAP is similar to calcitonin gene related peptide (CGRP), a known inhibitor of insulin secretion (Cooper et al., 1987). It therefore has the ability to inhibit insulin-stimulated glucose uptake while also suppressing glucose output from the liver (Molina et al., 1990), thereby causing hyperglycaemia (Molina et al., 1990). In humans, IAAP is secreted in obese individuals and amyloid is deposited into pancreatic β-cells. This causes insulin secretion to be inhibited in moments when insulin is required (i.e. after a meal, when glucose levels typically rise), which hampers insulin-stimulated glucose uptake. This results in glucose not being absorbed from the blood stream, leading to hyperglycaemia. Due to species variation, the human IAAP amino acid contains an amyloidogenic sequence whereas rat IAAP does not. As a result, rat IAAP cannot cause the deposition of amyloid into pancreatic β-cells (Asai *et al.*, 1989; Betsholtz, Christmansson, *et al.*, 1989; Betsholtz, Svensson, *et al.*, 1989; Stridsberg and Wilander, 1991). Glucose uptake is therefore not hampered in rats as it is in humans. The current study was partly able to show that this phenomenon is also evident in long-term obesity as a longer dietary intervention did not lead to hyperglycaemia and therefore type II diabetes in our model.

## 5.2. Effect of the long-term diet on male fertility

### 5.2.1. The effect of diet on testosterone, estrogen and other hormones

One of the interesting observations of this study is that chronic obesity did not affect testosterone concentrations, but rather caused supraphysiological estradiol concentrations. In addition, the diet led to higher visceral fat which, together with supraphysiological estradiol, correlated significantly with changes in sperm morphology, specifically a reduction in sperm head sizes. A correlation between serum estradiol levels and sperm morphology was also observed in a study by Müller *et al*. (2012) where increased estradiol concentrations were associated with a lower percentage of normal sperm. Likewise, Marchiani *et al*. (2015) provided a high fat diet to study animals and observed increases in visceral fat, estradiol concentrations and reduced normal sperm

morphology. In most cases, an increase in estradiol in human and animal models is linked to the aromatization of peripheral androgens into estrogens due to an increase in aromatase precipitated by increased body fat (Du Plessis *et al.*, 2010). As estrogen receptors are present in the testis, epididymis and sperm (Hess, 2003), and take part in spermatogenesis, sperm maturation, motility, capacitation, acrosome reaction and sperm fertilizing capacity (O'Donnell *et al.*, 2001; Guido *et al.*, 2011), changes in estrogen concentrations are likely to have an effect on sperm structure and function. The effect of estradiol on male physiology are further elaborated later in the text.

Regarding sperm parameters, smaller testes were significantly associated with reduced sperm viability irrespective of the DIO testis weight not being significantly lower compared to controls. Furthermore, the observed increase in visceral fat significantly lowered sperm morphology.

The haematocrit of the DIO animals was significantly reduced compared to controls, indicating a decreased percentage of circulating erythrocytes. Other studies have reported similar findings, where significant reductions in haematocrit were found in overweight and obese subjects (Tungtrongchitr *et al.*, 2001; Ferreira *et al.*, 2013) and in rats placed on diets high in fat (Fitzgerald *et al.*, 2001; Bertinato *et al.*, 2014). The current study found haematocrit (%) to be positively associated with testis size (g), a change also observed in an experiment by Hamilton and his co-investigators (1964). Studies have linked larger testes to higher production of testosterone (Berndtson and Jones, 1989; Kaplan and Mead, 1993; Garamszegi *et al.*, 2005; Preston *et al.*, 2012). Testosterone is a known stimulator of erythropoietin (Shahidi, 1973; Shahani *et al.*, 2009), a glycoprotein hormone produced by the kidneys. Erythropoietin stimulates erythrocyte precursors in the bone marrow, resulting in the increased production of red blood cells (Takeuchi and Kobata, 1991). As haematocrit is quantified as the percentage of red blood cells within the blood, the number of red blood cells stimulated by production of erythropoietin will have an effect on the haematocrit measurement. This mechanism explains the observed association.

The testosterone results observed in this study were contrary to those found in shorter term diets (Cattaneo et al., 1996; Wang et al., 2005; Vigueras-Villaseñor et al., 2011) but similar to that of a long-term diet study by Fernandez et al. (2011), possibly as an adaptive response to chronically low testosterone concentrations which are compensated over time. Similar to compensated hypogonadism, normal testosterone concentrations can be observed despite reductions in testis size (Tajar et al., 2010; Giannetta et al., 2012). This is attributed to possible readjustments to the HPG axis set-point to counteract deficits in testicular function or defects in the testosterone feedback mechanism at the hypothalamic-pituitary level (Liu et al., 2006). While the levels of testosterone in this study did not differ significantly compared to controls, a downward trend was observed in the obese animals. The testosterone concentrations correlated negatively with the AI. Furthermore, significant correlations show the rats with lower testosterone concentrations exhibited

lower haematocrit percentages and relatively smaller testis sizes. The importance of these findings is the consequence of a higher visceral fat mass on gonad size and hormone concentrations. Hormone concentrations are essential for gonad size and spermatogenesis, and a reduction in hormones such as testosterone can affect the volume of the seminiferous tubules, testicular interstitium and the number and structure of Leydig and Sertoli cells within the testes, all of which are necessary for proper spermatogenesis and reproductive function (Mendis-Handagama, Zirkin and Ewing, 1988; Berndtson and Jones, 1989). Furthermore, despite there not being a significant decrease in the testosterone concentrations of the current study, the slight reduction in testosterone combined with the increase in estradiol can impact spermatogenesis to some magnitude.

There was a tendency towards increased estradiol levels in DIO rats but these were not statistically significant. The concentrations found in the obese group were, however, higher than normal reference values, implying that chronic obesity can result in a hyperestrogenic state (Hemsell *et al.*, 1974; Schneider *et al.*, 1979, 1983). Similarly, several other studies have been able to correlate male obesity with elevated estrogen levels (Schneider *et al.*, 1979; Cohen, 1999; C. D. Fernandez *et al.*, 2011; Fan *et al.*, 2015). As these were short term diets, our study has shown that this change persists long-term. An increase in visceral fat stimulates estrogen production (Corona *et al.*, 2015), thereby disturbing the body's natural T:E ratio which is required for normal spermatogenesis (Bray, 1997; Pavlovich *et al.*, 2001; Raman and Schlegel, 2002) and optimal reproductive function. This is due to the release of aromatase. Aromatase is amplified by an increase in visceral fat, leading to an increased conversion of testosterone (aromatization) to estrogen (Cohen, 1999). High estrogen concentrations affect the hypothalamic-pituitary-gonadal (HPG) axis, thereby down-regulating the production of testosterone via a negative-feedback system (Wozniak *et al.*, 2009). The result is a reduction in testosterone, with even small changes in estrogen leading to low concentrations of this hormone. Male estrogen is vital for the regulation of several physiological functions including bone maturation and resorption, numerous brain functions and lipid metabolism (Vermeulen *et al.*, 2002). As estrogen receptors are highly expressed in the testes, changes in the level of this hormone could have a modulatory effect on the spermatogenic process (Heikinheimo *et al.*, 1995; Blanco-Rodríguez and Martínez-García, 1997).

### 5.2.2. The effect of diet on sperm morphology and histology of the testis and epididymis

Significantly lower percentages of morphologically normal sperm were observed in the DIO animals compared to controls. Sperm morphology is affected by testicular spermatogenic activity, which is directly linked to testicular size (Holstein, Schulze and Davidoff, 2003). With the high reactivity of male gonads to stress factors, increased rates of abnormal sperm production are a

reflection of negative stressors occurring within the body (Menkveld et al., 2011). Obesity can, therefore, affect sperm morphology severely due to the high susceptibility of testicular tissue to damage. In the present study, morphometric examination showed DIO spermatozoa to have significantly more head defects compared to controls. A high number of these sperm were borderline micro in size as characterised by van der Horst *et al*. (2018), a change found to negatively correlate with visceral fat weights and estradiol. Since the biological activity of estrogen is more potent than that of testosterone (Akingbemi et al., 2005), supraphysiological concentrations of estrogens are directly detrimental to spermatogenesis (Hammoud et al., 2006; du Plessis et al., 2010). Morphologically abnormal sperm are furthermore associated with DNA fragmentation, immature chromatin and abnormal number of chromosomes (Dadoune et al., 1988; Lee et al., 1996; Gandini et al., 2000; Devillard et al., 2002; Martin et al., 2003). Morphologically abnormal spermatozoa have a lower fertilizing capacity, leading to lower fertility rates and longer time to pregnancy.

The chronic hyperphagic diet reduced sperm viability significantly compared to controls. Sperm viability correlated positively with testicular weight, suggesting that the animals with larger testes produced more viable sperm.

As the absolute size of the testes was not significantly different between the two study groups, histological analysis of this organ was necessary to provide information on the microanatomical changes which may have been occurring within the testes of the obese subjects. The first observation is that the testes that are relatively smaller in size are likely to have smaller luminal diameters and higher relative interstitial volumes, according to the significant relationships observed in the current study. Second, the histopathological changes observed in the testes occurred to varying degrees. The most frequently observed histological pattern within the testicular tissue is a mosaic distribution of damage, where assessment of testicular histology at low magnification shows an array of damaged and normal seminiferous tubules (Figure 4.3). Damaged seminiferous tubules within the testes demonstrated significantly reduced tubular diameters. This tubular contraction could be the consequence of germ cell loss or a decline in tubular fluid secretion by the Sertoli cells, an androgen dependant process (Paniagua *et al.*, 1991; Stewart and Kim, 2011) . Seminiferous tubular fluid also maintains the size of tubular lumens (Sharpe *et al.*, 1994), which are significantly reduced in the diet group. Seminiferous epithelium was reduced in height; seminiferous tubules with a lower epithelial height were associated with higher levels of sperm head defects and the production of sperm with smaller heads. The damaged tubules showed degeneration of the pseudostratified epithelial layer (germ cell layer) evidenced by the significant decline in both the epithelial height and relative volume occupied by seminiferous epithelia. This compromises the testes' ability to adequately produce spermatozoa. The reduction in testicular epithelial height observed in this study has been negatively correlated with plasma

glucose levels, where a higher blood glucose was related to reductions in the epithelial height. Hyperglycaemia affects adenylate energy which is required for spermatogenesis (Amaral *et al.*, 2006). Moreover, glucose is required by the Sertoli cells to produce lactate, the primary substrate for ATP production in germ cells (Erkkilä *et al.*, 2002). Hyperglycaemia has been shown by previous studies to increase glycolysis, which in turn increases the NADH/NAD+ ratio, instigating a redox imbalance which increases the oxidation of tissue structures such as proteins and lipids (Ahmed, 2005). This can damage tissue structures and the correlation with glucose offers a mechanism for the observed reduction in testicular epithelia. Large vacuoles are evident between the germ cells at various depths of the seminiferous epithelium. Literature relates this to dilatations caused by degenerating germ cells being phagocytosed by Sertoli cells, giving rise to lipid droplets in Sertoli cell cytoplasm (Paniagua *et al.*, 1991). These are now visualized as patent spaces (Figure 4.3), as the fat droplets have been dissolved by the xylene during tissue fixation. Tubular vacuolation is also an early indicator of Sertoli cell injury (Creasy, 2002). The volume occupied by the testicular interstitium increased in the DIO group compared to controls. This is due to reductions in the seminiferous tubular diameters, giving a relative increase in interstitial tissue. Interstitial volume was negatively associated with relative testicular weights, implying that relatively smaller testes are likely to have a larger interstitial volume.

Microanatomic analysis of the epididymis showed no change in epithelial height and relative epithelial volume between the control and DIO group. Tubular diameter is reduced within the epididymides of DIO animals compared to controls, which points towards contraction of the epididymal tubules. Luminal diameter and relative luminal volume were expanded in the diet group compared to controls. This could be the result of reduced spermatogenesis and germ cell loss occurring within the testes. A greater luminal diameter and relative luminal volume is correlated with lower catalase activity in the epididymis. The resultant spermatozoa produced by the damaged tubules is likely to be morphologically abnormal and have reduced viability. This is correlated to high estradiol levels induced by the chronic diet. Furthermore, the male gonads are highly reactive to physiological stress, therefore the increased production of abnormal sperm is a reflection of negative stress factors within the body (Menkveld, Holleboom and Rhemrev, 2011).

The relative interstitial volume of DIO epididymides was enlarged in relation to the control epididymides. Literature points towards a possible edematous state that could exist within the interstitium as a result of an inflammatory process (Creasy, 2002) or an apparent increase in interstitium due to contraction of tubules. The current study has correlated the larger interstitial volume to lower catalase activity and less viable sperm.

### 5.2.3. Effect of diet on antioxidant capacity

Antioxidant activity was analysed to assess the capacity of the testes and epididymis to remove excess free radicals. The partial reduction of oxygen during metabolic processes gives rise to the production of radicals including $O_2^-$, $H_2O_2$, and the hydroxyl radical ($OH^•$) which in excess, may result in OS. This is a state where pro-oxidants (caused by ROS) overcome antioxidants' ability to reduce them, and has been implicated as a mechanism of damage in numerous conditions including obesity. Obesity produces OS via several mechanisms including mitochondrial and peroxisomal oxidation of fatty acids and the overconsumption of oxygen, resulting in ROS production via oxidation reactions and the generation of free radicals by the mitochondrial respiratory chain (Fernández-Sánchez *et al.*, 2011b). The current study measured the possible occurrence of OS within the study animals and assessed the possibility of oxidative damage within the reproductive organs. Assessment of the oxidative status of a system through the analysis of enzymatic antioxidant activity and the quantitation of cellular damage via the assessment of lipid peroxidation, etc., is necessary for the investigation of oxidative damage. As adipose tissue releases adipokines and cytokines, these can directly stimulate the production of free radicals (Fernández-Sánchez *et al.*, 2011b). These can also indirectly fuel ROS production by stimulating a low grade inflammatory state within obese individuals which, in turn, stimulates more ROS (Xu *et al.*, 2003).

The antioxidants typically analysed to assess antioxidant activity include CAT, SOD and glutathione. These are first line (CAT and SOD) and second line (GSH) antioxidants which neutralize and scavenge free radicals, respectively (Ighodaro and Akinloye, 2018). The current study monitored these antioxidants to assess the clearance of free radicals during the obese condition. CAT activity was lower in the testes of the obese animals, suggesting an inability to adequately remove $H_2O_2$ radicals from the system (Luck, 1965). An inability to reduce testicular $H_2O_2$ could imply an overabundance of this free radical, which can be attributed to the overproduction of ROS.

SOD is the most powerful enzymatic antioxidant (Ighodaro and Akinloye, 2018). As SOD is a metalloenzyme, a metal co-factor is required for its activity. Several metals are utilized by different forms of SOD, including iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu). This therefore creates 3 different isoforms which have different cellular locations. These are Fe-SOD which is predominant in prokaryotes and plant chloroplasts, Mn-SOD in prokaryotes and eukaryotic mitochondria, and Cu/Zn-SOD which is localized in the cytosol of eukaryotes as well as peroxisomes and chloroplasts. These are all encoded by different genes. (Fridovich, 1995; Dringen, Pawlowski and Hirrlinger, 2004; Gill and Tuteja, 2010; Karuppanapandian *et al.*, 2011). SOD activity was lower in the DIO group compared to controls, implying a decreased ability to

consume $O_2^-$ radicals. The significantly lowered CAT and SOD activities observed in the DIO testes are an indication of this tissue's reduced ability to remove access hydrogen peroxide ($H_2O_2$) and $O_2^-$ radicals, respectively. Lower antioxidant activity is a significant cause of cell injury and can cause damage to intracellular structures, which can ultimately lead to reduced cell function (Wiernsperger, 2003; King and Loeken, 2004; Suanarunsawat *et al.*, 2014).

GSH, an antioxidant highly abundant in aerobic cells, bodily fluids and tissue, protects cells and subcellular components from endogenous and exogenous ROS and reactive nitrogen species (RNS) (Rauhala, Andoh and Chiueh, 2005; Jozefczak *et al.*, 2012). GSH bears the brunt of an ROS attack by buffering high levels of free radicals (Meyer *et al.*, 2007) through its oxidation into a disulphide dimer, oxidised glutathione (GSSG) (Aquilano, Baldelli and Ciriolo, 2014). The loss of GSH via oxidative processes has been related to the pathophysiology of numerous disease processes (Ballatori *et al.*, 2009), including obesity (Cabler *et al.*, 2010; Du Plessis *et al.*, 2010). The lower concentrations of reduced glutathione (GSH) within the DIO testes implies the increased use of GSH to scavenge free radicals within this tissue. This may be related to obesity as an instigator of ROS production (Matsuda and Shimomura, 2013), a mechanism confirmed in the current study due to increased antioxidant activity and the increased oxidation of GSH.

The antioxidant activities of the reproductive organs have been correlated to changes in sperm, histological and molecular parameters. Lower antioxidant activity is a significant cause of cell injury and can cause damage to intracellular structures including sperm, which can ultimately lead to reduced cell function (Wiernsperger, 2003; King and Loeken, 2004; Suanarunsawat *et al.*, 2014). The negative association between relative epithelial volume in the testes and SOD offers a mechanism of damage. Lower SOD activity implies less clearance of superoxide ions by the testes, and a higher likelihood of damage to surrounding cellular structures and germ cells. The current study has associated smaller seminiferous tubular diameters with lower amounts of GSH. As more GSH has been oxidised by ROS, there is less available to buffer any changes in ROS levels within damaged seminiferous tubules, increasing damage to tubular structures and the developing germ cells. The reduced luminal diameters also correlated with reduced testosterone concentrations. Less testosterone produced by Leydig cells leads to reduced germ cell production as this hormone is necessary to stimulate and maintain spermatogenesis (Cheng and Mruk, 2010; Preston *et al.*, 2012). Reduced germ cell production could thus be the result of reduced testosterone production and increased damage from an overproduction of ROS. Although analysis of the epididymis showed no change in epithelial height and relative epithelial volume between the control and DIO group, the current study observed the relative epithelial volume to be negatively associated with lipid peroxidation, as reduced epithelial volume was related to higher levels of MDA. Increased lipid peroxidation could result in reduced cell viability due to destruction of sperm membranes, which are inherently susceptible to peroxidation due to the high amounts of

polyunsaturated fatty acids in their membranes. DNA damage, damage to sperm nuclei and loss of germ cells are additional consequences of lipid peroxidation (Sergio Oehninger *et al.*, 1995; Sikka, 2001; Aitken and Sawyer, 2003). The tubular diameters of the epididymides, which were reduced and therefore contracted in the DIO animals compared to controls, was associated with lower catalase activity. As previously mentioned, lower catalase activity indicates increased oxidation due to increased levels of superoxide radicals, which are likely to cause negative changes to the epididymal ultrastructure.

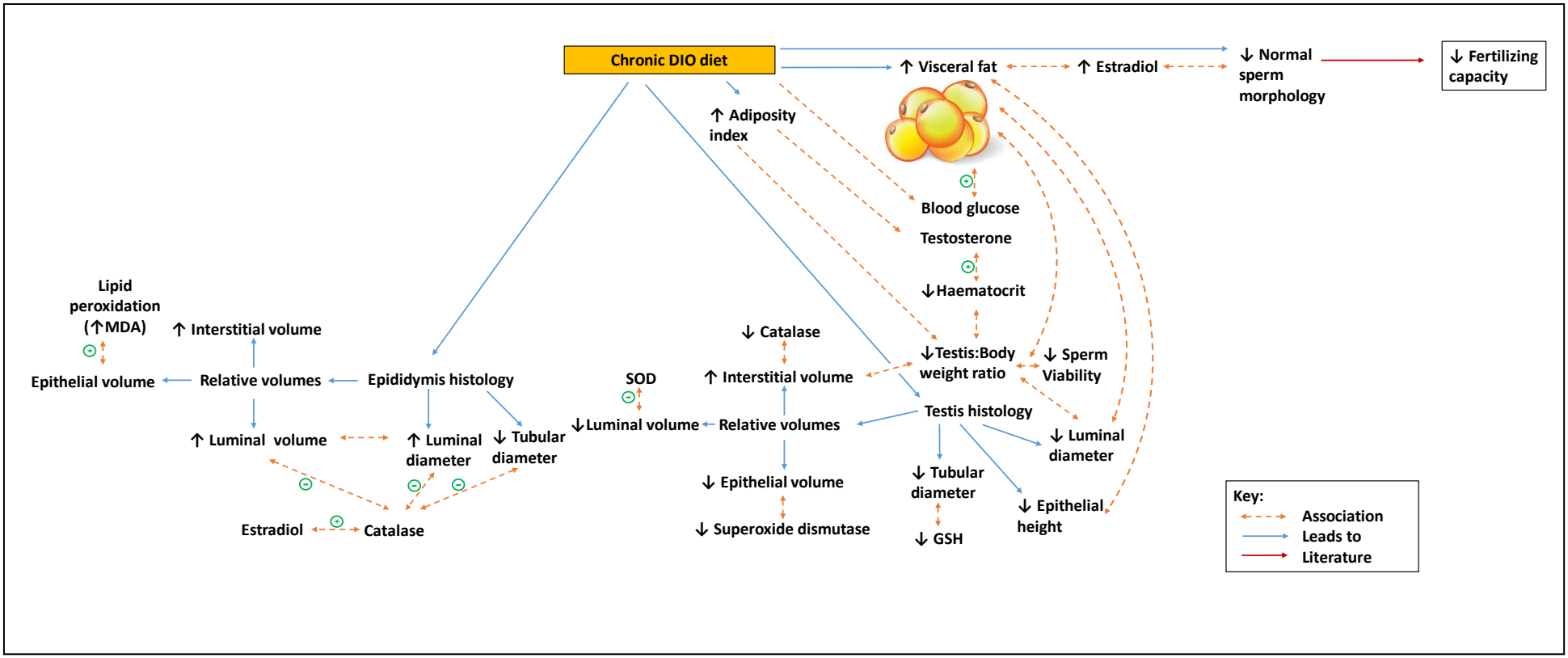



**Figure 5.1:** Summary of the study results and the associations between significantly different variables, as well as associations between variables that are significantly correlated. The chronic DIO diet is shown to affect anthropometric, macroscopic, microscopic and molecular variables directly or via a significant increase in visceral fat and the adiposity index. (MDA – malondialdehyde ; GSH – reduced glutathione).

## 5.3. The proteomics of diet-induced male infertility

The potential damaging effects of obesity can be further expanded using additional molecular assessments. The current study has analysed the reproductive organs and sperm of control and DIO animals using proteomics in an attempt to offer more insights into the broader underlying mechanisms through which obesity may be affecting sperm and body processes and thus male fertility. The findings of this section are illustrated in Figure 5.2 – 5.4, and thereafter summarised at the end of each subheading in Figures 5.5 – 5.9.

### 5.3.1. Proteins associated with glycolysis and oxidative phosphorylation

Proteomic analysis in the current study has shown a large number of the differentially expressed proteins between the DIO and control groups to be involved in metabolic processes (summarised in Figure 5.5). Spermatozoa have a high, fluctuating demand for energy (Wallimann, Wyss, Brdiczka, Nicolay & Eppenberger, 1992) which is attained via two key metabolic pathways, glycolysis and oxidative phosphorylation (Du Plessis *et al.*, 2015). Glycolysis predominantly takes place in the sperm head and principle piece of the flagella while oxidative phosphorylation is performed within the mitochondria. These two metabolic processes provide the sperm with adenosine triphosphate (ATP) which the cell specifically uses for numerous sperm functions including motility, capacitation, hyperactivation and the acrosome reaction (Miki, 2007; Mannowetz, Wandernoth and Wennemuth, 2012; Mukai and Travis, 2012). This ATP needs to be made available to the entire length of the sperm flagellum which requires the cell to utilize a variety of mechanisms and sources in order to generate it.

Glycolysis (Figure 5.2), the breakdown of monosaccharides from six to three carbon structures through a series of reactions catalysed by metabolic enzymes, results in the production of ATP (Villar-Palasi and Larner, 1970; Xu *et al.*, 2015). The 6-carbon monosaccharide predominantly metabolized is glucose, which is broken down into two 3-carbon pyruvate molecules. This process yields two ATP molecules for each glucose molecule metabolized. Pyruvate is then further oxidized into acetyl coenzyme A (acetyl-CoA), which can feed into the tricarboxylic acid (TCA) cycle to stimulate oxidative phosphorylation. Glycolytic energy substrates utilized by the sperm include glucose, fructose, mannose and sorbitol (Visconti, 2012). Fructose can be used directly for glycolysis or be converted into glyceraldehyde 3-phosphate via the Hers pathway before re-entering glycolysis.

Glycolysis and the TCA cycle (as well as fatty acid oxidation) create high-energy electron donors with a large electron transfer potential, namely nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), which donate electrons to oxygen and TCA cycle proteins to aid in the production of ATP (Berg, Tymoczko and Stryer, 2002).

Oxidative phosphorylation uses this donation of electrons by NADH and $FADH_2$ to create an accumulation of electrons between the inner and outer membranes of the mitochondria to generate an electron gradient that drives the movement of electrons from the mitochondrial matrix across the inner mitochondrial membrane. The movement of electrons across the inner mitochondrial membrane forms the electron transport chain, which allows electrons to move through complexes on the inner membrane. Electrons eventually return into the mitochondrial matrix through the final complex, complex IV, creating ATP with the assistance of ATP synthase (Erecińska and Wilson, 1977; Berg, Tymoczko and Stryer, 2002). Oxygen accepts electrons at the end of the electron transport chain as electrons return back into the mitochondrial matrix, and ATP synthase converts adenosine diphosphate (ADP) into ATP. Energy substrates used for oxidative phosphorylation include pyruvate, lactate and acetyl-CoA which are generated from glycolysis and beta (β) oxidation (Berg, Tymoczko and Stryer, 2002; Amaral *et al.*, 2013). Citrate is not permeable to mitochondria and is therefore not used as a primary energy source; it needs conversion by malate and ATP in order for it to enter the TCA cycle, therefore its use is less energy efficient (Visconti, 2012).

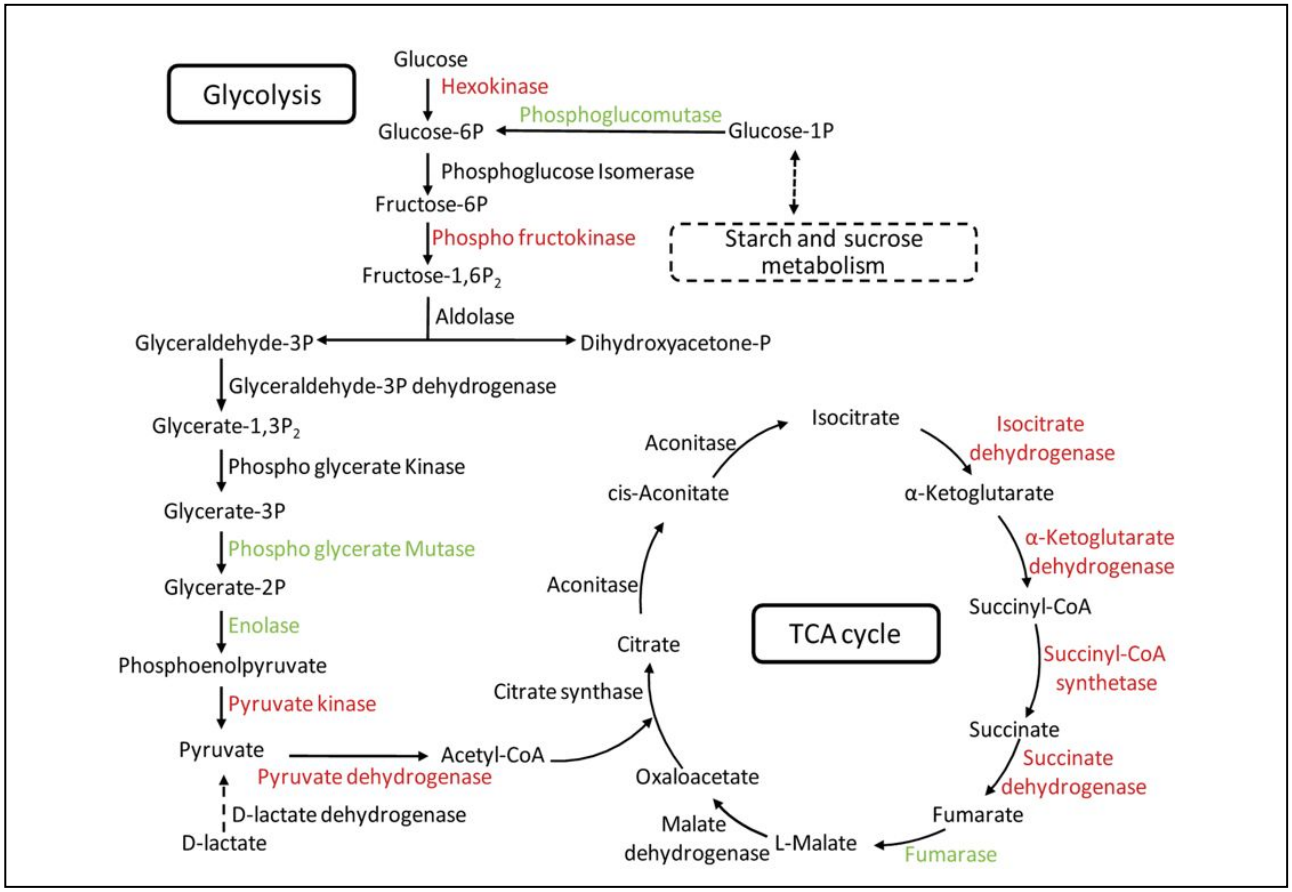



**Figure 5.2:** Diagram illustration of Glycolysis and the TCA cycle (Xu *et al.*, 2015).

The process of glycolysis was significantly affected in the current study, with numerous glycolytic proteins including phosphoglycerate kinase, enolase and hexokinase and pyruvate dehydrogenase within the epididymis (Krisfalusi *et al.*, 2006) showing differential expression between the control and DIO groups. These specific proteins have been found by previous studies to be expressed in the principle piece of spermatozoa in order to provide sufficient energy to cytoskeletal proteins, which bestow motility to sperm cells (Mukai and Okuno, 2004). Their position here aids in the provision of ATP to distal regions of the sperm tail. Furthermore, these proteins are not only concentrated in the principle piece but are also bound to the cytoskeletal proteins of the fibrous sheath. Their binding to cytoskeletal proteins allows them to provide the energy required for the active sliding of flagellar filaments during sperm movement (VandeBerg, Cooper and Close, 1973; Boer *et al.*, 1987). Phosphoglycerate kinase 2 (PGK2), a key enzyme in the glycolytic pathway, is expressed exclusively in male germ cells (Boer *et al.*, 1987). It is expressed by sperm cells which have undergone meiosis and the continued survival of these sperm appears to require glycolysis mediated by PGK2 (VandeBerg, Cooper and Close, 1973). The gene for this protein is located on the X chromosome and continues to be expressed after any possible inactivation of the X-chromosome during spermatogenesis (Monesi, 1971; VandeBerg, Cooper and Close, 1973). PGK catalyzes the first ATP generating step of glycolysis by converting 1,3-bisphosphoglycerate to 3-phosphoglycerate (Figure 5.2) and thus converting ADP to ATP (Danshina *et al.*, 2010). PGK2 is expressed exclusively during spermatogenesis; its transcription is triggered at the onset of meiosis by mRNA transcripts of PGK2 which are first expressed in preleptotene spermatocytes, increasing in pachytene spermatocytes and spermatids (McCarrey *et al.*, 1992). The PGK2 protein itself is only found in spermatids (Danshina *et al.*, 2010). A study by Danshina and colleagues (2010) which compared mice with an intact PGK2 gene (wild type, WT) with mice lacking the PGK2 gene ($pgk2^{-/-}$) observed $pgk2^{-/-}$ mice to have similar sperm motility to WT mice, although progressive motility was significantly reduced in the $pgk2^{-/-}$ group. ATP levels were also measured by incubating sperm under capacitating conditions and the ATP concentrations of the $pgk2^{-/-}$ sperm were observed to be < 10% of the WT levels within 30 minutes of incubation. In addition, the sperm from $pgk2^{-/-}$ and WT mice displayed similar levels of oxygen consumption during incubation in glucose-free medium containing pyruvate and lactate. Further incubation in media which contained substrates of the mitochondrial respiratory chain (i.e. succinate), showed that the reduction in progressive motility and ATP observed in the $pgk2^{-/-}$ mice were not due to a reduced activity of the mitochondrial respiratory chain as this process continued unabated. The stable total motility seen in $pgk2^{-/-}$ mice was postulated to be due the availability of other glycolytic enzymes such as acylphosphatase to catalyse the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate, bypassing the need for PGK2. The $pgk2^{-/-}$ mice also did not have altered testicular histology, sperm ultrastructure or sperm counts, which indicated that the loss of PGK2 is compensated for by other enzymes in the $pgk2^{-/-}$ mice and it is not necessarily required for the completion of

spermatogenesis. As both the DIO and control rats of the current study had high expression of PGK2, it can be deduced that sperm motility and ATP levels would be relatively conserved in both groups, although the DIO group showed higher expression of the PGK2 protein compared to controls. This is likely to be the result of the higher glucose levels present in the DIO plasma compared to controls. Glucose can act as a substrate for glycolysis and an excess amount can produce higher ATP concentrations. According to Danshina *et al*. (2010), higher ATP concentrations can result in higher progressive motility. This is plausible as mammalian sperm preferentially produce ATP from carbohydrate (fructose and glucose) fermentation during aerobic metabolism as opposed to gaining energy from sources such as lactate (Mann *et al.*, 1981). Higher sperm motility could also be a sign of sperm hyperactivation, which is an adverse event it if occurs prematurely. This is a high velocity, whiplash-like movement of sperm which is used by spermatozoa to burrow through cumulus cells that surround an oocyte just prior to fertilization (de Lamirande and Cagnon, 1993). Premature hyperactivation of sperm causes a reduction in fertility as this mechanism only happens once and cannot be initiated again should a sperm come into contact with an oocyte. Sperm motility was unfortunately not measured in the current study as the epididymides were placed directly on ice after extraction to preserve enzyme activity for subsequent antioxidant analyses, thus exposing sperm to low temperatures which lowered sperm motility. Comparisons between total and progressive motility as well as hyperactivation between the study animals could thus not be analysed to confirm this association, but the current higher expression of PGK2 seems to suggest a high amount of glycolysis occurring to make energy available for sperm cells, with DIO sperm possibly having higher motility. The postulation that hyperactivation is a possible reason for the increased motility is supported by the overexpression of PDHA (see later) in DIO sperm, a protein that has been shown to be active in sperm capacitation and hyperactivation (Lee, 2014).

#### 5.3.1.1. Other glycolytic and mitochondrial enzymes

Phosphoglycerate mutase 2 (PGAM2) and Enolase 1 (ENO-1), also glycolysis enzymes (Figure 5.2), similarly showed a higher expression in the epididymis of the DIO group, supporting the evidence for a higher rate of glycolysis in the DIO epididymis and epididymal sperm Figure 5.5). Hexokinase 2 (HK2) and Pyruvate dehydrogenase (PDHA), were, however, more abundantly expressed in the epididymis of the control animals. Hexokinases catalyse the first step of the glycolytic pathway by phosphorylating hexose sugars such as glucose and fructose (Wilson, 1997) to initiate their metabolism. The typical action of hexokinases is to convert glucose into glucose-6-phosphate (G-6-$PO_4$) (Figure 5.1). There are several hexokinase isoenzymes, namely hexokinase I-IV (Ureta, 1982). HK2 is the more insulin-sensitive isotype and is capable of metabolizing fructose, glucose and other hexose sugars such as mannose (Ureta, 1982) for use as energy substrates. The increased abundance of HK2 in the control epididymis of the current study implies

a better insulin sensitivity in the control animals and the use of other substrates including fructose and mannose for energy. HK2 has also been shown to be active in the synthesis of glycogen from G-6-$PO_4$ and in providing G-6-$PO_4$ for use by the pentose phosphate pathway (Wilson, 1985). The pentose phosphate pathway converts G-6-$PO_4$ into ribulose-5-phospate to generate NADPH for biosynthetic reactions, where NADPH is used for the biosynthesis of fatty acids, cholesterol, steroid hormones and nucleotides (Bhagavan, 2002; Bhagavan and Ha, 2015). Hexokinase IV, also known as Glucokinase (GK), is likewise more abundant in the epididymis of the control animals of the current study. This enzyme has been shown in pancreatic beta cells (β-cells) to sense and respond to fluctuations in plasma glucose concentrations and its phosphorylation of glucose is essential to the control of glycolytic flux in β-cells (Postic, Shiota and Magnuson, 2001). Other glucose responsive cells have been found to display functional changes in glucose metabolism based on GK activity. In the liver, GK has been shown to exert a strong influence on the utilization of glucose by hepatocytes and in glycogen synthesis (Ferre *et al.*, 1996; Hariharan *et al.*, 1997; Niswender *et al.*, 1997). Hariharan *et al*. (1997) were able to show in an experiment using transgenic mice expressing the human hepatic GK gene that increased GK activity results in the reduction of glucose plasma levels and in a lower BMI compared to control mice. The higher glucokinase (GK2 and GYKL1) expression in the epididymis of control animals of the current study also corresponds to the results observed by Hariharan *et al*. (1997) as the control rats had lower body weights compared to the DIO group. This implies better glucose uptake by cells, reducing plasma glucose and a better maintenance of body weight by the control animals. While the plasma glucose levels did not show significant changes between the control and DIO groups, the proteomic assessment of the epididymis points to higher glucokinase levels in the control animals, which according to the cited literature, points towards better glucose uptake in this tissue. The high rate of glycolysis in the DIO animals is therefore likely to be due to the use of other energy substrates which can feed into the pathway at the point after the hexokinase and GK dependant steps (see later). GK expression is also dependent on the presence of insulin and has been shown to regulate genes involved in fatty acid metabolism and lipogenesis (Girard, Ferré and Foufelle, 1997).

PDHA is localized in the mitochondrial matrix and stimulates the conversion of glucose to acetyl-CoA (Lissens *et al.*, 2000). It is the rate-limiting enzyme connecting glycolysis with the TCA cycle (Figure 5.1). Mammalian PDHA is present in two forms; somatic (PDHA1) and testis-specific (PDHA2) (Dahl *et al.*, 1990). PDHA is under regulation by metabolic, nutritional, developmental and hormonal control, and its expression can be down-regulated in stress conditions such as insulin-dependent diabetes and by high levels of free-fatty acids and ketone bodies (Wieland *et al.*, 1971). Succinyl-CoA:3-ketoacid coenzyme A transferase 1 (SCOT1), a key enzyme in the catabolism of ketone bodies, has been upregulated in the DIO epididymis of the current study.

SCOT catalyzes the conversion of acetoacetate, a major ketone body, into acetoacetyl-CoA which is further metabolized by the TCA cycle for energy production (Cong *et al.*, 2013). The use of ketone bodies for energy occurs when fat-derived energy is used by tissues other than the liver, which can result in ketoacidosis, the increased presence of ketones within plasma (Fukao *et al.*, 1997). The increased expression of SCOT1 observed in the DIO epididymis thus implies a possible increase in ketone bodies within the epididymis and plasma of the DIO group, which is a likely contributor to the reduced expression of PDHA1 in the DIO epididymis. The implications of the implied increase in ketones within the plasma of the DIO animals will be expanded on later in this discussion.

#### 5.3.1.2. Role of PDHA2 differential expression in energy production in testis compared to epididymis

The expression of PDHA2, the testis-specific form of PDHA, coincides with the onset of spermatogenesis, with significant expression being observed with the appearance of pachytene spermatocytes (Iannello, Young and Kola, 1994). This is similar to the expression of PGK, which is similarly observed in preleptotene spermatocytes at first before increasing within the more mature forms. PDHA1 is also present in the reproductive tissue, with expression noted in spermatogonia, Sertoli cells and Leydig cells, but it is limited to these regions. In white adipose tissue, the action of PDHA results in the provision of carbon from glucose, which becomes available for lipid synthesis (Maury *et al.*, 1995). The PDHA-stimulated increase in acetyl-CoA is also essential in the production of glycerol, fatty acids and amino acids (Lee, 2014). This bodes well for developing sperm as these products are necessary for the production of sperm membranes, in sperm energetics and in the attainment of cell fluidity that is necessary for processes such as capacitation and the acrosome reaction in mature sperm. PDHA2 expression differed between the epididymis and sperm homogenates in the current study. Its expression was less abundant in the DIO epididymides, which implies a lower availability of PDHA2 for sperm maturation within the epididymis of the DIO animals. This could compromise DIO sperm maturation and result in the creation of more immature sperm. PDHA2 expression was higher in the sperm homogenates of the DIO animals compared to controls. As these sperm have already gone through the maturation process, the actions of PDHA2 here are more likely for the purposes of sperm energy production. As most of the other glycolytic proteins have been observed to have higher expression in the DIO animals, it is logical that the expression of PDHA2 is also higher here. This increase in PDHA2, as the rate-limiting enzyme connecting glycolysis with the TCA cycle, implies a higher stimulation of the TCA cycle and therefore oxidative phosphorylation in the DIO sperm. Mannose also seems to be another energy substrate being used by the epididymis of the current study's animals due to the presence of beta-mannosidase (MANBA) in the epididymal homogenates of control and DIO animals, with lower expression observed in the DIO epididymis. It cleaves single (beta-linked)

mannose residues from non-reducing ends of all N-linked glycoprotein oligosaccharides. The DIO animals therefore seem to be less able to use this sugar as a metabolic substrate.

#### 5.3.1.3. Proteins involved in oxidative phosphorylation

Although sperm can essentially survive on glycolysis alone in terms of its energy requirements, it does require oxidative phosphorylation for differentiation and maturation (Barroso *et al.*, 1993; Spiropoulos, 2002; Nakada *et al.*, 2006; Du Plessis *et al.*, 2015). This is, however, species specific and mammals prefer differing metabolic pathways as sources of energy; glycolysis is essential for fertilization in human, hamster, mice and rat spermatozoa, but not for bovine sperm (Galantino-Homer *et al.*, 2004; Miki *et al.*, 2004). The mitochondria of spermatozoa, which are the site of oxidative phosphorylation, are tightly wrapped around the axoneme of the flagella at the principle piece. The binding of mitochondria to the axoneme is facilitated by selenoprotein and disulphide bridges which provide stability to the mitochondrial sheath (Ho and Suarez, 2003; Storey, 2008). These mitochondria, the source of ATP generation, are not located along the entire length of the sperm. This is due to their “bulky” nature which has the potential to negatively affect sperm motility at such locations. The ATP that they generate therefore has to be able to diffuse down the length of the entire flagellum in order to support the energy requirements of the tail (Serohijos *et al.*, 2006; Kamp *et al.*, 2007). Therefore, aberrations in the activities of mitochondrial respiratory enzymes can affect sperm motility (Luft, 1995).

Oxidative phosphorylation seems to be a highly used metabolic pathway in the animals of the current study (Figure 5.5), although several enzymes that are involved in this pathway differ in expression between the epididymis and sperm homogenates. In the epididymis, ubiquinol-cytochrome-c reductase complex core protein 2 (UQCRC2), succinate dehydrogenase (SDHA), Isocitrate dehydrogenase (IDH3B) and mitochondrial 2-oxoglutarate/malate carrier protein (SLC25A11) were significantly expressed in the epididymis, with lower expression observed in the DIO animals. On the other hand, oxidative phosphorylation enzymes UQCRC1, IDH3A, SDHA, NADH-ubiquinone oxidoreductase (NDUFS1), NADH dehydrogenase [ubiquinone] flavoprotein 1 (NDUFV1) and malic enzyme (ME2) showed higher expression in the DIO spermatozoa. These results point to a higher rate of oxidative phosphorylation used by the mature DIO spermatozoa than the epididymis itself.

### 5.3.2. Lipids and Fatty acids as an energy source in spermatozoa and the reproductive system

Spermatozoa have been shown to utilize fatty acid metabolites as another source of fuel. In a study by Amara *et al*. (2013), which performed proteomic analysis on human sperm tails, it was observed that a great proportion of the peptides identified within the sperm tails were enzymes

involved in lipid metabolism, as well as those involved in β-oxidation. They also observed peroxisome proteins known to participate in the oxidation of very long chain fatty acids, proving that the use of fatty acids as metabolic substrates may be more pronounced than previously believed. Their data was able to suggest that mitochondria within spermatozoa are able to oxidize saturated as well as unsaturated fatty acids, similar to other somatic cells. Furthermore, incubation of sperm in the absence of exogenous sugars did not reduce sperm motility; sperm were able to survive in this media for several days, implying that endogenous substrates of metabolism were made available by the sperm, although the nature of these were unknown. Due to the presence of enzymes of fatty acid metabolism, it was strongly postulated that sperm could possibly oxidize endogenous phospholipids to yield fatty acid substrates for use as energy substrates. Their data showed, possibly for the first time, that sperm may be able to metabolize very long chain fatty acids using peroxisomal enzymes.

Peroxisomes, which are single membrane organelles with a granular matrix, are known to contribute to the maintenance of cellular homeostasis (Islinger, Cardoso and Schrader, 2010). Very long chain fatty acids can only be degraded by peroxisomes, with evidence showing all peroxisomes to be capable of β-oxidation (Wanders and Waterham, 2006). Peroxisomes were initially believed to not be present in rodent reproductive tissue, but evidence has disproved this notion as these cells have been located in epididymal cells and were later also found to be present in Leydig cells and in spermatocytes themselves (Reisse *et al.*, 2001; Lüers *et al.*, 2006). Mitochondria and peroxisomes have been shown to be interconnected, with evidence of cross-linking between these organelles being found (Schrader and Fahimi, 2008). In addition, active vesicular transport occurs between mitochondria and peroxisomes. Somatic cells shorten very long chain fatty acids by metabolizing them via peroxisomal enzymes, and the resultant fatty acids can then be completely metabolized by mitochondria or become substrates for the synthesis of lipids and cholesterol. Based on the observed presence of peroxisome proteins, mitochondria and fatty acid oxidation enzymes, this process is also suggested to occur in the testis, epididymis and spermatozoa (Schrader and Fahimi, 2008).

The presence of several proteins involved in lipid metabolism have been observed in the present study which suggests a use of lipids as metabolic substrates in the study animals (summarised in Figure 5.5). These proteins include coatomer subunit beta (COPB1), long-chain-fatty-acid-CoA ligase (ACSBG1), tripeptidyl-peptidase 2 (TPP-2), acyl-CoA synthetase 2 (ACSF2), acyl-CoA-binding protein (ACBP) and hydroxyacyl-coenzyme A dehydrogenase (HADH) which stimulate or mediate lipolysis, lipid oxidation or adipogenesis. The presence of these proteins in our study corroborates the findings made by Amaral *et al*. (2013) that lipid metabolism is active within the reproductive system. Furthermore, peroxisomal proteins such as ATP-binding cassette sub-family D member 3 (ABCD3), 2-iminopropanoate deaminase (RIDA) and Isopentenyl-diphosphate delta-

isomerase 1 (IDI1) have been found to be significantly expressed in the epididymides of the present study, further pointing towards the presence of peroxisomes in the reproductive system, which was believed to not be the case for a significant amount of time.

COPB1, an enzyme involved in the control of adipocyte growth, was underexpressed in the DIO epididymis of the current study. It limits lipid storage within lipid droplets by promoting the association of the enzyme adipocyte triglyceride lipase (PNPLA2) with lipid droplet surfaces to mediate lipolysis. On the other hand, redox-regulatory protein FAM213A, an antioxidant, is a protein secreted by adipocytes and is located on the principle cells of the epididymis. Its increased expression in the epididymis of the DIO rats, together with the reduced expression of COPB1, implies a greater number and/or size of adipocytes within the DIO epididymides and a compensatory increase in antioxidant ability to assist in the adverse increase in ROS that can result from this higher abundance of adipocytes.

ACSBG1, a protein which mediates the synthesis of cellular lipids by activating long chain fatty acids as well as their degradation via β-oxidation, is significantly expressed in the epididymis of both the control and DIO rats, with a reduced expression in the DIO epididymis. Similarly, ACSF2, an acyl-CoA synthase, is less expressed in the DIO epididymis. This enzyme can catalyse the initial reaction of fatty acid metabolism, by forming a thioester with CoA. It has a preference for medium-chain fatty acids as energy substrates and plays a role in adipocyte differentiation. ACBP binds long and medium chain acyl-CoA esters and is active in β-oxidation; its expression, however, was higher in the DIO epididymis. HADH, a mitochondrial protein, plays an important role in the β-oxidation of short chain fatty acids, exerting its highest activity toward 3-hydroxybutyryl-CoA. This protein is also reduced in expression in the DIO epididymis. ABCD3, a peroxisomal membrane protein, is a transporter which has been postulated to be involved in the transport of branched-chain fatty acids and also shows a reduced expression in the epididymis of the DIO rats. This data points towards a lower amount of fatty acid catabolism in the DIO epididymis compared to controls. These fatty acids are possibly being metabolized in the controls to increase their availability as energy substrates for somatic cells and as well as the maturing of stored spermatozoa within the epididymis. They are also utilized for the synthesis of membrane lipids and cholesterol, which can be made available for sperm membranes and as hormone precursors. The apparently lower fatty acid metabolism in the DIO group implies a reduced usage of fatty acids as energy substrates of metabolism and for sperm maturation and hormone production within the epididymis. In the spermatozoa, acyl-CoA synthase 1 (ACSL1) is highly abundant in both the control and DIO sperm, although expression is lower in the DIO sperm. ACSL1 is a long-chain-fatty-acid-CoA ligase that stimulates fatty acid utilization through the metabolism of fatty acids into acyl-CoA and is activated by adiponectin. Its reduced expression by the DIO sperm suggests a lower availability of fatty acids for utilization by the DIO sperm. With that said, the increased expression of fatty acid transfer and

binding proteins carnitine O-palmitoyltransferase 2 (CPT 2), electron transfer flavoprotein subunit alpha (Alpha-ETF) and fatty acid-binding protein 3 (FABPH) (also known as aspartate aminotransferase) in the DIO sperm compared to controls implies a higher ability of DIO sperm to bind and transport the fatty acids that are available for use as energy and membrane substrates.

Furthermore, the spermatozoa of the DIO group having a higher expression of the ketone enzyme SCOT1 implies an additional mechanism through which DIO can make more energy substrates available when substrates from glycolysis and fatty acid oxidation are inadequate. Amaral *et al*. (2013) found the human homologue SCOT-t, a ketone catabolism enzyme, to be present in the sperm proteins that they quantified in their study, which literature suggests might be used to support sperm motility (Tanaka *et al.*, 2004). Ketones are normal metabolites produced by the liver for use by extrahepatic tissues (Cohen, 1937). They are created to replace glucose in times of cellular glucose deficiency such as starvation, insulin deprivation and insulin-insensitivity (Cahill *et al.*, 1966; Veech *et al.*, 2001; Cahill and Veech, 2003; Cahill, 2006). They are efficiently used by the mitochondria to produce ATP and may also protect glucose dependent cells from free radical damage (VanItallie and Nufert, 2003). Ketone utilization increases as their concentration rises in the blood (Mirsky and Broh-Kahn, 1937). In humans, the turnover time for ketone bodies in circulation is approximately two minutes (Wastney, Hall and Berman, 1984). The rate of ketone production is higher in obese individuals, and extrahepatic tissues absorb this metabolite for energy production but at a lower clearance rate than normal individuals (Hall *et al.*, 1984). This can result in high plasma ketone levels in obesity.

Based on the presence of proteins involved in ketone production and β-oxidation in the reproductive tissues of the current study (Figure 5.5), these mechanisms are implied to be extensively utilized by the control and DIO animals to produce metabolic substrates for ATP synthesis. A reduction in the availability or utilization of insulin in insulin-resistant states such as starvation, obesity and diabetes, stimulates a compensatory acceleration in the hepatic conversion of free fatty acids to ketones for use as energy substrates (Cahill, 1970). Ketones are produced from fatty acids which are mobilized from adipose tissue stores; these fatty acids enter the liver and are metabolized by acyl-CoA synthetases and will be further metabolized depending on the length of their fatty acid chains. Short and medium chain acyl-CoAs are permeable to the inner and outer mitochondrial membranes, and enter the mitochondria to undergo β-oxidation into acetoacetate and acetyl-CoA. Long chain fatty acyl-CoAs are permeable to the outer mitochondrial membrane, where they enter the mitochondria to be metabolized further into long chain acyl-carnitine before passing through the inner mitochondrial membrane to undergo β-oxidation. Very long chain acyl-CoAs and unsaturated fatty acids (as well as long and medium chain acyl-CoAs) are transported into peroxisomes for chain length reduction via β-oxidation; as peroxisomal β-

oxidation does not proceed to completion, peroxisomes transport medium chain and acylcarnitine compounds to the mitochondria for final β-oxidation (VanItallie and Nufert, 2003).

Mitochondrial β-oxidation (Figure 5.3) results in acetyl-CoA which enters the TCA cycle to be utilized for ATP generation (Abcam, 2019). When excess acetyl-CoA from β-oxidation cannot be metabolized, two acetyl-CoA molecules dimerize to form acetoacetyl-CoA, which begins ketogenesis (Figure 5.3 and 5.4). The result is two ketones, acetoacetate and β-hydroxybutyrate, being released by the mitochondria into circulation. From circulation, ketones can be taken up by extrahepatic tissues to use for energy production. Acetoacetate and β-hydroxybutyrate enter the mitochondria of extrahepatic tissues; β-hydroxybutyrate is further metabolized to acetoacetate by β-hydroxybutyrate dehydrogenase. Acetoacetate molecules are then broken down into two acetyl-CoAs which subsequently undergo oxidative phosphorylation within the TCA cycle (Figure 5.4). In the TCA cycle, NADH and $FADH_2$ are formed by the reduction of $NAD^+$ and FAD to NADH and $FADH_2$ (Figure 5.3). Electrons are then donated to complex I and II of the mitochondrial respiratory chain by NADH and $FADH_2$ which initiates the flow of electrons down the electron transport chain to oxygen. This allows complexes I, II and IV to pump $H^+$ out of the mitochondrial matrix into the intermembrane space. The movement of $H^+$ back into the mitochondrial matrix through complex V produces ATP (with the assistance of ATP synthase) (VanItallie and Nufert, 2003) (Figure 5.3). The implied lower rate of β-oxidation and higher expression of SCOT1 suggest ketones to be a source of acetyl-CoA for ATP generation within the DIO animals.

In an experiment by Lardy and Phillips (1945), which compared metabolic substrates utilized by mammalian spermatozoa, the two ketones acetoacetate and β-hydroxybutyrate were found to increase sperm motility at a lower oxygen consumption than the other metabolic substrates tested by these investigators. The mechanism of this result was not understood until studies by Kashiwaya *et al*. (1994; 1997) and Veech *et al*. (2001) were carried out on working heart perfusions. They compared the physiological effects of insulin versus those of ketones via the addition of glucose, ketones, insulin or a combination of these substrates to the heart perfusate. The efficiency of the hydraulic work performed by the heart to pump was increased by 28% after the addition of insulin and equally after the addition of ketones (Kashiwaya, King and Veech, 1997). The addition of ketones to the perfusate decreased the rate of conversion of glucose to pyruvate as ketones were used as an alternate fuel instead of glucose, but it did increase the concentration of G-6-$PO_4$. In addition, the activity of hexokinase was increased, but to a lesser degree than with the addition of insulin, which can explain the increase in G-6-$PO_4$. Acetyl-CoA was increased 15 fold compared to 9 fold when insulin was added. The insulin-mediated increase in acetyl-CoA was mediated by an increase in pyruvate dehydrogenase, whereas the ketone-stimulated increase was more likely due to the conversion of ketones themselves to acetyl-CoA through the de-dimerization of acetoacetate. The addition of ketone, furthermore, resulted in the

reduction of free mitochondrial $NAD^+$ to NADH and increased ATP production, as seen with the addition of insulin. The first 3 metabolites of the TCA cycle therefore increased, but succinate content was decreased while fumarate remained the same. Sato *et al*. (1995), who performed a similar experiment, noted a decrease in aspartate and oxaloacetate, and Kashiwaya *et al*. (1994) observed an increase in the action of glycerate mutase with the addition of ketones. The change in the succinate:fumarate content is interpreted as the oxidation of the mitochondrial coenzyme Q to co-enzyme $QH_2$; the combination of NAD/NADH and $Q/QH_2$ conversion results in an increase in electrons being ejected from the mitochondrial matrix, increasing the electron gradient to result in an elevated ATP production. These experiments can explain the results noted in the current study. The expression of hexokinase and pyruvate dehydrogenase being less abundant in the DIO epididymis compared to controls points to an insulin-stimulated increase in glycolysis in the control animals, which was not stimulated to the same extent in the DIO epididymis due to a higher dependence on ketones. The decreased succinate in the Sato *et al*. (1995) experiment is likely due to an increase in succinate dehydrogenase (SDHA), which has been observed in the DIO sperm of the current study, suggesting an alteration in the succinate:fumurate ratio as was seen with the addition of ketones in the aforementioned studies. The observed increase in acetyl-CoA in the hearts perfused with ketones is also a possible occurrence in the current study as the increase in ketone catabolism stimulated by SCOT1 can yield the breakdown of each molecule of acetoacetate into 2 molecules of acetyl-CoA. The change in the ratios of $NAD^+$/NADH and co-enzyme Q/co-enzyme $QH_2$ observed in the studies by Kashiwaya (1994; 1997) can be coupled to the respective increase in NDUFS1 and UQCRC1 expression observed in the DIO sperm, which increase the action of the electron transport chain to increase ATP. Thus the lower glycolysis noted in the DIO animals is likely to be compensated by an increase in ketone usage by the epididymis and sperm, which then increased the expression of TCA cycle enzymes and substrates to possibly yield an increase ATP production.

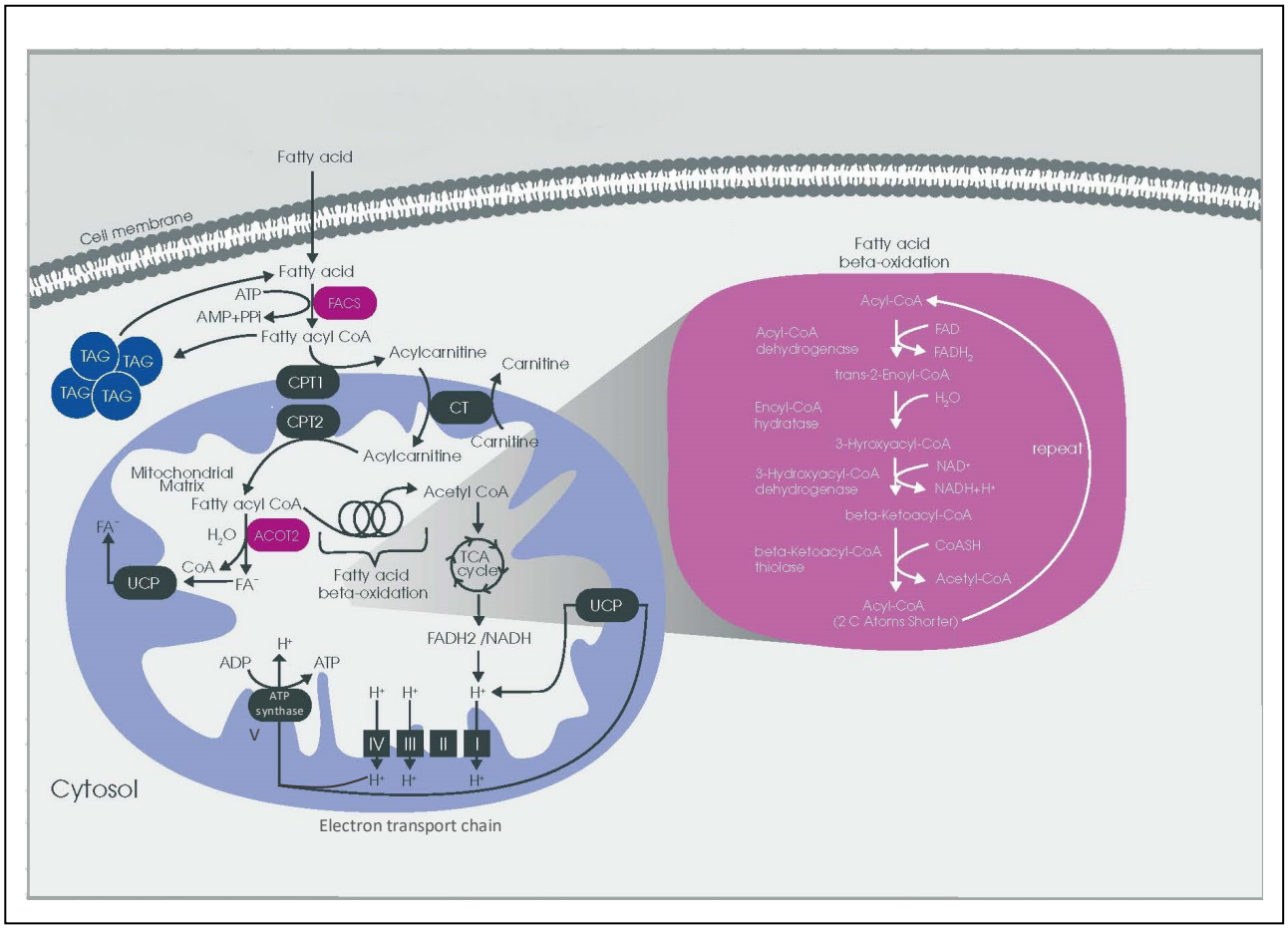



**Figure 5.3:** Image illustrating fatty acid metabolism, visualizing the collaboration between β-oxidation, the TCA cycle and the electron transport chain in the conversion of fatty acids into energy substrates and ATP. (FACS – Fatty-acyl-CoA synthase, CPT – carnitine palmitoyltransferase, CT – carnitine/acylcanitine translocase, ACOT2 – acyl-CoA thioesterase 2, UCP- uncoupling protein) Adapted from Abcam.com (Accessed 17/11/2019).

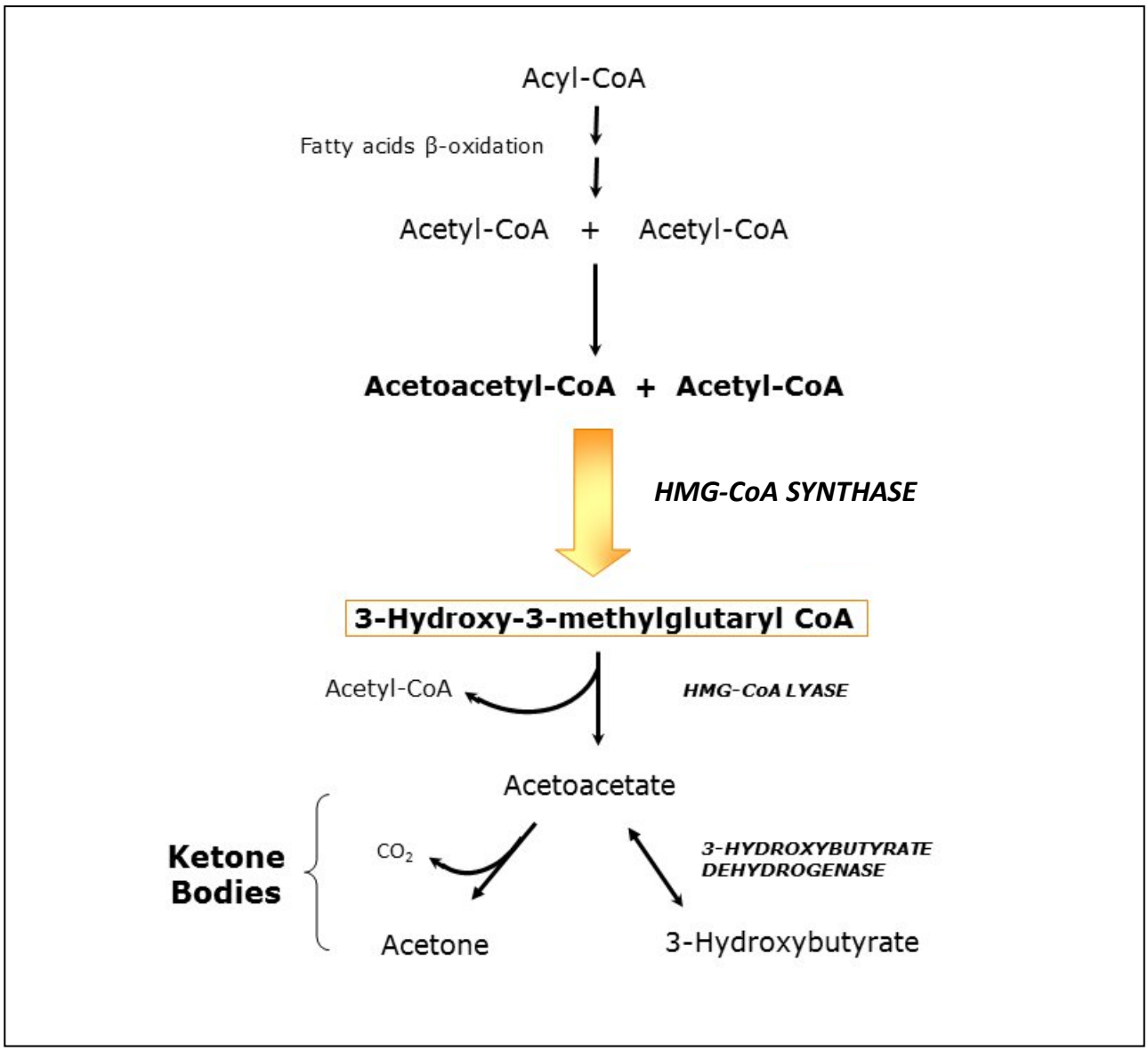



**Figure 5.4:** Diagram illustrating ketogenesis (Anedo *et al*. 2011)

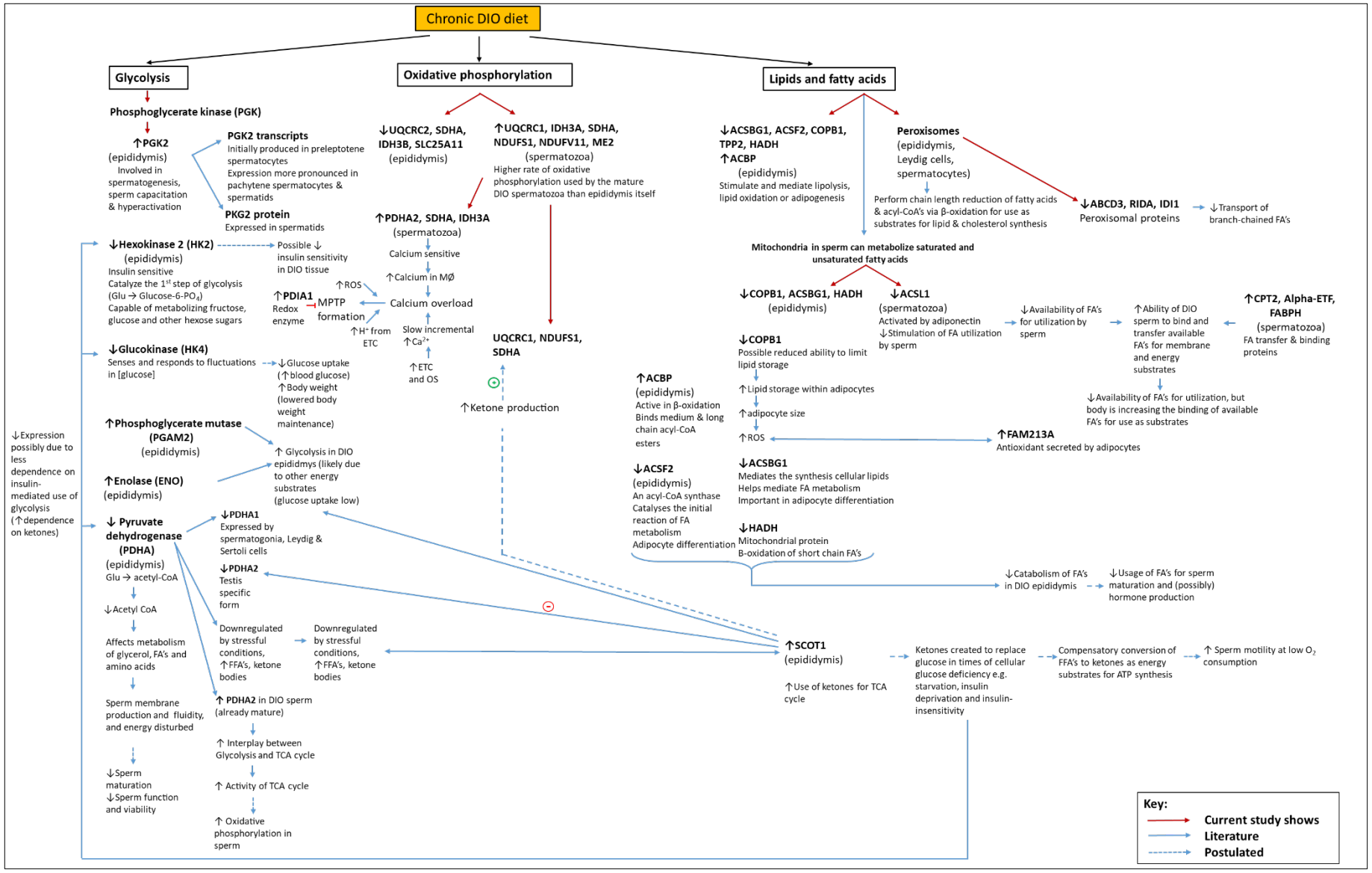



**Figure 5.5:** Summary of differentially expressed proteins involved in metabolic processes. Glu = glucose, FFF = free fatty acids, FA = fatty acids, MØ = mitochondria.

### 5.3.3. The role of creatinine kinase

Creatine kinase (CK) is an enzyme that is active in the transfer of ATP from mitochondria to sites of energy usage in cells such as sperm, muscles and neurons (Ford, 2006). It participates in what is known as the phospho-creatine (PCr) shuttle where it catalyses the transfer of an N-phosphoryl group from PCr to ADP, thereby regenerating ATP (Kaldis *et al.*, 1997). It is found in cells that have a high, fluctuating demand for energy (Wallimann *et al.*, 1992). This enzyme has been found in the current study to be less abundant in the epididymides of the DIO group (summarised in Figure 5.6). Carbonic anhydrase (Ca5b), on the other hand, is necessary to shuttle ATP substrates back to the mitochondria from the sites of ATP usage in order for more ATP to be produced for subsequent energy production (Ford, 2006). This protein was found in the current study to have a higher expression in the DIO groups compared to controls (Figure 5.6). The lower expression of CK and higher expression of Ca5b in the DIO animals implies that a lot of energy (ATP) is being used up in the DIO epididymides but this energy is replenished at a lesser rate than that of the controls. Thus the ratio of energy utilization vs. production could be compromised in the DIO animals. Energy transfer, especially in the sperm tails, could thus also compromised. The high rate of energy created by the TCA cycle therefore does not seem to be adequately transferred to the cells by the CK system or the increased Ca5b is a compensatory mechanism that allows the creation of more ATP via the TCA cycle due to a less than optimal ATP replenishment shunt that is likely to be present as a result of the lowered expression of CK.

CK has five isotypes, three are cytosolic namely brain-type (CK-BB), muscle-type (CK-MM), and a combination isotype (CK-MB) which occurs in dimers (Huszar, Vigue and Morshedi, 1992) and two are mitochondrial isotypes which are located in the intermembrane space, namely ubiquitous (CK-$Mi_a$) and sarcomeric (CK-$Mi_b$) mitochondrial CK (Wyss *et al.*, 1992). The CK-BB is localized in the sperm tail whilst the mitochondrial isotypes are localized in mitochondria within the sperm mid-piece (Kaldis *et al.*, 1997). Mitochondrial CK is, in all instances, co-expressed with at least one of the cytosolic CK enzymes. In this study, the epididymis appears to show co-expression with CK-MM (protein Ckm), which has a lower expression in the DIO epididymis. Inhibition of CK impedes sperm In view of this it can be inferred that the reduced CK indicates that ATP replenishment and transfer by the PCr shunt to sperm and high energy cells in the epididymis is inefficient and is likely compensated for by an increase in ATP via glycolysis.

The CK-MM protein levels in sperm of the current study's animals are, however, significantly more abundant in the DIO sperm compared to controls, the opposite of what was observed in the epididymis (Figure 5.6). According to Huszar and colleagues (1990; 1992), because CK-MM is a cytosolic protein, its concentrations are meant to decrease as sperm mature and reduce their cytoplasmic volume. High levels of CK-MM in mature sperm are thus a signal of higher amounts of

immature sperm with abnormal morphology. This can be compared to the higher levels of abnormal sperm morphology observed in the DIO sperm compared to controls.

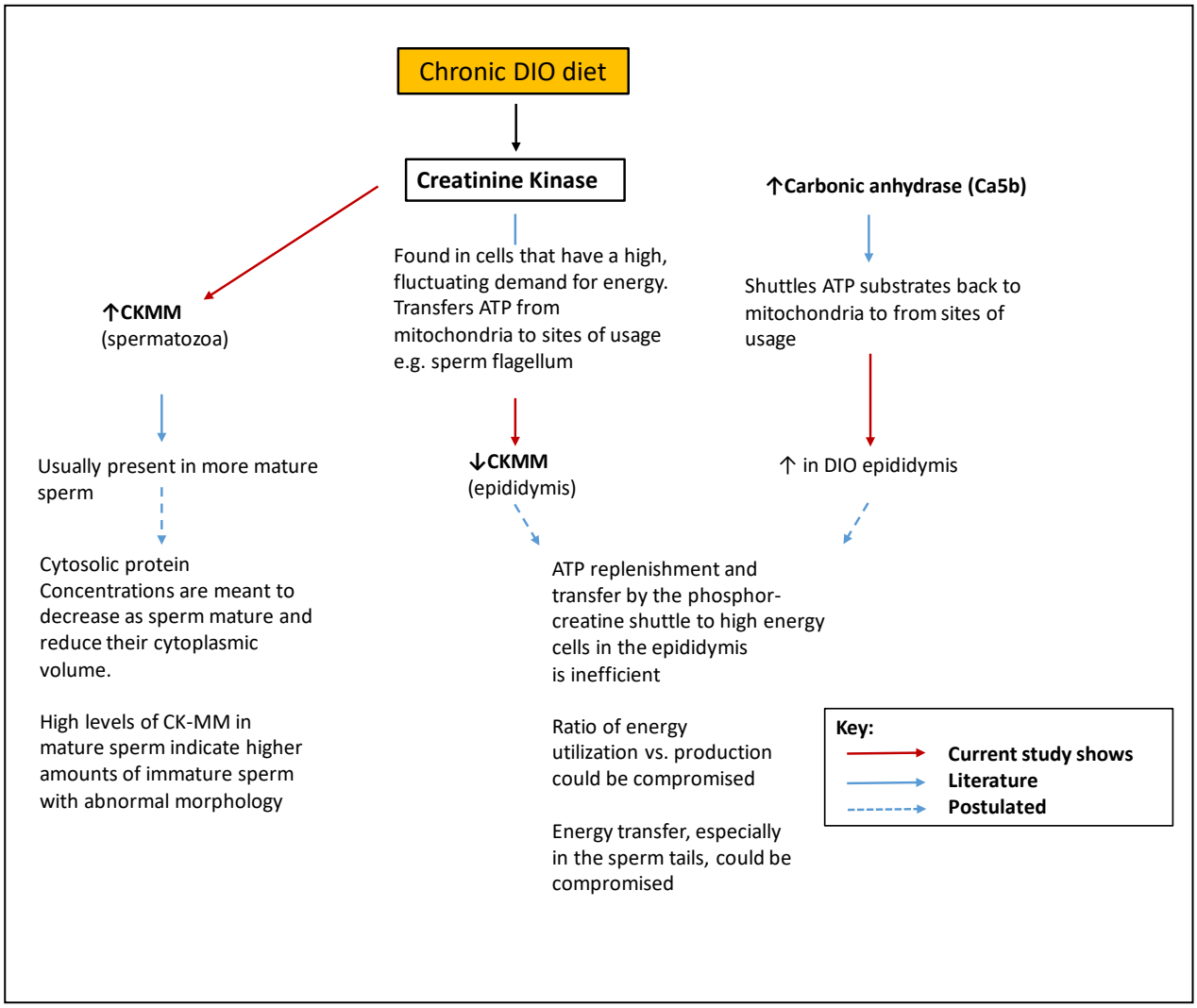



**Figure 5.6:** Summary of proteins involved in associated with creatinine kinases that were found to be differentially expressed in the current study, including the functions and possible interactions postulated based on literature. DIO = diet-induced obesity group.

### 5.3.4. The role of reactive oxygen species (ROS)

As oxidative phosphorylation uses large amounts of oxygen to create ATP, its consumption results in the production of large amounts of ROS. The partial reduction of oxygen during metabolism gives rise to the production of free radicals including $O_2^-$, $H_2O_2$, and the hydroxyl radical ($OH^\bullet$). While ROS molecules are necessary for normal sperm functions, an increased production of these highly reactive oxygen metabolites leads to OS, which can damage many cellular components including proteins, lipids and DNA (Francis and Stevenson, 2019). The products of ATP hydrolysis (ADP, inorganic phosphate and $H^+$) and ROS need to be efficiently removed in order to avoid cellular stress (Oberholzer *et al.*, 2007).

The passing of electrons from one mitochondrial complex to another within the ETC of the inner mitochondrial membrane is central to the production of ATP (Saraste, 1999). The main entry point for electrons into the ETC is Complex I, which stimulates the transfer of 2 electrons from NADH to ubiquinone (Wirth *et al.*, 2016; Leipnitz *et al.*, 2018). Proteins NDUFS1, SDHA and ETFA in spermatozoa as well as CDGSH iron-sulfur domain-containing protein 1 (CISD1) and ubiquinol-cytochrome-c-reductase complex core protein 2 (UQCRC2) in the epididymis are similarly involved in the ETC within mitochondria. The NDUFS1 gene codes for a major subunit in complex I (Bénit *et al.*, 2001) and CISD1 is an iron-containing protein located in the outer mitochondrial membrane that plays an essential role in complex I-driven respiration (Wiley *et al.*, 2007). These proteins being overexpressed in the DIO group of the current study implies an increase in electron translocation through complex I. SDHA codes for complex II, and is active in the TCA cycle (Brière *et al.*, 2005). It is overexpressed in the sperm of DIO animals, which reveals an increased activity of Complex II and an amplified ETC activity. ETFA transfers electrons to the mitochondrial respiratory chain via ETF dehydrogenase (ETF-ubiquinone oxidoreductase). UQCRC2 encodes Complex III, the most well-characterized complex of the ETC (Saraste, 1999) (summarised in Figure 5.7). Complex III transfers electrons from ubiquinol to cytochrome c, with this oxidation resulting in the transport of hydrogen ions ($H^+$) across the mitochondrial membrane (Sánchez *et al.*, 2013). Complex I and III have been identified as sites of $H_2O_2$ and $O_2^-$ radical formation (Boveris and Chance, 1973). As these free radicals attack nearby molecules to stabilize themselves, the proximity of these newly produced molecules to the mitochondria make these organelles susceptible to damage by free radicals (Petrosillo *et al.*, 2009). Free radicals attack mitochondrial complexes and mitochondrial membranes by virtue of their unsaturated fatty acid components. This can ultimately result in reduced mitochondrial ETC in the long term, thereby decreasing energy production. The result is a potential reduction in sperm function and motility, which are all dependent on mitochondria and ATP. The enzymes pyruvate dehydrogenase (PDHA2) and malate (malic acid, ME2) are molecules shown to drive flux (the rate of flow of electrons) through Complex I (Petrosillo *et al.*, 2009), and their overexpression in the spermatozoa

of the DIO group in the current study implicates an additional mechanism of ETC stimulation in obesity. The upregulation of PDHA2, through its activity in glycolysis and the TCA cycle, can further stimulate free radical production in DIO sperm. The increased release of NADPH produced via the conversion of malate to pyruvate by ME2 (Jiang *et al.*, 2013) is an additional source of stimulation of the TCA cycle and ETC, further driving the ability of these metabolic processes to increase free radical production.

Iron-containing proteins, such as CISD1, contain iron (Fe) and cysteinate sulfur (S) atoms in 2 to 4 iron-sulfur clusters (Lovenberg, 1973). In other words, they contain iron-sulfur (Fe-S) clusters of 2 to 4 atoms of iron and sulfur each (2Fe-2S to 4Fe-4S), that are bound to cysteine (Cys) protein residues (Rouault, 2012). They are used for numerous cellular functions including electron transport in the ETC, the regulation of enzymatic activity, DNA repair, transcription, cellular bioenergetics (Fontecave *et al.*, 2008) and in the synthesis of heme and iron–sulfur clusters (Crane, Siegel and Getzoff, 1995). Iron-containing proteins are redox-sensitive molecules and are readily converted into either [4Fe-4S] or [2Fe-2S] upon reduction or oxidation to accept or donate electrons accordingly (Karmi *et al.*, 2018). Fe-S clusters can thus bind to electron-rich enzyme substrates and accept or donate electrons to stabilize the protein conformations of numerous metabolic proteins as necessary for their activity (Beinert, Holm and Münck, 1997; Rouault, 2012).

Mitochondria can also synthesize Fe-S clusters and incorporate them into the TCA cycle (e.g. into aconitase and succinate dehydrogenase) and into mitochondrial complex I-III. CISD1, as an iron-containing protein, can therefore regulate the maximal capacity of electron transport and oxidative phosphorylation, thereby assisting to control maximal mitochondrial respiratory rates. CISD1 has also been postulated to take part in iron metabolism. In an experiment using the plant analogue, AtNEET, which is found in plant mitochondria and chloroplasts, AtNEET was shown to transfer Fe via an Fe-S/Fe cluster to mitochondria or an acceptor protein (Nechushtai *et al.*, 2012) leading to an accumulation of Fe within the plant. This result was also coupled to an increase in ROS production. Furthermore, the expression of AtNEET has been found to increase in response to an accumulation of the dioxygen molecule $O_2^-$ (Op Den Camp *et al.*, 2003), a function similarly observed in the mammalian equivalent (Beinert, Holm and Münck, 1997). The AtNEET protein has a common protein structure to the human analogue and its functions are thus transferrable to their human equivalent.

In the current study, CISD1 was overexpressed in the DIO epididymis compared to controls (Figure 5.7). In an experiment examining the effects of the overexpression of CISD1 (also known as MitoNEET) on adipocyte mitochondrial activity in mice, Kusminski *et al*. (2012) observed CISD1 overexpression to increase lipid uptake and storage, resulting in the expansion of adipose tissue mass within their study animals. These authors also found CISD1 overexpression to lower β-

oxidation rates due to the reduction of mitochondrial Fe transport into the matrix via direct inhibition of Fe transport by CISD1; this is a step which is a rate-limiting component of the β-oxidation pathway. This result is in agreement with the current study where a reduction in proteins involved in the β-oxidation pathway in the epididymis of DIO animals was observed. Furthermore, individuals suffering from obesity and type 2 diabetes are known to exhibit lower β –oxidation rates (Kelley *et al.*, 2002; Ritov *et al.*, 2005). The enhanced lipid uptake and lower β-oxidation rates stimulated by CISD1 suggests that it can contribute to the adjustment of energy stored vs. energy burnt by altering energy bioenergetics (Kusminski *et al.*, 2012). CISD1 was also found by Kusminski and co-investigators (2012) to lower the $NAD^+$/NADH ratio, resulting in a build-up of NADH. As NADH and $FADH_2$ are re-oxidized by the ETC into $NAD^+$ and FAD, the build-up of NADH caused by the overexpression of CISD1 could imply that a) high rates of glycolysis, which occur in compensation to a reduction in β-oxidation, generate surplus NADH, b) ETC activity during CISD1 overexpression is inefficient to re-oxidize NADH, c) CISD1 has an impact on the $NAD^+$/NADH ratio and d) the alteration of NAD shuttle, salvage and degradation by CISD1 compromises mitochondria performance which is further affected by the increased concentrations of NADH (Muoio and Newgard, 2006). A decrease in the ability of mitochondria to reduce the $NAD^+$/NADH ratio thereby disrupts the energy bioenergetics of mitochondria and their redox capabilities. An increased storage vs. usage of fat is also observed in the DIO animals based on the implied decrease in β-oxidation and increase in proteins necessary for energy storage.

Increased oxidative phosphorylation, the subsequent release of ROS by mitochondria and the possible damage incurred by mitochondria due to the ROS that they produce, instigates a vicious cycle which potentiates mitochondrial damage. This damage can alter mitochondrial dynamics (Kim and Song, 2016) and induce the mitochondrial permeability transition pore (MPTP) (Ellerby and Bredesen, 2000) at contact sites between the mitochondrial inner and outer membranes (Crompton, 1999). This increase in mitochondrial permeability could result in the release of cytochrome c which can stimulate the intrinsic apoptotic cascade via the activation of caspase 4, 8 and 9 (Pan, O'Rourke and Dixit, 1998; Osellame, Blacker and Duchen, 2012).

Pyruvate dehydrogenase, oxoglutarate dehydrogenase as well as isocitrate dehydrogenase are $Ca^{2+}$-sensitive enzymes. An increase in these enzymes is observed in the current study through the overexpression of PDHA2, SDHA and IDH3A in DIO sperm (Figure 5.7). Their increase can be linked to possible increases in $Ca^{2+}$ concentrations within mitochondria. OS and increased ETC cycling have been shown to induce a slow incremental rise in resting $Ca^{2+}$ concentrations within mitochondria (Crompton, 1978, 1976), which may result in $Ca^{2+}$ overload. This is due the release of hydrogen ions ($H^+$) from the respiratory chain, which leave mitochondria through transport systems on the inner mitochondrial membrane. The exit of $H^+$ from the mitochondrial matrix is exchanged for sodium ($Na^+$) entry into the mitochondria via a $Na^+/H^+$ exchanger. The $Na^+$ ions eventually exit

the mitochondria in exchange for an influx of $Ca^{2+}$ into the matrix by means of a $Na^{+}/Ca^{2+}$ exchanger (Crompton, Capano and Carafoli, 1976; Crompton, Künzi and Carafoli, 1977). This exchange sets up a gradient which can drive $Ca^{2+}$ influx into the matrix via a $Ca^{2+}$ uniporter (Crompton *et al.*, 1978). This allows $Ca^{2+}$ to be distributed into the mitochondrial matrix until a $Ca^{2+}$ overload occurs (Nichols and Crompton, 1980; Crompton, 1990). This is an important mechanism of cellular injury. Mitochondrial $Ca^{2+}$ overload can itself be harmless, but in combination with OS and a reduction in ATP due to mitochondrial damage, can lead to MPTP formation and cell death (Crompton, 1990). The intermembrane space between the inner and outer mitochondrial membranes is filled with apoptogenic proteins, thus a loss of the outer mitochondrial membrane due to damage by oxidants and $Ca^{2+}$ overload can lead to the release of these proteins and in accidental apoptosis (Crompton, 1990). Alterations in cytosolic $Ca^{2+}$ and thereby $Ca^{2+}$ cycling into mitochondrial matrix can increase the activity of PDHA2, SDHA and IDH3A (McCormack, Halestrap and Denton, 1990). Their increase, in response to increased mitochondrial $Ca^{2+}$, can serve as an additional stimulus for the ETC and further free radical production.

MPTP opening in the face of $Ca^{2+}$ overload and OS can also result in necrosis (Crompton, Costi and Hayat, 1987; Crompton, Ellinger and Costi, 1988). MPTP opening does not cause ATP synthesis but rather ATP hydrolysis, which impairs energy metabolism. This causes additional $Ca^{2+}$ dysregulation and further MPTP opening, creating a continuous vicious cycle which culminates in cell death (Crompton, Ellinger and Costi, 1988). Furthermore, the overexpression of the PDIA1 protein suggests a mechanism of protection against OS-induced apoptosis signaling. This protein is an abundant redox enzyme which may act as an anti-inflammatory via its downregulation of inflammatory mediators such as nuclear factor-kappa B (NF-κB) (Zhou *et al.*, 2008). Catalase can partially block MPTP formation in mitochondria (Kowaltowski, 1996), but the lower activity of this enzyme suggests the loss of this protective mechanism.

Heat shock proteins (HSP) are well conserved molecular chaperones which can be upregulated by stress conditions such as OS (Georgopoulos and Welch, 1993; Welch, 1993). They may be released by necrotic or stressed cells, or moved from the cytosol to cell surfaces where they can stimulate immune reactions (Vendetti *et al.*, 2000; Pfister *et al.*, 2005; Sapozhnikov *et al.*, 2007; Pockley, Muthana and Calderwood, 2008). HSP60, a prominent HSP isotype, is released from cells following metabolic and cellular stress, making it available for interaction with immune cells (Habich and Burkart, 2007; Pockley, Muthana and Calderwood, 2008). It is a chemoattractant for neutrophils, which degranulate upon stimulation to promote an inflammatory state (Osterloh *et al.*, 2009). It is also implicated as a stimulator of cytokine release from pre-adipocytes and adipocytes in obesity (Macaulay and Larkins, 1988; Gülden *et al.*, 2008, 2009). In so doing, it mediates adipocyte dysfunction and potentiates a low grade inflammatory state (Märker *et al.*, 2010). The overexpression of HPS60 in the DIO sperm (Figure 5.7) implies the instigation of an inflammatory

state during obesity, which is a stimulator of OS. Furthermore, the manganese isoform of SOD (MnSOD) seems to require HSP60 for proper protein folding, which could moreover explain the increase in HSP60 as it is required for MnSOD synthesis (Magnoni *et al.*, 2014).

Taken together, the changes in expression of the aforementioned proteins point to an increased rate of cellular metabolism and inflammation in the obese condition, which can increase the production of free radicals. These need to be detoxified by endogenous antioxidants.

The current study found an increase in the cytosolic SOD protein expression (SOD1) in the epididymis of DIO animals. This is contrary to the spectrophotometric measurement of SOD activity which evaluated the ability of SOD to inhibit the auto-oxidation of 6HD by the removal of superoxide radicals produced during the assay (Heikkila, 1985). SOD activity was lower in the DIO group compared to controls, implying a decreased ability to consume $O_2^-$ radicals. At the protein level, higher expression of SOD was observed, which seems to imply increased production of SOD in an attempt to cope with the increased demand. Chronic OS has been shown to deplete antioxidant enzyme, including SOD (Fernández-Sánchez *et al.*, 2011b). Due to the long-term nature of obesity and its persistent stimulation of OS, antioxidant sources become depleted, thereby reducing the activity of enzymes such as CAT and SOD (Amirkhizi *et al.*, 2010). Furthermore, SOD activity has also been previously found to be reduced in obese subjects compared to healthy controls (Ozata *et al.*, 2002; Fernández-Sánchez *et al.*, 2011b). In the event that SOD is depleted due to chronic OS or excessive ROS, *de novo* synthesis of SOD can be stimulated, resulting in increased production of the SOD protein (Miguel, Augusto and Gurgueira, 2009; Liu *et al.*, 2013; Nair *et al.*, 2015; Steinbacher and Eckl, 2015), as seen in the current study.

Glutathione-S-Transferases (GST) are a family of enzymes that stimulate the conjugation of GSH to electron-deficient molecules (electrophiles) (Strange, Jones and Fryer, 2000; Odbayar *et al.*, 2009). Cytosolic GSTs are found in all cellular life forms. In addition to their ability to assist in free radical detoxification, these enzymes are able to bind products of lipid peroxidation (Board and Menon, 2013). The most abundant in the cytosol is GSTM5, which has been under-expressed in the present study (Figure 5.7). This is a sperm specific isoform, the levels of which show rapid increases at weeks 6-7 of sperm development within the rat and are related to increased spermatogenesis (Rowe, Patskovsky, *et al.*, 1998). This isoform is easily broken down during OS (Rowe, Nieves and Listowsky, 1997), which explains the observed reduction of this protein in the current study. Furthermore, its decline implies a decrease in spermatogenesis due to the removal of this enzyme as a co-stimulant. Rats that have undergone hypohysectomy were demonstrated to have significantly lower GSTM5 expression after 5 weeks suggesting that the expression of this GST isoform is necessary for sperm production and maturation (Rowe, Tchaikovskaya, *et al.*, 1998). GSTM5 has been shown to anchor glycolytic enzymes, namely Hexokinase-1 and pyruvate

kinase, to sperm flagella (Krisfalusi *et al.*, 2006; Nakamura, Mori and Eddy, 2010). Its under-expression in this study implies an interruption in localization of glycolytic enzymes to sperm tails with a probable reduction in sperm motility in the long term due to reduced local delivery of ATP to distal regions of sperm flagella.

Membrane bound GSTs have also been located within mitochondrial membranes (Hayes and Pulford, 1995). The testes are rich with multiple forms of GSTs to afford the developing germ cells protection against free radical production (Hipler *et al.*, 2000). Testicular germ cells possess multiple isoforms of GST on their plasma membranes and their presence here is postulated to be beneficial to the removal of LPO intermediates (Hayes and Pulford, 1995) and harmful oxidants. The abundance of membrane-bound GSTs in haploid germ cells is necessary for the removal of surrounding toxins (Rao and Shaha, 2001) to which sperm are inherently vulnerable due to the abundance of polyunsaturated fatty acids (PUFAs) in sperm membranes (Kothari *et al.*, 2010). Their presence on cell membranes have also been linked to steroid binding which is necessary for cell function (Mukherjee *et al.*, 1999). Furthermore, their presence is confined to regions of the plasma membrane overlying the acrosome region in normal non-permeabilized germs cells at varying levels of maturity (Rao and Shaha, 2001). GSTA3, an isoform present on sperm plasma membranes, has been found to be overexpressed in the DIO epididymis of the current study. This implies an increased protection of DIO germ cells from membrane damage, possibly as a result of the implied increase in ROS molecules in the DIO animals. GSTM6L has not been well characterized, but shows glutathione transferase activity, and is under-expressed in the DIO epididymides.

Lipid peroxidation in both the testes and epididymides of the DIO animals was not significantly different to the amount occurring within the control animals. The reason for this could be the upregulation of proteins protecting the epididymis and sperm from peroxidation by free radicals including ALDH7A1, GSTA3, PRDX1 and PRDX6 (Figure 5.7). ALDH7A1, is a multifunctional enzyme which mediates protection against lipid peroxidation by metabolizing betaine aldehyde to betaine, an important cellular osmolyte and methyl donor (Brocker *et al.*, 2010). By doing so, it protects cells from OS by metabolizing a number of lipid peroxidation-derived aldehydes.

Vitamin D is a signaling molecule predominantly involved in the metabolism of calcium and phosphorus. It has multiple functions in reproduction including testosterone transport, the regulation of aromatase activity, calcium and phosphate transportation into spermatozoa, Leydig cells and the epididymis, and cholesterol homeostasis in Sertoli cells (Jensen, 2014). The vitamin D receptor (VDR) is present in male accessory glands and reproductive organs including the testes, epididymis, prostate and seminal vesicles. It is also present on Leydig cells, spermatozoa and the acrosomal cap (Olson Jr *et al.*, 1976; Addya *et al.*, 1991; Choudhary *et al.*, 2003, 2005;

Foresta *et al.*, 2011). The presence of the VDR and enzymes associated with vitamin D metabolism in the male reproductive system implies a local modulation of a response to vitamin D (Kidroni *et al.*, 1983). The testis and epididymis have a high binding affinity for the active form of vitamin D. Furthermore, vitamin D possesses antioxidant capabilities by decreasing lipid hydroperoxide levels and increasing total antioxidant capacity (Garcion *et al.*, 2002). Vitamin D-binding protein showed higher expression in the epididymis of DIO animals compared to controls (Figure 5.7). This implies a heightened use of vitamin D in protection by the body to help to increase antioxidant capacity.

Proteomic analysis of the control and obese tissues has revealed increased expression of additional antioxidant proteins during obesity, seemingly in order to increase antioxidant capacity of the reproductive organs. These include PDIA4, PARK7, PRDX1, PRDX6 and FAM213A (Figure 5.7).

Park 7 (Parkinson disease autosomal recessive, early onset 7) is an essential protein as it takes part in several cellular processes including autophagy and in the protection against oxidative stress and the maintenance of mitochondrial homeostasis (Wilson *et al.*, 2003; Kahle, Waak and Gasser, 2009). Upregulation of PARK7 is a protective mechanism to preserve mitochondrial and cell survival (McCoy and Cookson, 2011; Miller-Fleming *et al.*, 2014). This protein was observed to have increased expression in the epididymis of DIO animals compared to controls, implying an additional process through which the body may be attempting to reduce OS and increase cell survival.

The protein IMPACT is a regulator of protein translation and is used as a quick mechanism of adaptation to stress conditions (Roffé *et al.*, 2013). This protein was under-expressed in the epididymis of the DIO animals implying that the obese animals were not able to upregulate this protein in order to confer it's the protection. Furthermore, the downregulation of thioredoxin (TRX) in the DIO epididymis, which provides protection against nitric oxide (NO) insensitivity during periods of high NO exposure (Huang *et al.*, 2017) such as OS. This implies that the epididymis is insensitive to NO which can have downstream effects on NO in the DIO animals.

The data above implies that OS and inflammation are stimulated in the DIO animals, with a compensatory increase in antioxidant capacity via the increased expression of several antioxidant proteins in an attempt to reduce the possible damage that can be incurred by the potentiation of these processes. OS is a central mechanism through which many damage-inducing processes act and has been associated with obesity (Marseglia *et al.*, 2015). The implied existence of OS and inflammatory processes within the DIO animals could be a possible mechanism for cell damage as these have been implicated as significant causes of cellular injury which can result in damage to intracellular structures, ultimately leading to reduced cell function (Wiernsperger, 2003; King and

Loeken, 2004; Suanarunsawat *et al.*, 2014)(Wiernsperger *et al*., 2003; King and Loeken *et al*., 2004, Suanarunsawat *et al*., 2014).

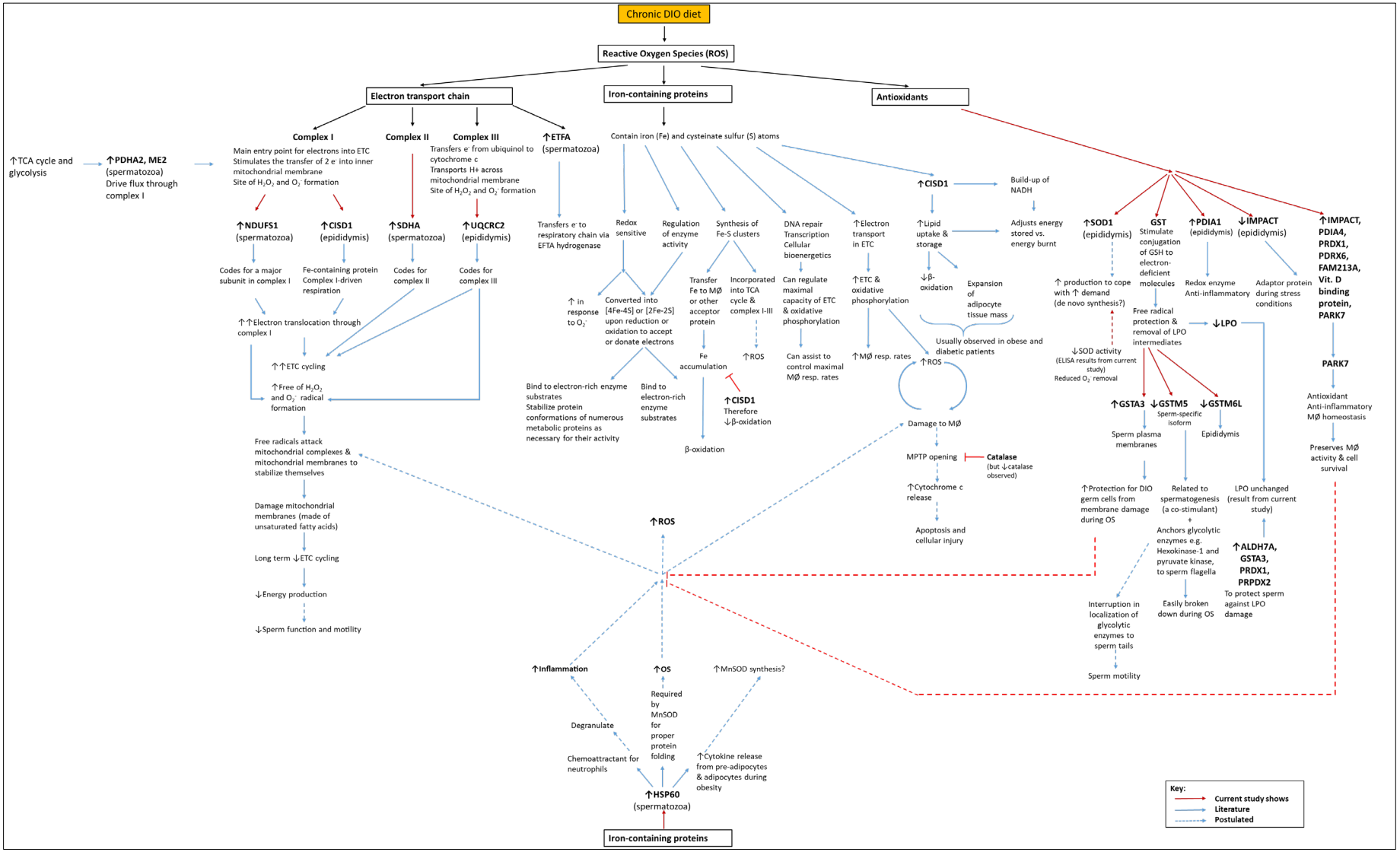



**Figure 5.7:** Summary of proteins related to the creation and elimination of ROS that were found to be differentially expressed in the current study. MØ = mitochondria.

### 5.3.5. Role of cytoskeletal proteins

Cytoskeletal proteins were variably affected by the OS and inflammation that appears to be present within the DIO animals. Actin proteins (ACTA1, ACTA2, ACTG2, ACTB), tubulins (TUBB4B, TUBB5), desmin (DES), vimentin (VIM), thymosin (TMSB4X), tropomyosin alpha-3 chain (TPM3) and microtubule-associated protein RP/EB family member 1 (MAPRE1) all showed higher expression in the DIO epididymis compared to controls (summarised in Figure 5.8). The proteins observed to be underexpressed in the DIO epididymis include plastin 3 (PLS3), fibrous sheath CABYR-binding protein (FSCB), lumican (LUM), catenin (CTNNA1), echinoderm microtubule-associated protein-like 2 (EML2), profilin-2 (PFN2), regulator of microtubule dynamics protein 1 (RMDN1) and surfeit 4 (SURF4) (Figure 5.8). Plastins are actinins that have been found in stereocillia and are responsible for the spatial arrangement of actin filaments within these structures (Daudet and Lebart, 2002). FSCB may be involved in the later stages of fibrous sheath generation. It binds calcium and has been localized to fibrous sheaths and the surface of longitudinal columns and ribs of the principal piece of sperm flagella. LUM is a proteoglycan protein, specifically a small leucine-rich proteoglycan, and has been reported to have an essential role in cell migration and proliferation during tissue growth, tissue repair and embryogenesis, and assists in the regulation of tissue hydration as well as collagen fibrillogenesis (Baba *et al.*, 2001; Onda *et al.*, 2002). It is also located in vascular smooth muscle cells, e.g. in the intima of blood vessels (Talusan *et al.*, 2005). CTNNA1 has been implicated in several important biological processes including ovarian follicle development, male gonad development and actin filament organisation. This protein is a catenin, which are cell adhesion proteins that combine with cadherins to form adherens junctions. Catenins are cytoplasmic anchoring (connecting) proteins that mediate the signalling of extracellular events to the interior of the cell. Additionally, catenins influence the architecture of differentiated tissue, as well as cell polarity, proliferation and in linking the cell surface to cytoplasmic and nuclear events through signal transduction (Aaltomaa *et al.*, 2005). Cadherins are plasma membrane proteins that participate in calcium-dependant cell adhesion. These proteins are essential for tissue development and differentiation in many organs and are important for tissue integrity and barrier functions (Sundfeldt *et al.*, 2000). The cytoplasmic portion of Epithelial cadherin (E-cadherin) binds to cytoplasmic anchoring proteins such as catenins to form adherens junctions (Piedra *et al.*, 2003). Beta catenins bind directly to E-catenin while alpha (α) catenins like CTNNA1 link E-cadherin bound β-catenins to the actin network of the cytoskeleton (Aaltomaa *et al.*, 2005). Cells that lack α-catenin expression are not able to form stable contacts even if β-catenins and E-cadherins are present (Hirano *et al.*, 1992). A study by Byers *et al*. (1994) which looked at the distribution of cadherins and catenins in rat testes found α- and β-catenins to be expressed in the seminiferous epithelium and prominently in Leydig cells, as well as in the basal part of seminiferous epithelia in the maturing testes. Another study (Zhang *et*

*al.*, 2006) located α-catenins in the acrosome of spermatids and in the cytoplasm of Leydig cells. Zhang *et al*. (2006) withdrew testosterone from adult rat testes with the use of testosterone undecanoate (TU) and assessed the expression of α-catenins. They found that α-catenins expression was reduced or absent in the TU-treated rat testes, resulting in Leydig cell atrophy. Ha *et al*. (2011) found ROS to significantly affect the expression of E-cadherin and α-catenin.

The expression of sperm proteins was predominantly not reduced in the sperm of the DIO group, with the exception of dynein light chain roadblock (DYNLRB2) and tubulin beta (TUBB1), which were under-expressed in the DIO sperm. Dyneins are motor proteins which aid in the movement of cilia and flagella (axonemes) and in driving movement of discrete structures along microtubules (Allan, 2011). They drive the sliding of microtubules and, if fixed to a cell structure, are able to pull on microtubules to reorganize the cell during migration and mitosis. Importantly, cells without dynein cannot divide properly (Allan, 2011). Tubulins are part of the microtubule family. Microtubules are filamentous structures that are responsible for directing the movement of vesicles, in organizing the endoplasmic reticulum (ER) and Golgi apparatus in the cytoplasm and in equally partitioning chromosomes before cell division (Bhattacharya and Cabral, 2004). EML2 is part of the echinoderm microtubule-associated protein (EMAP) family, a group of proteins involved in protein-protein interactions and active in cytoskeletal dynamics and signal transduction (Ly *et al.*, 2002). Furthermore, these proteins have been postulated to be an important link between signal transduction events and changes in the organization of microtubular structures during the cell cycle (Li and Suprenant, 1994). EMAPs are widely expressed in all peripheral tissues including the testes. They are also abundant in mitotic and interphase microtubule arrays (Li and Roberts, 2001) and are integral components of microtubules (Sloboda, Rosenbaum and Dentler, 1976). EML2 expression was reduced in the epididymis of DIO animals compared to controls

Taken together, this data suggests that while the expression of most cytoskeletal and structural proteins within the sperm and epididymis were predominantly unaffected in the DIO group, the functional proteins that are responsible for regulating their orientation and function were affected. The under-expression of essential proteins such as α-catenin, lumican and dynein have possibly comprised cell structure and function. Bearing in mind the histological changes observed in the tissue sections of the DIO and control epididymides, the reduced expression of plastin can be linked to the observed loss of definition within the stereocilia as this protein is necessary for the spatial arrangement of actin filaments within these structures. The reduction in α-catenin could be linked to the observed loss of adherence between the basement membrane and the epididymal epithelia, as the epithelia were seen to be less adherent to one another and to break away from the basement membrane. Furthermore, cell signalling between these structures could be altered as the loss of α-catenin has been shown to disrupt communication between cells and incoming extracellular signals which regulate cell movement and structure. The loss of cell adhesion could

also lead to cell atrophy and a disruption in cellular architecture. Lastly, the loss of α-catenin has been linked to acrosomal membranes. The reduction in this protein suggests either a loss of acrosomal membranes, implying an increased rate of acrosome reactions, or a loss of adhesion within the acrosomal membranes which could predispose spermatozoa and spermatids to premature acrosome reactions. This result can be coupled to the observed change in expression of an acrosomal protein named F-actin capping protein (CAPZA3) which is more abundantly expressed in the DIO sperm compared to controls. This protein belongs to a group of actin-capping proteins which are ubiquitously expressed components of mammalian cells (Hurst *et al.*, 1998). They are actin binding proteins which enable the actin cytoskeleton to rapidly respond to local or external signals and thereby promote changes in cell shape (Theriot, 1994). Capping proteins are co-localized with actin at the base of the acrosome in developing spermatocytes, almost as though to stabilize the position of the acrosome in relation to the nucleus (Fouquet and Kann, 1992). Furthermore, F-actin has been shown to break down during the acrosome reaction (Brener *et al.*, 2003). The increased expression of this protein in relation to the controls suggests that the acrosome is likely to be intact, therefore supporting that a decrease in α-catenin will likely affect the acrosomal membrane intactness but not necessarily in the of loss of acrosome membranes.

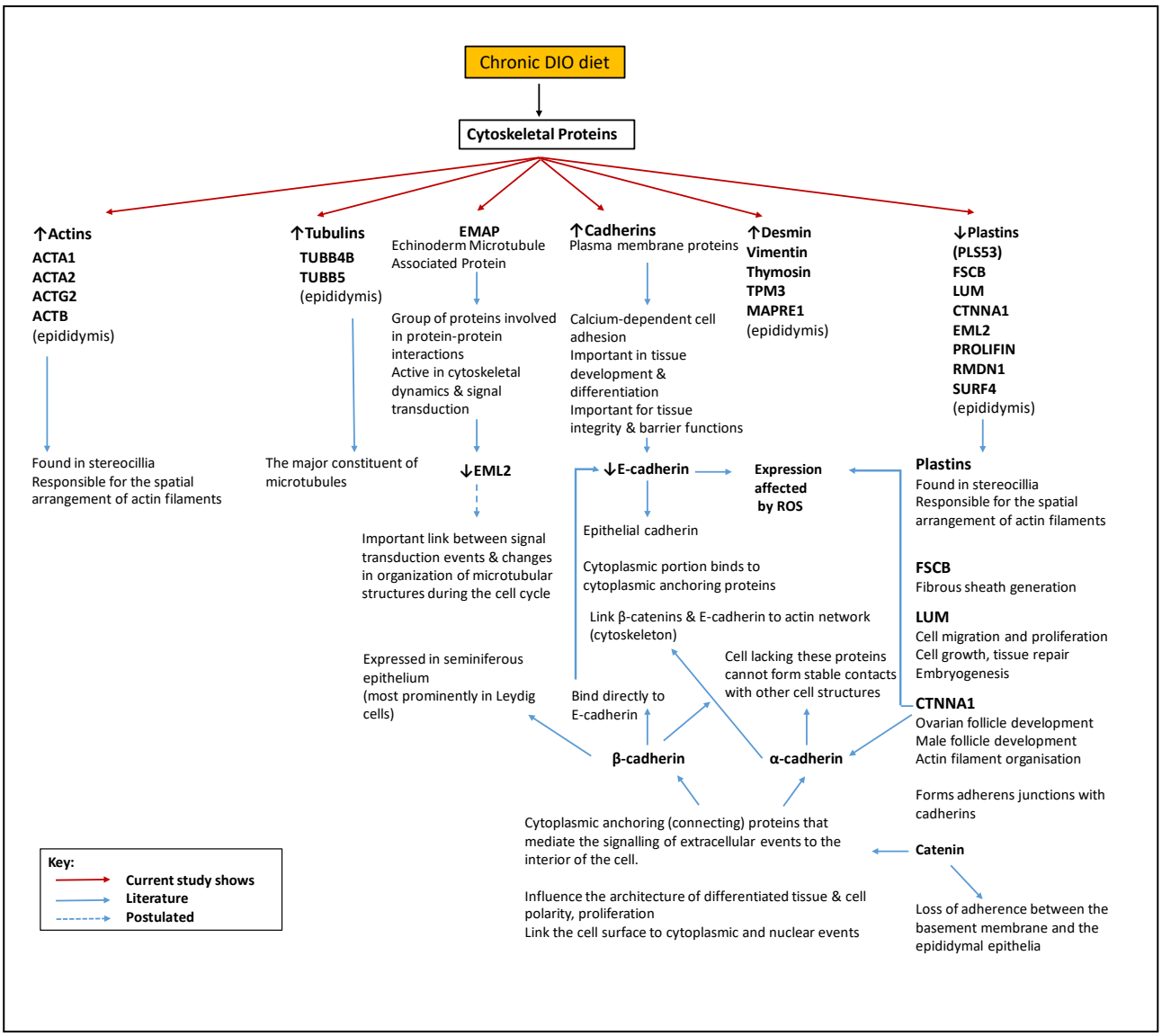



**Figure 5.8:** Summary of cytoskeletal proteins that were found to be differentially expressed in the current study.

### 5.3.6. Reproductive proteins

Several proteins specific to reproductive functions have been differentially expressed between the animals of the current study (summarised in Figure 5.9).

Slc25a11 is a mitochondrial oxidative phosphorylation protein that has also been shown to take part in spermatogenesis, and has been underexpressed in epididymis of the DIO group. Valosin-containing protein) (VCP) is a ubiquitous clathrin-binding protein that is involved in receptor-mediated endocytosis and Golgi sorting (Yeo and Yu, 2016). It has also been shown to have a catalytic similarity to chaperone proteins which indicates that it may modulate protein-protein interactions via membrane transport processes (Pleasure, Black and Keen, 1993). It recognises and co-localizes with ubiquitin-positive protein aggregates, thereby participating in the clearance of aggregates as part of its role in quality control of cellular proteins (Kobayashi *et al.*, 2002; Pécheur *et al.*, 2002). This function also pertains to abnormal protein aggregates (Kobayashi, Manno and Kakizuka, 2007). In addition to the removal of aggregates, the accumulation of misfolded proteins in the cytoplasm induces VCP to translocate to the nucleus and suppress the transcription of more misfolded proteins. This allows the cell to degrade and remove the existing misfolded proteins via cellular mechanisms such as autophagy, chaperones and proteasomes (Koike *et al.*, 2010) Importantly, it has also been implicated in the repair mechanism for double-stranded breaks within DNA (Panier and Boulton, 2014). VCP expression has been upregulated in the DIO epididymis of the current study, suggesting an increase in the repair of DNA strand breaks as well as the removal of misfolded proteins which could be resulting due to the observed increases in damage-inducing processes such as OS and inflammation induced by the obese phenotype. Its removal of misfolded proteins seems to be supported by other proteins including protein disulfide-isomerase (PDI), small ubiquitin-related modifier 2 (SUMO-2), HSP70 family protein 5 (HSPA5), 26S proteasome non-ATPase regulatory subunit 1 and 13 (PSMD1 and PSMD13) as well as proteasome subunit beta type-7 (PSMB7), which are all upregulated in the DIO epididymis.

Other sperm proteins affected by the DIO phenotype include sperm surface protein Sp17 (SPA17) and voltage-dependent anion-selective channel protein 2 (VDAC2). These proteins are necessary for the binding of sperm to the zona pellucida (ZP) and have been under-expressed in the epididymis of the DIO animals. Fertilization in mammals involves interactions by molecules present in both the egg and the sperm (Busso *et al.*, 2007). Cell-cell interactions between sperm and the oocyte's plasma membrane as well as cell-matrix interactions between the sperm and the cumulous cells surrounding oocyte, occur respectively during fertilization. SPA17 is a mannose-binding protein that is localized to the sperm head and flagella (Chiriva-Internati *et al.*, 2009). It has been shown to function upon acrosome reaction as a ZP binding protein, a function that has been corroborated by Chiriva-Internati *et al*. (2009) who observed immunoreaction of SPA17 with the ZP

matrix. SPA17 is therefore involved in a cell-matrix interaction during fertilization by binding to the carbohydrates in the extracellular matrix surrounding the oocyte (the ZP); the carbohydrates act as a receptor to allow cell recognition and attachment, thereby allowing SPA17 to bind sperm to the ZP with high affinity (Richardson, Yamasaki and O'rand, 1994). The binding of SPA17 within the flagella to AKAP (A-kinase anchoring protein), a scaffold protein which binds to protein kinase (PKA) and other components of signal transduction pathways, is postulated to involve this protein in the control of sperm motility (Lea, Widgren and O'Rand, 2004). VDAC2 is located at the flagella, in the head region overlying the acrosome and in the post-acrosomal region (Petit *et al.*, 2013). VDAC2 has been shown to interact with ZP proteins within the head region via its binding to zona pellucida proteins ZP2 and ZP3 in an experiment by Petite *et* al. (2013). Additionally, VDAC2 is a voltage-gated channel; at the head of the acrosome, it is postulated to be involved in the transport of ATP and calcium between the acrosome and the cytoplasm while on the flagella it is located on the outer mitochondrial membrane where it transports calcium, adenine nucleotides and other metabolites into the outer mitochondria, thereby participating in energy production (Petit *et al.*, 2013). Through these transport processes, VDAC has been implicated in capacitation, the acrosome reaction and in sperm motility (Liu, Zhang and Wang, 2010). The under-expression of these proteins suggests a reduced ability of the DIO sperm to capacitate and therefore undergo a timeous acrosome reaction as well as sperm-oocyte fusion. This reduce the ability of DIO sperm to fertilize an oocyte, thereby impacting on the fertility of the male.

COP9 signalosome subunit 3 (Cops3) is a protein which has been shown to have protein kinase activity and the control of ubiquitin ligases, a function through which it controls the degradation of several proteins involved in the cell cycle such as p53 and cyclin E as a controlling measure for the timeous expression of these proteins (Yan *et al.*, 2003). Disruptions in the function of this protein results in the accumulation of these proteins and cell cycle arrest, resulting in deficient cell proliferation and the death of early embryos (Lykke-Andersen *et al.*, 2003). COP9 is under-expressed in the DIO epididymis, suggesting that the DIO sperm have a reduced ability to undergo the appropriate cell cycle progression that is necessary for embryogenesis, which can predispose the DIO animals to early embryonic death.

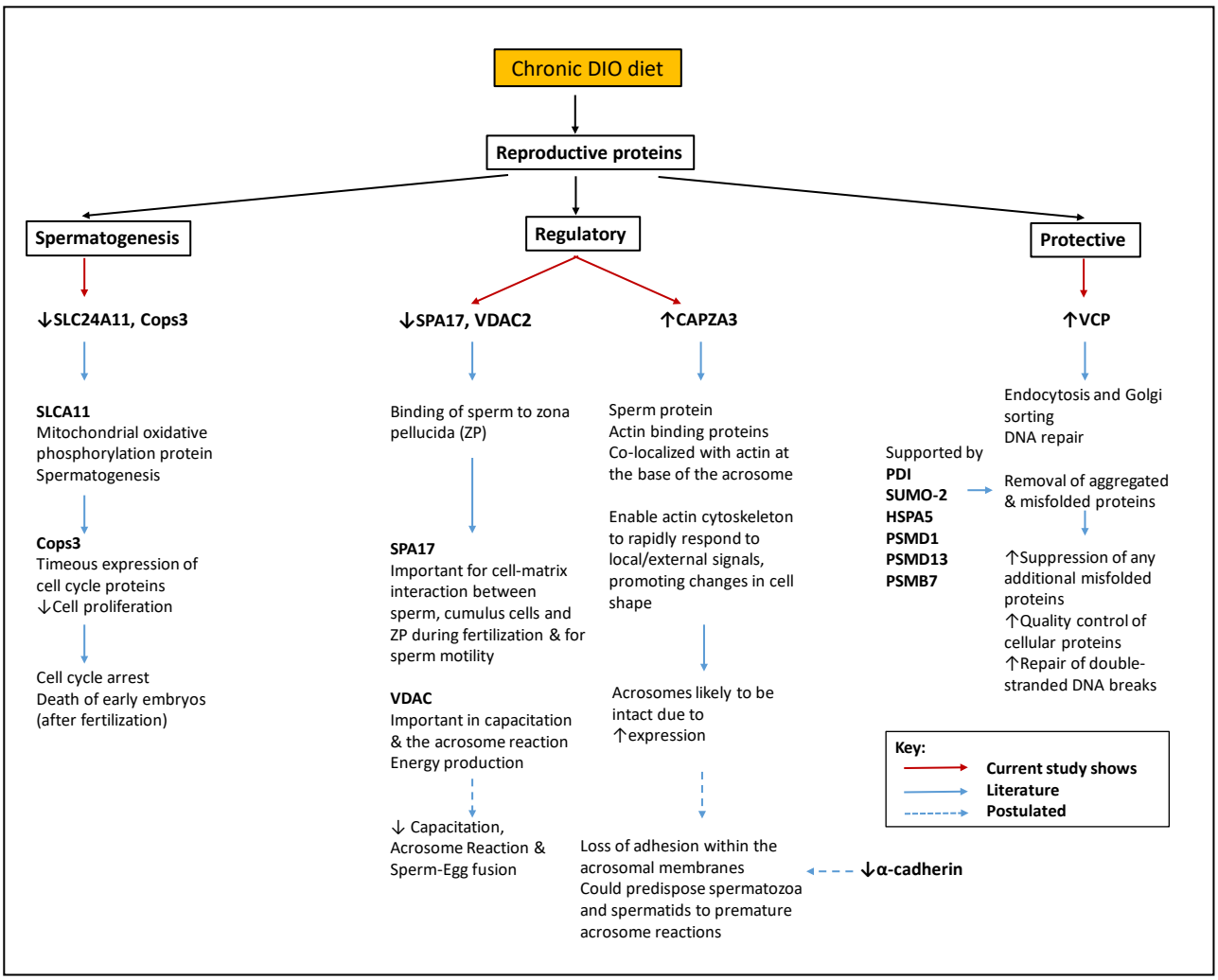



**Figure 5.9:** Summary of proteins involved in reproductive processes that were found to be differentially expressed in the current study, including the functions and possible interactions postulated based on literature.

## 5.4. Summary of protein networks affected by the DIO diet

Several protein networks were affected by the diet and the subsequent weight gain observed in the DIO animals. The predominant pathways affected were related to metabolism, specifically influencing proteins involved in glycolysis, β-oxidation and oxidative phosphorylation. Structural proteins involved in cell-to-cell communication, extracellular matrix organization, chromatin organization, protein localization, signal transduction, muscle contraction and cell motility showed differential expression after the introduction of the chronic obesogenic diet. These changes were possibly brought on by increases in stress proteins including redox, inflammatory and heat shock proteins. Regulatory pathways essential for cell transport, signal transduction, the immune system, organelle biogenesis and maintenance, homeostasis and the removal of aberrant cellular structures such as autophagy and programmed cell death, were dysregulated. In many cases the proteins involved in these pathways were overexpressed, possibly as a compensatory mechanism to stimulate the removal of misfolded proteins and detrimental cellular structures which can hinder the survival and well-being of DIO tissues. Cell cycle as well as DNA repair and replication proteins, which are essential for cell viability and the production of functional proteins, were affected by the diet. This influences the capacity of DIO organs to induce these multi-pathway, multi-enzyme systems which are necessary for the integrity of the cellular genome. Proper regulation of these pathways is essential for the survival of cells after cellular damage from metabolic by-products and environmental insults, by stimulating a variety of repair mechanisms including the repair of single- and double-stranded breaks prior to replication. Proteins essential for proper reproductive function were differentially expressed between the study groups. Reproduction includes sperm and oocyte production, fertilization as well as the early stages of embryonic development; the expression of proteins involved in the proper functioning of these pathways were negatively affected. Proteins necessary for the capacitation and acrosome reaction of sperm were underexpressed as well as those necessary for the facilitation of sperm-egg fusion. These are necessary for proper reproductive function and their aberrant expression can lead to infertility.

# Chapter 6: Conclusion

The induction of obesity via diet and a sedentary lifestyle provides similar metabolic responses in animals to those observed in humans, therefore diet-induced obesity animal models are effective in mimicking the pathophysiology of vascular and metabolic complications which have been shown to arise during obesity. This study found a chronic diet to be more effective in inducing obesity, and offers new data on the effects of a long-term obesogenic diet.

The increase in the adiposity of the diet treated animals brought along various physiological changes within the DIO group. Lower percentages of normal sperm morphology and viable sperm as well as supra-physiological levels of estradiol were observed. These changes show various relationships with each other and the other parameters that were measured within this study. This can assist in drawing links between the changes in sperm functions and reproductive parameters with other underlying physiological mechanisms.

Sperm morphology was seen in the current study to be associated with aberrant changes in testicular histology. Although the weights of the testes were not significantly different between the two study groups, adverse microscopic changes occurred within the DIO animals. These histological changes were related to lowered relative testicular weights, higher blood glucose levels, and changes in testosterone concentrations and antioxidant capacity. The changes in testicular histology were linked to alterations in sperm morphology and morphometry. Furthermore, while numerous seminiferous tubules displayed altered histology, some tubules with normal histology and qualitative and quantitative parameters were present. This implies that while damage can occur within the testicular tissue, unaffected seminiferous tubules could still produce normal spermatozoa. This can explain why obese humans and animals are still able to reproduce, as their fertility is lowered but not necessarily lost. The changes in epididymal histology of the DIO animals were linked to alterations in the redox state, where lowered catalase activity and higher lipid peroxidation were related to changes in epididymal diameters and relative volumes. These changes are more likely to reduce sperm viability and sperm morphometry. This study also found estradiol and not testosterone to be affected by chronic obesity. This crucial hormone has widespread effects on the male reproductive system and a case has now been made for its levels to be as closely monitored as testosterone in obese males.

Proteomic analysis in the current study has shown a large number of differentially expressed proteins to be involved in glycolysis, oxidative phosphorylation and in lipid metabolism. In the epididymis, glycolytic proteins essential in sensing and responding to fluctuations in glucose concentrations, in initiating an insulin-sensitive response to the presence of glucose, and in the metabolism of glucose, amino acids and glycerol, were downregulated. At the same time, the

epididymis appeared to compensate for these disturbances by increasing the expression of proteins that upregulate glycolysis but were not dependent on glucose as an energy substrate. Moreover, insulin sensitivity seems to be lowered in the DIO reproductive system, which can lower glucose uptake by cells and lead to an increase plasma glucose. Reductions in the proteins necessary for the metabolism of glycerol, fatty acids and amino acids for use in sperm membrane production and in the attainment of membrane fluidity, leads to a postulation that sperm maturation, function and viability have been compromised.

The presence of several proteins involved in lipid metabolism have been observed in the present study which suggests the use of lipids as metabolic substrates in the study animals. These proteins stimulate or mediate lipolysis, lipid oxidation, adipogenesis, adipocyte differentiation and β-oxidation. Furthermore, several key proteins involved in fatty acid metabolism were under expressed in the epididymis. This includes proteins which are involved in the control of adipocyte growth and in limiting lipid storage within lipid droplets. It also includes those that mediate the synthesis and degradation of cellular lipids, and catalyse the first step of fatty acid metabolism. On the other hand, peroxisomal proteins have been reduced in expression. This data points towards a lower amount of fatty acid catabolism in the DIO epididymis compared to controls. The DIO animals seem to have a lower ability to metabolize fatty acids for their use as energy substrates for somatic cells as well as the maturation of stored spermatozoa within the epididymis. The apparently lower fatty acid metabolism in the DIO epididymis therefore implies a reduced usage of fatty acids as energy substrates of metabolism and for sperm maturation and hormone production.

The reduction in the ability of the epididymis to limit adipocyte growth and lipid storage suggests the reduced ability to limit adipocyte growth, which implies a greater number and/or size of adipocytes within the DIO epididymides. On the other hand, antioxidant proteins secreted by adipocytes and located on the principle cells of the epididymis, were increased. This suggests a compensatory increase in antioxidant ability to assist in the adverse increase in ROS that can result from this higher abundance of adipocytes.

The spermatozoa of the DIO group having a higher expression of the ketone enzyme SCOT1 implies an additional mechanism through which diet-induced obesity can make more energy substrates available when substrates from glycolysis and fatty acid oxidation are inadequate. Thus the lower glycolysis noted in the DIO animals is likely to be compensated by an increase in ketone usage by the epididymis and sperm, which can then increase the expression of TCA cycle enzymes and substrates to possibly yield an increase ATP production.

Taken together, the changes in expression of the aforementioned proteins point to an increased rate of cellular metabolism and inflammation in the obese condition, which can increase the

production of free radicals. These changes seem to have an effect on numerous structural proteins within the epididymis and sperm of the DIO animals. Cytoskeletal proteins including actins, tubulins, cadherins and plastins were variably affected. These proteins are essential for cellular structure and function, as well as in cell motility. Our study data suggests that while the expression of most cytoskeletal and structural proteins within the sperm and epididymis were predominantly unaffected in the DIO group, the functional proteins that are responsible for regulating their orientation and function were influenced. In addition, the under-expression of essential proteins such as α-catenin, lumican and dynein have possibly comprised cell structure and function. Bearing in mind the histological changes observed in the tissue sections of the DIO and control epididymides, the reduced expression of these proteins can be linked to the observed loss of adherence between the basement membrane and the epididymal epithelia as well as the loss of definition within the stereocilia. The loss of cell adhesion could also lead to cell atrophy and a disruption in cellular architecture. Lastly, the loss of α-catenin has been linked to acrosomal membranes. The reduction in this protein suggests either a loss of acrosomal membranes, implying an increased rate of acrosome reactions, or a loss of adhesion within the acrosomal membranes which could predispose spermatozoa and spermatids to premature acrosome reactions.

Several reproductive parameters have been affected by the diet and the subsequent weight gain observed in the DIO animals. This includes those responsible for sperm production, fertilization as well as the early stages of embryonic development. It is therefore evident that long term obesity can impair male reproductive parameters and could be a contributing factor to the decline in male infertility by affecting sperm and reproductive parameters. This information allows us to accept our hypothesis, as the chronic diet-induced obesity model had a negative effect on sperm parameters, and on reproductive proteins that are responsible for normal reproductive function(s). Obesity in males is therefore a matter that needs continuing intervention as it may lead to changes in fertilizing capacity. Lifestyle changes are thus encouraged to reduce its incidence and prevalence. In addition, intervention strategies aiming to treat and/or prevent the anomalies caused by diet-induced obesity needs to target metabolic, inflammatory and ROS-generating pathways to prevent subsequent cellular damage in obese patients. Targeting some of the aforementioned proteins by means of targeted therapy, antioxidant therapy or anti-inflammatory mechanisms is worth exploration. This can help to alleviate some of the effects of obesity on body systems including the reproductive system.

## 6.1. Project limitations and future recommendations

There are several limitations in this study that need to be addressed by future studies. The measurement of obesity using the Lee Index (Lee, 1929) is recommended as it a sensitive

classification of animal obesity, which includes body length as a parameter to determine obesity. Fasted blood glucose can be used to reduce the interference of food consumption in the measurement of blood glucose concentrations. Sperm motility should be measured in subsequent studies. As the epididymides also had to be collected in the current study for antioxidant assays, organs were placed on ice to prevent metabolic reactions that can increase the production of free radicals after organ extraction. This, therefore, prevented the examination of sperm motility. Other hormone concentrations will need to be assessed, including but not limited to Gonadotropin Releasing Hormone (GnRH), Luteinizing Hormone and Follicle Stimulating Hormone (FSH) to better assess hormone alterations and to classify hypogonadism. Additional hormines to be assessed include leptin, ghrelin and steroidogenic hormones. Since metabolic and inflammatory mechanisms seem to have a role as underlying mechanisms affected by obesity, more extensive metabolic and inflammatory profiles need to be established in future experiments. Plasma or serum insulin, adipokines and metabolic substrates need to be measured to provide more information regarding underlying metabolic processes. Oral glucose tolerance tests also need to be performed throughout the experiment to determine insulin sensitivity. The measurement of metabolic proteins in urine and blood, including creatinine, ketones, glucose, nitrites and amino acids need to be assessed. This can allow for the comparison of molecular and proteomic data, and allow for more definite conclusions regarding causation to be ascertained. Inflammatory proteins can also be analysed via the assessment of e.g. multiplex assays that can give a better profile of the inflammatory mediators that are affected by obesity. A mating study can also be carried out to assess the effect of obesity on the number of live offspring.

## References

Aaltomaa, S. *et al.* (2005) 'Reduced α- and β-catenin expression predicts shortened survival in local prostate cancer', *Anticancer Research*, 25(6 C), pp. 4707–4712. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16334164 (Accessed: 10 November 2019).

Abate, N. *et al.* (1995) 'Relationships of generalized and regional adiposity to insulin sensitivity in men', *Journal of Clinical Investigation*, 96(1), pp. 88–98. doi: 10.1172/JCI118083.

Abcam (2019) *Fatty acid oxidation*. Available at: https://www.abcam.com/pathways/fatty-acid-oxidation (Accessed: 17 November 2019).

Addya, S. *et al.* (1991) 'Characterization of a female-specific hepatic mitochondrial cytochrome P-450 whose steady-state level is modulated by testosterone', *Biochemistry*, 30(34), pp. 8323–8330. doi: 10.1021/bi00098a007.

Adler, I. D. (1996) 'Comparison of the duration of spermatogenesis between male rodents and humans', *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, 352(1–2), pp. 169–172. doi: 10.1016/0027-5107(95)00223-5.

Aebersold, R. H. *et al.* (1987) 'Internal amino acid sequence analysis of proteins separated by one- or two-dimensional gel electrophoresis after in situ protease digestion on nitrocellulose.', *Proceedings of the National Academy of Sciences of the United States of America*, 84(20), pp. 6970–6974. doi: 10.1073/pnas.84.20.6970.

Afshin, A. *et al.* (2017) 'Health effects of overweight and obesity in 195 countries over 25 years', *New England Journal of Medicine*, 377(1), pp. 13–27. doi: 10.1056/NEJMoa1614362.

Afzelius, B. (1959) 'Electron Microscopy of the Sperm Tail Results Obtained with a New Fixative', *The Journal of Cell Biology*, 5(2), pp. 269–278. doi: 10.1083/jcb.5.2.269.

Ahima, R. S. *et al.* (1999) 'Distinct physiologic and neuronal responses to decreased leptin and mild hyperleptinemia', *Endocrinology*, 140(11), pp. 4923–4931. doi: 10.1210/endo.140.11.7105.

Ahlma, R. S. *et al.* (1996) 'Role of leptin in the neuroendocrine response to fasting', *Nature*, 382(6588), pp. 250–252. doi: 10.1038/382250a0.

Ahmed, R. G. (2005) 'The physiological and biochemical effects of diabetes on the balance between oxidative stress and antioxidant defense system', *Medical Journal of Islamic World Academy of Sciences*, 15(1), pp. 31–42.

Ailhaud, G. (1982) 'Adipose cell differentiation in culture', *Molecular and Cellular Biochemistry*, 49(1), pp. 17–31. doi: 10.1007/BF00230992.

Aitken, R. J. (1994) 'A free radical theory of male infertility', *Reproduction, Fertility and Development*, 6(1), pp. 19–24. doi: 10.1071/RD9940019.

Aitken, R. J. *et al.* (1997) *Male Infertility and Sexual Dysfunction*. Edited by W. J. G. Hellstrom. New York, United States of America: Springer Science+Business Media. doi: 10.1007/978-1-4612-1848-7.

Aitken, R. J. (1999) 'The Amoroso Lecture The human spermatozoon – a cell in crisis?', *Reproduction*, 115(1).

Aitken, R. J., Buckingham, D. and Harkiss, D. (1993) 'Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa', *Journal of Reproduction and Fertility*, 97, pp. 441–450.

Aitken, R. J. and Clarkson, J. S. (1987) 'Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa', *Reproduction*, 81(2), pp. 459–469.

Aitken, R. J. and Sawyer, D. (2003) 'The Human Spermatozoon — Not Waving but Drowning BT - Advances in Male Mediated Developmental Toxicity', in Robaire, B. and Hales, B. F. (eds) *Advances in Male Mediated Developmental Toxicity*. Boston, MA: Springer US, pp. 85–98. doi: 10.1007/978-1-4419-9190-4_8.

Ajuogu, P. K. *et al.* (2020) 'Effect of low- and high-protein maternal diets during gestation on reproductive outcomes in the rat: a systematic review and meta-analysis.', *Journal of animal science*, 98(1). doi: 10.1093/jas/skz380.

Alaba, O. and Chola, L. (2014) 'Socioeconomic inequalities in adult obesity prevalence in South Africa: A decomposition analysis', *International Journal of Environmental Research and Public Health*, 11(3), pp. 3387–3406. doi: 10.3390/ijerph110303387.

Albu, J. B. *et al.* (1997) 'Visceral fat and race-dependent health risks in obese nondiabetic premenopausal women', *Diabetes*, 46(3), pp. 456–462. doi: 10.2337/diab.46.3.456.

Allan, V. J. (2011) 'Cytoplasmic dynein', *Biochemical Society Transactions*, 39(5), pp. 1169–1178. doi: 10.1042/BST0391169.

Aly, J. M. and Polotsky, A. J. (2017) 'Paternal Diet and Obesity: Effects on Reproduction',

*Seminars in Reproductive Medicine*, 35(4), pp. 313–317. doi: 10.1055/s-0037-1602593.

Amaral, A. *et al.* (2013) 'Human sperm tail proteome suggests new endogenous metabolic pathways', *Molecular and Cellular Proteomics*, 12(2), pp. 330–342. doi: 10.1074/mcp.M112.020552.

Amaral, S. *et al.* (2006) 'Effects of hyperglycemia on sperm and testicular cells of Goto-Kakizaki and streptozotocin-treated rat models for diabetes', *Theriogenology*, 66(9), pp. 2056–2067. doi: https://doi.org/10.1016/j.theriogenology.2006.06.006.

Amatruda, J. M. *et al.* (1978) 'Depressed plasma testosterone and fractional binding of testosterone in obese males', *Journal of Clinical Endocrinology and Metabolism*, 47(2), pp. 268–271. doi: 10.1210/jcem-47-2-268.

Amirkhizi, F. *et al.* (2010) 'Is obesity associated with increased plasma lipid peroxidation and oxidative stress in women?', *Arya Atherosclerosis*, 2(4), pp. 189–192.

Anand, B. K. and Brobeck, J. R. (1951) 'Localization of a "Feeding Center" in the Hypothalamus of the Rat', *Proceedings of the Society for Experimental Biology and Medicine*, 77(2), pp. 323–325. doi: 10.3181/00379727-77-18766.

Andersen, I., Heitman, B. L. and Wagner, G. (2008) 'Obesity and sexual dysfunction in Younger Danish Men', *Journal of Sexual Medicine*, 5(9), pp. 2053–2060. doi: 10.1111/j.1743-6109.2008.00920.x.

Anderson, K., Nisenblat, V. and Norman, R. (2010) 'Lifestyle factors in people seeking infertility treatment - A review: Invited Review', *Australian and New Zealand Journal of Obstetrics and Gynaecology*, 50(1), pp. 8–20. doi: 10.1111/j.1479-828X.2009.01119.x.

Anderson, N. G. and Anderson, N. L. (1996) 'Twenty years of two-dimensional electrophoresis: Past, present and future', *Electrophoresis*, 17(3), pp. 443–453. doi: 10.1002/elps.1150170303.

Anderson, N. L., Matheson, A. D. and Steiner, S. (2000) 'Proteomics: Applications in basic and applied biology', *Current Opinion in Biotechnology*, 11(4), pp. 408–412. doi: 10.1016/S0958-1669(00)00118-X.

Anderson, W. A. and Personne, P. (1975) 'The Form and Function of Spermatozoa: A Comparative View', in Afzelius, B. A. (ed.) *Wenner-Gren Center International Symposium Series*. Oxford: Pergamon Press, Ltd, p. 3.

Angulo, M. A., Butler, M. G. and Cataletto, M. E. (2015) 'Prader-Willi syndrome: A review of

clinical, genetic, and endocrine findings', *Journal of Endocrinological Investigation*, 38(12), pp. 1249–1263. doi: 10.1007/s40618-015-0312-9.

Apalasamy, Y. D. and Mohamed, Z. (2015) 'Obesity and genomics: role of technology in unraveling the complex genetic architecture of obesity', *Hum Genet*, 134, pp. 361–374. doi: 10.1007/s00439-015-1533-x.

Aparicio, N. J. I. *et al.* (1980) 'The Effect of a Treatment with Doxycycline on Semen of Asthenozoospermic Patients with T-Mycoplasma Genital Infection', *Andrologia*, 12(6), pp. 521–524. doi: 10.1111/j.1439-0272.1980.tb01343.x.

Aquilano, K., Baldelli, S. and Ciriolo, M. R. (2014) 'Glutathione: new roles in redox signaling for an old antioxidant', *Frontiers in Pharmacology*, 5, p. 196. doi: 10.3389/fphar.2014.00196.

Araujo, A. B. *et al.* (2007) 'Prevalence of Symptomatic Androgen Deficiency in Men', *The Journal of Clinical Endocrinology & Metabolism*, 92(11), pp. 4241–4247. doi: 10.1210/jc.2007-1245.

Arsenescu, V. *et al.* (2008) 'Polychlorinated biphenyl-77 induces adipocyte differentiation and proinflammatory adipokines and promotes obesity and atherosclerosis', *Environmental Health Perspectives*, 116(6), pp. 761–768. doi: 10.1289/ehp.10554.

Asai, J. *et al.* (1989) 'Isolation and sequence determination of ratislet amyloid polypeptide', *Biochemical and Biophysical Research Communications*, 164(1), pp. 400–405. doi: 10.1016/0006-291X(89)91733-6.

Asai, J. *et al.* (1990) 'Regional distribution and molecular forms of rat islet amyloid polypeptide', *Biochemical and Biophysical Research Communications*, 169(2), pp. 788–795. doi: 10.1016/0006-291X(90)90400-H.

Auger, J. (2018) 'Spermatozoa and Sperm Structure', in *Encyclopedia of Reproduction*. Elsevier Inc., Academic Press, pp. 62–67.

Ayers, J. W. T. *et al.* (1985) 'Anthropomorphic, hormonal, and psychologic correlates of semen quality in endurance-trained male athletes**Supported by a grant from the Max Burnell Trust, Flint MacLaren Hospital, Flint, Michigan.', *Fertility and Sterility*, 43(6), pp. 917–921. doi: 10.1016/s0015-0282(16)48622-x.

Baba, H. *et al.* (2001) 'Expression and localization of lumican in the ischemic and reperfused rat heart', *Japanese Circulation Journal*, 65(5), pp. 445–450. doi: 10.1253/jcj.65.445.

Bacon, C. G. *et al.* (2003) 'Sexual Function in Men Older Than 50 Years of Age: Results from the

Health Professionals Follow-up Study', *Annals of Internal Medicine*, 139(3). doi: 10.7326/0003-4819-139-3-200308050-00005.

Bagatell, C. J. and Bremner, W. J. (1990) 'Sperm counts and reproductive hormones in male marathoners and lean controls', *Fertility and Sterility*, 53(4), pp. 688–692. doi: 10.1016/S0015-0282(16)53465-7.

Bagchi, M. and Stohs, S. J. (1993) 'In vitro induction of reactive oxygen species by 2,3,7,8-tetrachlorodibenzo-p-dioxin, endrin, and lindane in rat peritoneal macrophages, and hepatic mitochondria and microsomes', *Free Radical Biology and Medicine*, 14(1), pp. 11–18. doi: 10.1016/0891-5849(93)90504-N.

Bakos, H. W. *et al.* (2011a) 'The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model', *International Journal of Andrology*, 34(5 PART 1), pp. 402–410. doi: 10.1111/j.1365-2605.2010.01092.x.

Bakos, H. W. *et al.* (2011b) 'The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model', *International Journal of Andrology*, 34(5 PART 1), pp. 402–410. doi: 10.1111/j.1365-2605.2010.01092.x.

Ballatori, N. *et al.* (2009) 'Glutathione dysregulation and the etiology and progression of human diseases', *Biological Chemistry*, p. 191. doi: 10.1515/BC.2009.033.

Barouki, R. (2010) 'Linking long-term toxicity of xeno-chemicals with short-term biological adaptation', *Biochimie*, 92(9), pp. 1222–1226. doi: 10.1016/j.biochi.2010.02.026.

Barreira, T. V. *et al.* (2014) 'Relationship of anthropometric indices to abdominal and total body fat in youth: Sex and race differences', *Obesity*, 22(5), pp. 1345–1350. doi: 10.1002/oby.20714.

Barroso, N. *et al.* (1993) 'Respiratory chain enzyme activities in lymphocytes from untreated patients with Parkinson disease', *Clinical Chemistry*, 39(4), pp. 667–669.

Bassil, N., Alkaade, S. and Morley, J. E. (2009) 'The benefits and risks of testosterone replacement therapy: a review.', *Therapeutics and clinical risk management*, 5(3), pp. 427–48. doi: 10.2147/tcrm.s3025.

Bedford, J. M. (1990) 'Sperm dynamics in the epididymis', *Gamete physiology*, pp. 53–68.

Bedford, J. M. (1994) 'The status and the state of the human epididymis', *Human Reproduction*, 9(11), pp. 2187–2199. doi: 10.1093/oxfordjournals.humrep.a138416.

Bedrak, E., Chap, Z. and Fried, K. (1980) 'Factors for consideration in the interpretation of the adverse effects of elevated environmental temperatures on reproduction in the male rat', *International Journal of Biometeorology*, 24(2), pp. 117–128. doi: 10.1007/BF02253799.

Behre, H. M. *et al.* (2010) 'Diseases of the Hypothalamus and the Pituitary Gland', in Nieschlag, E., Behre, H. M., and Nieschlag, S. (eds) *Andrology: Male Reproductive Health and Dysfunction*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 169–192. doi: 10.1007/978-3-540-78355-8_12.

Beinert, H., Holm, R. H. and Münck, E. (1997) 'Iron-sulfur clusters: Nature's modular, multipurpose structures', *Science*, 277(5326), pp. 653–659. doi: 10.1126/science.277.5326.653.

Bell, C. G., Walley, A. J. and Froguel, P. (2005) 'The genetics of human obesity', *Nature Reviews Genetics*, 6(3), pp. 221–234. doi: 10.1038/nrg1556.

Bénit, P. *et al.* (2001) 'Large-Scale Deletion and Point Mutations of the Nuclear NDUFV1 and NDUFS1 Genes in Mitochondrial Complex I Deficiency', *The American Journal of Human Genetics*, 68(6), pp. 1344–1352. doi: https://doi.org/10.1086/320603.

Benzinou, M. *et al.* (2008) 'Common nonsynonymous variants in PCSK1 confer risk of obesity', *Nature Genetics*, 40(8), pp. 943–945. doi: 10.1038/ng.177.

Berg, J., Tymoczko, J. and Stryer, L. (2002) 'Oxidative Phosphorylation', in Freeman, W. H. (ed.) *Biochemistry*. 5th edn. New York: National Center for Biotechnology Information (NCBI). Available at: https://www.ncbi.nlm.nih.gov/books/NBK21208/ (Accessed: 7 November 2019).

Berger, R. E. *et al.* (1983) 'Improvement in the sperm penetration (hamster ova) assay (SPA) results after doxycycline treatment of infertile men.', *Journal of andrology*, 4(2), pp. 126–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6853357 (Accessed: 9 October 2019).

Bernardis, L. L. (1970) 'Prediction of carcass fat, water and lean body mass from Lee's "nutritive ratio" in rats with hypothalamic obesity', *Experientia*, 26(7), pp. 789–790. doi: 10.1007/BF02232553.

Bernardis, L. L. and Patterson, B. D. (1968) 'Correlation between "Lee index" and carcass fat content in weanling and adult female rats with hypothalamic lesions.', *The Journal of endocrinology*, 40(4), pp. 527–528. doi: 10.1677/joe.0.0400527.

Berndtson, W. E. and Jones, L. S. (1989) 'Relationship of intratesticular testosterone content of stallions to age, spermatogenesis, Sertoli cell distribution and germ cell–Sertoli cell ratios', *Reproduction*, 85(2), pp. 511–518.

Bertinato, J. *et al.* (2014) 'Diet-induced obese rats have higher iron requirements and are more vulnerable to iron deficiency', *European Journal of Nutrition*, 53(3), pp. 885–895. doi: 10.1007/s00394-013-0592-9.

Betsholtz, C., Svensson, V., *et al.* (1989) 'Islet amyloid polypeptide (IAPP): cDNA cloning and identification of an amyloidogenic region associated with the species-specific occurrence of age-related diabetes mellitus', *Experimental Cell Research*, 183(2), pp. 484–493. doi: 10.1016/0014-4827(89)90407-2.

Betsholtz, C., Christmansson, L., *et al.* (1989) 'Sequence divergence in a specific region of islet amyloid polypeptide (IAPP) explains differences in islet amyloid formation between species', *FEBS Letters*, 251(1–2), pp. 261–264. doi: 10.1016/0014-5793(89)81467-X.

Bhagavan, N. . (2002) 'Carbohydrate Metabolism II: Gluconeogenesis, Glycogen Synthesis and Breakdown, and Alternative Pathways', in *Medical Biochemistry*. Elsevier, pp. 275–305. doi: 10.1016/b978-012095440-7/50017-2.

Bhagavan, N. V and Ha, C.-E. (2015) *Essentials of Medical Biochemistry with Clinical Cases, Second Edition*. Academic Press, Elsevier Inc. doi: 10.1016/B978-0-12-095461-2.00014-X.

Bhasin, S. *et al.* (2010) 'Testosterone Therapy in Men with Androgen Deficiency Syndromes: An Endocrine Society Clinical Practice Guideline', *The Journal of Clinical Endocrinology & Metabolism*, 95(6), pp. 2536–2559. doi: 10.1210/jc.2009-2354.

Bhattacharya, R. and Cabral, F. (2004) 'A ubiqiutous β-tubulin disrupts microtubule assembly and inhibits cell proliferation', *Molecular Biology of the Cell*, 15(7), pp. 3123–3131. doi: 10.1091/mbc.E04-01-0060.

Bhattacharyya, A. K. and Kanjilal, S. (2003) 'Assessment of sperm functional competence and sperm-egg interaction', *Molecular and Cellular Biochemistry*, 253(1–2), pp. 255–261. doi: 10.1023/A:1026024202288.

Bjorntorp, P. *et al.* (1990) 'Regional obesity and steroid hormone interactions in human adipose tissue', in Bray, G., Ricquier, D., and Spiegelman, B. (eds) *Obesity: towards a molecular approach*. New York, NY: Wiley-Liss New York, NY, pp. 147–157.

Björntorp, P. (1990) '"Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes.', *Arteriosclerosis: An Official Journal of the American Heart Association, Inc.*, 10(4), pp. 493–496.

Björntorp, P. (1991) 'Metabolic implications of body fat distribution', *Diabetes Care*, 14(12), pp. 1132–1143. doi: 10.2337/diacare.14.12.1132.

Björntorp, P. (1995) 'Endocrine abnormalities of obesity', *Metabolism*, 44(SUPPL. 3), pp. 21–23. doi: 10.1016/0026-0495(95)90315-1.

Blanco-Rodríguez, J. and Martínez-García, C. (1997) 'Apoptosis pattern elicited by oestradiol treatment of the seminiferous epithelium of the adult rat', *Journal of Reproduction and Fertility*, 110(1), pp. 61–70. doi: 10.1530/jrf.0.1100061.

Blodgett, F. M. *et al.* (1956) 'Effects of Prolonged Cortisone Therapy on the Statural Growth, Skeletal Maturation and Metabolic Status of Children', *New England Journal of Medicine*, 254(14), pp. 636–641. doi: 10.1056/NEJM195604052541402.

Board, P. G. and Menon, D. (2013) 'Glutathione transferases, regulators of cellular metabolism and physiology', *Biochimica et Biophysica Acta - General Subjects*, 1830(5), pp. 3267–3288. doi: 10.1016/j.bbagen.2012.11.019.

Boer, P. H. *et al.* (1987) 'The testis-specific phosphoglycerate kinase gene pgk-2 is a recruited retroposon.', *Molecular and cellular biology*, 7(9), pp. 3107–12. doi: 10.1128/mcb.7.9.3107.

Borer, K. T. (2003) *Exercise endocrinology*. Human Kinetics.

Borgström, B. and Carlson, L. A. (1957) 'On the mechanism of the lipolytic action of the lipaemia-clearing factor', *BBA - Biochimica et Biophysica Acta*, 24(C), pp. 638–639. doi: 10.1016/0006-3002(57)90260-3.

Bouchard, C. and Pérusse, L. (1996) 'Current status of the human obesity gene map', *Obesity Research*, 4(1), pp. 81–90. doi: 10.1002/j.1550-8528.1996.tb00516.x.

Bouchard, C. and Tremblay, A. (1997) 'Genetic Influences on the Response of Body Fat and Fat Distribution to Positive and Negative Energy Balances in Human Identical Twins', *The Journal of Nutrition*, 127(5), pp. 943S-947S. doi: 10.1093/jn/127.5.943S.

Boveris, A. and Chance, B. (1973) 'The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen', *Biochemical Journal*, 134(3), pp. 707–716. doi: 10.1042/bj1340707.

Branigan, E. F. and Muller, C. H. (1994) 'Efficacy of treatment and recurrence rate of leukocytospermia in infertile men with prostatitis**Presented in part at the 41st Annual Meeting of the Pacific Coast Fertility Society, Palms Springs, California, April 14 to 18, 1993.', *Fertility and*

*Sterility*, 62(3), pp. 580–584. doi: 10.1016/s0015-0282(16)56949-0.

Bray, G. A. (1976) 'The Obese Patient, Major Problems', in *Internal Medicine*. WB Saunders Philadelphia.

Bray, G. A. (1977) 'The Zucker-fatty rat: a review.', *Federation proceedings*, 36(2), pp. 148–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/320051 (Accessed: 13 October 2019).

Bray, G. A. (1983) 'The energetics of obesity.', *Medicine and science in sports and exercise*, 15(1), pp. 32–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6341751 (Accessed: 25 September 2019).

Bray, G. A. (1987) 'Overweight Is Risking Fate: Definition, Classification, Prevalence, and Risks', *Annals of the New York Academy of Sciences*, 499(1), pp. 14–28. doi: 10.1111/j.1749-6632.1987.tb36194.x.

Bray, G. A. (1989) 'Obesity: Basic considerations and clinical approaches', *Disease-a-Month*, 35(7), pp. 454–537. doi: 10.1016/0011-5029(89)90007-2.

Bray, G. A. (1997) 'Obesity and Reproduction', *Human Reproduction*, 12(suppl 1), pp. 26–32. doi: 10.1093/humrep/12.suppl_1.26.

Bray, G. A. and York, D. A. (1997) 'Leptin and clinical medicine: A new piece in the puzzle of obesity', *Journal of Clinical Endocrinology and Metabolism*, 82(9), pp. 2771–2776. doi: 10.1210/jcem.82.9.4224.

Brener, E. *et al.* (2003) 'Remodeling of the Actin Cytoskeleton During Mammalian Sperm Capacitation and Acrosome Reaction', *Biology of Reproduction*, 68(3), pp. 837–845. doi: 10.1095/biolreprod.102.009233.

Bressler, R. (1963) 'The Biochemistry of Ketosis', *Annals of the New York Academy of Sciences*, 104(2), pp. 735–752. doi: 10.1111/j.1749-6632.1963.tb17705.x.

Brière, J. J. *et al.* (2005) 'Succinate dehydrogenase deficiency in human', *Cellular and Molecular Life Sciences*, 62(19–20), pp. 2317–2324. doi: 10.1007/s00018-005-5237-6.

Brocker, C. *et al.* (2010) 'Aldehyde Dehydrogenase 7A1 (ALDH7A1) Is a Novel Enzyme Involved in Cellular Defense against Hyperosmotic Stress', *Journal of Biological Chemistry* , 285(24), pp. 18452–18463. doi: 10.1074/jbc.M109.077925.

Brookings, C., Goldmeier, D. and Sadeghi-Nejad, H. (2013) 'Sexually Transmitted Infections and

Sexual Function in Relation to Male Fertility', *Korean J Urol*, 54(3), pp. 149–156. Available at: https://doi.org/10.4111/kju.2013.54.3.149.

Buettner, R., Schölmerich, J. and Bollheimer, L. C. (2007) 'High-fat diets: Modeling the metabolic disorders of human obesity in rodents', *Obesity*, pp. 798–808. doi: 10.1038/oby.2007.608.

Burger, H. G. (1988) 'Inhibin: definition and nomenclature, including related substances', *Journal of Endocrinology*, 117(2).

Burman, P., Ritzén, E. M. and Lindgren, A. C. (2001) 'Endocrine dysfunction in Prader-Willi syndrome: A review with special reference to GH', *Endocrine Reviews*, 22(6), pp. 787–799. doi: 10.1210/edrv.22.6.0447.

Busso, D. *et al.* (2007) 'A Novel Function for CRISP1 in Rodent Fertilization: Involvement in Sperm-Zona Pellucida Interaction1', *Biology of Reproduction*, 77(5), pp. 848–854. doi: 10.1095/biolreprod.107.061788.

Butler, M. G. *et al.* (2007) 'Energy expenditure and physical activity in Prader-Willi syndrome: Comparison with obese subjects', *American Journal of Medical Genetics, Part A*, 143(5), pp. 449–459. doi: 10.1002/ajmg.a.31507.

Byers, S. W. *et al.* (1994) 'Cadherins and cadherin-associated molecules in the developing and maturing rat testis', *Endocrinology*, 134(2), pp. 630–639. doi: 10.1210/endo.134.2.7507830.

Cabler, S. *et al.* (2010) 'Obesity: Modern man's fertility nemesis', *Asian Journal of Andrology*, 12(4), pp. 480–489. doi: 10.1038/aja.2010.38.

Cahill, G. F. *et al.* (1966) 'Hormone-fuel interrelationships during fasting.', *The Journal of clinical investigation*, 45(11), pp. 1751–1769.

Cahill, G. F. (1970) 'Starvation in Man', *New England Journal of Medicine*, 282(12), pp. 668–675. doi: 10.1056/NEJM197003192821209.

Cahill, G. F. (2006) 'Fuel Metabolism in Starvation', *Annual Review of Nutrition*, 26, pp. 1–22. doi: 10.1146/annurev.nutr.26.061505.111258.

Cahill, G. F. and Veech, R. L. (2003) 'Ketoacids? Good medicine?', *Transactions of the American Clinical and Climatological Association*, 114.

Caprio, M. *et al.* (1999) 'Expression of functional leptin receptors in rodent Leydig cells', *Endocrinology*, 140(11), pp. 4939–4947. doi: 10.1210/endo.140.11.7088.

Carlsen, E. *et al.* (1992) 'Evidence for decreasing quality of semen during past 50 years', *BMJ*, 305(6854), pp. 609–613. doi: 10.1136/bmj.305.6854.609.

Carlson, L. A. (1960) 'Chromatographic separation of serum lipoproteins on glass powder columns. Description of the method and some applications', *Clinica Chimica Acta*, 5(4), pp. 528–538. doi: 10.1016/0009-8981(60)90064-4.

Carlson, L. A., Boberg, J. and HÖgstedt, B. (2011) ' Some physiological and clinical implications of lipid mobilization from adipose tissue 1 ', in *Comprehensive Physiology*. John Wiley & Sons, Inc. doi: 10.1002/cphy.cp050163.

Carrell, D. T. (2008) 'Contributions of spermatozoa to embryogenesis: assays to evaluate their genetic and epigenetic fitness', *Reproductive BioMedicine Online*, 16(4), pp. 474–484. doi: https://doi.org/10.1016/S1472-6483(10)60454-3.

Carrell, D. T., De Jonge, C. and Lamb, D. J. (2006) 'The genetics of male infertility: A field of study whose time is now', *Systems Biology in Reproductive Medicine*, 52(4), pp. 269–274. doi: 10.1080/01485010500503603.

Carroll, J. F. *et al.* (2008) 'Visceral fat, waist circumference, and BMI: Impact of race/ethnicity', *Obesity*, 16(3), pp. 600–607. doi: 10.1038/oby.2007.92.

Carroll, J. F., Zenebe, W. J. and Strange, T. B. (2006) 'Cardiovascular function in a rat model of diet-induced obesity', *Hypertension*, 48(1), pp. 65–72. doi: 10.1161/01.HYP.0000224147.01024.77.

Castro, J. P. *et al.* (2003) 'Cardiometabolic syndrome: Pathophysiology and treatment', *Current Hypertension Reports*, 5(5), pp. 393–401. doi: 10.1007/s11906-003-0085-y.

Cattanach, B. M. *et al.* (1977) 'Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism', *Nature*, 269, p. 338. Available at: http://dx.doi.org/10.1038/269338a0.

Cecconello, A. L. *et al.* (2015) 'Sex-related differences in the effects of high-fat diets on DHEA-treated rats', *Endocrine*, 48(3), pp. 985–994. doi: 10.1007/s12020-014-0396-6.

Celis, J. E. and Gromov, P. (1999) '2D protein electrophoresis: Can it be perfected?', *Current Opinion in Biotechnology*, 10(1), pp. 16–21. doi: 10.1016/S0958-1669(99)80004-4.

Center for Disease Control (2003) *Reproductive Tract Infections: Reproductive Health Epidemiology Series Module 3*. Available at: https://www.cdc.gov/reproductivehealth/ProductsPubs/PDFs/Epi_Module_03A_Tag508.pdf.

Chambers, T. J. and Anderson, R. A. (2015) 'The impact of obesity on male fertility', *Hormones*, 14(4), pp. 563–568. doi: 10.14310/horm.2002.1621.

Chandel, N. S., Schumacker, P. T. and Arch, R. H. (2001) 'Reactive Oxygen Species Are Downstream Products of TRAF-mediated Signal Transduction', *Journal of Biological Chemistry*, 276(46), pp. 42728–42736. doi: 10.1074/jbc.M103074200.

Chang, S. *et al.* (1990) 'Metabolic differences between obesity-prone and obesity-resistant rats', *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 259(6 28-6). doi: 10.1152/ajpregu.1990.259.6.r1103.

Chen, Z. *et al.* (2002) 'Low bone density and high percentage of body fat among men who were treated with androgen deprivation therapy for prostate carcinoma.', *Cancer*, 95(10), pp. 2136–2144. doi: 10.1002/cncr.10967.

Cheng, C. Y. and Mruk, D. D. (2010) 'The biology of spermatogenesis: The past, present and future', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 365(1546), pp. 1459–1463. doi: 10.1098/rstb.2010.0024.

Cherry, K. (2018) 'The Five Levels of Maslow's Hierarchy of Needs: How Maslow's Famous Hierarchy Explains Human Motivation', *Very Well Mind*. Available at: https://www.verywellmind.com/what-is-maslows-hierarchy-of-needs-4136760.

Chiriva-Internati, M. *et al.* (2009) 'Sperm protein 17 is expressed in the sperm fibrous sheath', *Journal of Translational Medicine*, 7. doi: 10.1186/1479-5876-7-61.

Chitra, K. C., Latchoumycandane, C. and Mathur, P. P. (2003) 'Induction of oxidative stress by bisphenol A in the epididymal sperm of rats', *Toxicology*, 185(1–2), pp. 119–127. doi: 10.1016/S0300-483X(02)00597-8.

Choi Yoon-Jung Myung Seung-Kwon, L. J.-H. (2018) 'Light Alcohol Drinking and Risk of Cancer: A Meta-Analysis of Cohort Studies', *Cancer Res Treat*, 50(2), pp. 474–487. doi: 10.4143/crt.2017.094.

Chooi, Y. C., Ding, C. and Magkos, F. (2019) 'The epidemiology of obesity', *Metabolism: Clinical and Experimental*, 92, pp. 6–10. doi: 10.1016/j.metabol.2018.09.005.

Choudhary, D. *et al.* (2003) 'Comparative expression profiling of 40 mouse cytochrome P450 genes in embryonic and adult tissues', *Archives of Biochemistry and Biophysics*, 414(1), pp. 91–100. doi: https://doi.org/10.1016/S0003-9861(03)00174-7.

Choudhary, D. *et al.* (2005) 'Expression patterns of mouse and human CYP orthologs (families 1–4) during development and in different adult tissues', *Archives of Biochemistry and Biophysics*, 436(1), pp. 50–61. doi: https://doi.org/10.1016/j.abb.2005.02.001.

Chowdhary, R. and Tiwari, B. (2016) 'Understanding Male Infertility and Its Causes : A Review', *International Journal of Biomedical Research*, 7(10), pp. 702–707. doi: 10.7439/ijbr.

Chowdhury, A. K. and Steinberger, E. (1970) 'Early changes in the germinal epithelium of rat testes following exposure to heat', *Reproduction*, 22(2).

Christoffersen, B. *et al.* (2006) 'Evalution of the castrated male Sprague–Dawley rat as a model of the metabolic syndrome and type 2 diabetes', *International Journal of Obesity*, 30(8), pp. 1288–1297. doi: 10.1038/sj.ijo.0803261.

Chung, W. K. (2012) 'An overview of mongenic and syndromic obesities in humans', *Pediatric Blood and Cancer*, 58(1), pp. 122–128. doi: 10.1002/pbc.23372.

Clarke, W. (2016) 'Mass spectrometry in the clinical laboratory: Determining the need and avoiding pitfalls', in *Mass Spectrometry for the Clinical Laboratory*. Elsevier Inc., pp. 1–15. doi: 10.1016/B978-0-12-800871-3.00001-8.

Clement, K. (2006) 'Genetics of human obesity', *Comptes Rendus Biologies*, 329, pp. 605–653.

Cohen, B., Novick, D. and Rubinstein, M. (1996) 'Modulation of insulin activities by leptin', *Science*, 274(5290), pp. 1185–1188. doi: 10.1126/science.274.5290.1185.

Cohen, P. G. (1999) 'The hypogonadal-obesity cycle: Role of aromatase in modulating the testosterone-estradiol shunt - A major factor in the genesis of morbid obesity', *Medical Hypotheses*, 52(1), pp. 49–51. doi: 10.1054/mehy.1997.0624.

Cohen, P. P. (1937) 'Studies in ketogenesis', *Journal of Biological Chemistry*, 119(1), pp. 333–346. Available at: http://www.jbc.org/content/119/1/333.short.

Colborn, T., Vom Saal, F. S. and Soto, A. M. (1993) 'Developmental effects of endocrine-disrupting chemicals in wildlife and humans', *Environmental Health Perspectives*, 101(5), pp. 378–384. doi: 10.1289/ehp.93101378.

Collins, S. *et al.* (1996) 'Role of leptin in fat regulation', *Nature*, 380(6576), p. 677. doi: 10.1038/380677a0.

Collodel, G. *et al.* (2008) 'Effect of emotional stress on sperm quality', *Indian Journal of Medical*

*Research*, 128(3), p. 254.

Comhaire, F. H., De Kretser, D. and Farley, T. M. M. (1987) 'Towards more objectivity in the management of male infertility. The need for a standardized approach', *International Journal of Andrology*, 10(SUPPL. 7), pp. 1–2. Available at: https://www.scopus.com/inward/record.uri?eid=2-s2.0-0023267471&partnerID=40&md5=9fe79ecf44939b526976138210d7ac85.

Cone, R. D. (2006) 'Studies on the physiological functions of the melanocortin system', *Endocrine Reviews*, 27(7), pp. 736–749. doi: 10.1210/er.2006-0034.

Cong, W. *et al.* (2013) 'Metallothionein prevents diabetes-induced cardiac pathological changes, likely via the inhibition of succinyl-CoA:3-ketoacid coenzyme A transferase-1 nitration at Trp $^{374}$', *American Journal of Physiology-Endocrinology and Metabolism*, 304(8), pp. E826–E835. doi: 10.1152/ajpendo.00570.2012.

Considine, R. V. *et al.* (1996) 'Serum immunoreactive-leptin concentrations in normal-weight and obese humans', *New England Journal of Medicine*, 334(5), pp. 292–295. doi: 10.1056/NEJM199602013340503.

Corona, G. *et al.* (2006) 'Psychobiologic Correlates of the Metabolic Syndrome and Associated Sexual Dysfunction', *European Urology*, 50(3), pp. 595–604. doi: 10.1016/j.eururo.2006.02.053.

Corona, G. *et al.* (2015) 'Obesity and late-onset hypogonadism', *Molecular and Cellular Endocrinology*, 418, pp. 120–133. doi: 10.1016/j.mce.2015.06.031.

Cowley, M. A. *et al.* (2001) 'Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus', *Nature*, 411(6836), pp. 480–484. doi: 10.1038/35078085.

Craig Venter, J. *et al.* (2001) 'The sequence of the human genome', *Science*, 291(5507), pp. 1304–1351. doi: 10.1126/science.1058040.

Crane, B. R., Siegel, L. M. and Getzoff, E. D. (1995) 'Sulfite reductase structure at 1.6 Å: Evolution and catalysis for reduction of inorganic anions', *Science*, 270(5233), pp. 59–67. doi: 10.1126/science.270.5233.59.

Creasy, D. (2002) 'Histopathology of the Male Reproductive System II : Interpretation EVALUATION AND INTERPRETATION OF', *Current Protocols in Toxicology*, pp. 1–14.

Creasy, D. M. and Chapin, R. E. (2013) 'Male Reproductive System', in Haschek, W. M., Rousseaux, C. G., and Wallig, M. A. (eds) *Handbook of Toxicological Pathology*. 3rd edn. Elsevier Inc., Academic Press, pp. 2493–2598.

Crick, F. H. C. (1958) ‘On protein synthesis’, in *Symposia of the Society for Experimental Biology*, p. 8.

Crompton, M. *et al.* (1978) ‘The Interrelations between the Transport of Sodium and Calcium in Mitochondria of Various Mammalian Tissues’, *European Journal of Biochemistry*, 82(1), pp. 25–31. doi: 10.1111/j.1432-1033.1978.tb11993.x.

Crompton, M. (1990) ‘Calcium and the Heart’, in Langer, G. A. (ed.) *Calcium and the Heart*. New York: Raven Press, pp. 167–198.

Crompton, M. (1999) ‘The mitochondrial permeability transition pore and its role in cell death’, *Biochemical Journal*, 341(2), pp. 233–249. Available at: http://www.biochemj.org/content/341/2/233.abstract.

Crompton, M., Capano, M. and Carafoli, E. (1976) ‘The Sodium-Induced Efflux of Calcium from Heart Mitochondria’, *European Journal of Biochemistry*, 69(2), pp. 453–462. doi: 10.1111/j.1432-1033.1976.tb10930.x.

Crompton, M., Costi, A. and Hayat, L. (1987) ‘Evidence for the presence of a reversible Ca2+-dependent pore activated by oxidative stress in heart mitochondria’, *Biochemical Journal*, 245(3), pp. 915–918. doi: 10.1042/bj2450915.

Crompton, M., Ellinger, H. and Costi, A. (1988) ‘Inhibition by cyclosporin A of a Ca2+-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress’, *The Biochemical journal*, 255(1), pp. 357–360. Available at: https://www.ncbi.nlm.nih.gov/pubmed/3196322.

Crompton, M., Künzi, M. and Carafoli, E. (1977) ‘The Calcium-Induced and Sodium-Induced Effluxes of Calcium from Heart Mitochondria’, *European Journal of Biochemistry*, 79(2), pp. 549–558. doi: 10.1111/j.1432-1033.1977.tb11839.x.

Crovetti, R. *et al.* (1998) ‘The influence of thermic effect of food on satiety’, *European Journal of Clinical Nutrition*, 52(7), pp. 482–488. doi: 10.1038/sj.ejcn.1600578.

Cummings, D. E. *et al.* (2001) ‘A Preprandial Rise in Plasma Ghrelin Levels Suggests a Role in Meal Initiation in Humans’, *Diabetes*, 50(8), pp. 1714–1719. doi: 10.2337/diabetes.50.8.1714.

Cummings, D. E. *et al.* (2002) ‘Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery’, *New England Journal of Medicine*, 346(21), pp. 1623–1630. doi: 10.1056/NEJMoa012908.

Cummins, J. M. (1983) ‘Sperm Size, Body Mass and Reproduction in Mammals’, in *The Sperm*

*Cell*. Springer Netherlands, pp. 395–398. doi: 10.1007/978-94-009-7675-7_72.

Cummins, J. M. and Woodall, P. F. (1985) 'On mammalian sperm dimensions', *Journal of Reproduction and Fertility*, 75(1), pp. 153–175. doi: 10.1530/jrf.0.0750153.

Dabaja, A. A. and Schlegel, P. N. (2014) 'Medical treatment of male infertility', *Translational Andrology and Urology*, 3(1), p. 9. doi: 10.3978/j.issn.2223-4683.2014.01.06.

Dahl, H. H. M. *et al.* (1990) 'A testis-specific form of the human pyruvate dehydrogenase E1α subunit is coded for by an intronless gene on chromosome 4', *Genomics*, 8(2), pp. 225–232. doi: 10.1016/0888-7543(90)90275-Y.

Danshina, P. V. *et al.* (2010) 'Phosphoglycerate Kinase 2 (PGK2) Is Essential for Sperm Function and Male Fertility in Mice1', *Biology of Reproduction*, 82(1), pp. 136–145. doi: 10.1095/biolreprod.109.079699.

Das, S. *et al.* (2008) 'Cut-off value of reactive oxygen species for predicting semen quality and fertilization outcome', *Systems Biology in Reproductive Medicine*, 54(1), pp. 47–54. doi: 10.1080/19396360701883274.

Dattani, M. T. (2005) 'Growth hormone deficiency and combined pituitary hormone deficiency: does the genotype matter?', *Clinical Endocrinology*, 63(2), pp. 121–130. doi: 10.1111/j.1365-2265.2005.02289.x.

Daudet, N. and Lebart, M. C. (2002) 'Transient expression of the T-isoform of plastins/fimbrin in the stereocilia of developing auditory hair cells', *Cell Motility and the Cytoskeleton*, 53(4), pp. 326–336. doi: 10.1002/cm.10092.

Dauncey, M. J. and Bingham, S. A. (1983) 'Dependence of 24 h energy expenditure in man on the composition of the nutrient intake', *British Journal of Nutrition*. 2007/03/09, 50(1), pp. 1–13. doi: DOI: 10.1079/BJN19830066.

de Castro, J. M. (2004) 'Genes, the environment and the control of food intake', *British Journal of Nutrition*, 92(S1), pp. S59–S62. doi: 10.1079/bjn20041143.

De Grava Kempinas, W. and Klinefelter, G. R. (2015) 'Interpreting histopathology in the epididymis', *Spermatogenesis*, 4(2), pp. e979114–e979114. doi: 10.4161/21565562.2014.979114.

de Lamirande, E. and Cagnon, C. (1993) 'Human sperm hyperactivation and capacitation as parts of an oxidative process', *Free Radical Biology and Medicine*, 14(2), pp. 157–166. doi: 10.1016/0891-5849(93)90006-G.

De Lamirande, E. and Gagnon, C. (1992) 'Reactive Oxygen Species and Human Spermatozoa: I. Effects on the Motility of Intact Spermatozoa and on Sperm Axonemes', *Journal of Andrology*, 13(5), pp. 368–378. doi: 10.1002/j.1939-4640.1992.tb03327.x.

De Roos, A. J. *et al.* (2012) 'Adiposity, body composition, and weight change in relation to organochlorine pollutant plasma concentrations', *Journal of Exposure Science and Environmental Epidemiology*, 22(6), pp. 617–624. doi: 10.1038/jes.2012.43.

De Souza, M. *et al.* (1994) 'Gonadal Hormones and Semen Quality in Male Runners', *International Journal of Sports Medicine*, 15(07), pp. 383–391. doi: 10.1055/s-2007-1021075.

de Vries, J. W. A. *et al.* (2001) 'Absence of deleted in azoospermia (DAZ) genes in spermatozoa of infertile men with somatic DAZ deletions', *Fertility and Sterility*, 75(3), pp. 476–479. doi: https://doi.org/10.1016/S0015-0282(00)01758-1.

DeLany, J. P. *et al.* (2000) 'Differential oxidation of individual dietary fatty acids in humans', *The American Journal of Clinical Nutrition*, 72(4), pp. 905–911. doi: 10.1093/ajcn/72.4.905.

Derby, C. A. *et al.* (2006) 'Body mass index, waist circumference and waist to hip ratio and change in sex steroid hormones: The Massachusetts Male Ageing Study', *Clinical Endocrinology*, 65(1), pp. 125–131. doi: 10.1111/j.1365-2265.2006.02560.x.

Despres, J.-P. *et al.* (1989) 'Role of Deep Abdominal Fat in the Association Between Regional Adipose Tissue Distribution and Glucose Tolerance in Obese Women', *Diabetes*, 38(3), pp. 304–309. doi: 10.2337/diab.38.3.304.

Després, J. P. *et al.* (2000) 'Race, visceral adipose tissue, plasma lipids, and lipoprotein lipase activity in men and women: The health, risk factors, exercise training, and genetics (HERITAGE) family study', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 20(8), pp. 1932–1938. doi: 10.1161/01.ATV.20.8.1932.

Deurenberg, P., Deurenberg-Yap, M. and Guricci, S. (2002) 'Asians are different from Caucasians and from each other in their body mass index/body fat per cent relationship', *Obesity Reviews*, 3(3), pp. 141–146. doi: 10.1046/j.1467-789X.2002.00065.x.

Deurenberg, P., Yap, M. and Van Staveren, W. A. (1998) 'Body mass index and percent body fat: A meta analysis among different ethnic groups', *International Journal of Obesity*, 22(12), pp. 1164–1171. doi: 10.1038/sj.ijo.0800741.

Dewailly, Â. *et al.* (2000) 'Body weight loss increases plasma and adipose tissue concentrations of

potentially toxic pollutants in obese individuals.', *International Journal of Obesity*, 24(10), pp. 1272–1278. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11093288 (Accessed: 5 October 2019).

Dirinck, E. *et al.* (2015) 'Pivotal role for the visceral fat compartment in the release of persistent organic pollutants during weight loss', *Journal of Clinical Endocrinology and Metabolism*, 100(12), pp. 4463–4471. doi: 10.1210/jc.2015-2571.

Dockery, F. *et al.* (2003) 'The Relationship between Androgens and Arterial Stiffness in Older Men', *Journal of the American Geriatrics Society*, 51(11), pp. 1627–1632. doi: 10.1046/j.1532-5415.2003.51515.x.

Donahue, R. P. *et al.* (1987) 'Central Oobesity and Coronary Heart Disease in Men', *The Lancet*, 329(8537), pp. 821–824. doi: 10.1016/S0140-6736(87)91605-9.

Dong, J. Y., Zhang, Y. H. and Qin, L. Q. (2011) 'Erectile dysfunction and risk of cardiovascular disease: Meta-analysis of prospective cohort studies', *Journal of the American College of Cardiology*, 58(13), pp. 1378–1385. doi: 10.1016/j.jacc.2011.06.024.

Drago, F. *et al.* (1982) 'Aromatization of Testosterone by Adipose Tissue and Sexual Behavior of Castrated Male Rats', *Biology of Reproduction*, 27(4), pp. 765–770. doi: 10.1095/biolreprod27.4.765.

Dringen, R., Pawlowski, P. G. and Hirrlinger, J. (2004) 'Peroxide detoxification by brain cells', *Journal of Neuroscience Research*, 79(1-2), pp. 157–165. doi: 10.1002/jnr.20280.

Du, F., Higginbotham, D. A. and White, B. D. (2000) 'Food Intake, Energy Balance and Serum Leptin Concentrations in Rats Fed Low-Protein Diets', *The Journal of Nutrition*, 130(3), pp. 514–521. doi: 10.1093/jn/130.3.514.

Du Plessis, S. S. *et al.* (2010) 'The effect of obesity on sperm disorders and male infertility', *Nature Reviews Urology*, 7(3), pp. 153–161. doi: 10.1038/nrurol.2010.6.

Du Plessis, S. S. *et al.* (2015) 'Oxidative phosphorylation versus glycolysis: What fuel do spermatozoa use?', *Asian Journal of Andrology*, 17(2), pp. 230–235. doi: 10.4103/1008-682X.135123.

du Toit, E. F., Nabben, M. and Lochner, A. (2005) 'A potential role for angiotensin II in obesity induced cardiac hypertrophy and ischaemic/reperfusion injury', *Basic Research in Cardiology*, 100(4), pp. 346–354. doi: 10.1007/s00395-005-0528-5.

Dubern, B. *et al.* (2007) 'Homozygous Null Mutation of the Melanocortin-4 Receptor and Severe

Early-Onset Obesity'. doi: 10.1016/j.jpeds.2007.01.041.

Ducimetiere, P., Richard, J. and Cambien, F. (1986) 'The pattern of subcutaneous fat distribution in middle-aged men and the risk of coronary heart disease: the Paris Prospective Study.', *International journal of obesity*, 10(3), pp. 229–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3759330 (Accessed: 1 October 2019).

Durairajanayagam, D., Agarwal, A. and Ong, C. (2015) 'Causes, effects and molecular mechanisms of testicular heat stress', *Reproductive BioMedicine Online*, 30(1), pp. 14–27. doi: 10.1016/j.rbmo.2014.09.018.

Dursun, M. *et al.* (2018) 'Increased visceral adiposity index associated with sexual dysfunction in men', *Aging Male*, 21(3), pp. 187–192. doi: 10.1080/13685538.2017.1406468.

Dutta, S. and Sengupta, P. (2016) 'Men and mice: Relating their ages', *Life Sciences*, 152, pp. 244–248. doi: 10.1016/j.lfs.2015.10.025.

Dwyer, A. A. and Quinton, R. (2019) 'Anatomy and Physiology of the Hypothalamic-Pituitary-Gonadal (HPG) Axis', in *Advanced Practice in Endocrinology Nursing*. Springer International Publishing, pp. 839–852. doi: 10.1007/978-3-319-99817-6_43.

Eastwood, S. V. *et al.* (2015) 'Ethnic differences in associations between fat deposition and incident diabetes and underlying mechanisms: The SABRE study', *Obesity*, 23(3), pp. 699–706. doi: 10.1002/oby.20997.

Egawa, M., Yoshimatsu, H. and Bray, G. A. (1991) 'Neuropeptide Y suppresses sympathetic activity to interscapular brown adipose tissue in rats', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 260(2), pp. R328–R334. doi: 10.1152/ajpregu.1991.260.2.R328.

Ehling, U. H., Doherty, D. G. and Malling, H. V. (1972) 'Differential spermatogenic response of mice to the induction of dominant-lethal mutations by n-propyl methane-sulfonate and isopropyl methanesulfonate', *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, 15(2), pp. 175–184. doi: 10.1016/0027-5107(72)90030-9.

Elbers, J. M. H. *et al.* (1997) 'Long-Term Testosterone Administration Increases Visceral Fat in Female to Male Transsexuals [1]', *The Journal of Clinical Endocrinology & Metabolism*, 82(7), pp. 2044–2047. doi: 10.1210/jcem.82.7.4078.

Elbers, J. M. H. *et al.* (1999) 'Effects of sex steroid hormones on regional fat depots as assessed

by magnetic resonance imaging in transsexuals', *American Journal of Physiology-Endocrinology and Metabolism*, 276(2), pp. E317–E325. doi: 10.1152/ajpendo.1999.276.2.E317.

Elbers, J. M. H. *et al.* (2003) 'Effects of sex steroids on components of the insulin resistance syndrome in transsexual subjects', *Clinical Endocrinology*, 58(5), pp. 562–571. doi: 10.1046/j.1365-2265.2003.01753.x.

Ellerby, L. M. and Bredesen, D. E. (2000) 'Measurement of Cellular Oxidation, Reactive Oxygen Species, and Antioxidant Enzymes during Apoptosis', in Reed, J. C. B. T.-M. in E. (ed.) *Apoptosis*. Academic Press, pp. 413–421. doi: https://doi.org/10.1016/S0076-6879(00)22040-5.

Elshal, M. F. *et al.* (2009) 'Sperm head defects and disturbances in spermatozoal chromatin and DNA integrities in idiopathic infertile subjects: Association with cigarette smoking', *Clinical Biochemistry*, 42(7–8), pp. 589–594. doi: 10.1016/j.clinbiochem.2008.11.012.

Emery, B. R. and Carrell, D. T. (2006) 'The effect of epigenetic sperm abnormalities on early embryo-genesis', *Asian Journal of Andrology*, 8(2), pp. 131–142. doi: 10.1111/j.1745-7262.2006.00127.x.

Eng, J. K., McCormack, A. L. and Yates, J. R. (1994) 'An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database', *Journal of the American Society for Mass Spectrometry*, 5(11), pp. 976–989. doi: 10.1016/1044-0305(94)80016-2.

Engel, F. L. and Amatruda, T. T. (1963) 'HORMONAL ASPECTS OF KETOSIS', *Annals of the New York Academy of Sciences*, 104(2), pp. 753–771. doi: 10.1111/j.1749-6632.1963.tb17706.x.

Enzi, G. *et al.* (1986) 'Subcutaneous and visceral fat distribution according to sex, age, and overweight, evaluated by computed tomography', *American Journal of Clinical Nutrition*, 44(6), pp. 739–746. doi: 10.1093/ajcn/44.6.739.

Erecińska, M. and Wilson, D. F. (1977) 'On the mechanism of regulation of cellular respiration. The dependence of respiration on the cytosolic [ATP],[ADP] and [PI].', *Advances in Experimental Medicine and Biology*, 94, pp. 271–278. doi: 10.1007/978-1-4684-8890-6_36.

Erkkilä, K. *et al.* (2002) 'Lactate inhibits germ cell apoptosis in the human testis', *MHR: Basic science of reproductive medicine*, 8(2), pp. 109–117. doi: 10.1093/molehr/8.2.109.

Erlanson-Albertsson, C. (2005) 'How palatable food disrupts appetite regulation.', *Basic & clinical pharmacology & toxicology*, 97(2), pp. 61–73. doi: 10.1111/j.1742-7843.2005.pto_179.x.

Eskenazi, B. *et al.* (2005) 'Antioxidant intake is associated with semen quality in healthy men', *Human Reproduction*, 20(4), pp. 1006–1012. doi: 10.1093/humrep/deh725.

Esposito, K. *et al.* (2005) 'High proportions of erectile dysfunction in men with the metabolic syndrome', *Diabetes Care*, 28(5), pp. 1201–1203. doi: 10.2337/diacare.28.5.1201.

European Bioinformatics Institute (no date) *What is metabolomics?* Available at: https://www.ebi.ac.uk/training/online/course/introduction-metabolomics/what-metabolomics (Accessed: 10 October 2019).

Evans, D. J., Barth, J. H. and Burke, C. W. (1988) 'Body fat topography in women with androgen excess.', *International journal of obesity*, 12(2), pp. 157–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3384560 (Accessed: 22 September 2019).

Fan, Y. *et al.* (2015) 'Diet-induced obesity in male C57BL/6 mice decreases fertility as a consequence of disrupted blood-testis barrier', *PLoS ONE*, 10(4). doi: 10.1371/journal.pone.0120775.

Farley, C. *et al.* (2003) 'Meal pattern analysis of diet-induced obesity in susceptible and resistant rats', *Obesity Research*, 11(7), pp. 845–851. doi: 10.1038/oby.2003.116.

Farley, S. and Barnes, R. (1973) 'Stenosis of ejaculatory ducts treated by endoscopic resection.', *The Journal of urology*, 109(4), pp. 664–6. doi: 10.1016/s0022-5347(17)60510-x.

Farooqi, I. S. (2005) 'Genetic and hereditary aspects of childhood obesity', *Best Practice and Research: Clinical Endocrinology and Metabolism*, 19(3 SPEC. ISS.), pp. 359–374. doi: 10.1016/j.beem.2005.04.004.

Farooqi, I. S. *et al.* (2006) 'Heterozygosity for a POMC-null mutation and increased obesity risk in humans', *Diabetes*, 55(9), pp. 2549–2553. doi: 10.2337/db06-0214.

Farooqi, I. S. *et al.* (2007) 'Clinical and molecular genetic spectrum of congenital deficiency of the leptin receptor', *New England Journal of Medicine*, 356(3), pp. 237–247. doi: 10.1056/NEJMoa063988.

Faust, I M *et al.* (1978) 'Diet-induced adipocyte number increase in adult rats: a new model of obesity.', *American Journal of Physiology-Endocrinology and Metabolism*, 235(3), p. E279. doi: 10.1152/ajpendo.1978.235.3.E279.

Faust, I. M. *et al.* (1978) 'Diet-induced adipocyte number increase in adult rats: A new model of obesity', *American Journal of Physiology Endocrinology Metabolism and Gastrointestinal*

*Physiology*, 4(3). doi: 10.1152/ajpendo.1978.235.3.e279.

Fawcett, D. W. (1970) 'A Comparative View of Sperm Ultrastructure', *Biology of Reproduction*, 2(suppl_2), pp. 90–127. doi: 10.1095/biolreprod2.Supplement_2.90.

Fawcett, D. W. (1975) 'The mammalian spermatozoon', *Developmental Biology*, 44(2), pp. 394–436. doi: https://doi.org/10.1016/0012-1606(75)90411-X.

Feeley, R. J. and Traish, A. M. (2009) 'Obesity and erectile dysfunction: Is androgen deficiency the common link?', *TheScientificWorldJournal*, pp. 676–684. doi: 10.1100/tsw.2009.79.

Fejes, I. *et al.* (2006) 'Effect of body weight on testosterone/estradiol ratio in oligozoospermic patients', *Archives of Andrology*, 52(2), pp. 97–102. doi: 10.1080/01485010500315479.

Fenyö, D. (2000) 'Identifying the proteome: Software tools', *Current Opinion in Biotechnology*, 11(4), pp. 391–395. doi: 10.1016/S0958-1669(00)00115-4.

Ferlin, A., Raicu, F., *et al.* (2007) 'Male infertility: Role of genetic background', *Reproductive BioMedicine Online*, 14(6), pp. 734–745. doi: 10.1016/S1472-6483(10)60677-3.

Ferlin, A., Arredi, B., *et al.* (2007) 'Molecular and clinical characterization of Y chromosome microdeletions in infertile men: A 10-year experience in Italy', *Journal of Clinical Endocrinology and Metabolism*, 92(3), pp. 762–770. doi: 10.1210/jc.2006-1981.

Fernandes, M. R. *et al.* (2016) 'Animal models of obesity in rodents. An integrative review', *Acta Cirurgica Brasileira*, 31(12), pp. 840–844. doi: 10.1590/S0102-865020160120000010.

Fernández-Sánchez, A. *et al.* (2011a) 'Inflammation, oxidative stress, and obesity', *International Journal of Molecular Sciences*, pp. 3117–3132. doi: 10.3390/ijms12053117.

Fernández-Sánchez, A. *et al.* (2011b) 'Inflammation, oxidative stress, and obesity', *International Journal of Molecular Sciences*, 12(5), pp. 3117–3132. doi: 10.3390/ijms12053117.

Fernandez, C. D. *et al.* (2011) 'Diet-induced obesity in rats leads to a decrease in sperm motility', *Reproductive Biology and Endocrinology*, 9(1), p. 32. doi: 10.1186/1477-7827-9-32.

Fernandez, C. D. B. *et al.* (2011) 'Diet-induced obesity in rats leads to a decrease in sperm motility', *Reproductive Biology and Endocrinology*, 9(1), p. 32. doi: 10.1186/1477-7827-9-32.

Ferre, T. *et al.* (1996) 'Evidence from transgenic mice that glucokinase is rate limiting for glucose utilization in the liver', *FASEB Journal*, 10(10), pp. 1213–1218. doi: 10.1096/fasebj.10.10.8751724.

Ferreira, L. C. C. do N. *et al.* (2013) 'Relationship between lipid and hematological profiles with adiposity in obese adolescents', *Revista Brasileira de Hematologia e Hemoterapia*, 35(3), pp. 163–166. doi: 10.5581/1516-8484.20130051.

Festing, M. F. W. (1979) *Animal models of obesity*. Springer.

Fisher, W. A. *et al.* (2009) 'Erectile dysfunction (ED) is a shared sexual concern of couples I: Couple conceptions of ED', *Journal of Sexual Medicine*, 6(10), pp. 2746–2760. doi: 10.1111/j.1743-6109.2009.01457.x.

Fitzgerald, S. M. *et al.* (2001) 'Cardiovascular and renal responses to a high-fat diet in Osborne-Mendel rats', *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 281(2 50-2), pp. R547–R552. doi: 10.1152/ajpregu.2001.281.2.r547.

Fléchon, J.-E. (2016) 'The acrosome of eutherian mammals', *Cell and Tissue Research*, 363(1), pp. 147–157. doi: 10.1007/s00441-015-2238-0.

Flier, J. S. (1998) 'What's in a Name? In Search of Leptin's Physiologic Role [1]', *The Journal of Clinical Endocrinology & Metabolism*, 83(5), pp. 1407–1413. doi: 10.1210/jcem.83.5.4779.

Fogari, R., Preti, P., *et al.* (2002) 'Effect of antihypertensive treatment with valsartan or atenolol on sexual activity and plasma testosterone in hypertensive men', *European Journal of Clinical Pharmacology*, 58(3), pp. 177–180. doi: 10.1007/s00228-002-0456-3.

Fogari, R., Zoppi, A., *et al.* (2002) 'Sexual activity and plasma testosterone levels in hypertensive males', in *American Journal of Hypertension*, pp. 217–221. doi: 10.1016/S0895-7061(01)02280-4.

Fonseca-Alaniz, M. H. *et al.* (2007) 'O tecido adiposo como órgão endócrino: da teoria à prática', *Jornal de Pediatria*, 83(5), pp. S192–S203. doi: 10.1590/s0021-75572007000700011.

Fontecave, M. *et al.* (2008) 'From Iron and Cysteine to Iron-Sulfur Clusters: the Biogenesis Protein Machineries', *EcoSal Plus*, 3(1), pp. 1–24. doi: 10.1128/ecosalplus.3.6.3.14.

Ford, W. C. L. (2006) 'Glycolysis and sperm motility: Does a spoonful of sugar help the flagellum go round?', *Human Reproduction Update*, 12(3), pp. 269–274. doi: 10.1093/humupd/dmi053.

Foresta, C. *et al.* (2011) 'Bone Mineral Density and Testicular Failure: Evidence for a Role of Vitamin D 25-Hydroxylase in Human Testis', *The Journal of Clinical Endocrinology & Metabolism*, 96(4), pp. E646–E652. Available at: http://dx.doi.org/10.1210/jc.2010-1628.

Forouhi, N. G. (2006) 'Ethnicity and the Metabolic Syndrome', in *The Metabolic Syndrome*. John

Wiley & Sons, Ltd, pp. 43–84. doi: 10.1002/0470025131.ch2.

Fouquet, J. P. and Kann, M. L. (1992) 'Speciesspecific localization of actin in mammalian spermatozoa: Factor artifact?', *Microscopy Research and Technique*, 20(3), pp. 251–258. doi: 10.1002/jemt.1070200304.

Francis, H. M. and Stevenson, R. J. (2019) 'Fatty Acids and the Hippocampus', in *Omega Fatty Acids in Brain and Neurological Health*. Elsevier, pp. 117–129. doi: 10.1016/b978-0-12-815238-6.00008-0.

Frayling, T. M. *et al.* (2007) 'A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity', *Science*, 316(5826), pp. 889–894. doi: 10.1126/science.1141634.

Fridovich, I. (1995) 'Superoxide Radical and Superoxide Dismutases', *Annual Review of Biochemistry*, 64(1), pp. 97–112. doi: 10.1146/annurev.bi.64.070195.000525.

Friedman, J. M. *et al.* (1991) 'Molecular mapping of the mouse ob mutation', *Genomics*, 11(4), pp. 1054–1062. doi: 10.1016/0888-7543(91)90032-A.

Friedman, J. M. and Halaas, J. L. (1998) 'Leptin and the regulation of body weight in mammals', *Nature*, 395(6704), pp. 763–770. doi: 10.1038/27376.

Fukao, T. *et al.* (1997) 'Enzymes of ketone body utilization in human tissues: Protein and messenger RNA levels of succinyl-coenzyme A (CoA):3-ketoacid CoA transferase and mitochondrial and cytosolic acetoacetyl-CoA thiolases', *Pediatric Research*, 42(4), pp. 498–502. doi: 10.1203/00006450-199710000-00013.

Galantino-Homer, H. L. *et al.* (2004) 'Bovine Sperm Capacitation: Assessment of Phosphodiesterase Activity and Intracellular Alkalinization on Capacitation-Associated Protein Tyrosine Phosphorylation', *Molecular Reproduction and Development*, 67(4), pp. 487–500. doi: 10.1002/mrd.20034.

Garamszegi, L. Z. *et al.* (2005) 'Testosterone, testes size, and mating success in birds: A comparative study', *Hormones and Behavior*, 47(4), pp. 389–409. doi: 10.1016/j.yhbeh.2004.11.008.

Garcion, E. *et al.* (2002) '1,25-Dihydroxyvitamin D3 Regulates the Synthesis of γ-Glutamyl Transpeptidase and Glutathione Levels in Rat Primary Astrocytes', *Journal of Neurochemistry*, 73(2), pp. 859–866. doi: 10.1046/j.1471-4159.1999.0730859.x.

Garrow, J. S. (1988) 'Obesity and related disorders', *New York: Churchill Livingstone*.

Gartner, L. P. and Hiatt, J. L. (2000) 'Male Reproductive System', in *Colour Atlas of Histology*. 3rd edn. Lippincott, Williams and Wilkins, pp. 360–377.

Gaur, D. S., Talekar, M. and Pathak, V. P. (2007) 'Effect of cigarette smoking on semen quality of infertile men', *Singapore medical journal*, 48(2), p. 119.

Gautron, L. *et al.* (2010) 'Melanocortin-4 receptor expression in a vago-vagal circuitry involved in postprandial functions', *Journal of Comparative Neurology*, 518(1), pp. 6–24. doi: 10.1002/cne.22221.

Geller, F. *et al.* (2004) 'Melanocortin-4 Receptor Gene Variant I103 Is Negatively Associated with Obesity', *American Journal of Human Genetics*, 74(3), pp. 572–581. doi: 10.1086/382490.

Gentry, R. T. and Wade, G. N. (1976) 'Androgenic control of food intake and body weight in male rats.', *Journal of Comparative and Physiological Psychology*, 90(1), pp. 18–25. doi: 10.1037/h0077264.

Georgiou, I. *et al.* (2006) 'Genetic and epigenetic risks of intracytoplasmic sperm injection method', *Asian Journal of Andrology*, 8(6), pp. 643–673. doi: 10.1111/j.1745-7262.2006.00231.x.

Georgopoulos, C. and Welch, W. J. (1993) 'Role of the Major Heat Shock Proteins as Molecular Chaperones', *Annual Review of Cell Biology*, 9(1), pp. 601–634. doi: 10.1146/annurev.cb.09.110193.003125.

Gerhard, I. *et al.* (1989) 'Relationship of Sperm Acrosin Activity to Semen and Clinical Parameters in Infertile Patients/Die Beziehung der Akrosinaktivität zu verschiedenen Sperma- und klinischen Parametern bei infertilen Patienten', *Andrologia*, 21(2), pp. 146–154. doi: 10.1111/j.1439-0272.1989.tb02385.x.

Gerrits, T. *et al.* (2017) 'Infertility in the Global South: Raising awareness and generating insights for policy and practice.', *Facts, views & vision in ObGyn*, 9(1), pp. 39–44.

Geyer, H. J. *et al.* (1993) 'The Relevance of Fat Content in Toxicity of Lipophilic Chemicals to Terrestrial Animals with Special Reference to Dieldrin and 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)', *Ecotoxicology and Environmental Safety*, 26(1), pp. 45–60. doi: 10.1006/eesa.1993.1040.

Ghanayem, B. I. *et al.* (2010) 'Diet-Induced Obesity in Male Mice Is Associated with Reduced Fertility and Potentiation of Acrylamide-Induced Reproductive Toxicity1', *Biology of Reproduction*, 82(1), pp. 96–104. doi: 10.1095/biolreprod.109.078915.

Giagulli, V. A., Kaufman, J. M. and Vermeulen, A. (1994) 'Pathogenesis of the decreased androgen levels in obese men', *Journal of Clinical Endocrinology and Metabolism*, 79(4), pp. 997–1000. doi: 10.1210/jcem.79.4.7962311.

Giamarellou, H. *et al.* (1984) 'Infertility and Chronic Prostatitis', *Andrologia*, 16(5), pp. 417–422. doi: 10.1111/j.1439-0272.1984.tb00388.x.

Gibbons, I. R. (1996) 'The Role of Dynein in Microtubule-based Motility', *Cell Structure and Function*, 21, pp. 331–342.

Gil-Guzman, E. *et al.* (2001) 'Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation', *Human Reproduction*, 16(9), pp. 1922–1930. doi: 10.1093/humrep/16.9.1922.

Gill, S. S. and Tuteja, N. (2010) 'Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants', *Plant Physiology and Biochemistry*, 48(12), pp. 909–930. doi: https://doi.org/10.1016/j.plaphy.2010.08.016.

Gillum, R. F. (1999) 'Distribution of waist-to-hip ratio, other indices of body fat distribution and obesity and associations with HDL cholesterol in children and young adults aged 4-19 years: The Third National Health and Nutrition Examination Survey', *International Journal of Obesity*, 23(6), pp. 556–563. doi: 10.1038/sj.ijo.0800866.

Gimenes, F. *et al.* (2014) 'Male infertility: a public health issue caused by sexually transmitted pathogens', *Nature Reviews Urology*, 11, p. 672. Available at: https://doi.org/10.1038/nrurol.2014.285.

Girard, J., Ferré, P. and Foufelle, F. (1997) 'Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes', *Annual Review of Nutrition*, 17(1), pp. 325–352. doi: 10.1146/annurev.nutr.17.1.325.

Glass, A. R. *et al.* (1977) 'Low serum testosterone and sex-hormone-binding-globulin in massively obese men', *Journal of Clinical Endocrinology and Metabolism*, 45(6), pp. 1211–1219. doi: 10.1210/jcem-45-6-1211.

Glazier, A. M., Nadeau, J. H. and Aitman, T. J. (2002) 'Finding genes that underlie complex traits', *Science*, 298, pp. 2345–2349.

Glick, Z., Teague, R. J. and Bray, G. A. (1981) 'Brown adipose tissue: Thermic response increased by a single low protein, high carbohydrate meal', *Science*, 213(4512), pp. 1125–1127. doi:

10.1126/science.7268419.

Godsland, I. F. *et al.* (1987) 'Sex, plasma lipoproteins, and atherosclerosis: Prevailing assumptions and outstanding questions', *American Heart Journal*, 114(6), pp. 1467–1503. doi: 10.1016/0002-8703(87)90552-7.

Goodarzi, M. O. (2018) 'Genetics of obesity: what genetic association studies have taught us about the biology of obesity and its complications.', *The lancet. Diabetes & endocrinology*, 6(3), pp. 223–236. doi: 10.1016/S2213-8587(17)30200-0.

Goodpaster, B. H. *et al.* (1997) 'Subcutaneous abdominal fat and thigh muscle composition predict insulin sensitivity independently of visceral fat', *Diabetes*, 46(10), pp. 1579–1585. doi: 10.2337/diacare.46.10.1579.

Görg, A. *et al.* (2000) 'The current state of two-dimensional electrophoresis with immobilized pH gradients', *Electrophoresis*, pp. 1037–1053. doi: 10.1002/(SICI)1522-2683(20000401)21:6<1037::AID-ELPS1037>3.0.CO;2-V.

Görnig, V. M. and Schirren, C. (1996) '[Effect of exogenous toxins on fertility].', *Fortschritte der Medizin*, 114(14), pp. 169–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8707234 (Accessed: 7 October 2019).

Grandys, M. *et al.* (2009) 'Endurance training of moderate intensity increases testosterone concentration in young, healthy men', *International Journal of Sports Medicine*, 30(7), pp. 489–495. doi: 10.1055/s-0029-1202340.

Graves, P. R. and Haystead, T. A. J. (2002) 'Molecular Biologist's Guide to Proteomics', *Microbiology and Molecular Biology Reviews*, 66(1), pp. 39–63. doi: 10.1128/MMBR.66.1.39.

Gray, J. *et al.* (2006) 'Hyperphagia, severe obesity, impaired cognitive function, and hyperactivity associated with functional loss of one copy of the brain-derived neurotrophic factor (BDNF) gene', *Diabetes*, 55(12), pp. 3366–3371. doi: 10.2337/db06-0550.

Griffen, W. O. *et al.* (1981) 'Gastric bypass for morbid obesity', *World Journal of Surgery*, 5(6), pp. 817–822. doi: 10.1007/BF01657968.

Griffith, R. O. *et al.* (1990) 'Testicular function during exhaustive endurance training', *Physician and Sportsmedicine*, 18(5), pp. 54–64. doi: 10.1080/00913847.1990.11710041.

Griveau, J. F. and Lannou, D. L. E. (1997) 'Review Reactive oxygen species and human spermatozoa : physiology and pathology', 69, pp. 61–69.

Gu, N. H. *et al.* (2019) 'Comparative analysis of mammalian sperm ultrastructure reveals relationships between sperm morphology, mitochondrial functions and motility', *Reproductive Biology and Endocrinology*, 17(1). doi: 10.1186/s12958-019-0510-y.

Guido, C. *et al.* (2011) 'Human sperm physiology: Estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) influence sperm metabolism and may be involved in the pathophysiology of varicocele-associated male infertility', *Journal of Cellular Physiology*, 226(12), pp. 3403–3412. doi: 10.1002/jcp.22703.

Guilhaus, M., Mlynski, V. and Selby, D. (1997) 'Perfect Timing: Time-of-flight Mass Spectrometry', *Rapid Communications in Mass Spectrometry*, 11(9), pp. 951–962. doi: 10.1002/(sici)1097-0231(19970615)11:9<951::aid-rcm785>3.3.co;2-8.

Guilhaus, M., Selby, D. and Mlynski, V. (2000) 'Orthogonal acceleration time-of-flight mass spectrometry', *Mass Spectrometry Reviews*, 19(2), pp. 65–107. doi: 10.1002/(SICI)1098-2787(2000)19:2<65::AID-MAS1>3.0.CO;2-E.

Gülden, E. *et al.* (2008) 'Heat shock protein 60 induces inflammatory mediators in mouse adipocytes', *FEBS Letters*, 582(18), pp. 2731–2736. doi: 10.1016/j.febslet.2008.07.002.

Gülden, E. *et al.* (2009) 'Heat shock protein 60 : Evidence for receptor-mediated induction of proinflammatory mediators during adipocyte differentiation', *FEBS Letters*, 583(17), pp. 2877–2881. doi: 10.1016/j.febslet.2009.07.049.

Guo, F. *et al.* (2004) 'Leptin signaling targets the thyrotropin-releasing hormone gene promoter in vivo', *Endocrinology*, 145(5), pp. 2221–2227. doi: 10.1210/en.2003-1312.

Gupta, A. and Kumar, A. (2014) 'Pros and cons of the proteomics.', *Biomedical journal*, 37(3), pp. 163–164. doi: 10.4103/2319-4170.125652.

Gurr, M. I., Rothwell, N. J. and Stock, M. J. (1979) 'Effects of tube-feeding iso-energetic amounts of various dietary lipids on energy balance in the rat.', *The Proceedings of the Nutrition Society*, 38(1), p. 6A.

Guzik, T. J., Mangalat, D. and Korbut, R. (2006) 'Adipocytokines - novel link between inflammation and vascular function?', *Journal of Physiology and Pharmacology*, 57(4), pp. 505–528.

Ha, H. K., Park, H. J. and Park, N. C. (2011) 'Expression of E-cadherin and α-catenin in a varicocele-induced infertility rat model', *Asian Journal of Andrology*, 13(3), pp. 470–475. doi: 10.1038/aja.2010.94.

Habich, C. and Burkart, V. (2007) 'Heat shock protein 60: regulatory role on innate immune cells', *Cellular and Molecular Life Sciences*, 64(6), p. 742. doi: 10.1007/s00018-007-6413-7.

Hackney, A. (2005) 'Endurance Exercise Training and Reproductive Endocrine Dysfunction in Men Alterations in the Hypothalamic-Pituitary-Testicular Axis', *Current Pharmaceutical Design*, 7(4), pp. 261–273. doi: 10.2174/1381612013398103.

Hackney, A. C., Moore, A. W. and Brownlee, K. K. (2005) 'Testosterone and endurance exercise: Development of the "exercise-hypogonadal male condition"', *Acta Physiologica Hungarica*, 92(2), pp. 121–137. doi: 10.1556/APhysiol.92.2005.2.3.

Halaas, J. L. *et al.* (1995) 'Weight-reducing effects of the plasma protein encoded by the obese gene', *Science*, 269(5223), pp. 543–546. doi: 10.1126/science.7624777.

Hall, S. A. *et al.* (2008) 'Correlates of Low Testosterone and Symptomatic Androgen Deficiency in a Population-Based Sample', *The Journal of Clinical Endocrinology & Metabolism*, 93(10), pp. 3870–3877. doi: 10.1210/jc.2008-0021.

Hall, S. E. H. *et al.* (1984) 'Ketone body kinetics in humans: The effects of insulin-dependent diabetes, obesity, and starvation', *Journal of Lipid Research*, 25(11), pp. 1184–1194.

Halton, T. L. and Hu, F. B. (2004) 'The Effects of High Protein Diets on Thermogenesis, Satiety and Weight Loss: A Critical Review', *Journal of the American College of Nutrition*, 23(5), pp. 373–385. doi: 10.1080/07315724.2004.10719381.

Hamilton, J. B. *et al.* (1964) 'Effect of castration in man upon blood sedimentation rate, hematocrit and hemoglobin', *The Journal of clinical endocrinology and metabolism*, 24(6), pp. 506–511. doi: 10.1210/jcem-24-6-506.

Hammadeh, M. ., Filippos, A. and Hamma, M. . (2009) 'Reactive oxygen species ans antioxidant in seminal plasma and their impact on male fertility', *International Journal of Fertility and Sterility*, 3(3), pp. 87–110.

Hammoud, A. O. *et al.* (2008) 'Male obesity and alteration in sperm parameters', *Fertility and Sterility*, 90(6), pp. 2222–2225. doi: 10.1016/j.fertnstert.2007.10.011.

Hansen, B. C. (1979) 'Induction of obesity in nonhuman primate models of human obesity', in *Primates in Nutritional Research*. Academic Press New York, pp. 291–314.

Hariharan, N. *et al.* (1997) 'Expression of human hepatic glucokinase in transgenic mice liver results in decreased glucose levels and reduced body weight', *Diabetes*, 46(1), pp. 11–16. doi:

10.2337/diab.46.1.11.

Hariri, N. and Thibault, L. (2010) ‘High-fat diet-induced obesity in animal models’, *Nutrition Research Reviews*, 23(2), pp. 270–299. doi: 10.1017/S0954422410000168.

Harris, G. W. (1955) *Neural control of the pituitary gland*. E. Arnold.

Harrison, W. (2013) *Understanding How Testosterone Affects Men*, *NIH Research Matters*. Available at: www.nih.gov/news-events/nih-research-matters/understanding-how-testosterone-affects-men (Accessed: 28 September 2018).

Harrold, J. A., Williams, G. and Widdowson, P. S. (2000) ‘Early leptin response to a palatable diet predicts dietary obesity in rats: Key role of melanocortin-4 receptors in the ventromedial hypothalamic nucleus’, *Journal of Neurochemistry*, 74(3), pp. 1224–1228. doi: 10.1046/j.1471-4159.2000.741224.x.

Hausberger, F. X. and Hausberger, B. C. (1966) ‘Castration-induced obesity in mice. Body composition, histology of adrenal cortex and islets of Langerhans in castrated mice.’, *Acta endocrinologica*, 53(4), pp. 571–583.

Hayes, J. D. and Pulford, D. J. (1995) ‘The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance.’, *Critical reviews in biochemistry and molecular biology*, 30(6), pp. 445–600. doi: 10.3109/10409239509083491.

Haynes, W. G. *et al.* (1999) ‘Interactions Between the Melanocortin System and Leptin in Control of Sympathetic Nerve Traffic’, *Hypertension*, 33(1), pp. 542–547. doi: 10.1161/01.HYP.33.1.542.

Heffner, L. J. and Schust, D. J. (2010) *The reproductive system at a glance*. 4th edn. Oxford, United Kingdom: John Wiley & Sons.

Heikinheimo, O. *et al.* (1995) ‘Estrogen and progesterone receptor mRNA are expressed in distinct pattern in male primate reproductive organs’, *Journal of Assisted Reproduction and Genetics*, 12(3), pp. 198–204. doi: 10.1007/BF02211799.

Hemsell, D. L. *et al.* (1974) ‘Plasma precursors of estrogen. II. Correlation of the extent of conversion of plasma androstenedione to estrone with age’, *Journal of Clinical Endocrinology and Metabolism*, 38(3), pp. 476–479. doi: 10.1210/jcem-38-3-476.

Heo, M. *et al.* (2013) ‘Optimal scaling of weight and waist circumference to height for maximal association with DXA-measured total body fat mass by sex, age and race/ethnicity’, *International*

*Journal of Obesity*, 37(8), pp. 1154–1160. doi: 10.1038/ijo.2012.201.

Herberg, L. *et al.* (1974) 'Dietary-induced hypertrophic–hyperplastic obesity in mice', *Journal of Lipid Research* , 15(6), pp. 580–585. Available at: http://www.jlr.org/content/15/6/580.abstract.

Herbert, A. *et al.* (2006) 'A common genetic variant is associated with adult and childhood obesity', *Science*, 312(5771), pp. 279–283. doi: 10.1126/science.1124779.

Herman-Giddens, M. E., Wang, L. and Koch, G. (2001) 'Secondary sexual characteristics in boys: Estimates from the national health and nutrition examination survey iii, 1988-1994', *Archives of Pediatrics & Adolescent Medicine*, 155(9), pp. 1022–1028. doi: 10.1001/archpedi.155.9.1022.

Hess, R. A. (2003) 'Estrogen in the adult male reproductive tract : A review', *Reproductive Biology and Endocrinology*, 14(52), pp. 1–14.

Hess, R. A. and de Franca, L. R. (2005) 'History of the sertoli cell discovery', in Grisworld, M. and Skinner, M. (eds) *Sertoli Cell Biology*. New York, United States of America: Academic Press.

Hess, R. A. and de Franca, L. R. (2008) 'Spermatogenesis and cycle of the seminiferous epithelium', in *Molecular Mechanisms in Spermatogenesis*. Austin, Texas: Springer Science+Business Media, LLC, pp. 1–15.

Heymsfield, S. B. *et al.* (2016) 'Why are there race/ethnic differences in adult body mass index-adiposity relationships? A quantitative critical review', *Obesity Reviews*, 17(3), pp. 262–275. doi: 10.1111/obr.12358.

Hill, J. O. *et al.* (1984) 'Dietary obesity and exercise in young rats', *Physiology and Behavior*, 33(2), pp. 321–328. doi: 10.1016/0031-9384(84)90119-7.

Hinney, A. *et al.* (1999) 'Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans', *Journal of Clinical Endocrinology and Metabolism*, 84(4), pp. 1483–1486. doi: 10.1210/jcem.84.4.5728.

Hinney, A. *et al.* (2006) 'Prevalence, spectrum, and functional characterization of melanocortin-4 receptor gene mutations in a representative population-based sample and obese adults from Germany', *Journal of Clinical Endocrinology and Metabolism*, 91(5), pp. 1761–1769. doi: 10.1210/jc.2005-2056.

Hinney, A. and Hebebrand, J. (2008) 'Polygenic obesity in humans', *Obesity Facts*, 1(1), pp. 35–42. doi: 10.1159/000113935.

Hipler, U. C. *et al.* (2000) 'Stimulation and scavestrogen-induced inhibition of reactive oxygen species generated by rat sertoli cells.', *Archives of andrology*, 44(2), pp. 147–154.

Hirano, S. *et al.* (1992) 'Identification of a neural α-catenin as a key regulator of cadherin function and multicellular organization', *Cell*, 70(2), pp. 293–301. doi: 10.1016/0092-8674(92)90103-J.

Hjollund, N. H. I. *et al.* (2000) 'Diurnal scrotal skin temperature and semen quality', *International Journal of Andrology*, 23(5), pp. 309–318. doi: 10.1046/j.1365-2605.2000.00245.x.

Hjollund, N. H. I. *et al.* (2002) 'Impact of diurnal scrotal temperature on semen quality', *Reproductive Toxicology*, 16(3), pp. 215–221. doi: 10.1016/S0890-6238(02)00025-4.

Ho, H.-C. and Suarez, S. S. (2003) 'Characterization of the Intracellular Calcium Store at the Base of the Sperm Flagellum That Regulates Hyperactivated Motility1', *Biology of Reproduction*, 68(5), pp. 1590–1596. doi: 10.1095/biolreprod.102.011320.

Hoefnagel, D., Costello, P. J. and Hatoum, K. (1967) 'Prader-Willi Syndrome', *Journal of Intellectual Disability Research*, 11(1), pp. 1–11. doi: 10.1111/j.1365-2788.1967.tb00197.x.

Holder, J. L., Butte, N. F. and Zinn, A. R. (2000) 'Profound obesity associated with a balanced translocation that disrupts the SIM1 gene', *Human Molecular Genetics*, 9, pp. 102–108.

Hollifield, G. and Parson, W. (1959) 'Body composition and in vitro synthesis of lipids by adipose tissue of 11-dehydrocorticosterone-treated mice', *American Journal of Physiology-Legacy Content*, 197(1), pp. 105–107. doi: 10.1152/ajplegacy.1959.197.1.105.

Hollifield, G. U. Y. (1968) 'Glucocorticoid-induced obesity—a model and a challenge', *The American journal of clinical nutrition*, 21(12), pp. 1471–1474.

Holmäng, A. and Björntorp, P. (1992) 'The effects of testosterone on insulin sensitivity in male rats', *Acta Physiologica Scandinavica*, 146(4), pp. 505–510. doi: 10.1111/j.1748-1716.1992.tb09452.x.

Holstein, A. F., Schulze, W. and Davidoff, M. (2003) 'Understanding spermatogenesis is a prerequisite for treatment', *Reproductive Biology and Endocrinology*, 1(1), p. 107. doi: 10.1186/1477-7827-1-107.

Hoskins, R. G. (1925) 'Studies on Vigor', *American Journal of Physiology-Legacy Content*, 72(2), pp. 324–330. doi: 10.1152/ajplegacy.1925.72.2.324.

Hotchkiss, J. and Knobil, E. (1996) 'The hypothalamic pulse generator: the reproductive core',

*Reproductive endocrinology, surgery, and technology*, pp. 123–162.

Houseknecht, K. L. *et al.* (1998) 'The Biology of Leptin: A Review', *Journal of Animal Science*, 76(5), pp. 1405–1420. doi: 10.2527/1998.7651405x.

Houseknecht, K. L. and Portocarrero, C. P. (1998) 'Leptin and its receptors: Regulators of whole-body energy homeostasis', *Domestic Animal Endocrinology*, 15(6), pp. 457–475. doi: 10.1016/S0739-7240(98)00035-6.

Hruska, K. S. *et al.* (2000) 'Environmental Factors in Infertility', *Clinical Obstetrics and Gynecology*, 43(4). Available at: https://journals.lww.com/clinicalobgyn/Fulltext/2000/12000/Environmental_Factors_in_Infertility.14.aspx.

Huang, C. *et al.* (2017) 'Guanylyl cyclase sensitivity to nitric oxide is protected by a thiol oxidation-driven interaction with thioredoxin-1.', *The Journal of biological chemistry*, 292(35), pp. 14362–14370. doi: 10.1074/jbc.M117.787390.

Huang, X. *et al.* (2013) 'Effects of dietary protein to carbohydrate balance on energy intake, fat storage, and heat production in mice', *Obesity*, 21(1), pp. 85–92. doi: 10.1002/oby.20007.

Hue, O. *et al.* (2006) 'Increased plasma levels of toxic pollutants accompanying weight loss induced by hypocaloric diet or by bariatric surgery', *Obesity Surgery*, 16(9), pp. 1145–1154. doi: 10.1381/096089206778392356.

Huhtaniemi, I. T. (1999) 'Endocrine regulation of male reproduction', in Wang, C. (ed.) *Male Reproductive Function*. Kluwer Academic Publishers, pp. 1–17.

Hull, M. G. R. *et al.* (1985) 'Population study of causes, treatment, and outcome of infertility', *British Medical Journal*, 291(December), pp. 1693–1697. doi: 10.1093/gerona/glt257.

Hurst, S. *et al.* (1998) 'Expression of a testis-specific putative actin-capping protein associated with the developing acrosome during rat spermiogenesis', *Molecular Reproduction and Development*, 49(1), pp. 81–91. doi: 10.1002/(SICI)1098-2795(199801)49:1<81::AID-MRD9>3.0.CO;2-K.

Huszar, G. and Vigue, L. (1990) 'Spermatogenesis-related change in the synthesis of the creatine kinase B-type and M-type isoforms in human spermatozoa', *Molecular Reproduction and Development*, 25(3), pp. 258–262. doi: 10.1002/mrd.1080250307.

Huszar, G., Vigue, L. and Morshedi, M. (1992) 'Sperm creatine phosphokinase M-isoform ratios and fertilizing potential of men: A blinded study of 84 couples treated with in vitro fertilization', in

*Fertility and Sterility*, pp. 882–888. doi: 10.1016/S0015-0282(16)54975-9.

Iannaccone, P. M. and Jacob, H. J. (2009) 'Rats ! Why the rat ? Stem cell beginnings', *Disease models & mechanisms*, 2,5-6, pp. 206–10. doi: 10.1242/dmm.002733.

Iannello, R. C., Young, J. C. and Kola, I. (1994) 'Pdha-2: A model for studying transcriptional regulation in early spermatocytes', *Molecular Reproduction and Development*, 39(2), pp. 194–199. doi: 10.1002/mrd.1080390212.

Ighodaro, O. M. and Akinloye, O. A. (2018) 'First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid', *Alexandria Journal of Medicine*, 54(4), pp. 287–293. doi: 10.1016/j.ajme.2017.09.001.

Immler, S. *et al.* (2007) 'By hook or by crook? Morphometry, competition and cooperation in rodent sperm', *PLoS ONE*. Edited by T. Pizzari, 2(1), p. e170. doi: 10.1371/journal.pone.0000170.

Ishikawa, T. *et al.* (2007) 'Expression of leptin and leptin receptor in the testis of fertile and infertile patients', *Andrologia*, 39(1), pp. 22–27. doi: 10.1111/j.1439-0272.2006.00754.x.

Isidori, A. M. *et al.* (1999) 'Leptin and Androgens in Male Obesity: Evidence for Leptin Contribution to Reduced Androgen Levels [1]', *The Journal of Clinical Endocrinology & Metabolism*, 84(10), pp. 3673–3680. doi: 10.1210/jcem.84.10.6082.

Isken, F. *et al.* (2010) 'Effects of long-term soluble vs. insoluble dietary fiber intake on high-fat diet-induced obesity in C57BL/6J mice.', *The Journal of nutritional biochemistry*, 21(4), pp. 278–284. doi: 10.1016/j.jnutbio.2008.12.012.

Islinger, M., Cardoso, M. J. R. and Schrader, M. (2010) 'Be different-The diversity of peroxisomes in the animal kingdom', *Biochimica et Biophysica Acta - Molecular Cell Research*, 1803(8), pp. 881–897. doi: 10.1016/j.bbamcr.2010.03.013.

Jackson, A. S. *et al.* (2002) 'The effect of sex, age and race on estimating percentage body fat from body mass index: The Heritage Family Study', *International Journal of Obesity*, 26(6), pp. 789–796. doi: 10.1038/sj.ijo.0802006.

Jackson, E. *et al.* (2017) 'Adipose tissue as a site of toxin accumulation', *Comprehensive Physiology*, 7(4), pp. 1085–1135. doi: 10.1002/cphy.c160038.

Jacob, H. J. (2010) 'The Rat: A Model Used in Biomedical Research BT - Rat Genomics: Methods and Protocols', in Anegon, I. (ed.). Humana Press, pp. 1–11. doi: 10.1007/978-1-60327-389-3_1.

Jambor, T., Tvrda, E. and Lukac, N. (2015) 'Leydig Cells as a Model of Male Reproduction', (January).

James, W. (2008) 'The epidemiology of obesity: the size of the problem', *Journal of Internal Medicine*, 263(4), pp. 336–352. doi: 10.1111/j.1365-2796.2008.01922.x.

James, W. P. T. (2008) 'WHO recognition of the global obesity epidemic', *International Journal of Obesity*, 32, pp. S120–S126. doi: 10.1038/ijo.2008.247.

Jandacek, R. J. *et al.* (2005) 'Effects of yo-yo diet, caloric restriction, and olestra on tissue distribution of hexachlorobenzene', *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 288(2 51-2). doi: 10.1152/ajpgi.00285.2004.

Jauniaux, E. (2001) 'An introduction to reproduction in pharaonic Egypt', *Reproductive BioMedicine Online*, 2(2), pp. 106–112. doi: 10.1016/S1472-6483(10)62234-1.

Jensen, M. B. (2014) 'Vitamin D and male reproduction', *Nature Reviews Endocrinology*, 10, p. 175. Available at: https://doi.org/10.1038/nrendo.2013.262.

Jensen, T. K. *et al.* (2004) 'Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men', *Fertility and Sterility*, 82(4), pp. 863–870. doi: 10.1016/j.fertnstert.2004.03.056.

Jequier, A. M. (2008) 'The Structure and Function of the Human Spermatozoon, Sperm Transport and Fertilization', in *Male infertility: a guide for the clinician*. John Wiley & Sons, pp. 26–35.

Jéquier, E. (2002) 'Pathways to obesity', *International Journal of Obesity*, 26(2), pp. S12–S17. doi: 10.1038/sj.ijo.0802123.

Jia, Y. F. *et al.* (2018) 'Obesity impairs male fertility through long-term effects on spermatogenesis', *BMC Urology*, 18(1). doi: 10.1186/s12894-018-0360-5.

Jiang, P. *et al.* (2013) 'Reciprocal regulation of p53 and malic enzymes modulates metabolism and senescence.', *Nature*, 493(7434), pp. 689–693. doi: 10.1038/nature11776.

Johannes, C. B. *et al.* (2000) 'Incidence of erectile dysfunction in men 40 to 69 years old: Longitudinal results from the Massachusetts male aging study', *Journal of Urology*, 163(2), pp. 460–463. doi: 10.1016/S0022-5347(05)67900-1.

Johnson, A. R. *et al.* (2016) 'Cafeteria diet-induced obesity causes oxidative damage in white adipose.', *Biochemical and biophysical research communications*, 473(2), pp. 545–550. doi:

10.1016/j.bbrc.2016.03.113.

Johnson, L. *et al.* (2002) 'The pesticide methoxychlor given orally during the perinatal/juvenile period, reduced the spermatogenic potential of males as adults by reducing their Sertoli cell number', *Reproduction Nutrition Development*, 42(6), pp. 573–580. doi: 10.1051/rnd:2002043.

Jones, C. A. and Sills, E. S. (2013) 'Contrasting selected reproductive challenges of today with those of antiquity-the past is prologue', *Ulster Medical Journal*, 82(3), pp. 150–156.

Jones, R., Mann, T. and Sherins, R. (1979) 'Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma', *Fertility and Sterility*, 31(5), pp. 531–537. doi: 10.1016/S0015-0282(16)43999-3.

Jozefczak, M. *et al.* (2012) 'Glutathione Is a Key Player in Metal-Induced Oxidative Stress Defenses', *International Journal of Molecular Sciences*, 13(3), pp. 3145–3175. doi: 10.3390/ijms13033145.

Kadowaki, T. *et al.* (2006) 'Japanese men have larger areas of visceral adipose tissue than Caucasian men in the same levels of waist circumference in a population-based study', *International Journal of Obesity*, 30(7), pp. 1163–1165. doi: 10.1038/sj.ijo.0803248.

Kahle, P. J., Waak, J. and Gasser, T. (2009) 'DJ-1 and prevention of oxidative stress in Parkinson's disease and other age-related disorders.', *Free radical biology & medicine*, 47(10), pp. 1354–1361. doi: 10.1016/j.freeradbiomed.2009.08.003.

Kahn, B. B. and Flier, J. S. (2000) 'Obesity and insulin resistance', *The Journal of Clinical Investigation*, 106(4), pp. 473–481. doi: 10.1172/JCI10842.

Kaldis, P. *et al.* (1997) 'Functions of Creatine Kinase Isoenzymes in Spermatozoa', *Advances in Developmental Biology (1992)*, 5, pp. 275–312. doi: 10.1016/S1566-3116(08)60040-7.

Kallman, F. J., Schoenfeld, W. A. and Barrera, S. E. (1944) 'No Title', *Am. J. Ment. Defic.*, 48, pp. 203–236.

Kamischke, A. and Nieschlag, E. (1999) 'Analysis of medical treatment of male infertility', in *Human Reproduction*. Oxford University Press, pp. 1–23. doi: 10.1093/humrep/14.suppl_1.1.

Kamp, G. *et al.* (2007) 'Regulatory properties of 6-phosphofructokinase and control of glycolysis in boar spermatozoa', *Reproduction*, 133(1), pp. 29–40. doi: 10.1530/REP-06-0082.

Kanarek, R. B. and Marks-Kaufman, R. (1979) 'Developmental aspects of sucrose-induced obesity

in rats', *Physiology and Behavior*, 23(5), pp. 881–885. doi: 10.1016/0031-9384(79)90195-1.

Kaplan, J. B. and Mead, R. A. (1993) 'Influence of season on seminal characteristics, testis size and serum testosterone in the western spotted skunk (Spilogale gracilis)', *Reproduction*, 98(2), pp. 321–326.

Karas, M. and Hillenkamp, F. (1988) 'Laser Desorption Ionization of Proteins with Molecular Masses Exceeding 10 000 Daltons', *Analytical Chemistry*, 60(20), pp. 2299–2301. doi: 10.1021/ac00171a028.

Karastergiou, K. *et al.* (2012) 'Sex differences in human adipose tissues - The biology of pear shape', *Biology of Sex Differences*, 3(1). doi: 10.1186/2042-6410-3-13.

Karmi, O. *et al.* (2018) 'The unique fold and lability of the [2Fe-2S] clusters of NEET proteins mediate their key functions in health and disease', *Journal of Biological Inorganic Chemistry*, 23(4), pp. 599–612. doi: 10.1007/s00775-018-1538-8.

Karnehed, N. *et al.* (2006) 'Physical activity, diet and gene-environment interactions in relation to body mass index and waist circumference: The Swedish Young Male Twins Study', *Public Health Nutrition*, 9(7), pp. 851–858. doi: 10.1017/PHN2005926.

Karst, H. *et al.* (1984) 'Diet-Induced Thermogenesis in Man: Thermic Effects of Single Proteins, Carbohydrates and Fats Depending on Their Energy Amount', *Annals of Nutrition and Metabolism*, 28(4), pp. 245–252. doi: 10.1159/000176811.

Karuppanapandian, T. *et al.* (2011) 'Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms', *Australian Journal of Crop Science*, 5(6).

Kashiwagi, E. *et al.* (2020) 'Relationship between body composition and hormone sensitivity for androgen deprivation therapy in patients with metastatic prostate cancer.', *Prostate international*, 8(1), pp. 22–26. doi: 10.1016/j.prnil.2019.11.002.

Kashiwaya, Y. *et al.* (1994) 'Control of glucose utilization in working perfused rat heart.', *Journal of Biological Chemistry*, 269(41), pp. 25502–25514. Available at: http://www.jbc.org/content/269/41/25502.abstract.

Kashiwaya, Y., King, M. T. and Veech, R. L. (1997) 'Substrate signaling by insulin: A ketone bodies ratio mimics insulin action in heart', in *American Journal of Cardiology*. Elsevier Inc., pp. 50A-64A. doi: 10.1016/S0002-9149(97)00458-X.

Kasturi, S. S., Tannir, J. and Brannigan, R. E. (2008) 'The metabolic syndrome and male infertility',

*Journal of Andrology*, 29(3), pp. 251–259. doi: 10.2164/jandrol.107.003731.

Katib, A. (2015) 'Mechanisms linking obesity to male infertility', *Central European Journal of Urology*, 68(1), pp. 79–85. doi: 10.5173/ceju.2015.01.435.

Kaukua, J. *et al.* (2003) 'Sex hormones and sexual function in obese men losing weight', *Obesity Research*, 11(6), pp. 689–694. doi: 10.1038/oby.2003.98.

Kaur, Y. *et al.* (2017) 'A systematic review of genetic syndromes with obesity', *Obesity Reviews*, 18(6), pp. 603–634. doi: 10.1111/obr.12531.

Kelley, D. E. *et al.* (2000) 'Subdivisions of subcutaneous abdominal adipose tissue and insulin resistance', *American Journal of Physiology-Endocrinology and Metabolism*, 278(5), pp. E941–E948. doi: 10.1152/ajpendo.2000.278.5.E941.

Kelley, D. E. *et al.* (2002) 'Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes', *Diabetes*, 51(10), pp. 2944–2950. doi: 10.2337/diabetes.51.10.2944.

Kelly, T. L., Wilson, K. E. and Heymsfield, S. B. (2009) 'Dual energy X-ray absorptiometry body composition reference values from NHANES', *PLoS ONE*, 4(9). doi: 10.1371/journal.pone.0007038.

Keltz, J. *et al.* (2010) 'Overweight Men: Clinical pregnancy after ART is decreased in IVF but not in ICSI cycles', *Journal of Assisted Reproduction and Genetics*, 27(9–10), pp. 539–544. doi: 10.1007/s10815-010-9439-y.

Kemnitz, J. W., Goy, R. W. and Keesey, R. E. (1977) 'Effects of gonadectomy on hypothalamic obesity in male and female rats.', *International journal of obesity*, 1(3), pp. 259–270.

Kern, P. A. *et al.* (2002) 'The stimulation of tumor necrosis factor and inhibition of glucose transport and lipoprotein lipase in adipose cells by 2,3,7,8-Tetrachlorodibenzo-p-dioxin', *Metabolism: Clinical and Experimental*, 51(1), pp. 65–68. doi: 10.1053/meta.2002.28088.

Keto, C. J. *et al.* (2012) 'Obesity is associated with castration-resistant disease and metastasis in men treated with androgen deprivation therapy after radical prostatectomy: results from the SEARCH database.', *BJU international*, 110(4), pp. 492–498. doi: 10.1111/j.1464-410X.2011.10754.x.

Khalil, S. F., Mohktar, M. S. and Ibrahim, F. (2014) 'The theory and fundamentals of bioimpedance analysis in clinical status monitoring and diagnosis of diseases', *Sensors (Switzerland)*, 14(6), pp. 10895–10928. doi: 10.3390/s140610895.

Khaw, K. T. and Barrett-Connor, E. (1988) 'Blood pressure and endogenous testosterone in men: an inverse relationship.', *Journal of hypertension*, 6(4), pp. 329–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3379300 (Accessed: 3 October 2019).

Kidroni, G. *et al.* (1983) 'Vitamin D3 metabolites in rat epididymis: High 24,25-dihydroxy vitamin D3 levels in the cauda region', *Biochemical and Biophysical Research Communications*, 113(3), pp. 982–989. doi: https://doi.org/10.1016/0006-291X(83)91095-1.

Kim, B. and Song, Y. S. (2016) 'Mitochondrial dynamics altered by oxidative stress in cancer', *Free Radical Research*, 50(10), pp. 1065–1070. doi: 10.1080/10715762.2016.1210141.

Kim, H. C. and Oh, S. M. (2013) 'Noncommunicable Diseases: Current Status of Major Modifiable Risk Factors in Korea', *J Prev Med Public Health*, 46, pp. 165–172. doi: 10.3961/jpmph.2013.46.4.165.

Kim, M. J. *et al.* (2011) 'Fate and complex pathogenic effects of dioxins and polychlorinated biphenyls in obese subjects before and after drastic weight loss', *Environmental Health Perspectives*, 119(3), pp. 377–383. doi: 10.1289/ehp.1002848.

Kim, M. J. *et al.* (2012) 'Inflammatory pathway genes belong to major targets of persistent organic pollutants in adipose cells.', *Environmental health perspectives*, 120(4), pp. 508–14. doi: 10.1289/ehp.1104282.

King, B. M. (1991) 'Ventromedial hypothalamic obesity: a reexamination of the irritative hypothesis.', *Neuroscience and biobehavioral reviews*, 15(3), pp. 341–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1956603 (Accessed: 13 October 2019).

King, B. M. (2006) 'The rise, fall, and resurrection of the ventromedial hypothalamus in the regulation of feeding behavior and body weight.', *Physiology & behavior*, 87(2), pp. 221–44. doi: 10.1016/j.physbeh.2005.10.007.

King, G. L. and Loeken, M. R. (2004) 'Hyperglycemia-Induced Oxidative Stress in Diabetic Complications', *Histochemistry and Cell Biology*, 122(4), pp. 333–338.

Kishi, T. and Elmquist, J. K. (2005) 'Body weight is regulated by the brain: A link between feeding and emotion', *Molecular Psychiatry*, 10(2), pp. 132–146. doi: 10.1038/sj.mp.4001638.

Kissebah, A. H. *et al.* (1982) 'Relation of Body Fat Distribution to Metabolic Complications of Obesity*', *The Journal of Clinical Endocrinology & Metabolism*, 54(2), pp. 254–260. doi: 10.1210/jcem-54-2-254.

Klaiber, E. L. *et al.* (1987) 'Interrelationships of cigarette smoking, testicular varicoceles, and seminal fluid indexes', *Fertility and Sterility*, 47(3), pp. 481–486. doi: 10.1016/s0015-0282(16)59059-1.

Kley, H. K. *et al.* (1980) 'Enhanced conversion of androstenedione to estrogens in obese males', *Journal of Clinical Endocrinology and Metabolism*, 51(5), pp. 1128–1132. doi: 10.1210/jcem-51-5-1128.

Knittle, J. L. *et al.* (1979) 'The growth of adipose tissue in children and adolescents. Cross-sectional and longitudinal studies of adipose cell number and size', *Journal of Clinical Investigation*, 63(2), pp. 239–246. doi: 10.1172/JCI109295.

Kobayashi, T. *et al.* (2002) 'Functional ATpase activity of p97/Valosin-containing Protein (VCP) is required for the quality control of endoplasmic reticulum in neuronally differentiated mammalian PC12 cells', *Journal of Biological Chemistry*, 277(49), pp. 47358–47365. doi: 10.1074/jbc.M207783200.

Kobayashi, T., Manno, A. and Kakizuka, A. (2007) 'Involvement of valosin-containing protein (VCP)/p97 in the formation and clearance of abnormal protein aggregates', *Genes to Cells*, 12(7), pp. 889–901. doi: 10.1111/j.1365-2443.2007.01099.x.

Kohn, D. F. and Clifford, C. B. (2002) 'Biology and diseases of rats', *Laboratory animal medicine*, 2, pp. 121–167.

Koike, M. *et al.* (2010) 'Valosin-containing protein (VCP) in novel feedback machinery between abnormal protein accumulation and transcriptional suppression', *Journal of Biological Chemistry*, 285(28), pp. 21736–21749. doi: 10.1074/jbc.M109.099283.

Kojima, M. *et al.* (1999) 'Ghrelin is a growth-hormone-releasing acylated peptide from stomach', *Nature*, 402(6762), pp. 656–660. doi: 10.1038/45230.

Koloszár, S. *et al.* (2005) 'Effect of body weight on sperm concentration in normozoospermic males', *Archives of Andrology*, 51(4), pp. 299–304. doi: 10.1080/01485010590919701.

Kotani, K. *et al.* (1994) 'Sexual dimorphism of age-related changes in whole-body fat distribution in the obese.', *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity*, 18(4), pp. 207–202.

Kothari, S. *et al.* (2010) 'Free radicals : Their beneficial and detrimental effects on sperm function', *Indian Journal of Experimental Biology*, 48, pp. 425–435.

Kottler, M.-L., Counis, R. and Bouchard, P. (1999) 'Mutations of the GnRH Receptor Gene: A New Cause of Autosomal-Recessive Hypogonadotropic Hypogonadism', *Archives of Medical Research*, 30(6), pp. 481–485. doi: https://doi.org/10.1016/S0188-4409(99)00072-7.

Kraemer, W. J. and Ratamess, N. A. (2005) 'Hormonal responses and adaptations to resistance exercise and training', *Sports Medicine*, 35(4), pp. 339–361. doi: 10.2165/00007256-200535040-00004.

Krisfalusi, M. *et al.* (2006) 'Multiple Glycolytic Enzymes Are Tightly Bound to the Fibrous Sheath of Mouse Spermatozoa1', *Biology of Reproduction*, 75(2), pp. 270–278. doi: 10.1095/biolreprod.105.049684.

Krotkiewski, M. *et al.* (1983) 'Impact of obesity on metabolism in men and women. Importance of regional adipose tissue distribution', *Journal of Clinical Investigation*, 72(3), pp. 1150–1162. doi: 10.1172/JCI111040.

Krotkiewski, M., Kral, J. G. and Karlsson, J. (1980) 'Effects of castration and testosterone substitution on body composition and muscle metabolism in rats', *Acta Physiologica Scandinavica*, 109(3), pp. 233–237. doi: 10.1111/j.1748-1716.1980.tb06592.x.

Kujala, U. M., Alen, M. and Huhtaniemi, I. T. (1990) 'Gonadotrophin-releasing hormone and human chorionic gonadotrophin tests reveal that both hypothalamic and testicular endocrine functions are suppressed during acute prolonged physical exercise', *Clinical Endocrinology*, 33(2), pp. 219–226. doi: 10.1111/j.1365-2265.1990.tb00486.x.

Kuk, J. L. *et al.* (2005) 'Waist circumference and abdominal adipose tissue distribution: influence of age and sex', *The American Journal of Clinical Nutrition*, 81(6), pp. 1330–1334. doi: 10.1093/ajcn/81.6.1330.

Kurabayashi, T. *et al.* (2016) 'Ovarian infertility is associated with cardiovascular disease risk factors in later life: A Japanese cross-sectional study.', *Maturitas*, 83, pp. 33–39. doi: 10.1016/j.maturitas.2015.08.015.

Kushnir, V. A. *et al.* (2017) 'Systematic review of worldwide trends in assisted reproductive technology 2004-2013', *Reproductive Biology and Endocrinology*. BioMed Central Ltd. doi: 10.1186/s12958-016-0225-2.

Kusminski, C. M. *et al.* (2012) 'MitoNEET-driven alterations in adipocyte mitochondrial activity reveal a crucial adaptive process that preserves insulin sensitivity in obesity', *Nature Medicine*, 18(10), pp. 1539–1551. doi: 10.1038/nm.2899.

La Merrill, M. *et al.* (2013) 'Toxicological function of adipose tissue: Focus on persistent organic pollutants', *Environmental Health Perspectives*, pp. 162–169. doi: 10.1289/ehp.1205485.

Lamberts, S. W. J., de Herder, W. W. and van der Lely, A. J. (1998) 'Pituitary insufficiency', *The Lancet*, 352(9122), pp. 127–134. doi: 10.1016/S0140-6736(98)85043-5.

Langdon, R. G. (1957) 'The biosynthesis of fatty acids in rat liver', *Journal of Biological Chemistry*, 226(2), pp. 615–629.

Langley-Evans, S. C. (2000) 'Critical differences between two low protein diet protocols in the programming of hypertension in the rat.', *International journal of food sciences and nutrition*, 51(1), pp. 11–17. doi: 10.1080/096374800100859.

Lanska, D. J. *et al.* (1985) 'Factors influencing anatomic location of fat tissue in 52,953 women.', *International journal of obesity*, 9(1), pp. 29–38. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3874840 (Accessed: 22 September 2019).

Lanzafame, F. *et al.* (2009) 'Oxidative stress and medical antioxidant treatment in male infertility', *Reproductive BioMedicine Online*. Elsevier Ltd, pp. 638–659. doi: 10.1016/j.rbmo.2009.09.014.

Lapidus, L. *et al.* (1984) 'Distribution of adipose tissue and risk of cardiovascular disease and death: a 12 year follow up of participants in the population study of women in Gothenburg, Sweden.', *BMJ*, 289(6454), pp. 1257–1261. doi: 10.1136/bmj.289.6454.1257.

Lardy, H. and Phillips, P. (1945) 'Studies of fat and carbohydrate oxidation in mammalian spermatozoa', *Archives of Biochemistry*, 6(1), pp. 53–61.

Larsen, U. (2005) 'Research on infertility: Which definition should we use?', *Fertility and Sterility*, 83(4), pp. 846–852. doi: 10.1016/j.fertnstert.2004.11.033.

Larsson, B. *et al.* (1984) 'Abdominal adipose tissue distribution, obesity, and risk of cardiovascular disease and death: 13 year follow up of participants in the study of men born in 1913.', *BMJ*, 288(6428), pp. 1401–1404. doi: 10.1136/bmj.288.6428.1401.

Laurell, S. (1956) 'Plasma free fatty acids in diabetic acidosis and starvation', *Scandinavian Journal of Clinical and Laboratory Investigation*, 8(1), pp. 81–82. doi: 10.3109/00365515609049249.

Le Magnen, J. and Devos, M. (1970) 'Metabolic correlates of the meal onset in the free food intake of rats', *Physiology & Behavior*, 5(7), pp. 805–814. doi: https://doi.org/10.1016/0031-9384(70)90284-2.

Lea, I. A., Widgren, E. E. and O'Rand, M. G. (2004) 'Association of sperm protein 17 with A-kinase anchoring protein 3 flagella', *Reproductive Biology and Endocrinology*, 2. doi: 10.1186/1477-7827-2-57.

Lear, S. A. *et al.* (2009) 'Ethnic variation in fat and lean body mass and the association with insulin resistance', *Journal of Clinical Endocrinology and Metabolism*, 94(12), pp. 4696–4702. doi: 10.1210/jc.2009-1030.

Lee, I. K. (2014) 'The role of pyruvate dehydrogenase kinase in diabetes and obesity', *Diabetes and Metabolism Journal*, 38(3), pp. 181–186. doi: 10.4093/dmj.2014.38.3.181.

Lee, M. O. (1929) 'Determination of the surface area of the white rat with its application to the expression of metabolic results', *American Journal of Physiology-Legacy Content*, 89(1), pp. 24–33. doi: 10.1152/ajplegacy.1929.89.1.24.

Légràdi, G. *et al.* (1997) 'Leptin prevents fasting-induced suppression of prothyrotropin-releasing hormone messenger ribonucleic acid in neurons of the hypothalamic paraventricular nucleus', *Endocrinology*, 138(6), pp. 2569–2576. doi: 10.1210/endo.138.6.5209.

Leipnitz, G. *et al.* (2018) 'Evaluation of mitochondrial bioenergetics , dynamics , mitochondria crosstalk , and reactive oxygen species in fibroblasts from patients with complex I deficiency', *Scientific Reports*, 8(1165), pp. 1–14. doi: 10.1038/s41598-018-19543-3.

Lemonnier, D. (1972) 'Effect of age, sex, and site on the cellularity of the adipose tissue in mice and rats rendered obese by a high-fat diet', *The Journal of Clinical Investigation*, 51(11), pp. 2907–2915. doi: 10.1172/JCI107115.

Lenzi, A., Gandini, L. and Picardo, M. (1998) 'A rationale for glutathione therapy.', *Human reproduction (Oxford, England)*, 13(6), pp. 1419–1422.

Leopoldo, A. S. *et al.* (2016) 'Classification of different degrees of adiposity in sedentary rats', *Brazilian Journal of Medical and Biological Research*, 49(4). doi: 10.1590/1414-431X20155028.

Levin, B. E. *et al.* (2003) 'A new obesity-prone, glucose-intolerant rat strain (F.DIO)', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 285(5), pp. R1184–R1191. doi: 10.1152/ajpregu.00267.2003.

Levin, B. E. and Dunn-Meynell, A. A. (2002) 'Defense of body weight depends on dietary composition and palatability in rats with diet-induced obesity', *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 282(1 51-1). doi:

10.1152/ajpregu.2002.282.1.r46.

Leyton, J., Drury, P. J. and Crawford, M. A. (1987) 'Differential oxidation of saturated and unsaturated fatty acids in vivo in the rat', *British Journal of Nutrition*. 2007/03/09, 57(3), pp. 383–393. doi: DOI: 10.1079/BJN19870046.

Li, D. and Roberts, R. (2001) 'WD-repeat proteins: Structure characteristics, biological function, and their involvement in human diseases', *Cellular and Molecular Life Sciences*, 58(14), pp. 2085–2097. doi: 10.1007/PL00000838.

Li, Q. and Suprenant, K. A. (1994) 'Molecular characterization of the 77-kDa echinoderm microtubule-associated protein. Homology to the beta-transducin family.', *Journal of Biological Chemistry* , 269(50), pp. 31777–31784. Available at: http://www.jbc.org/content/269/50/31777.abstract.

Li, W., Vogel, C. F. A. and Matsumura, F. (2007) 'Studies on the cell treatment conditions to elicit lipolytic responses from 3T3-L1 adipocytes to TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin', *Journal of Cellular Biochemistry*, 102(2), pp. 389–402. doi: 10.1002/jcb.21303.

Libretexts Biology (2019) *Investigation: Rat dissection*, *Biology Corner*. Available at: https://bio.libretexts.org/Bookshelves/Ancillary_Materials/Worksheets/Book%3A_The_Biology_Corner_(Worksheets)/Anatomy_Worksheets/Investigation%3A_Rat_Dissection (Accessed: 13 October 2019).

LibreTexts Chemistry (no date) *Mass Analyzers (Mass Spectrometry)*. Available at: https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_(Analytical_Chemistry)/Instrumental_Analysis/Mass_Spectrometry/Mass_Spectrometers_(Instrumentation)/Mass_Analyzers_(Mass_Spectrometry) (Accessed: 11 October 2019).

Lieberman, H. R., Yeghiayan, S. K. and Maher, T. J. (2005) 'A low-protein diet alters rat behavior and neurotransmission in normothermic and hyperthermic environments', *Brain Research Bulletin*, 66(2), pp. 149–154. doi: https://doi.org/10.1016/j.brainresbull.2005.04.011.

Lim, U. *et al.* (2019) 'Propensity for Intra-abdominal and Hepatic Adiposity Varies Among Ethnic Groups', *Gastroenterology*, 156(4), pp. 966-975.e10. doi: 10.1053/j.gastro.2018.11.021.

Lindemann, C. B. and Lesich, K. A. (2016) 'Functional anatomy of the mammalian sperm flagellum', *Cytoskeleton*, 73, pp. 652–669. doi: 10.1002/cm.21338.

Link, A. J. *et al.* (1999) 'Direct analysis of protein complexes using mass spectrometry : Article :

Nature Biotechnology', *Nature Biotechnology*, 17(7), pp. 676–682. doi: 10.1038/10890.

Lissens, W. *et al.* (2000) 'Mutations in the X-linked pyruvate dehydrogenase (E1) α subunit gene (PDHA1) in patients with a pyruvate dehydrogenase complex deficiency', *Human Mutation*, 15(3), pp. 209–219. doi: 10.1002/(SICI)1098-1004(200003)15:3<209::AID-HUMU1>3.0.CO;2-K.

Liu, B., Zhang, W. and Wang, Z. (2010) 'Voltage-dependent anion channel in mammalian spermatozoa', *Biochemical and Biophysical Research Communications*, 397(4), pp. 633–636. doi: 10.1016/j.bbrc.2010.06.025.

Liu, Y. *et al.* (2013) 'Oxidative damage associated with obesity is prevented by overexpression of CuZn- or Mn-superoxide dismutase', *Biochemical and Biophysical Research Communications*, 438(1), pp. 78–83. doi: 10.1016/j.bbrc.2013.07.029.

Löllmann, B. *et al.* (1997) 'Detection and quantification of the leptin receptor splice variants Ob-Ra, b, and, e in different mouse tissues', *Biochemical and Biophysical Research Communications*, 238(2), pp. 648–652. doi: 10.1006/bbrc.1997.7205.

Lopushnyan, N. A. and Walsh, T. J. (2012) 'Surgical techniques for the management of male infertility', *Asian Journal of Andrology*, pp. 94–102. doi: 10.1038/aja.2011.62.

Lotter, E. C. and Woods, S. C. (1977) 'Injections of insulin and changes of body weight', *Physiology and Behavior*, 18(2), pp. 293–297. doi: 10.1016/0031-9384(77)90136-6.

Lovejoy, J. C. *et al.* (1996) 'Abdominal fat distribution and metabolic risk factors: Effects of race', *Metabolism: Clinical and Experimental*, 45(9), pp. 1119–1124. doi: 10.1016/S0026-0495(96)90011-6.

Lovenberg, W. (1973) *Iron sulfur proteins*, *Academic Press*. New York.

Lowe, R. *et al.* (2017) 'Transcriptomics technologies', *PLoS Computational Biology*, 13(5). doi: 10.1371/journal.pcbi.1005457.

Luboshitzky, R. *et al.* (2001) 'Disruption of the nocturnal testosterone rhythm by sleep fragmentation in normal men', *Journal of Clinical Endocrinology and Metabolism*, 86(3), pp. 1134–1139. doi: 10.1210/jcem.86.3.7296.

Luboshitzky, R. *et al.* (2005) 'Altered luteinizing hormone and testosterone secretion in middle-aged obese men with obstructive sleep apnea', *Obesity Research*, 13(4), pp. 780–786. doi: 10.1038/oby.2005.88.

Luckman, S. M., Rosenzweig, I. and Dickson, S. L. (1999) 'Activation of arcuate nucleus neurons by systemic administration of leptin and growth hormone-releasing peptide-6 in normal and fasted rats', *Neuroendocrinology*, 70(2), pp. 93–100. doi: 10.1159/000054463.

Lüers, G. H. *et al.* (2006) 'Peroxisomes are present in murine spermatogonia and disappear during the course of spermatogenesis', *Histochemistry and Cell Biology*, 125(6), pp. 693–703. doi: 10.1007/s00418-005-0114-9.

Luft, R. (1995) 'Proceedings of the Nobel Symposium 90: Mitochondrial Diseases. Saltsjobaden, Sweden, 2-6 July 1994.', in *Biochimica et biophysica acta*, pp. 1–292. doi: 10.1016/0925-4439(95)00002-L.

Lukacova, J. *et al.* (2015) 'Dose- and time-dependent effects of bisphenol A on bovine spermatozoa in vitro', *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, 50(7), pp. 669–676. doi: 10.1080/10934529.2015.1011963.

Lukinius, A. *et al.* (1989) 'Co-localization of islet amyloid polypeptide and insulin in the B cell secretory granules of the human pancreatic islets', *Diabetologia*, 32(4), pp. 240–244. doi: 10.1007/BF00285291.

Lunsky, I. O. and Meyre, D. (2019) 'Decoding Mendelian obesity', *Current Opinion in Endocrine and Metabolic Research*, 4, pp. 21–28. doi: 10.1016/j.coemr.2018.10.002.

Lutz, T. A. and Woods, S. C. (2012) 'Overview of animal models of obesity', *Current Protocols in Pharmacology*, 58(1). doi: 10.1002/0471141755.ph0561s58.

Ly, D. *et al.* (2002) 'Identification of rat EMAP, a δ-glutamate receptor binding protein', *Biochemical and Biophysical Research Communications*, 291(1), pp. 85–90. doi: 10.1006/bbrc.2002.6413.

Lyetjens, M. C. and Weinbauer, G. F. (2012) 'Testosterone: biosynthesis, transprt, metabolism and (non-gemonic) actions', in Nieschlag, E., Behre, H. M., and Nieschlag, S. (eds) *Testosterone: action, deficiency, substitution*. Cambridge University Press, pp. 15–29.

Lykke-Andersen, K. *et al.* (2003) 'Disruption of the COP9 Signalosome Csn2 Subunit in Mice Causes Deficient Cell Proliferation, Accumulation of p53 and Cyclin E, and Early Embryonic Death', *Molecular and Cellular Biology*, 23(19), pp. 6790–6797. doi: 10.1128/mcb.23.19.6790-6797.2003.

Macaulay, S. L. and Larkins, R. G. (1988) 'Impaired insulin action in adipocytes of New Zealand

Obese mice: A role for postbinding defects in pyruvate dehydrogenase and insulin mediator activity', *Metabolism - Clinical and Experimental*, 37(10), pp. 958–965. doi: 10.1016/0026-0495(88)90153-9.

MacDonald, A. A. *et al.* (2009) 'The impact of body mass index on semen parameters and reproductive hormones in human males: A systematic review with meta-analysis', *Human Reproduction Update*, 16(3), pp. 293–311. doi: 10.1093/humupd/dmp047.

Madsen, L., Petersen, R. K. and Kristiansen, K. (2005) 'Regulation of adipocyte differentiation and function by polyunsaturated fatty acids', *Biochimica et Biophysica Acta*, 1740(2), pp. 266–286. doi: https://doi.org/10.1016/j.bbadis.2005.03.001.

Magnoni, R. *et al.* (2014) 'The Hsp60 folding machinery is crucial for manganese superoxide dismutase folding and function', *Free Radical Research*, 48(2), pp. 168–179. doi: 10.3109/10715762.2013.858147.

Magnusdottir, E. V. *et al.* (2005) 'Persistent organochlorines, sedentary occupation, obesity and human male subfertility', *Human Reproduction*, 20(1), pp. 208–215. doi: 10.1093/humrep/deh569.

Mahfouz, R. Z. *et al.* (2010) 'Sperm viability, apoptosis, and intracellular reactive oxygen species levels in human spermatozoa before and after induction of oxidative stress', *Fertility and Sterility*, 93(3), pp. 814–821. doi: 10.1016/j.fertnstert.2008.10.068.

Maling, H. M. and Highman, B. (1958) 'Exaggerated Ventricular Arrhythmias and Myocardial Fatty Changes After Large Doses of Norepinephrine and Epinephrine in Unanesthetized Dogs', *American Journal of Physiology-Legacy Content*, 194(3), pp. 590–596. doi: 10.1152/ajplegacy.1958.194.3.590.

Malta, A. *et al.* (2014) 'Low-protein diet in adult male rats has long-term effects on metabolism', *Journal of Endocrinology*, 221(2), pp. 285–295. doi: 10.1530/JOE-13-0473.

Manandhar, G., Schatten, H. and Sutovsky, P. (2005) 'Centrosome Reduction During Gametogenesis and Its Significance1', *Biology of Reproduction*, 72(1), pp. 2–13. doi: 10.1095/biolreprod.104.031245.

Mandenoff, A., Lenoir, T. and Apfelbaum, M. (1982) 'Tardy occurrence of adipocyte hyperplasia in cafeteria-fed rat', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 242(3), pp. R349–R351. doi: 10.1152/ajpregu.1982.242.3.R349.

Mann, T. *et al.* (1981) 'Male Reproductive Function and the Composition of Semen: General

Considerations', in *Male Reproductive Function and Semen*. Springer London, pp. 1–37. doi: 10.1007/978-1-4471-1300-3_1.

Mannowetz, N., Wandernoth, P. M. and Wennemuth, G. (2012) 'Glucose is a pH-dependent motor for sperm beat frequency during early activation', *PLoS ONE*, 7(7). doi: 10.1371/journal.pone.0041030.

Manzoni, C. *et al.* (2018) 'Genome, transcriptome and proteome: The rise of omics data and their integration in biomedical sciences', *Briefings in Bioinformatics*, 19(2), pp. 286–302. doi: 10.1093/BIB/BBW114.

Marchiani, S. *et al.* (2015) 'Metabolic syndrome-associated sperm alterations in an experimental rabbit model: Relation with metabolic profile, testis and epididymis gene expression and effect of tamoxifen treatment', *Molecular and Cellular Endocrinology*, 401, pp. 12–24. doi: 10.1016/j.mce.2014.11.005.

Marinelli, D. *et al.* (2004) 'Mini-review of studies on the effect of smoking and drinking habits on semen parameters', *International Journal of Hygiene and Environmental Health*, 207(3), pp. 185–192. doi: 10.1078/1438-4639-00283.

Märker, T. *et al.* (2010) 'Heat shock protein 60 and adipocytes: Characterization of a ligand-receptor interaction', *Biochemical and Biophysical Research Communications*, 391(4), pp. 1634–1640. doi: 10.1016/j.bbrc.2009.12.104.

Marques, C. *et al.* (2016) 'High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat', *Adipocyte*, 5(1), pp. 11–21. doi: 10.1080/21623945.2015.1061723.

Marseglia, L. *et al.* (2015) 'Oxidative Stress in Obesity : A Critical Component in Human Diseases', *International Journal of Molecular Sciences*, 16, pp. 378–400. doi: 10.3390/ijms16010378.

Martínez-Hernández, A. *et al.* (2007) 'Genetics of obesity', *Public Health Nutrition*, 10(10A), pp. 1138–1144. doi: 10.1017/S1368980007000626.

Martinez, J. A. (2000) 'Body-weight regulation: Causes of obesity', in *Proceedings of the Nutrition Society*. CAB International, pp. 337–345. doi: 10.1017/S0029665100000380.

Martini, A. C. *et al.* (2004) 'Effects of alcohol and cigarette consumption on human seminal quality', *Fertility and Sterility*, 82(2), pp. 374–377. doi: 10.1016/j.fertnstert.2004.03.022.

Maslow, A. H. (1943) 'A theory of human motivation', *Psychological Review*, 50(4), pp. 370–396. doi: 10.1037/h0054346.

Masson, P. and Brannigan, R. E. (2014) 'The varicocele', *Urologic Clinics of North America*, 41(1), pp. 129–144. doi: 10.1016/j.ucl.2013.08.001.

Mathur, D. N., Toriola, A. L. and Dada, O. A. (1986) 'Serum cortisol and testosterone levels in conditioned male distance runners and nonathletes after maximal exercise.', *The Journal of sports medicine and physical fitness*, 26(3), pp. 245–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3795918 (Accessed: 7 October 2019).

Mathur, P. P. and D'Cruz, S. C. (2011) 'The effect of environmental contaminants on testicular function', *Asian Journal of Andrology*, 13(4), pp. 585–591. doi: 10.1038/aja.2011.40.

Mathur, P., Saradha, B. and Vaithinathan, S. (2008) 'Impact of Environmental Toxicants on Testicular Function', *Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry*, 8(1), pp. 79–90. doi: 10.2174/187152208783790705.

Matsuda, M. and Shimomura, I. (2013) 'Increased oxidative stress in obesity : Implications for metabolic syndrome , diabetes , hypertension , dyslipidemia , atherosclerosis , and cancer', *Obesity Research & Clinical Practice*, 7(5), pp. e330–e341. doi: 10.1016/j.orcp.2013.05.004.

Mattison, D. R. and Thomford, P. J. (1987) 'Selection of animals for male reproductive toxicology studies: an evaluation of selected assumptions in reproductive toxicity and risk assessment', in Roloff, M. V. et al. (eds) *Human risk assessment - the role of animal selection and extrapolation*. London: Taylor & Francis, pp. 195–213.

Mauras, N. *et al.* (1998) 'Testosterone Deficiency in Young Men: Marked Alterations in Whole Body Protein Kinetics, Strength, and Adiposity1', *The Journal of Clinical Endocrinology & Metabolism*, 83(6), pp. 1886–1892. doi: 10.1210/jcem.83.6.4892.

Maury, J. *et al.* (1995) 'Pretranslational regulation of pyruvate dehydrogenase complex subunits in white adipose tissue during the suckling-weaning transition in the rat', *Biochemical Journal*, 311(2), pp. 531–535. doi: 10.1042/bj3110531.

Mawhinney, M. and Angelo, M. (2013) 'Physiology , pathology and pharmacology of the male reproductive system', *Periodontology*, 61, pp. 232–251.

McArdle, W. D., Katch, F. I. and Katch, V. L. (2006) *Essentials of exercise physiology*. Lippincott Williams & Wilkins.

McCarrey, J. R. *et al.* (1992) 'Differential transcription of Pgk genes during spermatogenesis in the mouse', *Developmental Biology*, 154(1), pp. 160–168. doi: 10.1016/0012-1606(92)90056-M.

McCormack, J. G., Halestrap, A. P. and Denton, R. M. (1990) 'Role of calcium ions in regulation of mammalian intramitochondrial metabolism', *Physiological Reviews*, 70(2), pp. 391–425. doi: 10.1152/physrev.1990.70.2.391.

McCoy, M. K. and Cookson, M. R. (2011) 'DJ-1 regulation of mitochondrial function and autophagy through oxidative stress.', *Autophagy*, 7(5), pp. 531–532.

McGhee, J. D. and Felsenfeld, G. (1980) 'Nucleosome Structure', *Annual Review of Biochemistry*, 49(1), pp. 1115–1156. doi: 10.1146/annurev.bi.49.070180.005343.

McTigue, K. M., Garrett, J. M. and Popkin, B. M. (2002) 'The natural history of the development of obesity in a cohort of young U.S. adults between 1981 and 1998', *Annals of Internal Medicine*, 136(12), pp. 857–864. doi: 10.7326/0003-4819-136-12-200206180-00006.

Meethal, S. V. and Atwood, C. S. (2005) 'The role of hypothalamic-pituitary-gonadal hormones in the normal structure and functioning of the brain', *Cellular and Molecular Life Sciences*, (62), pp. 257–270. doi: 10.1007/s00018-004-4381-3.

Mehta, A. *et al.* (2016) 'Limitations and barriers in access to care for male factor infertility', *Fertility and Sterility*, 105(5), pp. 1128–1137. doi: https://doi.org/10.1016/j.fertnstert.2016.03.023.

Mendis-Handagama, S. M. L. C., Zirkin, B. R. and Ewing, L. L. (1988) 'Comparison of components of the testis interstitium with testosterone secretion in hamster, rat, and guinea pig testes perfused in vitro', *American Journal of Anatomy*, 181(1), pp. 12–22. doi: 10.1002/aja.1001810103.

Menkveld, R., Holleboom, C. A. G. and Rhemrev, J. P. T. (2011) 'Measurement and significance of sperm morphology', *Asian Journal of Andrology*, 13(1), pp. 59–68. doi: 10.1038/aja.2010.67.

Meyer, A. J. *et al.* (2007) 'Redox-sensitive GFP in Arabidopsis thaliana is a quantitative biosensor for the redox potential of the cellular glutathione redox buffer', *The Plant Journal*, 52(5), pp. 973–986. doi: 10.1111/j.1365-313X.2007.03280.x.

Mickelsen, O., Takahashi, S. and Craig, C. (1955) 'Experimental obesity. 1. Production of obesity in rats by feeding high-fat diets.', *Journal of Nutrition*, 57, pp. 541–554.

Mieusset, R. and Bujan, L. (1995) 'Testicular heating and its possible contributions to male infertility: a review', *International Journal of Andrology*, 18(4), pp. 169–184. doi: 10.1111/j.1365-2605.1995.tb00408.x.

Miguel, F., Augusto, A. C. and Gurgueira, S. A. (2009) 'Effect of acute vs chronic H2O2-induced oxidative stress on antioxidant enzyme activities', *Free Radical Research*, 43(4), pp. 340–347. doi:

10.1080/10715760902751894.

Miki, K. *et al.* (2004) 'Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility', *Proceedings of the National Academy of Sciences of the United States of America*, 101(47), pp. 16501–16506. doi: 10.1073/pnas.0407708101.

Miki, K. (2007) 'Energy metabolism and sperm function.', *Society of Reproduction and Fertility supplement*, 65, pp. 309–325.

Miller-Fleming, L. *et al.* (2014) 'Yeast DJ-1 superfamily members are required for diauxic-shift reprogramming and cell survival in stationary phase.', *Proceedings of the National Academy of Sciences of the United States of America*, 111(19), pp. 7012–7017. doi: 10.1073/pnas.1319221111.

Mirsky, I. A. and Broh-Kahn, R. H. (1937) 'The influence of increased metabolism on β-hydroxybutyric acid utilization', *American Journal of Physiology-Legacy Content*, 120(3), pp. 446–450. doi: 10.1152/ajplegacy.1937.120.3.446.

Misra, A. (1997) 'Relationship of anterior and posterior subcutaneous abdominal fat to insulin sensitivity in nondiabetic men', *Obesity Research*, 5(2), pp. 93–99. doi: 10.1002/j.1550-8528.1997.tb00648.x.

Misra, A. and Khurana, L. (2009) 'The metabolic syndrome in South Asians: Epidemiology, determinants, and prevention', *Metabolic Syndrome and Related Disorders*, 7(6), pp. 497–514. doi: 10.1089/met.2009.0024.

Mitsukawa, T. *et al.* (1990) 'Islet amyloid polypeptide response to glucose, insulin, and somatostatin analogue administration', *Diabetes*, 39(5), pp. 639–642. doi: 10.2337/diab.39.5.639.

Monesi, V. (1971) 'Chromosome activities during meiosis and spermiogenesis.', *Journal of reproduction and fertility. Supplement*, 13, p. Suppl 13:1-14. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5292798 (Accessed: 30 October 2019).

Montague, C. T. *et al.* (1997) 'Congenital leptin deficiency is associated with severe early-onset obesity in humans', *Nature*, 387(6636), pp. 903–908. doi: 10.1038/43185.

Montorsi, F. *et al.* (2010) 'Summary of the recommendations on sexual dysfunctions in men', *Journal of Sexual Medicine*, 7(11), pp. 3572–3588. doi: 10.1111/j.1743-6109.2010.02062.x.

Morice, P. *et al.* (1995) 'History of infertility', *Human reproduction update*, 1(5), pp. 497–504. doi:

10.1093/humupd/1.5.497.

Morris, H. G. *et al.* (1968) 'Metabolic effects of human growth hormone in corticosteroid-treated children', *The Journal of Clinical Investigation*, 47(3), pp. 436–451. doi: 10.1172/JCI105740.

Mountjoy, K. G. *et al.* (1994) 'Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain.', *Molecular Endocrinology*, 8(10), pp. 1298–1308. doi: 10.1210/mend.8.10.7854347.

Mounzih, K., Lu, R. and Chehab, F. F. (1997) 'Leptin treatment rescues the sterility of genetically obese ob/ob males', *Endocrinology*, 138(3), pp. 1190–1193. doi: 10.1210/endo.138.3.5024.

Moussavi, N., Gavino, V. and Receveur, O. (2008) 'Could the Quality of Dietary Fat, and Not Just Its Quantity, Be Related to Risk of Obesity?', *Obesity*, 16(1), pp. 7–15. doi: 10.1038/oby.2007.14.

Movérare-Skrtic, S. *et al.* (2006) 'Dihydrotestosterone Treatment Results in Obesity and Altered Lipid Metabolism in Orchidectomized Mice', *Obesity*, 14(4), pp. 662–672. doi: 10.1038/oby.2006.75.

Mukai, C. and Okuno, M. (2004) 'Glycolysis Plays a Major Role for Adenosine Triphosphate Supplementation in Mouse Sperm Flagellar Movement', *Biology of Reproduction*, 71(2), pp. 540–547. doi: 10.1095/biolreprod.103.026054.

Mukai, C. and Travis, A. J. (2012) 'What sperm can teach us about energy production', *Reproduction in Domestic Animals*, 47(SUPPL.4), pp. 164–169. doi: 10.1111/j.1439-0531.2012.02071.x.

Mukherjee, S. B. *et al.* (1999) 'Secretion of glutathione S-transferase isoforms in the seminiferous tubular fluid, tissue distribution and sex steroid binding by rat GSTM1.', *The Biochemical journal*, 340 ( Pt 1, pp. 309–320.

Müller, G. *et al.* (2012) 'Testicular testosterone: Estradiol ratio in domestic cats and its relationship to spermatogenesis and epididymal sperm morphology', *Theriogenology*, 78(6), pp. 1224–1234. doi: 10.1016/j.theriogenology.2012.05.018.

Muoio, D. M. *et al.* (1997) 'Leptin directly alters lipid partitioning in skeletal muscle', *Diabetes*, 46(8), pp. 1360–1363. doi: 10.2337/diab.46.8.1360.

Muoio, D. M. and Newgard, C. B. (2006) 'Obesity-Related Derangements in Metabolic Regulation', *Annual Review of Biochemistry*, 75(1), pp. 367–401. doi: 10.1146/annurev.biochem.75.103004.142512.

Murshidi, M. M., Choy, J. T. and Eisenberg, M. L. (2020) 'Male Infertility and Somatic Health', *Urologic Clinics of North America*, 47(2), pp. 211–217. doi: 10.1016/j.ucl.2019.12.008.

Muscogiuri, G. *et al.* (2019) 'Prader- Willi syndrome: An uptodate on endocrine and metabolic complications', *Reviews in Endocrine and Metabolic Disorders*. Springer New York LLC, pp. 239–250. doi: 10.1007/s11154-019-09502-2.

Mutch, D. M. and Clément, K. (2006) 'Unraveling the genetics of human obesity', *PLoS Genetics*, 2(12), pp. 1956–1963. doi: 10.1371/journal.pgen.0020188.

Muthusami, K. R. and Chinnaswamy, P. (2005) 'Effect of chronic alcoholism on male fertility hormones and semen quality', *Fertility and Sterility*, 84(4), pp. 919–924. doi: 10.1016/j.fertnstert.2005.04.025.

Mutsumoto, A. M. and Bremner, W. J. (2016) 'Testicular disorders', in Melmed, S. et al. (eds) *Williams Textbook of Endocrinology*. 13th edn. Elsevier Ltd.

Mvo, Z. (1999) 'Perceptions of overweight African women about acceptable body size of women and children', *Curationis*, 22(2). doi: 10.4102/curationis.v22i2.719.

Nair, A. R. *et al.* (2015) 'Glutathione and mitochondria determine acute defense responses and adaptive processes in cadmium-induced oxidative stress and toxicity of the kidney', *Archives of Toxicology*, 89(12), pp. 2273–2289. doi: 10.1007/s00204-014-1401-9.

Nakada, K. *et al.* (2006) 'Mitochondria-related male infertility', *Proceedings of the National Academy of Sciences of the United States of America*, 103(41), pp. 15148–15153. doi: 10.1073/pnas.0604641103.

Nakamura, N., Mori, C. and Eddy, E. M. (2010) 'Molecular Complex of Three Testis-Specific Isozymes Associated with the Mouse Sperm Fibrous Sheath: Hexokinase 1, Phosphofructokinase M, and Glutathione S-Transferase mu class 51', *Biology of Reproduction*, 82(3), pp. 504–515. doi: 10.1095/biolreprod.109.080580.

Nascimento, A. F. *et al.* (2008) 'A hypercaloric pellet-diet cycle induces obesity and co-morbidities in wistar rats', *Arquivos Brasileiros de Endocrinologia e Metabologia*, 52(6), pp. 968–974. doi: 10.1590/s0004-27302008000600007.

Nasim, S., Bilal, S. and Qureshi, M. (2019) 'Psycho-social aspects of infertility-a review of current trends.', *The Professional Medical Journal*, 26(09), pp. 1537–1541. doi: 10.29309/tpmj/2019.26.09.4019.

Natarajan, P. and Khan, S. D. (2019) 'Ejaculatory Dysfunction', in Gunasekaran, K. and Khan, S. D. (eds) *Sexual Medicine : Principles and Practice*. Singapore: Springer Singapore, pp. 33–45. doi: 10.1007/978-981-13-1226-7_4.

National Institutes of Health National Library of Medicine (2019) *What is epigenetics?*, *How Genes Work*. Available at: https://ghr.nlm.nih.gov/ (Accessed: 9 September 2019).

Naugler, W. E. and Karin, M. (2008) 'The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer', *Trends in Molecular Medicine*, 14(3), pp. 109–119. doi: 10.1016/j.molmed.2007.12.007.

Naz, M. and Kamal, M. (2017) 'Classification, causes, diagnosis and treatment of male infertility: a review', *Oriental Pharmacy and Experimental Medicine*, 17(2), pp. 89–109. doi: 10.1007/s13596-017-0269-7.

Nechushtai, R. *et al.* (2012) 'Characterization of Arabidopsis NEET reveals an ancient role for NEET proteins in iron metabolism', *Plant Cell*, 24(5), pp. 2139–2154. doi: 10.1105/tpc.112.097634.

Nemeroff, C. B., Lipton, M. A. and Kizer, J. S. (1978) 'Models of neuroendocrine regulation: use of monosodium glutamate as an investigational tool.', *Developmental neuroscience*, 1(2), pp. 102–9. doi: 10.1159/000112561.

Nichols, D. G. and Crompton, M. (1980) 'Mitochondrial Ca2+ transport', *FEBS Lett*, 111, pp. 261–268.

Nieschlag, E. *et al.* (2010) *Disorders at the testicular level*, *Andrology: Male Reproductive Health and Dysfunction*. Springer Berlin Heidelberg. doi: 10.1007/978-3-540-78355-8_13.

Nishiumi, S. *et al.* (2010) '2,3,7,8-Tetrachlorodibenzo-p-dioxin impairs an insulin signaling pathway through the induction of tumor necrosis factor-α in adipocytes', *Toxicological Sciences*, 115(2), pp. 482–491. doi: 10.1093/toxsci/kfq052.

Niskanen, L. *et al.* (2004) 'Changes in sex hormone-binding globulin and testosterone during weight loss and weight maintenance in abdominally obese men with the metabolic syndrome', *Diabetes, Obesity and Metabolism*, 6(3), pp. 208–215. doi: 10.1111/j.1462-8902.2004.00335.x.

Niswender, K. D. *et al.* (1997) 'Cell-specific expression and regulation of a glucokinase gene locus transgene', *Journal of Biological Chemistry*, 272(36), pp. 22564–22569. doi: 10.1074/jbc.272.36.22564.

Norman, A. W. and Henry, H. H. (2015) 'Androgens', in Norman, A. W. and Henry, H. H. (eds)

*Hormones*. 3rd edn. Elsevier Inc., Academic Press.

O'Donnell, L. *et al.* (2001) 'Estrogen and spermatogenesis', *Endocrine Reviews*, 22(3), pp. 289–318. doi: 10.1210/er.22.3.289.

O'Farrell, P. H. (1975) 'High resolution two-dimensional electrophoresis of proteins.', *Journal of biological chemistry*, 250(10), pp. 4007–4021.

O'Flynn O'Brien, K. L., Varghese, A. C. and Agarwal, A. (2010) 'The genetic causes of male factor infertility: A review', *Fertility and Sterility*, 93(1), pp. 1–12. doi: 10.1016/j.fertnstert.2009.10.045.

Oates, R. D. (1997) 'Genetics for the Clinician', in Hellstrom, W. J. G. (ed.) *Male Infertility and Sexual Dysfunction*. New York: Springer Science+Business Media.

Oberholzer, M. *et al.* (2007) 'Trypanosomes and mammalian sperm: one of a kind?', *Trends in Parasitology*, 23(2), pp. 71–77. doi: 10.1016/j.pt.2006.12.002.

Odbayar, T.-O. *et al.* (2009) 'Isoenzyme-specific up-regulation of glutathione transferase and aldo-keto reductase mRNA expression by dietary quercetin in rat liver', *Molecular and Cellular Biochemistry*, 325(1), pp. 121–130. doi: 10.1007/s11010-009-0026-4.

Oehninger, S *et al.* (1995) 'Effects of hydrogen peroxide on human spermatozoa', *Journal of Assisted Reproduction and Genetics*. Kluwer Academic Publishers-Plenum Publishers, pp. 41–47. doi: 10.1007/BF02214128.

Oehninger, Sergio *et al.* (1995) 'Effects of hydrogen peroxide on human spermatozoa', *Journal of Assisted Reproduction and Genetics*, 12(1), pp. 41–47. doi: 10.1007/BF02214128.

Ohmiya, Y. and Nakai, K. (1977) 'Effect of Starvation on Excretion, Distribution and Metabolism of DDT in Mice', *The Tohoku Journal of Experimental Medicine*, 122(2), pp. 143–153. doi: 10.1620/tjem.122.143.

Oiwa, K., Kamimura, S. and Iwamoto, H. (2009) 'X-ray Fiber Diffraction Studies on Flagellar Axonemes', in King, S. M. and Pazour, G. J. (eds) *Cilia: Structure and Motility*. Academic Press (Methods in Cell Biology), pp. 89–109. doi: https://doi.org/10.1016/S0091-679X(08)91005-0.

Okhura, S., Tsukamura, H. and Maeda, K. (2000) 'Endocrinology', in Krinke, G. J. (ed.) *The Laboratory Rat*. Academic Press, pp. 406–408.

Olivares, A. *et al.* (2010) 'Reproductive axis function and gonadotropin microheterogeneity in a male rat model of diet-induced obesity', *General and Comparative Endocrinology*, 166(2), pp. 356–

364. doi: 10.1016/j.ygcen.2009.12.007.

Ollmann, M. M. *et al.* (1997) 'Antagonism of Central Melanocortin receptors in vitro and in vivo by agouti-related protein', *Science*, 278(5335), pp. 135–138. doi: 10.1126/science.278.5335.135.

Olsen, J. *et al.* (1997) 'Does moderate alcohol intake reduce fecundability? A European multicenter study on infertility and subfecundity', *Alcoholism: Clinical and Experimental Research*, 21(2), pp. 206–212. doi: 10.1111/j.1530-0277.1997.tb03751.x.

Olson Jr, E. B. *et al.* (1976) 'The effect of hepatectomy on the synthesis of 25-hydroxyvitamin D3.', *The Journal of Clinical Investigation*, 57(5), pp. 1213–1220. doi: 10.1172/JCI108389.

Onda, M. *et al.* (2002) 'Expression of lumican in thickened intima and smooth muscle cells in human coronary atherosclerosis', *Experimental and Molecular Pathology*, 72(2), pp. 142–149. doi: 10.1006/exmp.2002.2425.

Oosterman, J. E. *et al.* (2015) 'Timing of fat and liquid sugar intake alters substrate oxidation and food efficiency in male Wistar rats', *Chronobiology International*, 32(2), pp. 289–298. doi: 10.3109/07420528.2014.971177.

Op Den Camp, R. G. L. *et al.* (2003) 'Rapid Induction of Distinct Stress Responses after the Release of Singlet Oxygen in Arabidopsis', *Plant Cell*, 15(10), pp. 2320–2332. doi: 10.1105/tpc.014662.

Opiteck, G. J. *et al.* (1997) 'Comprehensive On-Line LC/LC/MS of Proteins', *Analytical Chemistry*, 69(8), pp. 1518–1524. doi: 10.1021/ac961155l.

Orahilly, S. (2009) 'Human genetics illuminates the paths to metabolic disease', *Nature*, 462(7271), pp. 307–314. doi: 10.1038/nature08532.

Organization, W. H. and others (2014) 'Sexual and reproductive health: infertility is a global public health issue'. Available at: http://www.who.int/reproductivehealth/topics/infertility/en/.

Oscai, L. B. (1982) 'Dietary-induced severe obesity: A rat model', *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 11(2), pp. R212–R215. doi: 10.1152/ajpregu.1982.242.3.r212.

Osellame, L. D., Blacker, T. S. and Duchen, M. R. (2012) 'Cellular and molecular mechanisms of mitochondrial function', *Best Practice & Research Clinical Endocrinology & Metabolism*, 26(6), pp. 711–723. doi: https://doi.org/10.1016/j.beem.2012.05.003.

Osterloh, A. *et al.* (2009) 'Heat shock protein 60 ( HSP60 ) stimulates neutrophil effector functions', *Journal of Leukocyte Biology*, 86, pp. 423–434. doi: 10.1189/jlb.0109011.

Ostrowski, L. E. *et al.* (2002) 'A Proteomic Analysis of Human Cilia', *Molecular & Cellular Proteomics*, 1(6), pp. 451–465. doi: 10.1074/mcp.M200037-MCP200.

Ouerghi, D., Rivest, S. and Richard, D. (1992) 'Adrenalectomy Attenuates the Effect of Chemical Castration on Energy Balance in Rats', *The Journal of Nutrition*, 122(2), pp. 369–373. doi: 10.1093/jn/122.2.369.

Oyeyipo, I. P. *et al.* (2011) 'Effects of nicotine on sperm characteristics and fertility profile in adult male rats: a possible role of cessation.', *Journal of reproduction & infertility*, 12(3), pp. 201–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23926503 (Accessed: 7 October 2019).

Oyeyipo, I. P., Maartens, P. J. and du Plessis, S. S. (2014) 'In vitro effects of nicotine on human spermatozoa', *Andrologia*, 46(8), pp. 887–892. doi: 10.1111/and.12169.

Ozata, M. *et al.* (2002) 'Increased oxidative stress and hypozincemia in male obesity', *Clinical Biochemistry*, 35(8), pp. 627–631. doi: 10.1016/S0009-9120(02)00363-6.

Paasch, U. *et al.* (2010) 'Obesity and age affect male fertility potential', *Fertility and Sterility*, 94(7), pp. 2898–2901. doi: 10.1016/j.fertnstert.2010.06.047.

Pakrashi, A. and Chatterjee, S. (1995) 'Effect of tobacco consumption on the function of male accessory sex glands', *International Journal of Andrology*, 18(5), pp. 232–236. doi: 10.1111/ijan.1995.18.5.232.

Pan, G., O'Rourke, K. and Dixit, V. M. (1998) 'Caspase-9, Bcl-XL, and Apaf-1 Form a Ternary Complex ', *Journal of Biological Chemistry* , 273(10), pp. 5841–5845. Available at: http://www.jbc.org/content/273/10/5841.abstract.

Paniagua, R. *et al.* (1991) 'Ultrastructure of the aging human testis', *Journal of Electron Microscopy Technique*, 19(2), pp. 241–260. doi: 10.1002/jemt.1060190209.

Panier, S. and Boulton, S. J. (2014) 'Double-strand break repair: 53BP1 comes into focus', *Nature Reviews Molecular Cell Biology*, 15(1), pp. 7–18. doi: 10.1038/nrm3719.

Pantel, J. and Cone, R. D. (2013) 'Melanocortin System', in *Encyclopedia of Biological Chemistry: Second Edition*. Elsevier Inc., pp. 24–27. doi: 10.1016/B978-0-12-378630-2.00488-6.

Parasuraman, S. *et al.* (2010) 'Anti-hyperlipidemic effect of triglize, a polyherbal formulation',

*International Journal of Pharmacy and Pharmaceutical Sciences*, 2(3), pp. 118–122.

Parasuraman, S. *et al.* (2013) 'Antihyperlipidemic effect of Angiosifa, a polyherbal formulation, in Sprague-Dawley rats', *Pharmacognosy Journal*, 5(5), pp. 221–227. doi: 10.1016/j.phcgj.2013.08.001.

Park, K. S. *et al.* (1991) 'Intra-abdominal fat is associated with decreased insulin sensitivity in healthy young men', *Metabolism*, 40(6), pp. 600–603. doi: 10.1016/0026-0495(91)90050-7.

Pasquali, R. *et al.* (1995) 'Insulin regulates testosterone and sex hormone-binding globulin concentrations in adult normal weight and obese men', *Journal of Clinical Endocrinology and Metabolism*, 80(2), pp. 654–658. doi: 10.1210/jcem.80.2.7852532.

Pasquali, R. (2006) 'Obesity, fat distribution and infertility', *Maturitas*, 54(4), pp. 363–371. doi: 10.1016/j.maturitas.2006.04.018.

Pavlovich, C. P. *et al.* (2001) 'Evidence of a treatable endocrinopathy in infertile men', *Journal of Urology*, 165(3), pp. 837–841. doi: 10.1016/S0022-5347(05)66540-8.

Pécheur, E. I. *et al.* (2002) 'Phospholipid species act as modulators in p97/p47-mediated fusion of Golgi membranes', *Biochemistry*, 41(31), pp. 9813–9823. doi: 10.1021/bi0259195.

Peckham, S. C., Entenman, C. and Carroll, H. W. (1962) 'The influence of a hypercaloric diet on gross body and adipose tissue composition in the rat', *The Journal of Nutrition*, 77(2), pp. 187–197. doi: 10.1093/jn/77.2.187.

Pellati, D. *et al.* (2008) 'Genital tract infections and infertility', *European Journal of Obstetrics and Gynecology and Reproductive Biology*. Elsevier Ireland Ltd, pp. 3–11. doi: 10.1016/j.ejogrb.2008.03.009.

Perkins, D. N. *et al.* (1999) 'Probability-based protein identification by searching sequence databases using mass spectrometry data', in *Electrophoresis*. Wiley-VCH Verlag, pp. 3551–3567. doi: 10.1002/(SICI)1522-2683(19991201)20:18<3551::AID-ELPS3551>3.0.CO;2-2.

Petit, F. M. *et al.* (2013) 'Identification of sperm head proteins involved in zona pellucida binding', *Human Reproduction*, 28(4), pp. 852–865. doi: 10.1093/humrep/des452.

Petricoin, E. F. *et al.* (2002) 'Clinical proteomics: Translating benchside promise into bedside reality', *Nature Reviews Drug Discovery*, pp. 683–695. doi: 10.1038/nrd891.

Petrosillo, G. *et al.* (2009) 'Mitochondrial complex I dysfunction in rat heart with aging: critical role

of reactive oxygen species and cardiolipin', *Free Radical Biology and Medicine*, 46(1), pp. 88–94. doi: 10.1016/j.freeradbiomed.2008.09.031.

Petrunkina, A. M. *et al.* (2007) 'Determinants of sperm quality and fertility in domestic species', *Reproduction*, 134(1), pp. 3–17.

Pezeshki, A. *et al.* (2016) 'Low protein diets produce divergent effects on energy balance', *Scientific Reports*, 6(1), p. 25145. doi: 10.1038/srep25145.

Pfister, G. *et al.* (2005) 'Detection of HSP60 on the membrane surface of stressed human endothelial cells by atomic force and confocal microscopy', *Journal of Cell Science*, 118(8), pp. 1587–1594. doi: 10.1242/jcs.02292.

Phillips, G. B. *et al.* (1993) 'Sex hormones and hemostatic risk factors for coronary heart disease in men with hypertension', *Journal of Hypertension*, 11(7), pp. 699–702. doi: 10.1097/00004872-199307000-00003.

Pi-Sunyer, F. X. (2002) 'The Obesity Epidemic: Pathophysiology and Consequences of Obesity', *Obesity Research*, 10(S12), pp. 97S-104S. doi: 10.1038/oby.2002.202.

Piché, M.-E. *et al.* (2018) 'Overview of Epidemiology and Contribution of Obesity and Body Fat Distribution to Cardiovascular Disease: An Update', *Progress in Cardiovascular Diseases*, 61(2), pp. 103–113. doi: https://doi.org/10.1016/j.pcad.2018.06.004.

Piedra, J. *et al.* (2003) 'p120 Catenin-Associated Fer and Fyn Tyrosine Kinases Regulate Beta-Catenin Tyr-142 Phosphorylation and Beta-Catenin-Alpha-Catenin Interaction', *Molecular and Cellular Biology*, 23(7), pp. 2287–2297. doi: 10.1128/mcb.23.7.2287-2297.2003.

Pilatz, A. *et al.* (2016) 'Infektionen und Infertilität', *Der Urologe*, 55(7), pp. 883–889. doi: 10.1007/s00120-016-0151-0.

Pinkney, J. and Kopelman, P. (2004) 'Handbook of obesity: etiology and pathophysiology', in Bray, G. A. and Bouchard, C. (eds) *Handbook of obesity. Etiology and pathophysiology*. New York: Marcel Dekker, pp. 655–670.

Pitteloud, N. *et al.* (2005) 'Increasing insulin resistance is associated with a decrease in Leydig cell testosterone secretion in men', *Journal of Clinical Endocrinology and Metabolism*, 90(5), pp. 2636–2641. doi: 10.1210/jc.2004-2190.

Pleasure, I. T., Black, M. M. and Keen, J. H. (1993) 'Valosin-containing protein, VCP, is a ubiquitous clathrin-binding protein', *Nature*, 365(6445), pp. 459–462. doi: 10.1038/365459a0.

Plebani, M. (2005) ‘Proteomics: The next revolution in laboratory medicine?’, *Clinica Chimica Acta*, 357(2), pp. 113–122. doi: 10.1016/j.cccn.2005.03.017.

Pockley, A. G., Muthana, M. and Calderwood, S. K. (2008) ‘The dual immunoregulatory roles of stress proteins’, *Trends in Biochemical Sciences*, 33(2), pp. 71–79. doi: https://doi.org/10.1016/j.tibs.2007.10.005.

Pond, C. M. (1992) ‘An evolutionary and functional view of mammalian adipose tissue’, *Proceedings of the Nutrition Society*, 51(3), pp. 367–377. doi: 10.1079/pns19920050.

Postic, C., Shiota, M. and Magnuson, M. A. (2001) ‘Cell-specific roles of glucokinase in glucose homeostasis’, *Recent progress in hormone research*, 56, pp. 195–218.

Pouliot, M. C. *et al.* (1992) ‘Visceral obesity in men: Associations with glucose tolerance, plasma insulin, and lipoprotein levels’, *Diabetes*, 41(7), pp. 826–834. doi: 10.2337/diab.41.7.826.

Pouliot, M. C. *et al.* (1994) ‘Waist circumference and abdominal sagittal diameter: Best simple anthropometric indexes of abdominal visceral adipose tissue accumulation and related cardiovascular risk in men and women’, *The American Journal of Cardiology*, 73(7), pp. 460–468. doi: 10.1016/0002-9149(94)90676-9.

Practice Committee of the American Society for Reproductive Medicine (2013) ‘Definitions of infertility and recurrent pregnancy loss: a committee opinion’, *Fertility and sterility*, 99(1), p. 63. doi: 10.1016/j.fertnstert.2012.09.023.

Practice Committee of the American Society for Reproductive Medicine (2017) ‘Guidance on the limits to the number of embryos to transfer: a committee opinion.’, *Fertility and sterility*, 107(4), pp. 901–903. doi: 10.1016/j.fertnstert.2017.02.107.

Prader, A. (1956) ‘Ein syndrom von adipositas, kleinwuchs, kryptorchismus und oligophrenie nach myatonieartigem zustand im neugeborenenalter.’, *Schweiz Med Wochenschr*, 86, pp. 1260–1261.

Preguiça, I. *et al.* (2020) ‘Diet-induced rodent models of obesity-related metabolic disorders—A guide to a translational perspective’, *Obesity Reviews*, pp. 1–29. doi: 10.1111/obr.13081.

Pressat, R. and Wilson, C. (1985) *The dictionary of demography*, *The dictionary of demography*. Basil Blackwell. Available at: https://www.scopus.com/inward/record.uri?eid=2-s2.0-85040876325&partnerID=40&md5=fdfad6c2788766f824a7789bf4c3a464.

Preston, B. T. *et al.* (2012) ‘Testes size, testosterone production and reproductive behaviour in a natural mammalian mating system’, *Journal of Animal Ecology*, 81(1), pp. 296–305. doi:

10.1111/j.1365-2656.2011.01907.x.

Pryor, J. P. and Hendry, W. F. (1991) 'Ejaculatory duct obstruction in subfertile males: Analysis of 87 patients', *Fertility and Sterility*, 56(4), pp. 725–730. doi: 10.1016/S0015-0282(16)54606-8.

Puig, K. L. *et al.* (2012) 'Amyloid precursor protein and proinflammatory changes are regulated in brain and adipose tissue in a murine model of high fat diet-induced obesity', *PLoS ONE*. Edited by T. Ikezu, 7(1), p. e30378. doi: 10.1371/journal.pone.0030378.

Puoane, T. *et al.* (2002) 'Obesity in South Africa: The South African Demographic and Health Survey', *Obesity Research*, 10(10), pp. 1038–1048. doi: 10.1038/oby.2002.141.

Pusztai, P. *et al.* (2008) 'Ghrelin: A new peptide regulating the neurohormonal system, energy homeostasis and glucose metabolism', *Diabetes/Metabolism Research and Reviews*, pp. 343–352. doi: 10.1002/dmrr.830.

Qin, D. D. *et al.* (2007) 'Do reproductive hormones explain the association between body mass index and semen quality?', *Asian Journal of Andrology*, 9(6), pp. 827–834. doi: 10.1111/j.1745-7262.2007.00268.x.

Quadroni, M. and James, P. (1999) 'Proteomics and automation', *Electrophoresis*, 20(4–5), pp. 664–677. doi: 10.1002/(SICI)1522-2683(19990101)20:4/5<664::AID-ELPS664>3.0.CO;2-A.

Quennell, J. H. *et al.* (2009) 'Leptin indirectly regulates gonadotropin-releasing hormone neuronal function', *Endocrinology*, 150(6), pp. 2805–2812. doi: 10.1210/en.2008-1693.

Quinn, R. (2005) 'Comparing rat's to human's age: How old is my rat in people years?', *Nutrition*, 21(6), pp. 775–777. doi: 10.1016/j.nut.2005.04.002.

Raman, J. D. and Schlegel, P. N. (2002) 'Aromatase inhibitors for male infertility', *Journal of Urology*, 167(2 Part 1), pp. 624–629. doi: 10.1016/S0022-5347(01)69099-2.

Ramlau-Hansen, C. H. *et al.* (2007) 'Subfecundity in overweight and obese couples', *Human Reproduction*, 22(6), pp. 1634–1637. doi: 10.1093/humrep/dem035.

Randle, P. J. (1963) 'Endocrine Control of Metabolism', *Annual Review of Physiology*, 25(1), pp. 291–324. doi: 10.1146/annurev.ph.25.030163.001451.

Rankinen, T. *et al.* (2006) 'The human obesity gene map: The 2005 update', *Obesity*, 14(4), pp. 529–644. doi: 10.1038/oby.2006.71.

Rao, A. V. S. K. and Shaha, C. (2001) 'Multiple glutathione S-transferase isoforms are present on

male germ cell plasma membrane', *FEBS Letters*, 507(2), pp. 174–180. doi: 10.1016/S0014-5793(01)02958-1.

Rauhala, P., Andoh, T. and Chiueh, C. C. (2005) 'Neuroprotective properties of nitric oxide and S-nitrosoglutathione', *Toxicology and Applied Pharmacology*, 207(2, Supplement), pp. 91–95. doi: https://doi.org/10.1016/j.taap.2005.02.028.

Ravussin, E. *et al.* (1988) 'Reduced Rate of Energy Expenditure as a Risk Factor for Body-Weight Gain', *New England Journal of Medicine*, 318(8), pp. 467–472. doi: 10.1056/NEJM198802253180802.

Ravussin, E. (2005) 'A NEAT way to control weight?', *Science*, 307(5709), pp. 530–531. doi: 10.1126/science.1108597.

Razak, F. *et al.* (2007) 'Defining obesity cut points in a multiethnic population', *Circulation*, 115(16), pp. 2111–2118. doi: 10.1161/CIRCULATIONAHA.106.635011.

Regal, M. *et al.* (2002) 'Prevalence and incidence of hypopituitarism in an adult Caucasian population in northwestern Spain', *Clinical Endocrinology*, 55(6), pp. 735–740. doi: 10.1046/j.1365-2265.2001.01406.x.

Reisse, S. *et al.* (2001) 'Peroxisomes and Ether Lipid Biosynthesis in Rat Testis and Epididymis', *Biology of Reproduction*, 64(6), pp. 1689–1694. doi: 10.1095/biolreprod64.6.1689.

Richardson, R. T., Yamasaki, N. and O'rand, M. G. (1994) 'Sequence of a rabbit sperm zona pellucida binding protein and localization during the acrosome reaction', *Developmental Biology*, 165(2), pp. 688–701. doi: 10.1006/dbio.1994.1285.

Ringler, D. H. and Dabich, L. (2013) 'Hematology and Clinical Biochemistry', in Baker, H. J., Lindsey, J. R., and Wesibroth, S. H. (eds) *The Laboratory Rat: Biology and Diseases*. Elsevier, p. 113.

Ritov, V. B. *et al.* (2005) 'Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes', *Diabetes*, 54(1), pp. 8–14. doi: 10.2337/diabetes.54.1.8.

Rivier, C. and Rivest, S. (1991) 'Effect of Stress on the Activity of the Hypothalamic-Pituitary-Gonadal Axis: Peripheral and Central Mechanisms1', *Biology of Reproduction*, 45(4), pp. 523–532. doi: 10.1095/biolreprod45.4.523.

Rivlin, J. *et al.* (2004) 'Role of Hydrogen Peroxide in Sperm Capacitation and Acrosome Reaction1', *Biology of Reproduction*, 70(2), pp. 518–522. doi: 10.1095/biolreprod.103.020487.

Roberts, K. P. and Pryor, J. L. (1997) 'Anatomy and Physiology of the Male Reproductive System', in Hellstrom, W. J. G. (ed.) *Male Infertility and Sexual Dysfunction*. New York, United States of America: Springer Science+Business Media. doi: 10.1007/978-1-4612-1848-7.

Robins, G. (1993) *Women in ancient Egypt*. Cambridge, Massechusettes: Harvard University Press.

Roffé, M. *et al.* (2013) 'IMPACT is a developmentally regulated protein in neurons that opposes the eukaryotic initiation factor 2βkinase GCN2 in the modulation of neurite outgrowth', *Journal of Biological Chemistry*, 288(15), pp. 10860–10869. doi: 10.1074/jbc.M113.461970.

Rogers, K. (2011) 'Anatomy of the Human Reproductive System', in Rogers, K. (ed.) *The Reproductive System*. New York, United States of America: Britannica Educational Publishing, pp. 19–35.

Rogers, P. and Webb, G. P. (1980) 'Estimation of body fat in normal and obese mice', *British Journal of Nutrition*, 43(1), pp. 83–86. doi: 10.1079/bjn19800066.

Rolls, B. J. (2009) 'The relationship between dietary energy density and energy intake.', *Physiology & behavior*, 97(5), pp. 609–615. doi: 10.1016/j.physbeh.2009.03.011.

Rolls, B. J., Rowe, E. A. and Turner, R. C. (1980) 'Persistent obesity in rats following a period of consumption of a mixed, high energy diet.', *The Journal of Physiology*, 298(1), pp. 415–427. doi: 10.1113/jphysiol.1980.sp013091.

Rosini, T. C., Ramos da Silva, A. S. and de Moraes, C. (2012) 'Diet-induced obesity: rodent model for the study of obesity-related disorders', *Revista da Associação Médica Brasileira (English Edition)*, 58(3), pp. 383–387. doi: 10.1016/S2255-4823(12)70211-4.

Ross, R. *et al.* (1992) 'Quantification of adipose tissue by MRI: relationship with anthropometric variables', *Journal of Applied Physiology*, 72(2), pp. 787–795. doi: 10.1152/jappl.1992.72.2.787.

Roth, M. Y., Amory, J. K. and Page, S. T. (2008) 'Treatment of male infertility secondary to morbid obesity', *Nature Clinical Practice Endocrinology and Metabolism*, 4(7), pp. 415–419. doi: 10.1038/ncpendmet0844.

Rothwell, N J, Saville, M. E. and Stock, M. J. (1982) 'Acute And Chronic Effects Of Sucrose And Glucose On Thermogenic Responses And Weight-Gain In The Rat', in *Proceedings of the Nutrition Society*, p. A8.

Rothwell, N. J., Saville, M. E. and Stock, M. J. (1982) 'Effects of feeding a "cafeteria" diet on

energy balance and diet induced thermogenesis in four strains of rat', *Journal of Nutrition*, 112(8), pp. 1515–1524. doi: 10.1093/jn/112.8.1515.

Rothwell, N. J. and Stock, M. J. (1984) '1 - The development of obesity in animals: The role of dietary factors', *Clinics in Endocrinology and Metabolism*, 13(3), pp. 437–449. doi: https://doi.org/10.1016/S0300-595X(84)80032-8.

Rouault, T. A. (2012) 'Biogenesis of iron-sulfur clusters in mammalian cells: New insights and relevance to human disease', *Disease Models and Mechanisms*, 5(2), pp. 155–164. doi: 10.1242/dmm.009019.

Rousseaux, S. *et al.* (2008) 'Epigenetic reprogramming of the male genome during gametogenesis and in the zygote', *Reproductive BioMedicine Online*, 16(4), pp. 492–503. doi: https://doi.org/10.1016/S1472-6483(10)60456-7.

Rowe, J. D., Patskovsky, Y. V, *et al.* (1998) 'Rationale for Reclassification of a Distinctive Subdivision of Mammalian Class Mu Glutathione S-Transferases That Are Primarily Expressed in Testis ', *Journal of Biological Chemistry* , 273(16), pp. 9593–9601. Available at: http://www.jbc.org/content/273/16/9593.abstract.

Rowe, J. D., Tchaikovskaya, T., *et al.* (1998) 'Selective Expression of a Glutathione S-Transferase Subclass During Spermatogenesis', *Journal of Andrology*, 19(5), pp. 558–567. doi: 10.1002/j.1939-4640.1998.tb02057.x.

Rowe, J. D., Nieves, E. and Listowsky, I. (1997) 'Subunit diversity and tissue distribution of human glutathione S-transferases: interpretations based on electrospray ionization-MS and peptide sequence-specific antisera', *Biochemical Journal*, 325(2), pp. 481–486. Available at: http://www.biochemj.org/content/325/2/481.abstract.

Rumbold, A. R. *et al.* (2019) 'Impact of male factor infertility on offspring health and development', *Fertility and Sterility*, 111(6), pp. 1047–1053. doi: https://doi.org/10.1016/j.fertnstert.2019.05.006.

Russell, J. C. *et al.* (1993) 'Effect of castration on hyperlipidemic, insulin resistant JCR:LA-corpulent rats.', *Atherosclerosis*, 100(1), pp. 113–122. doi: 10.1016/0021-9150(93)90073-4.

Rusz, A. *et al.* (2012) 'Influence of urogenital infections and inflammation on semen quality and male fertility', *World Journal of Urology*, 30(1), pp. 23–30. doi: 10.1007/s00345-011-0726-8.

Sajjad, Y. (2010) 'Development of the genital ducts and external genitalia in the early human embryo', *Journal of Obstetrics and Gynaecology Research*, 36(5), pp. 929–937. doi:

10.1111/j.1447-0756.2010.01272.x.

Saleh, R. *et al.* (2010) 'Histopathologic patterns of testicular biopsies in infertile azoospermic men with varicocele', *Fertility and Sterility*, 94(6). doi: 10.1016/j.fertnstert.2010.03.026.

Saleh, R. A. *et al.* (2002) 'Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study', *Fertility and Sterility*, 78(3), pp. 491–499. doi: 10.1016/S0015-0282(02)03294-6.

Salie, R., Huisamen, B. and Lochner, A. (2014) 'High carbohydrate and high fat diets protect the heart against ischaemia/reperfusion injury', *Cardiovascular Diabetology*, 13(1), pp. 1–12. doi: 10.1186/s12933-014-0109-8.

Sampey, B. P. *et al.* (2011) 'Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet.', *Obesity (Silver Spring, Md.)*, 19(6), pp. 1109–1117. doi: 10.1038/oby.2011.18.

Sánchez, E. *et al.* (2013) 'LYRM7/MZM1L is a UQCRFS1 chaperone involved in the last steps of mitochondrial Complex III assembly in human cells', *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1827(3), pp. 285–293. doi: https://doi.org/10.1016/j.bbabio.2012.11.003.

Saper, C. B., Chou, T. C. and Elmquist, J. K. (2002) 'The need to feed: Homeostatic and hedonic control of eating', *Neuron*, 36(2), pp. 199–211. doi: 10.1016/S0896-6273(02)00969-8.

Sapozhnikov, A. M. *et al.* (2007) 'Spontaneous apoptosis and expression of cell surface heat-shock proteins in cultured EL-4 lymphoma cells', *Cell Proliferation*, 32(6), pp. 363–378. doi: 10.1111/j.1365-2184.1999.tb01354.x.

Saradha, B. and Mathur, P. P. (2006) 'Effect of environmental contaminants on male reproduction', *Environmental Toxicology and Pharmacology*, 21(1), pp. 34–41. doi: 10.1016/j.etap.2005.06.004.

Saraste, M. (1999) 'Oxidative Phosphorylation at the fin de siècle', *Science*, 283(5407), pp. 1488–1493. doi: 10.1126/science.283.5407.1488.

Saravanan, G. *et al.* (2014) 'Anti-obesity action of gingerol: Effect on lipid profile, insulin, leptin, amylase and lipase in male obese rats induced by a high-fat diet', *Journal of the Science of Food and Agriculture*, 94(14), pp. 2972–2977. doi: 10.1002/jsfa.6642.

Saris, W. H. M. *et al.* (2000) 'Randomized controlled trial of changes in dietary carbohydrate/fat ratio and simple vs complex carbohydrates on body weight and blood lipids: the CARMEN study', *International Journal of Obesity*, 24(10), pp. 1310–1318. doi: 10.1038/sj.ijo.0801451.

Sarkar, O. *et al.* (2011) 'Impact of inflammation on male fertility', *Fronteirs in Bioscience (Elite edition)*, 3, pp. 89–95.

Sato, K. *et al.* (1995) 'Insulin, ketone bodies, and mitochondrial energy transduction', *FASEB Journal*, 9(8), pp. 651–658. doi: 10.1096/fasebj.9.8.7768357.

Schafer, F. Q. and Buettner, G. R. (2001) 'Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple', *Free Radical Biology and Medicine*, 30(11), pp. 1191–1212. doi: 10.1016/S0891-5849(01)00480-4.

Scheffer, P. G. *et al.* (1997) 'Measurement of low-density lipoprotein particle size by high-performance gel-filtration chromatography', *Clinical chemistry*, 43(10), pp. 1904–1912.

Schemmel, R., Mickelsen, O. and Tolgay, Z. (1969) 'Dietary obesity in rats: influence of diet, weight, age, and sex on body composition.', *The American journal of physiology*, 216(2), pp. 373–379. doi: 10.1152/ajplegacy.1969.216.2.373.

Schlegel, P. N. (2004) 'Causes of azoospermia and their management', *Reproduction, Fertility and Development*, 16(5), pp. 561–572. Available at: https://doi.org/10.1071/RD03087.

Schneider, G. *et al.* (1979) 'Increased estrogen production in obese men', *Journal of Clinical Endocrinology and Metabolism*, 48(4), pp. 633–638. doi: 10.1210/jcem-48-4-633.

Schneider, J. *et al.* (1983) 'Effects of Obesity on Estradiol Metabolism: Decreased Formation of Nonuterotropic Metabolites', *Journal of Clinical Endocrinology and Metabolism*, 56(5), pp. 973–978. doi: 10.1210/jcem-56-5-973.

Schrader, M. and Fahimi, H. D. (2008) 'The peroxisome: Still a mysterious organelle', *Histochemistry and Cell Biology*, 129(4), pp. 421–440. doi: 10.1007/s00418-008-0396-9.

Schulster, M., Bernie, A. M. and Ramasamy, R. (2006) 'The role of estradiol in male reproductive function', *Asian Journal of Andrology*, (18), pp. 435–440. doi: 10.4103/1008-682X.173932.

Schuppe, H.-C. *et al.* (2017) 'Urogenital Infection as a Risk Factor for Male Infertility', *Dtsch Arztebl International*, 114(19), pp. 339–346. doi: 10.3238/arztebl.2017.0339.

Schuster, D. P. (2009) 'Changes in physiology with increasing fat mass', *Seminars in Pediatric Surgery*, 18(3), pp. 126–135. doi: 10.1053/j.sempedsurg.2009.04.002.

Schwanzel-Fukuda, M., Bick, D. and Pfaff, D. W. (1989) 'Luteinizing hormone-releasing hormone (LHRH)-expressing cells do not migrate normally in an inherited hypogonadal (Kallmann)

syndrome', *Molecular Brain Research*, 6, pp. 311–326.

Sclafani, A. (1978) 'Dietary obesity', *Recent advances in obesity research*, 2, pp. 123–132.

Sclafani, A. (1984) 'Animal models of obesity : Classification and characterization', *International Journal of Obesity*, 8(February), pp. 491–508.

Sclafani, A. and Springer, D. (1976) 'Dietary obesity in adult rats: Similarities to hypothalamic and human obesity syndromes', *Physiology and Behavior*, 17(3), pp. 461–471. doi: 10.1016/0031-9384(76)90109-8.

Scotellaro, P. A. *et al.* (1991) 'Body fat accretion: a rat model', *Medicine & Science in Sports & Exercise*, 23(3), pp. 275–279. Available at: https://journals.lww.com/acsm-msse/Fulltext/1991/03000/Body_fat_accretion__a_rat_model.2.aspx.

Seftel, A. D., Sun, P. and Swindle, R. (2004) 'The prevalence of hypertension, hyperlipidemia, diabetes mellitus and depression in men with erectile dysfunction', *Journal of Urology*, 171(6 I), pp. 2341–2345. doi: 10.1097/01.ju.0000125198.32936.38.

Segal, K. R. *et al.* (1985) 'Estimation of human body composition by electrical impedance methods: a comparative study', *Journal of Applied Physiology*, 58(5), pp. 1565–1571. doi: 10.1152/jappl.1985.58.5.1565.

Segula, D. (2014) 'Complications of obesity in adults: a short review of the literature.', *Malawi medical journal : the journal of Medical Association of Malawi*, 26(1), pp. 20–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24959321 (Accessed: 23 September 2019).

Seidell, J. *et al.* (1988) 'Abdominal fat depots measured with computed tomography: effects of degree of obesity, sex, and age.', *European journal of clinical nutrition*, 42(9), pp. 805–815.

Seidell, J. C. *et al.* (1990) 'Visceral fat accumulation in men is positively associated with insulin, glucose, and C-peptide levels, but negatively with testosterone levels', *Metabolism*, 39(9), pp. 897–901. doi: https://doi.org/10.1016/0026-0495(90)90297-P.

Selfridge, J. E. *et al.* (2014) 'Effect of one month duration ketogenic and non-ketogenic high fat diets on mouse brain bioenergetic infrastructure', *Journal of Bioenergetics and Biomembranes*, 47(1–2). doi: 10.1007/s10863-014-9570-z.

Sengupta, P. (2013) 'The laboratory rat: Relating its age with human's', *International Journal of Preventive Medicine*, 4(6), pp. 624–630.

Sermondade, N. *et al.* (2013) 'BMI in relation to sperm count: An updated systematic review and collaborative meta-analysis', *Human Reproduction Update*, 19(3), pp. 221–231. doi: 10.1093/humupd/dms050.

Serohijos, A. W. R. *et al.* (2006) 'A structural model reveals energy transduction in dynein', *Proceedings of the National Academy of Sciences of the United States of America*, 103(49), pp. 18540–18545. doi: 10.1073/pnas.0602867103.

Setchell, B. P. (1998) 'The Parkes Lecture Heat and the testis', *Reproduction*, 114(2).

Shafik, A. and Olfat, S. (1981) 'Lipectomy in the Treatment of Scrotal Lipomatosis', *British Journal of Urology*, 53(1), pp. 55–61. doi: 10.1111/j.1464-410X.1981.tb03129.x.

Shah, T. R. (2011) *Challenges in Delivery of Therapeutic Genomics and Proteomics*, *Challenges in Delivery of Therapeutic Genomics and Proteomics*. Elsevier. doi: 10.1016/B978-0-12-384964-9.00008-6.

Shahani, S. *et al.* (2009) 'Androgens and erythropoiesis: Past and present', *Journal of Endocrinological Investigation*, 32(8), pp. 704–716. doi: 10.1007/BF03345745.

Shahidi, N. T. (1973) 'Androgens and Erythropoiesis', *New England Journal of Medicine*, 289(2), pp. 72–80. doi: 10.1056/NEJM197307122890205.

Shao, W. *et al.* (2012) 'Curcumin prevents high fat diet induced insulin resistance and obesity via attenuating lipogenesis in liver and inflammatory pathway in adipocytes', *PLoS ONE*. Edited by R. Schneider-Stock, 7(1), p. e28784. doi: 10.1371/journal.pone.0028784.

Sharman, M. and Volek, J. (2004) 'Weight loss leads to reductions in inflammatory biomarkers after a very-low-carbohydrate diet and a low-fat diet in overweight men', *ECU Publications Pre. 2011*. Available at: https://ro.ecu.edu.au/ecuworks/5170 (Accessed: 9 October 2019).

Sharpe, R. M. (1944) 'Regulation of Spermatogenesis', in Knobil, E. and Neill, J. D. (eds) *The Physiology of Reproduction*. New York: Raven Press, pp. 1–72.

Sharpe, R. M. *et al.* (1994) 'Temporal relationship between androgen-dependent changes in the volume of seminiferous tubule fluid, lumen size and seminiferous tubule protein secretion in rats', *Reproduction*, 101(1), pp. 193–198. doi: 10.1530/jrf.0.1010193.

Shimokata, H. *et al.* (1989) 'Studies in the Distribution of Body Fat: I. Effects of Age, Sex, and Obesity', *Journal of Gerontology*, 44(2), pp. M66–M73. doi: 10.1093/geronj/44.2.M66.

Siddiq, F. M. and Sigman, M. (2002) 'A new look at the medical management of infertility', *Urologic Clinics of North America*, 29(4), pp. 949–963. doi: 10.1016/S0094-0143(02)00085-X.

Sikka, S. C. (1996) 'Oxidative stress and role of antioxidants in normal and abnormal sperm function', *Frontiers in Biosceince*, 1, pp. e78-86.

Sikka, S. C. (2001) 'Relative Impact of Oxidative Stress on Male Reproductive Function', *Current Medicinal Chemistry*, 8(7), pp. 85–98.

Silverthorn, D. U. *et al.* (2014) *Human physiology: an integrated approach*. Pearson/Benjamin Cummings San Francisco, CA:

Simmonds, M. (1914) 'Ueber Hypophysisschwund mit tödlichem Ausgang', *Dtsch med Wochenschr*, 40(7), pp. 322–323. doi: 10.1055/s-0029-1190185.

Sinawat, S. (2000) 'The environmental impact on male fertility.', *Journal of the Medical Association of Thailand = Chotmaihet thangphaet*, 83(8), pp. 880–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10998841 (Accessed: 6 October 2019).

Skinner, M. K., McLachlan, R. I. and Bremner, W. J. (1989) 'Stimulation of Sertoli cell inhibin secretion by the testicular paracrine factor PModS', *Molecular and Cellular Endocrinology*, 66(2), pp. 239–249. doi: https://doi.org/10.1016/0303-7207(89)90036-1.

Sloboda, R. D., Rosenbaum, J. L. and Dentler, W. L. (1976) 'Microtubule-Associated Proteins and the Stimulation of Tubulin Assembly in Vitro', *Biochemistry*, 15(20), pp. 4497–4505. doi: 10.1021/bi00665a026.

Slutsky, M., Levin, J. L. and Levy, B. S. (1999) 'Azoospermia and oligospermia among a large cohort of DBCP applicators in 12 countries', *International Journal of Occupational and Environmental Health*, 5(2), pp. 116–122. doi: 10.1179/oeh.1999.5.2.116.

Smith, S. R. *et al.* (2001) 'Contributions of total body fat, abdominal subcutaneous adipose tissue compartments, and visceral adipose tissue to the metabolic complications of obesity', *Metabolism: Clinical and Experimental*, 50(4), pp. 425–435. doi: 10.1053/meta.2001.21693.

Smith, S. R. and Zachwieja, J. J. (1999) 'Visceral adipose tissue: A critical review of intervention strategies', *International Journal of Obesity*, 23(4), pp. 329–335. doi: 10.1038/sj.ijo.0800834.

Smith, W. B. *et al.* (2014) 'Sexual Issues: Role of Sexually Transmitted Infections on Male Factor Fertility BT - Male Infertility: A Complete Guide to Lifestyle and Environmental Factors', in du Plessis, S. S., Agarwal, A., and Sabanegh Edmund S., J. (eds) *Male Infertility*. New York, NY:

Springer New York, pp. 127–140. doi: 10.1007/978-1-4939-1040-3_9.

Snevechenko, A. *et al.* (1996) 'Mass spectrometric sequencing of proteins silver-stained polyacrilamide gels', *Analytical Chemistry*, 68(5), pp. 850–858.

Sniderman, A. D. *et al.* (2007) 'Why might South Asians be so susceptible to central obesity and its atherogenic consequences? The adipose tissue overflow hypothesis', *International Journal of Epidemiology*, 36(1), pp. 220–225. doi: 10.1093/ije/dyl245.

Sobal, J. and Stunkard, A. J. (1989) 'Socioeconomic status and obesity: A review of the literature', *Psychological Bulletin*, 105(2), pp. 260–275. Available at: https://psycnet.apa.org/doiLanding?doi=10.1037%2F0033-2909.105.2.260 (Accessed: 29 September 2019).

Solon-Biet, S. M. *et al.* (2015) 'Dietary Protein to Carbohydrate Ratio and Caloric Restriction: Comparing Metabolic Outcomes in Mice', *Cell Reports*, 11(10), pp. 1529–1534. doi: https://doi.org/10.1016/j.celrep.2015.05.007.

Sower, S. A., Freamat, M. and Kavanaugh, S. I. (2009) 'The origins of the vertebrate hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) endocrine systems: New insights from lampreys', *General and Comparative Endocrinology*, 161(1), pp. 20–29. doi: 10.1016/j.ygcen.2008.11.023.

Sowers, J. R., Whaley-Connell, A. and Hayden, M. R. (2011) 'The role of overweight and obesity in the cardiorenal syndrome', *CardioRenal Medicine*, pp. 5–12. doi: 10.1159/000322822.

Speakman, J. *et al.* (2008) 'The contribution of animal models to the study of obesity', *Lab Animal*, 42, pp. 413–432.

Spiegelman, B. M. and Flier, J. S. (1996) 'Adipogenesis and obesity: Rounding out the big picture', *Cell*, 87(3), pp. 377–389. doi: 10.1016/S0092-8674(00)81359-8.

Spiropoulos, J. (2002) 'Can mitochondrial DNA mutations cause sperm dysfunction?', *Molecular Human Reproduction*, 8(8), pp. 719–721. doi: 10.1093/molehr/8.8.719.

Steinbacher, P. and Eckl, P. (2015) 'Impact of oxidative stress on exercising skeletal muscle', *Biomolecules*, 5(2), pp. 356–377. doi: 10.3390/biom5020356.

Stewart, A. F. and Kim, E. D. (2011) 'Fertility concerns for the aging male', *Urology*, 78(3), pp. 496–499. doi: 10.1016/j.urology.2011.06.010.

Stewart, J. D. *et al.* (2019) 'Contemporary Management of Unexplained Infertility', *Clinical Obstetrics and Gynecology*, 62(2), pp. 282–292. doi: 10.1097/GRF.0000000000000450.

Steyn, K. and Damasceno, A. (2006) 'Lifestyle and related risk factors for chronic diseases', in Makgoba, M. et al. (eds) *Disease and Mortality in Sub-Saharan Africa*. The World Bank, pp. 247–265. doi: 10.1596/978-0-8213-6397-3.

Storey, B. T. (2008) 'Mammalian sperm metabolism: Oxygen and sugar, friend and foe', *International Journal of Developmental Biology*, 52(5–6), pp. 427–437. doi: 10.1387/ijdb.072522bs.

Strain, G. W. *et al.* (1982) 'Mild hypogonadotropic hypogonadism in obese men', *Metabolism*, 31(9), pp. 871–875. doi: 10.1016/0026-0495(82)90175-5.

Strange, R. C., Jones, P. W. and Fryer, A. A. (2000) 'Glutathione S-transferase: Genetics and role in toxicology', in *Toxicology Letters*, pp. 357–363. doi: 10.1016/S0378-4274(99)00230-1.

Stridsberg, M. and Wilander, E. (1991) 'Islet amyloid polypeptide (IAPP) a short review', *Acta Oncologica*, 30(4), pp. 451–456. doi: 10.3109/02841869109092400.

Strömblad, G. and Björntorp, P. (1986) 'Reduced hepatic insulin clearance in rats with dietary-induced obesity', *Metabolism*, 35(4), pp. 323–327. doi: 10.1016/0026-0495(86)90148-4.

Stunkard, A. J. *et al.* (1986) 'An Adoption Study of Human Obesity', *New England Journal of Medicine*, 314(4), pp. 193–198. doi: 10.1056/NEJM198601233140401.

Suanarunsawat, T. *et al.* (2014) 'Anti-Oxidative , Anti-Hyperglycemic and Lipid-Lowering Effects of Aqueous Extracts of Ocimum sanctum L . Leaves in Diabetic Rats', *Food and Nutrition Sciences*, 5, pp. 801–811. doi: 10.4236/fns.2014.59090.

Sullivan, R. and Mieusset, R. (2016) 'The human epididymis: Its function in sperm maturation', *Human Reproduction Update*, 22(5), pp. 574–587. doi: 10.1093/humupd/dmw015.

Sun, H. *et al.* (2019) 'Global, regional, and national prevalence and disability-adjusted life-years for infertility in 195 countries and territories, 1990–2017: results from a global burden of disease study, 2017', *Aging*, 11(23), pp. 10952–10991. doi: 10.18632/aging.102497.

Sundaram, K. and Witorsch, R. (1995) 'Toxic effects on the testes', in *Reproductive toxicology*. Raven Press New York, pp. 99–122.

Sundfeldt, K. *et al.* (2000) 'E-cadherin-catenin complex in the rat ovary: Cell-specific expression during folliculogenesis and luteal formation', *Journal of Reproduction and Fertility*, 118(2), pp. 375–

385. doi: 10.1530/reprod/118.2.375.

Svedberg, J. *et al.* (1991) 'Fatty acids in the portal vein of the rat regulate hepatic insulin clearance', *Journal of Clinical Investigation*, 88(6), pp. 2054–2058. doi: 10.1172/JCI115534.

Swerdloff, R. S., Batt, R. A. and Bray, G. A. (1976) 'Reproductive hormonal function in the genetically obese (ob/ob) mouse', *Endocrinology*, 98(6), pp. 1359–1364. doi: 10.1210/endo-98-6-1359.

Switzar, L., Giera, M. and Niessen, W. M. A. (2013) 'Protein digestion: An overview of the available techniques and recent developments', *Journal of Proteome Research*, 12(3), pp. 1067–1077. doi: 10.1021/pr301201x.

Takeuchi, M. and Kobata, A. (1991) 'Structures and functional roles of the sugar chains of human erythropoietins', *Glycobiology*, 1(4), pp. 337–346. doi: 10.1093/glycob/1.4.337.

Talusan, P. *et al.* (2005) 'Analysis of intimal proteoglycans in atherosclerosis-prone and atherosclerosis-resistant human arteries by mass spectrometry', *Molecular and Cellular Proteomics*, 4(9), pp. 1350–1357. doi: 10.1074/mcp.M500088-MCP200.

Tanaka, H. *et al.* (2004) 'Ketone bodies could support the motility but not the acrosome reaction of mouse sperm', *International Journal of Andrology*, 27(3), pp. 172–177. doi: 10.1111/j.1365-2605.2004.00471.x.

Taxvig, C. *et al.* (2012) 'Differential effects of environmental chemicals and food contaminants on adipogenesis, biomarker release and PPARγ activation', *Molecular and Cellular Endocrinology*, 361(1–2), pp. 106–115. doi: 10.1016/j.mce.2012.03.021.

Tchernof, A. *et al.* (1996) 'The dense LDL phenotype: Association with plasma lipoprotein levels, visceral obesity, and hyperinsulinemia in men', *Diabetes Care*, 19(6), pp. 629–637. doi: 10.2337/diacare.19.6.629.

Tchernof, A. and Després, J. P. (2013) 'Pathophysiology of human visceral obesity: An update', *Physiological Reviews*, 93(1), pp. 359–404. doi: 10.1152/physrev.00033.2011.

Teerds, K. J., Rooij, D. G. De and Keijer, J. (2011) 'Functional relationship between obesity and male reproduction : from humans to animal models', 17(5), pp. 667–683. doi: 10.1093/humupd/dmr017.

Tehrani, A. B. *et al.* (2012) 'Obesity and its associated disease: A role for microbiota?', *Neurogastroenterology and Motility*, 24(4), pp. 305–311. doi: 10.1111/j.1365-2982.2012.01895.x.

Telöken, C., Juncal, S. and Graziottin, T. M. (2012) 'Environmental Factors', in *Male Infertility*. New York, NY: Springer New York, pp. 283–295. doi: 10.1007/978-1-4614-3335-4_27.

*Testicular Causes of Male Infertility* (2018) *Stanford Health Care*. Available at: https://stanfordhealthcare.org/medical-conditions/mens-health/male-infertility/causes/testicular.html (Accessed: 6 October 2018).

Theriot, J. A. (1994) 'Regulation of the actin cytoskeleton in living cells', *Seminars in Cell and Developmental Biology*, 5(3), pp. 193–199. doi: 10.1006/scel.1994.1024.

Thompson, A., Agarwal, A. and Plessis, S. S. (2013) 'Physiological Role of Reactive Oxygen Species in Sperm Function : A Review Endogenous ROS Production by Sperm', pp. 69–89.

Truett, G. E. *et al.* (1991) 'Rat obesity gene fatty (fa) maps to chromosome 5: Evidence for homology with the mouse gene diabetes (db)', *Proceedings of the National Academy of Sciences of the United States of America*, 88(17), pp. 7806–7809. doi: 10.1073/pnas.88.17.7806.

Tungtrongchitr, R. *et al.* (2001) 'Leptin concentration in relation to body mass index (BMI) and hematological measurements in Thai obese and overweight subjects', *Southeast Asian Journal of Tropical Medicine and Public Health*, 10(4), pp. 787–794.

Twigger, S. N. (2004) 'Of rats and men.', *Genome biology*, 5(3), p. 314. doi: 10.1186/gb-2004-5-3-314.

Ureta, T. (1982) 'The comparative isozymology of vertebrate hexokinases', *Comparative Biochemistry and Physiology -- Part B: Biochemistry and*, 71(4), pp. 549–555. doi: 10.1016/0305-0491(82)90461-8.

US EPA, O. (no date) *Persistent Organic Pollutants: A Global Issue, A Global Response*. Available at: https://www.epa.gov/international-cooperation/persistent-organic-pollutants-global-issue-global-response (Accessed: 5 October 2019).

Vaamonde, D. *et al.* (2006) 'Reproductive profile of physically active men after exhaustive endurance exercise', *International Journal of Sports Medicine*, 27(9), pp. 680–689. doi: 10.1055/s-2005-872906.

Vaamonde, D. *et al.* (2012) 'Physically active men show better semen parameters and hormone values than sedentary men', *European Journal of Applied Physiology*, 112(9), pp. 3267–3273. doi: 10.1007/s00421-011-2304-6.

Vaamonde Martin, D. M. *et al.* (2014) 'The Impact of Physical Exercise on Male Fertility', in *Male*

*Infertility*. Springer New York, pp. 47–60. doi: 10.1007/978-1-4939-1040-3_4.

Vague, J. (1947) 'La differentiation sexuelle facteur determinant des formes de l'obesite', *Presse Med*, 30, pp. 339–340.

Vaisse, C. *et al.* (1998) 'A frameshift mutation in human MC4R is associated with a dominant form of obesity', *Nature Genetics*, 20(2), pp. 113–114. doi: 10.1038/2407.

van Ahlen, H. and Hertle, L. (2001) 'Disorders of Sperm Deposition', in Nieschlag, E. and Behre, H. M. (eds) *Andrology: Male Reproductive Health and Dysfunction*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 191–222. doi: 10.1007/978-3-662-04491-9_10.

Van der Horst, G. *et al.* (2018) 'Cut-off values for normal sperm morphology and toxicology for automated analysis of rat sperm morphology and morphometry', *Biotechnic and Histochemistry*, 93(1), pp. 49–58. doi: 10.1080/10520295.2017.1380842.

VandeBerg, J. L., Cooper, D. W. and Close, P. J. (1973) 'Mammalian testis phosphoglycerate kinase.', *Nature: New biology*, 243(123), pp. 48–50. doi: 10.1038/physci243048a0.

VanItallie, T. B. and Nufert, T. H. (2003) 'Ketones: Metabolism's ugly duckling', *Nutrition Reviews*, 61(10), pp. 327–341. doi: 10.1301/nr.2003.oct.327-341.

Vashishat, N., Dhanju, C. K. and Cheema, R. S. (2012) 'Morphology and morphometry of cauda epididymal spermatozoa in Rattus rattus L.', *Proceedings of the National Academy of Sciences India Section B - Biological Sciences*, 82(2), pp. 285–290. doi: 10.1007/s40011-012-0020-6.

Veech, R. L. *et al.* (2001) 'Ketone bodies, potential therapeutic uses', *IUBMB Life*, 51(4), pp. 241–247. doi: 10.1080/152165401753311780.

Vendetti, S. *et al.* (2000) 'Induction and membrane expression of heat shock proteins in heat-treated HPC-4 cells is correlated with increased resistance to LAK-mediated lysis', *Journal of experimental & clinical cancer research : CR*, 19(3), p. 329—334. Available at: http://europepmc.org/abstract/MED/11144526.

Vermeulen, A. *et al.* (2002) 'Estradiol in elderly men', *Aging Male*, 5(2), pp. 98–102. doi: 10.1080/tam.5.2.98.102.

Vermeulen, A., Kaufman, J. M. and Giagulli, V. A. (1996) 'Influence of some biological indexes on sex hormone-binding globulin and androgen levels in aging or obese males.', *The Journal of Clinical Endocrinology & Metabolism*, 81(5), pp. 1821–1826. doi: 10.1210/jcem.81.5.8626841.

Vigueras-Villaseñor, R. M. *et al.* (2011) 'Alterations in the spermatic function generated by obesity in rats', *Acta Histochemica*, 113(2), pp. 214–220. doi: 10.1016/j.acthis.2009.10.004.

Villalta, J. *et al.* (1997) 'Testicular Function in Asymptomatic Chronic Alcoholics: Relation to Ethanol Intake', *Alcoholism: Clinical and Experimental Research*, 21(1), pp. 128–134. doi: 10.1111/j.1530-0277.1997.tb03740.x.

Villar-Palasi, C. and Larner, J. (1970) 'Glycogen Metabolism and Glycolytic Enzymes', *Annual Review of Biochemistry*, 39(1), pp. 639–672. doi: 10.1146/annurev.bi.39.070170.003231.

Viru, A. and Viru, M. (2003) *Análisis y control del rendimiento deportivo*. Editorial Paidotribo.

Visconti, P. E. (2012) 'Sperm Bioenergetics in a Nutshell', *Biology of Reproduction*, 87(3). doi: 10.1095/biolreprod.112.104109.

Wade, G. N. and Gray, J. M. (1979) 'Gonadal effects on food intake and adiposity: A metabolic hypothesis', *Physiology and Behavior*, 22(3), pp. 583–593. doi: 10.1016/0031-9384(79)90028-3.

Wajchenberg, B. L. (2000) 'Subcutaneous and visceral adipose tissue: Their relation to the metabolic syndrome', *Endocrine Reviews*, 21(6), pp. 697–738. doi: 10.1210/edrv.21.6.0415.

Walker, A. R. P., Adam, F. and Walker, B. F. (2001) 'World pandemic of obesity: The situation in Southern African populations', *Public Health*, 115(6), pp. 368–372. doi: 10.1038/sj.ph.1900790.

Wallimann, T. *et al.* (1992) 'Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: The "phosphocreatine circuit" for cellular energy homeostasis', *Biochemical Journal*, 281(1), pp. 21–40. doi: 10.1042/bj2810021.

Wanders, R. J. A. and Waterham, H. R. (2006) 'Biochemistry of Mammalian Peroxisomes Revisited', *Annual Review of Biochemistry*, 75(1), pp. 295–332. doi: 10.1146/annurev.biochem.74.082803.133329.

Wang, B. and Trayhurn, P. (2006) 'Acute and prolonged effects of TNF-α on the expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture', *Pflugers Archiv European Journal of Physiology*, 452(4), pp. 418–427. doi: 10.1007/s00424-006-0055-8.

Wang, M. Y. *et al.* (1996) 'A novel leptin receptor isoform in rat', *FEBS Letters*, 392(2), pp. 87–90. doi: 10.1016/0014-5793(96)00790-9.

Wannamethee, S. G. *et al.* (2007) 'Decreased muscle mass and increased central adiposity are independently related to mortality in older men', *American Journal of Clinical Nutrition*, 86(5), pp. 1339–1346. doi: 10.1093/ajcn/86.5.1339.

Ward, S. and Coffey, D. S. (1991) 'DNA Packaging and Organization in Mammalian Spermatozoa: Comparison with Somatic Cell', *Biology of Reproduction*, 44(4), pp. 569–574. doi: 10.1095/biolreprod44.4.569.

Wasinger, V. C. *et al.* (1995) 'Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium', *ELECTROPHORESIS*, 16(1), pp. 1090–1094. doi: 10.1002/elps.11501601185.

Wastney, M. E., Hall, S. E. and Berman, M. (1984) 'Ketone body kinetics in humans: a mathematical model.', *Journal of lipid research*, 25(2), pp. 160–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6707525 (Accessed: 13 November 2019).

Watson, A. E. N., Bruggeman, E. C. and Parent, M. B. (2012) 'Wearing memory thin: the effects of high fat diet on neuroinflammation and memory', *DISCOVERY: Georgia State Honors College Undergraduate Research Journal*, 1(1), p. 5.

Welch, W. J. (1993) 'How Cells Respond to Stress', *Scientific American*, 268(5), pp. 56–64. Available at: http://www.jstor.org/stable/24941478.

Werdelin, L. and Nilsonne, Å. (1999) 'The evolution of the scrotum and testicular descent in mammals: A phylogenetic view', *Journal of Theoretical Biology*, 196(1), pp. 61–72. doi: 10.1006/jtbi.1998.0821.

Westermark, P. *et al.* (1986) 'A novel peptide in the calcitonin gene related peptide family as an amyloid fibril protein in the endocrine pancreas', *Biochemical and Biophysical Research Communications*, 140(3), pp. 827–831. doi: 10.1016/0006-291X(86)90708-4.

Westermark, P. *et al.* (1987) 'Islet amyloid polypeptide-like immunoreactivity in the islet B cells of Type 2 (non-insulin-dependent) diabetic and non-diabetic individuals', *Diabetologia*, 30(11), pp. 887–892. doi: 10.1007/BF00274799.

Westerterp, K. R., Wilson, S. A. J. and Rolland, V. (1999) 'Diet induced thermogenesis measured over 24h in a respiration chamber: effect of diet composition', *International Journal of Obesity*, 23(3), pp. 287–292. doi: 10.1038/sj.ijo.0800810.

Wheeler, G. D. *et al.* (1991) 'Endurance training decreases serum testosterone levels in men without change in luteinizing hormone pulsatile release', *Journal of Clinical Endocrinology and*

*Metabolism*, 72(2), pp. 422–425. doi: 10.1210/jcem-72-2-422.

Whirledge, S. and Cidlowski, J. A. (2010) 'Glucocorticoids, stress, and fertility.', *Minerva endocrinologica*, 35(2), pp. 109–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20595939 (Accessed: 9 October 2019).

White, B. D., Porter, M. H. and Martin, R. J. (2000) 'Protein selection, food intake, and body composition in response to the amount of dietary protein', *Physiology & Behavior*, 69(4), pp. 383–389. doi: https://doi.org/10.1016/S0031-9384(99)00232-2.

Whorton, D. *et al.* (1977) 'Infertility in male pesticide workers', *The Lancet*, 310(8051), pp. 1259–1261. doi: 10.1016/S0140-6736(77)92665-4.

Wieland, O. *et al.* (1971) 'Active and inactive forms of pyruvate dehydrogenase in rat heart and kidney: Effect of diabetes, fasting, and refeeding on pyruvate dehydrogenase interconversion', *Archives of Biochemistry and Biophysics*, 143(2), pp. 593–601. doi: 10.1016/0003-9861(71)90244-X.

Wiernsperger, N. F. (2003) 'Oxidative stress as a therapeutic target in diabetes: revisiting the controversy', *Diabetes & Metabolism*, 29(6), pp. 579–585. doi: https://doi.org/10.1016/S1262-3636(07)70072-1.

Wild, R. A. (1995) 'Obesity, lipids, cardiovascular risk, and androgen excess', *The American Journal of Medicine*, 98(1 SUPPL. 1). doi: 10.1016/S0002-9343(99)80056-4.

Wilding, J. P. H. (2001) 'Causes of obesity', *Practical Diabetes International*, 18(8), pp. 288–292. doi: 10.1002/pdi.277.

Wilding, J. P. H. (2006) 'Pathophysiology and aetiology of obesity', *Medicine*, 34(12), pp. 501–505. doi: 10.1053/j.mpmed.2006.09.004.

Wilding, J., Widdowson, P. and Williams, G. (1997) 'Neurobiology', *British Medical Bulletin*, 53(2), pp. 286–306. doi: 10.1093/oxfordjournals.bmb.a011614.

Wiley, S. E. *et al.* (2007) 'MitoNEET is an iron-containing outer mitochondrial membrane protein that regulates oxidative capacity', *Proceedings of the National Academy of Sciences of the United States of America*, 104(13), pp. 5318–5323. doi: 10.1073/pnas.0701078104.

Wilkins, M. R. *et al.* (1996) 'Progress with proteome projects: Why all proteins expressed by a genome should be identified and how to do it', *Biotechnology and Genetic Engineering Reviews*, 13(1), pp. 19–50. doi: 10.1080/02648725.1996.10647923.

Willesen, M. G., Kristensen, P. and Rømer, J. (1999) 'Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat', *Neuroendocrinology*, 70(5), pp. 306–316. doi: 10.1159/000054491.

Wilson, J. E. (1985) 'Regulation of mammalian hexokinase activity', in *Regulation of carbohydrate metabolism*. CRC Press Boca Raton, pp. 45–85.

Wilson, J. E. (1997) 'An introduction to the isoenzymes of mammalian hexokinase types I-III', in *Biochemical Society Transactions*. Portland Press Ltd, pp. 103–107. doi: 10.1042/bst0250103.

Wilson, M. A. *et al.* (2003) 'The 1.1-A resolution crystal structure of DJ-1, the protein mutated in autosomal recessive early onset Parkinson's disease.', *Proceedings of the National Academy of Sciences of the United States of America*, 100(16), pp. 9256–9261. doi: 10.1073/pnas.1133288100.

Wirth, C. *et al.* (2016) 'Biochimica et Biophysica Acta Structure and function of mitochondrial complex I ☆', *Biochimica et Biophysica Acta j*, 1857, pp. 902–914.

Wisniewski, P. *et al.* (2015) 'Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic-pituitary-testicular axis', *Toxicology*, 329, pp. 1–9. doi: 10.1016/j.tox.2015.01.002.

Womersley, J. and Durnin, J. V. G. A. (1977) 'A comparison of the skinfold method with extent of "overweight" and various weight-height relationships in the assessment of obesity', *British Journal of Nutrition*, 38(2), pp. 271–284. doi: 10.1079/bjn19770088.

Wong, T. W. (1974) 'Testicular biopsy in the study of male infertility. III. Pretesticular causes of infertility', *Archives of pathology & laboratory medicine.*, 98(1), pp. 1–8.

Wong, T. W. *et al.* (1978) 'Pathological aspects of the infertile testis', *Urologic Clinics of North America*, 5(3), pp. 503–530. Available at: https://www.scopus.com/inward/record.uri?eid=2-s2.0-0018158944&partnerID=40&md5=cbb308e6e5ebb11faa34cba08f231808.

Woods, S. C. *et al.* (2003) 'A Controlled High-Fat Diet Induces an Obese Syndrome in Rats', *The Journal of Nutrition*, 133(4), pp. 1081–1087. doi: 10.1093/jn/133.4.1081.

World Health Organization (2009) *Infertility definitions and terminology*. Available at: http://www.who.int/reproductivehealth/topics/infertility/definitions/en/ (Accessed: 5 July 2017).

World Health Organization (2010) *WHO laboratory Manual for the Examination and Processing of Human Semen*. Switzerland.

World Health Organization (2016a) *Final report of the Commission on Ending Childhood Obesity*. Available at: http://www.who.int/endchildhood-%0Aobesity/final-report/en/ (Accessed: 3 September 2019).

World Health Organization (2016b) *WHO definitions of genetics and genomics*, *Human Genomics in Global Health*. World Health Organization. Available at: https://www.who.int/genomics/geneticsVSgenomics/en/ (Accessed: 10 October 2019).

World Health Organization (2018a) *Global Health Observatory (GHO) data*. Available at: https://www.who.int/gho/ncd/mortality_morbidity/en/ (Accessed: 3 September 2019).

World Health Organization (2018b) *Non-communicable diseases country profiles 2018*, *World Health Organization fact sheet*.

World Health Organization (2018c) *Obesity and overweight*. Available at: https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight (Accessed: 22 September 2019).

World Health Organization (2019) *Noncommunicable diseases and their risk factors*. Available at: https://www.who.int/ncds/en/ (Accessed: 3 September 2019).

Wozniak, S. E. *et al.* (2009) 'Adipose tissue: The new endocrine organ? a review article', *Digestive Diseases and Sciences*, 54(9), pp. 1847–1856. doi: 10.1007/s10620-008-0585-3.

Wright, P. C. *et al.* (2012) 'A review of current proteomics technologies with a survey on their widespread use in reproductive biology investigations', *Theriogenology*, 77(4), pp. 738–765. doi: 10.1016/j.theriogenology.2011.11.012.

Wyss, M. *et al.* (1992) 'Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism', *BBA - Bioenergetics*, 1102(2), pp. 119–166. doi: 10.1016/0005-2728(92)90096-K.

Xu, H. *et al.* (2003) 'Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance', *The Journal of Clinical Investigation*, 112(12), pp. 1821–1830. doi: 10.1172/JCI19451.

Xu, H. X. *et al.* (2015) 'Transcriptional responses of invasive and indigenous whiteflies to different host plants reveal their disparate capacity of adaptation', *Scientific Reports*, 5(10774). doi: 10.1038/srep10774.

Yan, J. *et al.* (2003) 'COP9 Signalosome Subunit 3 Is Essential for Maintenance of Cell Proliferation in the Mouse Embryonic Epiblast', *Molecular and Cellular Biology*, 23(19), pp. 6798–

6808. doi: 10.1128/mcb.23.19.6798-6808.2003.

Yates, J. R. (1998) ‘Mass spectrometry and the age of the proteome’, *Journal of Mass Spectrometry*, 33(1), pp. 1–19. doi: 10.1002/(SICI)1096-9888(199801)33:1<1::AID-JMS624>3.0.CO;2-9.

Yeo, B. K. and Yu, S.-W. (2016) ‘Valosin-containing protein (VCP): structure, functions, and implications in neurodegenerative diseases’, *Animal Cells and Systems*, 20(6), pp. 303–309. doi: 10.1080/19768354.2016.1259181.

Yeo, G. S. H. *et al.* (2003) ‘Mutations in the human melanocortin-4 receptor gene associated with severe familial obesity disrupts receptor function through multiple molecular mechanisms’, *Human Molecular Genetics*, 12(5), pp. 561–574. doi: 10.1093/hmg/ddg057.

Yeo, G. S. H. *et al.* (2004) ‘A de novo mutation affecting human TrkB associated with severe obesity and developmental delay’, *Nature Neuroscience*, 7(11), pp. 1187–1189. doi: 10.1038/nn1336.

Yeung, C. H. *et al.* (2009) ‘Coiled sperm from infertile patients: Characteristics, associated factors and biological implication’, *Human Reproduction*, 24(6), pp. 1288–1295. doi: 10.1093/humrep/dep017.

Yildiz, B. O. and Haznedaroglu, I. C. (2006) ‘Rethinking leptin and insulin action: Therapeutic opportunities for diabetes’, *International Journal of Biochemistry and Cell Biology*, 38(5–6), pp. 820–830. doi: 10.1016/j.biocel.2005.09.013.

Yoshio, O. *et al.* (1987) ‘Aromatase’, *Journal of Steroid Biochemistry*, 27(4–6), pp. 781–789. doi: 10.1016/0022-4731(87)90150-6.

Youn, J. *et al.* (2014) ‘Role of vascular oxidative stress in obesity and metabolic syndrome’, *Diabetes*, 63(7), pp. 2344–2355. doi: 10.2337/db13-0719.

Yu, W. H. *et al.* (1997) ‘Role of leptin in hypothalamic-pituitary function’, *Proceedings of the National Academy of Sciences of the United States of America*, 94(3), pp. 1023–1028. doi: 10.1073/pnas.94.3.1023.

Zalata, A. A. *et al.* (1998) ‘The fatty acid composition of phospholipids of spermatozoa from infertile patients’, *Molecular Human Reproduction*, 4(2), pp. 111–118.

Zava, D. T., Blen, M. and Duwe, G. (1997) ‘Estrogenic activity of natural and synthetic estrogens in human breast cancer cells in culture.’, *Environmental Health Perspectives*, 105(suppl 3), pp. 637–

645. doi: 10.1289/ehp.97105s3637.

Zavos Ed.S., Ph.D., P. M. *et al.* (1998) 'An Electron Microscope Study of the Axonemal Ultrastructure in Human Spermatozoa From Male Smokers and Nonsmokers', *Fertility and Sterility*, 69(3), pp. 430–434. doi: 10.1016/s0015-0282(97)00563-3.

Zegers-Hochschild, F. *et al.* (2017) 'The international glossary on infertility and fertility care, 2017', *Human Reproduction*, 32(9), pp. 1786–1801. doi: 10.1093/humrep/dex234.

Zhang, J.-P. *et al.* (2000) 'Effect of smoking on semen quality of infertile men in Shandong, China', *Asian Journal of Andrology*, 2(2), pp. 143–146.

Zhang, R.-D. *et al.* (2006) 'Effect of intra-testicular testosterone withdrawal on the expression of alpha-catenin in the adult rat testis', *Zhonghua nan ke xue = National journal of andrology*, 12(5), pp. 394–6, 400. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16755864 (Accessed: 10 November 2019).

Zhang, Y. *et al.* (1994) 'Positional cloning of the mouse obese gene and its human homologue', *Nature*, 372(6505), pp. 425–432. doi: 10.1038/372425a0.

Zhou, M. *et al.* (2008) 'Downregulation of protein disulfide isomerase in sepsis and its role in tumor necrosis factor-alpha release', *Critical Care*, 12(4), p. R100. doi: 10.1186/cc6977.

Zitzmann, M., Faber, S. and Nieschlag, E. (2006) 'Association of Specific Symptoms and Metabolic Risks with Serum Testosterone in Older Men', *The Journal of Clinical Endocrinology & Metabolism*, 91(11), pp. 4335–4343. doi: 10.1210/jc.2006-0401.

Zumoff, B. *et al.* (1990) 'Plasma Free and Non-Sex-Hormone-Binding-Globulin Bound Testosterone Are Decreased in Obese Men in Proportion to Their Degree of Obesity', *Journal of Clinical Endocrinology and Metabolism*, 71(4), pp. 929–931. doi: 10.1210/jcem-71-4-929.

Zurbano, R. *et al.* (2004) 'Estudios sobre obesidad de origen monogénico en humanos', *Rev Esp Obes*, 2(5), pp. 269–278.

# Appendix

**Table A.1:** Full table of significantly expressed proteins in the epididymis, divided into each of their functional categories. Annotated using the Uniprot protein database (https://www.uniprot.org/)

| Accession number | Gene name | Protein name | Control Mean | Diet Mean | Adjusted p value | Function |
|---|---|---|---|---|---|---|
| **Metabolic proteins** | | | | | | |
| Q5XIV1 | Phosphoglycerate kinase | Pgk2 | 100 | 118 | 0.017 | Carbohydrate degradation; glycolysis; conversion of pyruvate from D-glyceraldehyde 3-phosphate. Flagellated sperm motility |
| P04764 | Alpha-enolase (Enolase 1) | Eno1 Eno-1 | 97 | 113 | 0.023 | Involved in glycolysis. Has a role in several processes e.g. growth control and allergic responses. Is a plasminogen receptor and activator on the cell surface. Stimulates immunoglobulin production |
| P00507 | Aspartate aminotransferase mitochondrial (mAspAT) Glutamate oxaloacetate transaminase 2 (Got2) | Got2 Maat | 61 | 79 | 0.004 | Amino acid metabolism. Essential for metabolite exchange between mitochondria and then cytosol. A fatty acid-binding protein that enables cellular uptake of long-chain free fatty acids |

| | | | | | | |
|---|---|---|---|---|---|---|
| P16290 | Phosphoglycerate mutase 2 | Pgam2 | 32 | 52 | <0.001 | Gluconeogenesis, glycolytic process, regulation of pentose-phosphate shunt. Interconversion of 3- and 2-phosphoglycerate with 2,3-bisphosphoglycerate as the primer<br><br>Spermatogenesis |
| P11030 | Acyl-CoA-binding protein (ACBP) (Diazepam-binding inhibitor) (DBI) | Dbi | 27 | 49 | <0.001 | High-affinity binding to medium- and long-chain acyl-CoA esters and possibly functions as an intracellular carrier of acyl-CoA esters. Displaces diazepam from the recognition site on GABA type A receptors |
| P01026 | Complement C3 | C3 | 66 | 45 | 0.008 | Activation of the complement system. Stimulates histamine release from basophilic leukocytes and mast cells. In chronic inflammation, it is a chemoattractant for neutrophils. Adipogenic hormone which participates in stimulation of triglyceride synthesis and glucose transport in adipocytes. Therefore regulates fat storage and postprandial TG removal. Appears to stimulate triglyceride synthesis |
| P27881 | Hexokinase-2 (EC 2.7.1.1) (Hexokinase type II) (HK II) | Hk2 | 27 | 17 | 0.029 | Hexokinases stimulate the glycolytic (e.g. glucose and fructose) thereby initiating their metabolism. HK2 is the insulin-sensitive and is able to metabolize fructose, glucose and other hexose sugars |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q4FZV0 | Beta-mannosidase (EC 3.2.1.25) (Lysosomal beta A mannosidase) (Mannanase) (Mannase) | Manba | 38 | 16 | <0.001 | Exoglycosidase. Involved in the hydrolysis of beta-D-mannose residues of all glycoprotein oligosaccharides (N-linked) on their non-reducing ends |
| Q62867 | Gamma-glutamyl hydrolase (EC 3.4.19.9) (Conjugase) (GH) (Gamma-Glu-X carboxypeptidase ) | Ggh | 24 | 13 | 0.006 | Possibly plays a role in the bioavailability of dietary pteroylpolyglutamates (derivatives of folic acid) as well as the metabolism of pteroylpolyglutamates and antifolates. Has either endo- or exopeptidase activity depending on its tissue of origin. When secreted, it primarily exhiits endopeptidase activity |
| P23514 | Coatomer subunit beta (Beta-coat protein) (Beta-COP) | Copb1 Copb | 21 | 11 | 0.015 | Necessary for the limiting of lipid storage within lipid droplets. Involved in lipid homeostasis and promoting the association of adipocyte triglyceride lipase with the lipid droplet surface in order to mediate lipolysis. Retrograde Golgi-to-ER transport of proteins. Involved in Golgi disassembly and reassembly processes during the cell cycle. Takes part in autophagy via early endosome function/development. Has a role in organellar compartmentalization of secretory compartments. |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q4QQW8 | Putative phospholipase B-like 2 (EC 3.1.1.-) (LAMA-like protein 2) (Lamina ancestor homolog 2) (Phospholipase B domain-containing protein 2) | Plbd2 RDCR-0918-3 | 13 | 6 | 0.036 | Lipid metabolism and degradation |
| Q924N5 | Long-chain-fatty-acid--CoA ligase ACSBG1 (EC 6.2.1.3) (Acyl-CoA synthetase bubblegum family member 1) (Gonadotropin-regulated long chain acyl CoA synthetase) (GR-LACS) | Acsbg1 Grlacs | 16 | 6 | 0.001 | Facilitates the activation of long-chain fatty acids for the synthesis and degradation of cellular lipids, via beta-oxidation. Can activate long-chain fatty acids. Able to activate a range of saturated, monosaturated and polyunsaturated fatty acids |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q64560 | Tripeptidyl-peptidase 2 (TPP-2) (EC 3.4.14.10) (Tripeptidyl aminopeptidase) (Tripeptidyl-peptidase II) (TPP-II) | Tpp2 | 15 | 5 | <0.001 | Stimulates adipogenesis |
| P32551 | (Complex III subunit 2) (Core protein II) (Ubiquinol-cytochrome-c reductase complex core protein 2) | Uqcrc2 | 10 | 4 | 0.049 | Part of the mitochondrial respiratory chain. Is required for the assembly of the ubiquinol-cytochrome c reductase complex |
| D3Z8F7 | Glucokinase activity, related sequence 1 (Predicted) (Glycerol kinase-like 1) | Gykl1 Gk-rs1_predicted rCG_46978 | 10 | 4 | 0.026 | Implicated in the first step of the subpathway that synthesizes sn-glycerol 3-phosphate from glycerol |

| | | | | | | |
|---|---|---|---|---|---|---|
| P16970 | ATP-binding cassette sub-family D member 3 (70 kDa peroxisomal membrane protein) (PMP70) | Abcd3 Pmp70 Pxmp1 | 11 | 3 | <0.001 | Probable transporter involved in the transport of branched-chain fatty acids and bile acids (C27) into the peroxisome; which is essential in bile acid biosynthesis. |
| Q4FZZ4 | Pyruvate dehydrogenase E1 component subunit alpha (EC 1.2.4.1) | Pdha1 LOC100365902 rCG_36458 | 8 | 3 | 0.015 | A pyruvate dehydrogenase complex which catalyzes the overall conversion of pyruvate to acetyl-CoA and $CO_2$ |
| Q920L2 | Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial (EC 1.3.5.1) (Flavoprotein | Sdha | 7 | 2 | 0.003 | A subunit of succinate dehydrogenase (SDH) that is takes part in mitochondrial electron transport chain via complex II by transferring electrons from succinate to ubiquinone (coenzyme Q) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | subunit of complex II) (Fp) | | | | | |
| Q499N5 | Acyl-CoA synthetase family member 2, mitochondrial (EC 6.2.1.-) | Acsf2 | 6 | 2 | 0.025 | Part of a group of enzymes which catalyze the first reaction in fatty acid metabolism, via the formation of a thioester with CoA. Involved in adipocyte differentiation |
| Q9WVK7 | Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial (HCDH) (EC 1.1.1.35) (Medium and short-chain L-3-hydroxyacyl-coenzyme A dehydrogenase) (Short-chain 3-hydroxyacyl-CoA dehydrogenase) | Hadh Had Hadhsc Schad | 6 | 2 | 0.018 | Plays an important role in the mitochondrial beta-oxidation of short chain fatty acids, with the highest activity toward 3-hydroxybutyryl-CoA |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q06437 | Pyruvate dehydrogenase E1 component subunit alpha, testis-specific form, mitochondrial (EC 1.2.4.1) (PDHE1-A type II) | Pdha2 | 6 | 1 | 0.005 | Stimulates the overall conversion of pyruvate to acetyl-CoA and $CO_2$, and thereby linking the glycolytic pathway to the tricarboxylic (TCA) cycle |
| Q68FX0 | Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial (Isocitric dehydrogenase subunit beta) (NAD(+)-specific ICDH subunit beta) | Idh3B | 4 | 1 | 0.049 | Facilitates the assembly and full activity of the enzyme which catalyzes the decarboxylation of isocitrate (ICT) into alpha-ketoglutarate |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q9ERD9 | Indoleamine 2,3-dioxygenase 1 (IDO-1) (EC 1.13.11.52) (Indoleamine-pyrrole 2,3-dioxygenase) | Ido1 Ido Indo | 4 | 1 | 0.049 | Catalyzes the initial, rate limiting catabolism of the tryptophan using the kynurenine pathway. Limits the growth of intracellular pathogens by depriving tryptophan. The tryptophan shortage blocks the division of T lymphocytes while the accumulation of tryptophan catabolites causes T-cell apoptosis and the differentiation of regulatory T-cells. Protects the fetus from maternal immune rejection. Involved in peripheral immune tolerance; this contributes to homeostasis via prevention of possible autoimmunity from uncontrolled/overreacting immune responses |
| P00564 | Creatine kinase M-type (EC 2.7.3.2) (Creatine kinase M chain) (Creatine phosphokinase M-type) (CPK-M) (M-CK) | Ckm Ckmm | 5 | 1 | 0.004 | Reversibly stimulates the transfer of phosphate between ATP and other phosphogens (e.g. creatine phosphate). Creatine kinase isoenzymes play an essential role in energy transduction within tissues that have large, fluctuating energy demands, such as skeletal muscle, heart, brain and spermatozoa |
| D4A781 | Importin 5 | Ipo5 | 6 | 1 | <0.001 | Protein transport into the nucleus |
| Q68FP7 | Glucokinase activity, related sequence 2 | Gk2 Gk-rs2 | 5 | 1 | <0.001 | Glycerol-3-phosphate biosynthesis. Involved in the subpathway that is involved in glycerol degradation via glycerol kinase pathway |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (Glycerol kinase 2) | | | | | |
| P13221 | Aspartate aminotransferase, cytoplasmic (cAspAT) (Cysteine transaminase, cytoplasmic) (cCAT) (Glutamate oxaloacetate transaminase 1) (Transaminase A) | Got1 | 11 | 1 | <0.001 | Regulates levels of glutamate, a major excitatory neurotransmitter of the central nervous system in vertebrates. Provides brain neuroprotection by scavenging glutamate. The aspartate aminotransferase activity is involved in glucose synthesis within the liver during development, and in adipocyte glyceroneogenesis |
| P97700 | Mitochondrial 2-oxoglutarate/malate carrier protein (OGCP) (Solute carrier family 25 member 11) | Slc25a11 Slc20a4 | 4 | 0 | <0.001 | Active (and essential) in several metabolic processes, including gluconeogenesis from lactate, the oxoglutarate/isocitrate shuttle, malate-aspartate shuttle, and in nitrogen metabolism. Maintains mitochondrial fusion and fission events, as well as the organization and morphology of cristae. Involved in the regulation of apoptosis<br><br>Spermatogenesis |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Structural proteins** | | | | | | |
| P63269 | Actin, gamma-enteric smooth muscle (Alpha-actin-3) (Gamma-2-actin) (Smooth muscle gamma-actin) | Actg2 Acta3 Actsg | 171 | 215 | <0.001 | Cell motility. Ubiquitously expressed in all eukaryotic cells |
| P60711 | Actin, cytoplasmic 1 (Beta-actin) | Actb | 153 | 190 | <0.001 | Cell motility. Ubiquitously expressed in all eukaryotic cells |
| P68136 | Alpha-actin-1 | Acta1 | 158 | 203 | <0.001 | Cell motility. Ubiquitously expressed in all eukaryotic cells |
| Q6P9T8 | Tubulin beta-4B chain (Tubulin beta-2C chain) | Tubb4b Tubb2c | 77 | 93 | 0.020 | The major component of microtubules |
| P31000 | Vimentin | Vim | 37 | 58 | <0.001 | Vimentins are class-III intermediate filaments found in several non-epithelial cells, especially mesenchymal cells. Located in the endoplasmic reticulum,mitochondria and attached to the nucleus. Is involved in the stabilization of type I collagen mRNAs |
| P58775 | Tropomyosin beta chain (Beta- | Tpm2 | 26 | 69 | <0.001 | Binds to actin filaments in muscle and non-muscle cells. Plays a essential role in calcium dependent contraction in striated muscle. The contraction of smooth muscle is regulated by an |

| | | | | | | |
|---|---|---|---|---|---|---|
| | tropomyosin) (Tropomyosin-2) | | | | | interaction with caldesmon. In non-muscle cells it is implicated in the stabilization of cytoskeletal actin filaments |
| P48675 | Desmin | Des | 36 | 59 | <0.001 | A type III intermediate filament necessary for proper muscle structure and function. Plays an important role in the maintenance of the structure of sarcomeres, Z-disks and in the formation of myofibrils. It links them to the sarcolemmal cytoskeleton, the nucleus and mitochondria, thereby providing strength for the muscle fiber during activity |
| P31000 | Vimentin | Vim | 37 | 58 | <0.001 | Class-III intermediate filaments found in several non-epithelial cells, especially mesenchymal cells. Located in the endoplasmic reticulum, mitochondria and attached to the nucleus. Is involved in stabilizing type I collagen mRNAs |
| Q10758 | Keratin, type II cytoskeletal 8 | Krt8 Krt2-8 | 24 | 49 | <0.001 | Assists in linking the contractile apparatus to in striated muscle |
| Q6IRK8 | Spectrin alpha chain, non-erythrocytic 1 (Spna2 protein) | Sptan1 Spna2 | 67 | 44 | 0.003 | Calcium ion binding |
| F1LPK7 | Plastin 3 (T-isoform), isoform CRA_a (Plastin-3) | Pls3 rCG_23133 | 45 | 29 | 0.016 | Actin binding, calcium ion binding |

| | | | | | | |
|---|---|---|---|---|---|---|
| P62329 | Thymosin beta-4 (T beta 4) [Cleaved into: Hematopoietic system regulatory peptide (Seraspenide)] | Tmsb4 x Thyb4 Tmsb4 | 8 | 28 | <0.001 | Plays an essental role in the organizing the cytoskeleton. Binds to and sequesters actin monomers (G actin) and thereby inhibiting actin polymerization. Blocks the entry of hematopoietic pluripotent stem cells into the S-phase of cell division |
| Q08290 | Calponin-1 (Basic calponin) (Calponin H1, smooth muscle) | Cnn1 | 16 | 26 | 0.004 | Protein associated with the thin filament. Is involved in regulating and modulating smooth muscle contraction. Can bind to actin, calmodulin, troponin C and tropomyosin |
| Q63610 | Tropomyosin alpha-3 chain (Gamma-tropomyosin) (Tropomyosin-3) (Tropomyosin-5) | Tpm3 Tpm-5 Tpm5 | 11 | 20 | 0.002 | Binds to actin filaments in muscle and non-muscle cells. Plays an important role in the regulation of striated muscle contraction (in association with the troponin complex). Smooth muscle contraction is regulated by interaction with caldesmon. In non-muscle cells it stabilizes cytoskeletal actin filaments |
| Q6AZ25 | Tropomyosin 1, alpha, isoform CRA_m | Tpm1 | 8 | 19 | <0.001 | Actin crosslink formation, actin filament bundle assembly |

| | | | | | | |
|---|---|---|---|---|---|---|
| A0A0G2K014 | Lymphocyte cytosolic protein 1 | Lcp1 | 30 | 17 | 0.005 | Actin crosslink formation, actin filament bundle assembly |
| Q4V7A4 | Fibrous sheath CABYR-binding protein | Fscb | 24 | 14 | 0.029 | May be involved in the later stages of fibrous sheath biogenesis. Binds calcium |
| P51886 | Lumican (Keratan sulfate proteoglycan lumican) (KSPG lumican) | Lum Lcn Ldc | 24 | 14 | 0.028 | Binds to laminin. collagen fibril organization |
| P97536 | Cullin-associated NEDD8-dissociated protein 1 (Cullin-associated and neddylation-dissociated protein 1) (TBP-interacting protein of 120 kDa A) (TBP-interacting | Cand1 Tip120 Tip120 a | 25 | 10 | <0.001 | Acts as an F-box protein exchange factor. Possibly enhances transcription via various types of promoters (indirectly) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | protein 120A) (p120 CAND1) | | | | | |
| Q66HR2 | Microtubule-associated protein RP/EB family member 1 (APC-binding protein EB1) (End-binding protein 1) (EB1) | Mapre1 | 1 | 5 | <0.001 | Binds to microtubules and regulates the dynamics of the microtubule cytoskeleton. Promotes the nucleation and elongation of cytoplasmic microtubules. May be involved in spindle function by stabilizing microtubules and anchoring them at centrosomes. Tether non-centrosomal microtubules to the Golgi, an important step for polarized cell movement. |
| Q5U302 | Catenin (Cadherin associated protein), alpha 1 (Catenin (Cadherin-associated protein), alpha 1, isoform CRA_b) (Catenin alpha 1) | Ctnna1 Catna1 rCG_49560 | 10 | 4 | 0.017 | Acrosomal vesicle protein. Located at cell-cell adherens junctions |
| Q6P6T4 | Echinoderm microtubule-associated | Eml2 Emap2 | 8 | 2 | 0.004 | Tubulin binding protein. Inhibits microtubule nucleation and growth, resulting in shorter microtubules |

| | | | | | | |
|---|---|---|---|---|---|---|
| | protein-like 2 (EMAP-2) | | | | | |
| D4A626 | Calmin | Clmn | 6 | 2 | 0.0463 | Actin binding |
| D4A1D8 | Surfeit 4 (Surfeit 4, isoform CRA_a) | Surf4 rCG_45751 | 5 | 1 | 0.049 | Golgi organ organisation |
| Q9ES40 | PRA1 family protein 3 (ADP-ribosylation factor-like protein 6-interacting protein 5) (ARL-6-interacting protein 5) (Aip-5) (Glutamate transporter EAAC1-interacting protein) (Prenylated Rab acceptor protein 2) | Arl6ip5 Gtrap3-18 Jwa Pra2 Praf3 | 4 | 1 | 0.049 | Involved in regulating intracellular concentrations of taurine and glutamate. Negatively controls glutamate transport activity by decreasing its affinity for glutamate. May be involved in membrane trafficking |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q9EPC6 | Profilin-2 (Profilin II) | Pfn2 | 3 | 1 | 0.046 | Binds to actin to affect the structure of the cytoskeleton. Prevents the polymerization of actin at high concentrations, but enhances it at low concentrations |
| P18614 | Integrin alpha-1 (CD49 antigen-like family member A) (Laminin and collagen receptor) (VLA-1) (CD antigen CD49a) | Itga1 | 7 | 0 | <0.001 | A receptor for laminin and collagen. Takes part in the negative regulation of EGF-stimulated cell growth |
| Q4G069 | Regulator of microtubule dynamics protein 1 (RMD-1) (Protein FAM82B) | Rmdn1 Fam82 b | 3 | 0 | 0.008 | Interacts with microtubules. Localizes to the cytoplasm in interphase, and to the spindle microtubules and spindle poles during mitosis |
| **Reproduction specific** | | | | | | |
| Q5XIV1 | Phosphoglycerate kinase | Pgk2 | 100 | 118 | 0.017 | Flagellated sperm motility, glycolytic process |
| P12020 | Cysteine-rich secretory protein | Crisp1 Aeg | 100 | 151 | <0.001 | May help spermatozoa to undergo functional maturation while they migrate from the testis to the ductus deferens |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 1 (32 kDa epididymal protein) (Acidic epididymal glycoprotein)) | | | | | |
| P0DP29 | Calmodulin-1 | Cam1, Cam2, Cam3 | 37 | 48 | 0.027 | Facilitates the control of numerous enzymes, ion channels, aquaporins and other proteins via calcium-binding. Stimulates protein kinases and phosphatases using a calcium-dependant mechanism. Together with centrin, it takes part in the genetic regulation of the centrosome cycle and progression through cytokinesis. Positively controls calcium-activated potassium channel activity |
| P04182 | Ornithine aminotransferase, mitochondrial (Ornithine--oxo-acid aminotransferase) | Oat | 32 | 44 | 0.012 | Expressed in the head and flagella of epididymal sperm (at protein level) .Amino-acid biosynthesis; L-proline biosynthesis; L-glutamate 5-semialdehyde from L-ornithine |
| P14668 | Annexin A5 (Anchorin CII) (Endonexin II) (Lipocortin V) | Anxa5 Anx5 | 17 | 25 | 0.049 | An anticoagulant protein. Acts as an indirect inhibitor of a thromboplastin-specific complex that is involved in blood coagulation |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (Placental anticoagulant protein 4) (PP4)) | | | | | |
| A7VJC2 | Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2/B1) | Hnrnpa2b1 Hnrnp Hnrpa2b1 | 15 | 7 | 0.016 | In the testis, isoform A2 and isoform B1 are expressed in spermatogonia and spermatocytes, but not spermatids or sperm. Condenses and stabilizes mRNA transcripts thereby minimizing tangling and knotting. Protects telomeric DNA repeat against endonuclease digestion by specifically binding to single-stranded telomeric DNA sequences, Helps to regulate the efficiency of mRNA splicing |
| P81155 | Voltage-dependent anion-selective channel protein 2 (VDAC-2) (Outer mitochondrial membrane protein porin 2) | Vdac2 | 14 | 6 | 0.020 | Expressed in the head region of epididymal sperm. Facilitates the diffusion of small hydrophilic molecules by forming a channel through the mitochondrial outer membrane |
| Q9R1Z0 | Voltage-dependent anion-selective channel protein 3 (VDAC- | Vdac3 | 7 | 2 | 0.022 | Facilitates the diffusion of small hydrophilic molecules by forming a channel through the mitochondrial outer membrane |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 3) (rVDAC3) (Outer mitochondrial membrane protein porin 3) | | | | | |
| Q9Z1K2 | Sperm surface protein Sp17 (Sperm autoantigenic protein 17) | Spa17 sp17 rCG_58879 | 6 | 1 | 0.006 | Sperm surface zona pellucida binding protein. Facilitates the high affinity binding of spermatozoa to the zona pellucida. Possibly functions to bind the zona pellucida and carbohydrates |
| Q68FW9 | COP9 signalosome complex subunit 3 (SGN3) (Signalosome subunit 3) | Cops3 Csn3 | 4 | 1 | 0.049 | Takes part in numerous cellular and developmental processes. Essential in the regulation of the ubiquitin (Ubl) conjugation pathway. Important in maintain the survival of epiblast cells and the development of a postimplantation embryo |
| D4AE30 | Prostate and testis expressed C (RCG22807) (RCG22807-like) | LOC100361828 rCG_22807 | 4 | 0 | <0.001 | Not classified |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q6P685 | Eukaryotic translation initiation factor 2 subunit beta (Eukaryotic translation initiation factor 2, subunit 2 (Beta)) (Eukaryotic translation initiation factor 2, subunit 2 (Beta), isoform CRA_a) | Eif2s2 rCG_37232 | 3 | 0 | 0.008 | Male germ cell proliferation, male gonad development |
| **Antioxidant/pro-oxidant** | | | | | | |
| P04904 | Glutathione S-transferase alpha-3 (Glutathione S-transferase Yc-1) (GST Yc1) | Gsta3 Gstyc1 | 60 | 83 | <0.001 | Conjugates reduced glutathione (GSH) to exogenous and endogenous hydrophobic electrophiles to detoxify the system |
| O35244 | Peroxiredoxin-6 (Acidic calcium- | Prdx6 Aipla2 | 36 | 50 | 0.006 | Plays a role in cell protection against oxidative stress by detoxifying peroxides and via phospholipid homeostasis. |

| | | | | | | |
|---|---|---|---|---|---|---|
| | independent phospholipase A2) (Antioxidant protein 2) | Aop2 Tsa | | | | Catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively. Can also reduce $H_2O_2$ and short chain organic, fatty acid, and phospholipid hydroperoxides. Also has phospholipase activity. |
| Q63716 | Peroxiredoxin-1 (EC 1.11.1.15) (HBP23) (Heme-binding 23 kDa protein) (Thioredoxin peroxidase 2) (Thioredoxin-dependent peroxide reductase 2) | Prdx1 Tdpx2 | 22 | 32 | 0.148 | Conjugates reduced glutathione to a variety of exogenous and endogenous hydrophobic electrophiles |
| P07632 | Superoxide dismutase [Cu-Zn] | Sod1 | 13 | 29 | <0.001 | Destroys free radicals which are usually produced within toxic cells |
| Q63716 | Peroxiredoxin-1 (HBP23) (Heme-binding 23 kDa protein) | Prdx1 Tdpx2 | 22 | 32 | 0.015 | Thiol-specific peroxidase that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively. Facilitates cell protection against oxidative stress via the detoxifying peroxides and the sensing of |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (Thioredoxin peroxidase 2) | | | | | hydrogen peroxide-mediated signaling events. May regulate the intracellular concentrations of $H_2O_2$ to control signalling cascades of growth factors and tumour necrosis factor-alpha |
| Q64057 | Alpha-aminoadipic semialdehyde dehydrogenase (Alpha-AASA dehydrogenase) (Aldehyde dehydrogenase family 7 member A1) | Aldh7a1 Ald7a1 | 22 | 12 | 0.019 | Protects cells from oxidative stress by metabolizing numerous aldehydes derived from lipid peroxidation. Participates in lysine catabolism. Multifunctional enzyme which mediates important protective effects |
| Q6AXX6 | Redox-regulatory protein FAM213A (Peroxiredoxin-like 2 activated in M-CSF stimulated monocytes) (Protein PAMM) | Fam213a Pamm Shp1 | 12 | 19 | 0.041 | Involved in redox regulation of the cell. Acts as an antioxidant. Inhibits macrophage production of inflammatory cytokines. Expressed by the principal cells of the epididymis. Detected in the head region of epididymal sperm (at protein level) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (Sperm head protein 1) | | | | | |
| P11507 | Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2) (SR Ca(2+)-ATPase 2) (EC 3.6.3.8) (Calcium pump 2) (Calcium-transporting ATPase sarcoplasmic reticulum type, slow twitch skeletal muscle isoform) (Endoplasmic reticulum class 1/2 Ca(2+) ATPase) | Atp2a2 | 24 | 14 | 0.029 | Magnesium-dependent enzyme. Production of mitochondrial ROS, thereby triggering $Ca^{2+}$ signaling cascades that promote osteoclast differentiation and activation |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q64057 | Alpha-aminoadipic semialdehyde dehydrogenase (Alpha-AASA dehydrogenase) (EC 1.2.1.31) (Aldehyde dehydrogenase family 7 member A1) (EC 1.2.1.3) (Antiquitin-1) (Betaine aldehyde dehydrogenase) (EC 1.2.1.8) (Delta1-piperideine-6-carboxylate dehydrogenase) (P6c dehydrogenase) | Aldh7a1 Ald7a1 | 22 | 12 | 0.019 | Multifunctional enzyme mediating important protective effects. Metabolizes betaine aldehyde to betaine, an important cellular osmolyte and methyl donor. Protects cells from oxidative stress by metabolizing numerous aldehydes derived from lipid peroxidation |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q9Z1B2 | Glutathione S-transferase Mu 5 (EC 2.5.1.18) (GST class-mu 5) | Gstm5 | 18 | 8 | 0.003 | Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles |
| B0K020 | CDGSH iron-sulfur domain-containing protein 1 (MitoNEET) | Cisd1 | 3 | 8 | 0.021 | Plays a key role in regulating maximal capacity for electron transport and oxidative phosphorylation. May be involved in Fe-S cluster shuttling and/or in redox reactions |
| P14942 | Glutathione S-transferase alpha-4 (EC 2.5.1.18) (GST 8-8) (GST A4-4) (GST K) (Glutathione S-transferase Yk) (GST Yk) | Gsta4 | 14 | 7 | 0.028 | Conjugation of reduced glutathione to numerous exogenous and endogenous hydrophobic electrophiles |
| A0A0G2K6L4 | Glutathione S-transferase, mu 6 (Predicted), isoform CRA_a (Glutathione S- | Gstm6l Gstm6_predicted rCG_29071 | 12 | 5 | <0.001 | Conjugation of reduced glutathione to numerous exogenous and endogenous hydrophobic electrophiles |

| | | | | | | |
|---|---|---|---|---|---|---|
| | transferase, mu 6-like) | | | | | |
| P11232 | Thioredoxin (Trx) | Txn Txn1 | 12 | 3 | <0.001 | Participates in various redox reactions. Plays a role in the reversible S-nitrosylation of cysteine residues in target proteins, and thereby contributes to the response to intracellular nitric oxide. Inhibits caspase-3 activity. Induces DNA binding activity in ionizing radiation (IR) cells through its oxidation/reduction status |
| P22985 | Xanthine dehydrogenase/oxidase [Includes: Xanthine dehydrogenase (XD) (EC 1.17.1.4); Xanthine oxidase (XO) (EC 1.17.3.2) (Xanthine oxidoreductase) (XOR)] | Xdh | 4 | 0 | 0.003 | Key enzyme in purine degradation. Catalyzes the oxidation of hypoxanthine to xanthine. Catalyzes the oxidation of xanthine to uric acid. Plays a role in the generation of reactive oxygen species |
| **Regulatory** | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| P02770 | Serum albumin | Alb | 449 | 536 | <0.001 | A protein with a binding capacity for water, plasma ions, fatty acids, hormones, bilirubin and drugs. A major zinc transporter (±80%) in plasma |
| P31044 | Phosphatidylethanolamine-binding protein 1 (PEBP-1) | Pebp1 Pbp Pebp | 264 | 311 | <0.001 | Binds ATP, opioids and phosphatidylethanolamine. Serine protease inhibitor which inhibits thrombin, neuropsin and chymotrypsin. Spermatid development; sperm capacitation |
| P12346 | Serotransferrin (Transferrin) (Beta-1 metal-binding globulin) | Tf | 113 | 132 | 0.013 | Iron binding transport proteins which bind two $Fe^{3+}$ ions in association with an anion, usually bicarbonate. Transports iron atoms from sites of absorption and heme degradation to sites of storage and utilization |
| Q66HD0 | Endoplasmin (94 kDa glucose-regulated protein) (GRP-94) (Heat shock protein 90 kDa beta member 1) | Hsp90b1 Grp94 Tra1 | 72 | 89 | 0.012 | Molecular chaperone - processes and transports secreted proteins. Involved in endoplasmic reticulum associated degradation. Has ATPase activity.<br>Acute-phase response |
| P06761 | Endoplasmic reticulum chaperone BiP (78 kDa glucose- | Hspa5 Grp78 | 84 | 111 | <0.001 | Endoplasmic reticulum chaperone. Necessary for correct folding of proteins and degradation of misfolded proteins |

| | | | | | | |
|---|---|---|---|---|---|---|
| | regulated protein) (GRP-78) (Binding-immunoglobulin protein) (BiP) (Heat shock protein family A member 5) | | | | | Cellular response to glucose starvation, cellular response to interleukin-4 |
| F7FJQ3 | NPC intracellular cholesterol transporter 2 | Npc2 | 43 | 55 | 0.026 | Cholesterol efflux, cholesterol homeostasis, intracellular cholesterol transport |
| P02091 | Hemoglobin subunit beta-1 (Beta-1-globin) (Hemoglobin beta chain, major-form) (Hemoglobin beta-1 chain) | Hbb | 60 | 72 | 0.047 | Oxygen transport from the lung to peripheral tissues |
| Q7TN19 | Serine proteinase inhibitor HongrES1 | Serpina 16 LOC29 9271 | 51 | 22 | <0.001 | Endopeptidase activity |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q68FY4 | Group specific component (Group specific component, isoform CRA_b) (Vitamin D-binding protein) | Gc rCG_60501 | 35 | 50 | 0.003 | Involved in vitamin D transport and storage, scavenging of extracellular G-actin, enhancement of the chemotactic activity of C5 alpha for neutrophils in inflammation and macrophage activation |
| D4A4R7 | RCG21015, isoform CRA_a (Serine (or cysteine) peptidase inhibitor, clade A, member 1F) | Serpina 1f rCG_21015 | 64 | 37 | <0.001 | Endopeptidase activity |
| G3V7Q7 | IQ motif containing GTPase activating protein 1 (Predicted), isoform CRA_b (IQ motif-containing | Iqgap1 Iqgap1 _predicted rCG_24856 | 26 | 13 | 0.001 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | GTPase-activating protein 1) | | | | | |
| P62944 | AP-2 complex subunit beta (AP105B) (Adaptor protein complex AP-2 subunit beta) (Adaptor-related protein complex 2 subunit beta) (Beta-2-adaptin) (Beta-adaptin) (Clathrin assembly protein complex 2 beta large chain) (Plasma membrane adaptor HA2/AP2 | Ap2b1 Clapb1 | 11 | 2 | <0.001 | Adaptor protein complexes take part in protein transport via transport vesicles in different membrane traffic pathways. Are vesicle coat components and appear to be involved in cargo selection and vesicle formation. Clathrin-dependent endocytosis where cargo proteins are incorporated into vesicles surrounded by clathrin (clathrin-coated vesicles) and are destined for fusion with the early endosome. |

| | | | | | | |
|---|---|---|---|---|---|---|
| | adaptin beta subunit) | | | | | |
| Q5XI62 | Protein MENT (Methylated in normal thymocytes protein) | Ment | 3 | 0 | 0.014 | Involved in control of cellular proliferation. Onconcogenic modifier contributing to tumor suppressor function. |
| Q6P7A7 | Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 | Rpn1 rCG_56194 | 15 | 8 | 0.048 | Protein modification, protein glycosylation. |
| P50398 | Rab GDP dissociation inhibitor alpha (Rab GDI alpha) (Guanosine diphosphate dissociation inhibitor 1) (GDI-1) | Gdi1 Rabgdia | 25 | 15 | 0.047 | Controls the GDP/GTP exchange reaction of most Rab proteins by inhibiting the dissociation of GDP from these proteins, and the binding of GTP to them. Promotes the dissociation of GDP-bound Rab proteins from the membrane and inhibits their activation |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q80U96 | Exportin-1 (Exp1) (Chromosome region maintenance 1 protein homolog) | Xpo1 Crm1 | 5 | 1 | 0.017 | Mediates the nuclear export of cellular proteins (cargos) with a leucine-rich nuclear export signal (NES) and of RNAs. Involved in RNA transport from Cajal bodies to nucleoli |
| P62260 | 14-3-3 protein epsilon (14-3-3E) (Mitochondrial import stimulation factor L subunit) (MSF L) | Ywhae | 20 | 10 | 0.007 | Adapter protein associated with the regulation of numerous general and specialized signaling pathways. Binds to a large number of partners, usually by recognition of a phosphoserine or phosphothreonine motif. Binding generally results in the modulation of the activity of the binding partner. Positively regulates phosphorylated protein HSF1 nuclear export to the cytoplasm |
| O88761 | 26S proteasome non-ATPase regulatory subunit 1 (26S proteasome regulatory subunit RPN2) (26S proteasome regulatory subunit S1) (26S | Psmd1 | 12 | 4 | 0.002 | A component of the 26S proteasome, a complex of numerous proteins involved in ATP-dependent degradation of ubiquitinated proteins. This complex maintains protein homeostasis by removing misfolded or damaged proteins, that can impair cellular functions, and by removing proteins whose functions are no longer required. Therefore, the proteasome participates in numerous cellular processes, including cell cycle progression, apoptosis, or DNA damage repair |

| | | | | | | |
|---|---|---|---|---|---|---|
| | proteasome subunit p112) | | | | | |
| P21139 | Alpha-mannosidase 2C1 (Alpha-D-mannoside mannohydrolase) (Mannosidase alpha class 2C member 1) | Man2c1 | 9 | 2 | <0.001 | Cleaves alpha 1,2-, alpha 1,3-, and alpha 1,6-linked mannose residues from glycoproteins. Involved in the degradation of free oligosaccharides in the cytoplasm |
| Q6AYZ4 | Serine/threonine-protein phosphatase | Ppp1ccrCG_21849 | 20 | 5 | <0.001 | Protein phosphatase that associates with > 200 regulatory proteins to form highly specific holoenzymes that dephosphorylate numerous of biological targets. Protein phosphatase (PP1) is essential for cell division, by participating in the regulation of glycogen metabolism, muscle contraction and protein synthesis. Involved in the control of chromatin structure and cell cycle progression during the transition from mitosis into interphase |
| P62142 | Serine/threonine-protein phosphatase PP1-beta catalytic subunit (PP-1B) | Ppp1cb | 17 | 6 | <0.001 | |
| P62138 | Serine/threonine-protein phosphatase PP1-alpha | Ppp1caPpp1a | 16 | 4 | <0.001 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | catalytic subunit (PP-1A) | | | | | |
| A0A0G2JWX1 | 26S proteasome non-ATPase regulatory subunit 11 (Proteasome (Prosome, macropain) 26S subunit, non-ATPase, 11 (Predicted), isoform CRA_b) | Psmd11 Psmd11_predicted rCG_32504 | 12 | 5 | 0.015 | Component of the 26S proteasome, a complex involved in the ATP-dependent degradation of ubiquitinated proteins. The complex plays an important role in maintaining protein homeostasis via the removal of misfolded or damaged proteins that could impair cellular functions, and by removing proteins whose functions are no longer required. Therefore, the proteasome participates in numerous cellular processes, including cell cycle progression, apoptosis, or DNA damage repair |
| F1LN42 | Tensin 1 | Tns1 | 15 | 8 | 0.037 | Intracellular signal transduction |
| Q6P9V7 | Proteasome (Prosome, macropain) activator subunit 1 (Proteasome activator complex subunit 1) (RCG23530) | Psme1 rCG_23530 | 21 | 9 | <0.001 | Predicted to have endopeptidase activator activity. Predicted to be involved in antigen processing and the presentation of exogenous antigen to immune cells; regulates the G1/S transition of mitotic cell cycle; and regulates proteolysis. Predicted to be localized to the cytoplasm and nucleoplasm |

| | | | | | | |
|---|---|---|---|---|---|---|
| P68255 | 14-3-3 protein theta (14-3-3 protein tau) | Ywhaq | 20 | 11 | 0.028 | Adapter protein involved in the regulation of a numerous general and specialized signaling pathways. Binds to a large number of partners, usually by recognition of a phosphoserine or phosphothreonine motif. Binding generally results in the modulation of the activity of the binding partner |
| Q4G061 | Eukaryotic translation initiation factor 3 subunit B (eIF3b) (Eukaryotic translation initiation factor 3 subunit 9) (eIF-3-eta) | Eif3b Eif3s9 | 8 | 2 | 0.008 | RNA-binding component of the eukaryotic translation initiation factor 3 (eIF-3) complex, that is required for several steps in the initiation of protein synthesis. The eIF-3 complex stimulates mRNA recruitment and scanning of the mRNA for AUG recognition. This complex is also required for the disassembly and recycling of post-termination ribosomal complexes and thereby preventing the premature joining of the 40S and 60S ribosomal subunits prior to initiation. The eIF-3 complex specifically targets and initiates translation of a subset of mRNAs involved in cell proliferation, including cell cycling, differentiation and apoptosis, and uses different modes of RNA stem-loop binding to exert either translational activation or repression |
| B0BN93 | 26S proteasome non-ATPase regulatory subunit 13 (26S | Psmd13 | 7 | 2 | 0.023 | Component of the 26S proteasome, a multiprotein complex involved in the ATP-dependent degradation of ubiquitinated proteins. This complex plays an important role in the maintenance of protein homeostasis via the removal of |

| | | | | | | |
|---|---|---|---|---|---|---|
| | proteasome regulatory subunit RPN9) (26S proteasome regulatory subunit S11) (26S proteasome regulatory subunit p40.5) | | | | | misfolded or damaged proteins, which could impair cellular functions, and via the removal of proteins whose functions are no longer required. Therefore, the proteasome participates in numerous cellular processes, including cell cycle progression, apoptosis, or DNA damage repair |
| G3V8A5 | Vacuolar protein sorting-associated protein 35 | Vps35 Vps35_mapped rCG_44281 | 5 | 1 | 0.025 | Plays a role in vesicular protein sorting |
| D4A8H3 | Similar to RIKEN cDNA 5730469D23 (Predicted) (Ubiquitin-like modifier-activating enzyme 6) | Uba6 RGD1308324_predicted rCG_56897 | 6 | 1 | <0.001 | Involved in the pathway protein ubiquitination, which is part of Protein modification. |

| | | | | | | |
|---|---|---|---|---|---|---|
| G3V762 | Tissue specific transplantation antigen P35B (Predicted), isoform CRA_a (Tissue-specific transplantation antigen P35B) | Tsta3 Tsta3_predicted rCG_59877 | 11 | 4 | 0.002 | Catalyzes the two-step NADP-dependent conversion of GDP-4-dehydro-6-deoxy-D-mannose to GDP-fucose, involving an epimerase and a reductase reaction |
| P61206 | ADP-ribosylation factor 3 (Liver regeneration-related protein LRRG202) | Arf3 Ac1-253 | 22 | 12 | 0.015 | Involved in protein trafficking; may modulate vesicle budding and uncoating within the Golgi apparatus. |
| Q68FS2 | COP9 signalosome complex subunit 4 (SGN4) (Signalosome subunit 4) (JAB1-containing signalosome subunit 4) | Cops4 Csn4 | 5 | 2 | 0.035 | Component of the COP9 signalosome complex (CSN), a complex involved in numerous cellular and developmental processes. The CSN complex is important in the regulation of the ubiquitin (Ubl) conjugation pathway. Involved in phosphorylation of p53/TP53, c-jun/JUN, IkappaBalpha/NFKBIA, ITPK1, IRF8/ICSBP and SNAPIN. CSN-dependent phosphorylation of TP53 and JUN promotes and protects degradation by the Ubl system, respectively. |

| | | | | | | |
|---|---|---|---|---|---|---|
| P84083 | ADP-ribosylation factor 5 | Arf5 | 16 | 7 | 0.005 | GTP-binding protein that functions as an allosteric activator of the cholera toxin catalytic subunit, an ADP-ribosyltransferase. Involved in protein trafficking; may modulate vesicle budding and uncoating within the Golgi apparatus |
| Q66HG5 | Transmembrane 9 superfamily member 2 | Tm9sf2 | 12 | 1 | <0.001 | In the intracellular compartments, may function as a channel or small molecule transporter |
| | | | | | 0.023 | Adapter protein involved in the regulation of various general and specialized signaling pathways. Binds to a numerous partners, usually by recognition of a phosphoserine or phosphothreonine motif. Binding generally results in the modulation of the activity of the binding partner |
| B5DFC8 | Eukaryotic translation initiation factor 3 subunit C (eIF3c) (Eukaryotic translation initiation factor 3 subunit 8) (eIF3 p110) | Eif3c Eif3s8 | 3 | 1 | 0.046 | Component of the eukaryotic translation initiation factor 3 (eIF-3) complex, which is necessary for numerous steps in the initiation of protein synthesis. The eIF-3 complex is required for disassembly and recycling of post-termination ribosomal complexes and thereby preventing premature joining of the 40S and 60S ribosomal subunits prior to initiation. The eIF-3 complex specifically targets and initiates translation of a subset of mRNAs involved in cell proliferation, including cell cycling, differentiation and apoptosis, and uses different modes of RNA |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | stem-loop binding to exert either translational activation or repression |
| O88506 | STE20/SPS1-related proline-alanine-rich protein kinase (Ste-20-related kinase) (Pancreatic serine/threonine-protein kinase) (PS/TK) (PSTK1) (Serine/threonine-protein kinase 39) | Stk39 Pask Spak | 3 | 0 | 0.001 | May act as a mediator of stress-activated signals |
| G3V6S3 | Calumenin (RCG28015, isoform CRA_b) | Calu rCG_28015 | 7 | 2 | 0.023 | Calcium ion binding. Involved in regulation of vitamin K-dependent carboxylation of multiple N-terminal glutamate residues |
| Q66H80 | Coatomer subunit delta (Archain) (Delta-coat protein) (Delta-COP) | Arcn1 Copd | 6 | 11 | 0.037 | Cytosolic protein complex that reversibly associates with Golgi non-clathrin-coated vesicles to mediate protein transport from the ER to the trans Golgi network. Required for budding from Golgi membranes, and is important in the retrograde Golgi-to-ER transport of dilysine-tagged proteins. Recruited by |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | membranes associated to ADP-ribosylation factors (ARFs), which are small GTP-binding proteins; thereby influencing the Golgi structural integrity, as well as the processing, activity, and endocytic recycling of LDL receptors |
| P02401 | 60S acidic ribosomal protein P2 | Rplp2 Rp2 | 9 | 16 | 0.023 | Plays an essential role in the elongation step of protein synthesis |
| Q5PPJ6 | Leucyl-tRNA synthetase | Lars | 3 | 0 | 0.014 | Protein biosynthesis. Catalyzes the specific attachment of an amino acid to its cognate tRNA in a two-step reaction: the amino acid (AA) is first activated by ATP to form AA-AMP and then transferred to the acceptor end of the tRNA |
| Q5U206 | Calmodulin-like protein 3 | Calml3 | 5 | 16 | <0.001 | May function as a specific light chain of unconventional myosin-10 (MYO10). Enhances MYO10 translation, possibly via its action as a chaperone for the emerging MYO10 heavy chain protein. May compete with calmodulin by binding, with different affinities, to cellular substrates |
| Q811Q2 | Chloride intracellular channel protein 6 | Clic6 Clic6b | 10 | 3 | 0.009 | Possibly inserts into membranes to form chloride ion channels. May play an important role in water-secreting cells, possibly through the regulation of chloride ion transport |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q66HL0 | 5' nucleotidase, ecto (5' nucleotidase, ecto, isoform CRA_a) (5'-nucleotidase) | Nt5e rCG_25342 | 11 | 5 | 0.040 | Hydrolyzes extracellular nucleotides into membrane permeable nucleosides |
| D3ZAB1 | Lactotransferrin (Lactotransferrin (Predicted)) | Ltf Ltf_predicted rCG_25565 | 13 | 3 | <0.001 | A major iron-binding and multifunctional protein found in exocrine fluids e.g. mucosal secretions. Has antimicrobial activity. Involved in nnate immune response. Regulation of cytokine production |
| Q4KLM9 | Asparaginyl-tRNA synthetase | Nars | 6 | 1 | 0.002 | Protein synthesis. Catalyzes the attachment of asparagine to tRNA(Asn) |
| Q66HM2 | AP-2 complex subunit alpha | Ap2a2 | 7 | 2 | 0.023 | Adaptor protein complexes which function in protein transport via transport vesicles in different membrane traffic pathways. Adaptor protein complexes are vesicle coat components and are implicated in cargo selection and vesicle formation. AP-2 is directly involved in clathrin-dependent endocytosis where cargo proteins are incorporated into vesicles surrounded by clathrin (clathrin-coated vesicles) that are destined for fusion with the early endosome |

| | | | | | | |
|---|---|---|---|---|---|---|
| P05964 | Protein S100-A6 (Calcyclin) (Prolactin receptor-associated protein) (S100 calcium-binding protein A6) | S100a6 Cacy | 7 | 14 | 0.013 | May function as calcium sensor and modulator, contributing to cellular calcium signaling. May function by interacting with other proteins, and indirectly play a role in other physiological processes including reorganization of the actin cytoskeleton and cell motility. Binds 2 calcium ions. |
| P16446 | Phosphatidylinositol transfer protein alpha isoform (PI-TP-alpha) (PtdIns transfer protein alpha) (PtdInsTP alpha) | Pitpna Pitpn | 5 | 0 | <0.001 | Catalyzes the transfer of PtdIns and phosphatidylcholine between membranes |
| Q68FZ8 | Propionyl coenzyme A carboxylase, beta polypeptide (Propionyl-CoA carboxylase beta | Pccb rCG_25130 | 8 | 3 | 0.040 | Ligase. ATP binding. Fatty acid catabolism. Is one of the 2 subunits of the biotin-dependent propionyl-CoA carboxylase (PCC), a mitochondrial enzyme involved in the catabolism of odd chain fatty acids, branched-chain amino acids, isoleucine, threonine, methionine, valine and other metabolites |

| | | | | | | |
|---|---|---|---|---|---|---|
| | chain, mitochondrial) | | | | | |
| Q924C3 | Ectonucleotide pyrophosphatase/ phosphodiesterase family member 1 (E-NPP 1) (Phosphodiesterase I/nucleotide pyrophosphatase 1) (Plasma-cell membrane glycoprotein PC-1) [Includes: Alkaline phosphodiesterase I;Nucleotide pyrophosphatase (NPPase)<br><br>(Nucleotide diphosphatase)] | Enpp1 Npps Pc1 Pdnp1 | 12 | 4 | 0.005 | Regulate pyrophosphate levels, controls bone mineralization and soft tissue calcification. PPi inhibits mineralization by binding to nascent hydroxyapatite (HA) crystals, thereby preventing further growth of these crystals. Preferentially hydrolyzes ATP, but can also hydrolyze other nucleoside 5' triphosphates such as GTP, CTP, TTP and UTP to their corresponding monophosphates with release of pyrophosphate and diadenosine polyphosphates, and also 3',5'-cAMP to AMP. May also be involved in the regulation of the availability of nucleotide sugars in the endoplasmic reticulum and Golgi, and the regulation of purinergic signaling. Appears to modulate insulin sensitivity |

| | | | | | | |
|---|---|---|---|---|---|---|
| G3V8Q1 | Coatomer subunit epsilon | Cope Cope_predicted rCG_38784 | 11 | 5 | 0.013 | Cytosolic protein complex that binds to dilysine motifs and reversibly associates with Golgi non-clathrin-coated vesicles, which mediate biosynthetic protein transport from the ER, via the Golgi up to the trans Golgi network. Required for budding from Golgi membranes, and is essential for the retrograde Golgi-to-ER transport of dilysine-tagged proteins |
| P15865 | Histone H1.4 (H1d) | Hist1h1e H1f4 | 1 | 4 | 0.008 | Histone H1 protein binds to linker DNA between nucleosomes forming the macromolecular structure known as the chromatin fiber. Histones H1 are necessary for the condensation of nucleosome chains into higher-order structured fibers. Acts also as a regulator of individual gene transcription through chromatin remodeling, nucleosome spacing and DNA methylation |
| Q6AYS4 | Plasma alpha-L-fucosidase (EC 3.2.1.51) (Alpha-L-fucoside fucohydrolase 2) (Alpha-L-fucosidase 2) | Fuca2 | 11 | 1 | <0.001 | Alpha-L-fucosidase is responsible for hydrolyzing the alpha-1,6-linked fucose joined to the reducing-end N-acetylglucosamine of the carbohydrate moieties of glycoproteins |
| P62982 | Ubiquitin-40S ribosomal protein S27a (Ubiquitin | Rps27a Uba80 Ubcep1 | 11 | 4 | 0.011 | Ubiquitin: Exists either covalently attached to another protein, or free (unanchored). DNA repair; involved in ERAD (endoplasmic reticulum-associated degradation) and cell-cycle regulation; |

| | | | | | | |
|---|---|---|---|---|---|---|
| | carboxyl extension protein 80) [Cleaved into: Ubiquitin; 40S ribosomal protein S27a] | | | | | lysosomal degradation; kinase modification; protein degradation via the proteasome; endocytosis, DNA-damage responses as well as in signaling processes leading to activation of the transcription factor NF-kappa-B. Linear polymer chains formed via attachment by the initiator Met lead to cell signaling. When polyubiquitin is free (unanchored-polyubiquitin), it also has distinct roles, such as in activation of protein kinases, and in signaling. Component of the 40S subunit of the ribosome |
| P61983 | 14-3-3 protein gamma [Cleaved into: 14-3-3 protein gamma, N-terminally processed] | Ywhag | 11 | 5 | 0.036 | Adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathways. Binds to a large number of partners, usually by recognition of a phosphoserine or phosphothreonine motif. Binding generally results in the modulation of the activity of the binding partner |
| D3ZZL3 | N-acetyl-alpha-glucosaminidase (RCG33377, isoform CRA_b) | Naglu rCG_33377 | 11 | 3 | 0.002 | Glycosidase and hydrolase. Lysosome organisation |
| O35760 | Isopentenyl-diphosphate Delta-isomerase 1 (Isopentenyl | Idi1 | 7 | 2 | 0.040 | Isomerase. Cholesterole biosynthetic process. Involved in step 1 of the subpathway that synthesizes dimethylallyl diphosphate from isopentenyl diphosphate |

| | | | | | | |
|---|---|---|---|---|---|---|
| | pyrophosphate isomerase 1) (IPP isomerase 1) (IPPI1) | | | | | |
| A0A0G2K654 | Histone cluster 1 H1 family member c (RCG45246) | Hist1h1c LOC684681 rCG_45246 | 2 | 6 | 0.012 | Chromosome condensation |
| P28075 | Proteasome subunit beta type-5 (EC 3.4.25.1) (Macropain epsilon chain) (Multicatalytic endopeptidase complex epsilon chain) (Proteasome chain 6) (Proteasome | Psmb5 | 5 | 1 | 0.003 | Component of the 20S core proteasome complex involved in the proteolytic degradation of numerous intracellular proteins. The 26S proteasome plays an important role in the maintenance of protein homeostasis by removing misfolded or damaged proteins that could impair cellular functions, and by removing proteins whose functions are no longer required. Associated with 20S proteasome mediates of ubiquitin-independent protein degradation. This type of proteolysis is required in several pathways including spermatogenesis or generation of a subset of MHC class I-presented antigenic peptides. Within the 20S core complex, PSMB5 displays a chymotrypsin-like activity |

| | | | | | | |
|---|---|---|---|---|---|---|
| | epsilon chain) (Proteasome subunit X) | | | | | |
| P62703 | 40S ribosomal protein S4, X isoform | Rps4x Rps4 | 3 | 0 | 0.022 | Protein translation, RNA binding |
| P61751 | ADP-ribosylation factor 4 | Arf4 | 13 | 5 | 0.005 | GTP-binding protein that functions as an allosteric activator of the cholera toxin catalytic subunit, an ADP-ribosyltransferase. Involved in protein trafficking; may modulate vesicle budding and uncoating within the Golgi apparatus. |
| B0BNK1 | RAB5C, member RAS oncogene family (RCG32615, isoform CRA_a) (Rab5c protein) | Rab5c rCG_32615 | 7 | 2 | 0.006 | Plasma membrane to endosome transport. Endocytosis, intracellular protein transport. Neutrophil degranulation |
| Q9R0J8 | Legumain (EC 3.4.22.34) (Asparaginyl endopeptidase) | Lgmn Prsc1 | 11 | 4 | 0.004 | Has a strict specificity for hydrolysis of asparaginyl bonds. Can also cleave aspartyl bonds slowly, especially under acidic conditions. Required for normal lysosomal protein degradation in renal proximal tubules. Required for normal degradation of internalized EGFR. Plays a role in the regulation of cell proliferation via its role in EGFR degradation. May be involved in |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (Protease, cysteine 1) | | | | | the processing of proteins for MHC class II antigen presentation in the lysosomal/endosomal system |
| B2RYD7 | RCG25591, isoform CRA_a (RGD1311563 protein) (STT3B, catalytic subunit of the oligosaccharyltransferase complex) | Stt3b RGD1311563 rCG_25591 | 6 | 0 | <0.001 | Involved in the pathway protein glycosylation, which is part of Protein modification |
| G3V8M5 | Serine/threonine-protein phosphatase (EC 3.1.3.16) | Ppp4c rCG_39762 | 5 | 0 | <0.001 | Regulation of double-stranded DNA break repair via homologous recombination |
| B0BN52 | Mitochondrial carrier 2 (Mitochondrial carrier homolog 2 (C. elegans) (Predicted), isoform CRA_a) | Mtch2 Mtch2_predicted rCG_26739 | 5 | 1 | 0.002 | Protein localization to mitochondrial membrane |

| | | | | | | |
|---|---|---|---|---|---|---|
| B2RYN6 | AP-1 complex subunit gamma | Ap1g1 rCG_51678 | 4 | 1 | 0.049 | Golgi to lysosome transport. Microtubule cytoskeleton organization |
| Q6ZYN8 | Alpha-2,6-sialyltransferase ST6GalNAc II (ST6 (Alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2) (ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 2) (Fragment) | St6galnac2 siat7B rCG_34304 | 6 | 2 | 0.024 | Protein glycosylation and sialylation. Catalyzes the transfer of N-acetylneuraminyl groups onto glycan chains in glycoproteins |
| G3V9T7 | ATPase Asna1 (EC 3.6.-.-) (Arsenical pump- | Asna1 ASNA1 | 5 | 1 | 0.003 | ATPase required for the post-translational delivery of tail-anchored (TA) proteins to the endoplasmic reticulum. |

| | | | | | | |
|---|---|---|---|---|---|---|
| | driving ATPase) (Arsenite-stimulated ATPase) | rCG_51373 | | | | Recognizes and selectively binds the transmembrane domain of TA proteins in the cytosol. |
| P62859 | 40S ribosomal protein S28 | Rps28 | 1 | 6 | 0.006 | RNA binding and protein translation |
| O70513 | Galectin-3-binding protein (Cyp-C-associated protein) (CyCAP) (Lectin galactoside-binding soluble 3-binding protein) (Mac-2-binding protein) (MAC2BP) (Mac-2 BP) (Protein 90K) | Lgals3bp | 8 | 1 | <0.001 | Promotes integrin-mediated cell adhesion. May stimulate host defense against viruses and tumour cells. |
| A0A0G2JZH0 | Calcium-binding protein 39 | Cab39 | 4 | 0 | <0.001 | Signal transduction by protein phosphorylation. Protein serine/threonine kinase activator |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q9JHW0 | Proteasome subunit beta type-7 (Macropain chain Z) (Multicatalytic endopeptidase complex chain Z) (Proteasome subunit Z) | Psmb7 | 1 | 3 | 0.029 | Component of the 20S core proteasome complex involved in the proteolytic degradation of numerous intracellular proteins. Associates with different regulatory particles. Participates in the ATP-dependent degradation of ubiquitinated proteins. The 26S proteasome plays an important role in protein homeostasis by removing misfolded or damaged proteins that could impair cellular functions, and removing proteins whose functions are no longer required. The 20S proteasome mediates ubiquitin-independent protein degradation required in several pathways including spermatogenesis or generation of a subset of MHC class I-presented antigenic peptides. Displays a trypsin-like activity |
| P17074 | 40S ribosomal protein S19 | Rps19 | 3 | 8 | 0.028 | Required for pre-rRNA processing and maturation of 40S ribosomal subunits |
| P36972 | Adenine phosphoribosyltransferase (APRT) | Aprt | 8 | 0 | <0.001 | Catalyzes a salvage reaction resulting in the formation of AMP, that is energically less costly than de novo synthesis |
| B0BN46 | Glyoxylate and hydroxypyruvate reductase (Grhpr protein) | Grhpr rCG_54768 | 5 | 2 | 0.035 | Oxidoreductase. Oxidation-reduction process. Carboxylic acid binding, glycerate dehydrogenase activity |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (RCG54768, isoform CRA_a) | | | | | |
| Q68FT8 | RCG33981, isoform CRA_a (Serine (Or cysteine) peptidase inhibitor, clade F, member 2) | Serpinf2 rCG_33981 | 6 | 2 | 0.017 | Serine protease inhibitor. The major targets of this inhibitor are plasmin and trypsin |
| P36860 | Ras-related protein Ral-B | Ralb Ral-b | 1 | 4 | 0.021 | Multifunctional GTPase involved in a variety of cellular processes including gene expression, cell migration, cell proliferation, oncogenic transformation and membrane trafficking. Accomplishes its multiple functions by interacting with distinct downstream effectors. Acts as a GTP sensor for GTP-dependent exocytosis of dense core vesicles. Required both to stabilize the assembly of the exocyst complex and to localize functional exocyst complexes to the leading edge of migrating cells. Required for suppression of apoptosis. In late stages of cytokinesis, upon completion of the bridge formation between dividing cells, mediates exocyst recruitment to the midbody to drive abscission |

| | | | | | | |
|---|---|---|---|---|---|---|
| P52759 | 2-iminobutanoate/2-iminopropanoate deaminase (Liver perchloric acid-soluble protein) (L-PSP) (Reactive intermediate imine deaminase A homolog) (Translation inhibitor L-PSP ribonuclease) (UK114 antigen homolog) (rp14.5) | Rida Psp1 | 5 | 0 | <0.001 | Catalyzes the hydrolytic deamination of enamine/imine intermediates that form during the course of normal metabolism. May facilitate the release of ammonia from these potentially toxic reactive metabolites, reducing their impact on cellular components. It may act on enamine/imine intermediates formed by several types of pyridoxal-5'-phosphate-dependent dehydratases including L-threonine dehydratase. May also function as an endoribonuclease, cleaving mRNA phosphodiester bonds of single-stranded RNA. Thereby, may inhibit protein translation |
| A0A077S6M4 | Lysozyme | Lyzl6 Lyzb Lyzl6_predicted rCG_32936 | 4 | 1 | 0.013 | May be involved sperm-egg plasma membrane adhesion and fusion during fertilization. Exhibits bacteriolytic activity *in vitro*. Lysozyme activity |

| | | | | | | |
|---|---|---|---|---|---|---|
| P62271 | 40S ribosomal protein S18 | Rps18 | 2 | 7 | 0.002 | Located at the top of the head of the 40S subunit, it contacts several helices of the 18S rRN |
| Q6AYR1 | RCG52996, isoform CRA_a (Trk-fused gene) (Trk-fused protein) | Tfg rCG_52996 | 4 | 1 | 0.049 | Plays a role in the normal dynamic function of the endoplasmic reticulum (ER) and its associated microtubules. Required for secretory cargo traffic from the endoplasmic reticulum to the Golgi apparatus |
| Q5PQW5 | Calcium-transporting ATPase (EC 3.6.3.8) | Atp2c1 rCG_25874 | 1 | 4 | 0.004 | This magnesium-dependent enzyme catalyzes the hydrolysis of ATP coupled with the transport of calcium |
| D3ZAZ0 | Eukaryotic translation initiation factor 3 subunit M (eIF3m) | Eif3m EIF3M RGD1565840_predicted rCG_27120 | 4 | 0 | <0.001 | Component of the eukaryotic translation initiation factor 3 (eIF-3) complex, required for several steps in the initiation of protein synthesis. The eIF-3 complex stimulates mRNA recruitment and scanning of the mRNA for AUG recognition. The complex is also required for disassembly and recycling of post-termination ribosomal complexes and subsequently prevents premature joining of the 40S and 60S ribosomal subunits prior to initiation. Specifically targets and initiates translation of a subset of mRNAs involved in cell proliferation, including cell cycling, differentiation and apoptosis |

| | | | | | | |
|---|---|---|---|---|---|---|
| P08753 | Guanine nucleotide-binding protein G(k) subunit alpha (G(i) alpha-3) | Gnai3 Gnai-3 | 6 | 1 | 0.004 | Heterotrimeric guanine nucleotide-binding proteins (G proteins) function as transducers downstream of G protein-coupled receptors (GPCRs) in numerous signaling cascades. Signaling by an activated GPCR promotes GDP release and GTP binding. Both GDP release and GTP hydrolysis are modulated by numerous regulatory proteins. Signaling is mediated via effector proteins, such as adenylate cyclase. Inhibits adenylate cyclase activity, leading to decreased intracellular cAMP levels. Stimulates the activity of receptor-regulated K(+) channels. The active GTP-bound form prevents the association of RGS14 with centrosomes and is required for the translocation of RGS14 from the cytoplasm to the plasma membrane. May play a role in cell division |
| B4F7E8 | Niban-like protein 1 (Protein FAM129B) | Fam129b | 5 | 1 | 0.036 | May play a role in apoptosis suppression |
| M0R9L0 | Nascent polypeptide-associated complex subunit alpha | Naca | 3 | 1 | 0.029 | Prevents inappropriate targeting of non-secretory polypeptides to the endoplasmic reticulum (ER). Binds to nascent polypeptide chains as they emerge from the ribosome and blocks their interaction with the signal recognition particle (SRP), which normally targets nascent secretory peptides to the ER. Also reduces the inherent affinity of ribosomes for protein |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | translocation sites in the ER membrane (M sites) (By similarity). Isoform 1 and isoform 2 appear to bind DNA and play roles in transcription. Isoform 1 may function as a specific coactivator for JUN, acting to stabilize the interaction of JUN homodimers with promoter elements |
| Q924K2 | FAS-associated factor 1 | Faf1 | 3 | 1 | 0.029 | Ubiquitin-binding protein. Required for the progression of DNA replication forks |
| P62332 | ADP-ribosylation factor 6 | Arf6 | 4 | 0 | <0.001 | GTP-binding protein involved in protein trafficking that regulates endocytic recycling and cytoskeleton remodelling. Required for normal completion of mitotic cytokinesis. May also modulate vesicle budding and uncoating within the Golgi apparatus. Plays an important role in membrane trafficking, during junctional remodelling and epithelial polarization. Regulates surface levels of adherens junction proteins |
| O88422 | Polypeptide N-acetylgalactosaminyltransferase 5 (Polypeptide GalNAc transferase 5) (GalNAc-T5) (pp-GaNTase 5) | Galnt5 | 4 | 0 | <0.001 | Catalyzes the initial reaction in O-linked oligosaccharide biosynthesis, which involves the transfer of an N-acetyl-D-galactosamine residue to a serine or threonine residue on the protein receptor. |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (Protein-UDP acetylgalactosaminyltransferase 5) (UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase 5) | | | | | |
| Q920A6 | Retinoid-inducible serine carboxypeptidase (Serine carboxypeptidase 1) | Scpep1 Risc | 5 | 1 | 0.025 | May be involved in vascular wall and kidney homeostasis. |
| P61959 | Small ubiquitin-related modifier 2 (SUMO-2) (SMT3 homolog 2) (Sentrin-2) (Ubiquitin-like protein SMT3A) (Smt3A) | Sumo2 Smt3a Smt3h2 | 0 | 3 | <0.001 | Ubiquitin-like protein that can be covalently attached to proteins as a monomer or as a lysine-linked polymer. Post-translational modification on lysine residues of proteins plays an important role in a number of cellular processes such as nuclear transport, DNA replication and repair, mitosis and signal transduction. Polymeric SUMO2 chains are also susceptible to polyubiquitination which functions as a signal for proteasomal degradation of modified proteins. |

| | | | | | | |
|---|---|---|---|---|---|---|
| B2RYQ5 | Enhancer of rudimentary homolog | Erh rCG_20904 | 1 | 4 | 0.013 | May have a role in the cell cycle |
| G3V9K3 | Coxsackie virus and adenovirus receptor-like 1 (Coxsackievirus and adenovirus receptor-like) | LOC100369007 0 Cxadrl1 rCG_25143 | 3 | 0 | 0.001 | Receptor |
| Q5GFD9 | Protein IMPACT (Imprinted and ancient gene protein homolog) | Impact | 4 | 0 | <0.001 | Translational regulator that ensures constant high levels of translation upon a variety of stress conditions, such as amino acid starvation, UV-C irradiation, proteasome inhibitor treatment and glucose deprivation. Plays a role as a negative regulator of kinase activity; impairs eIF-2-alpha phosphorylation and subsequent down-regulation of protein synthesis |
| O35078 | D-amino-acid oxidase (DAAO) (DAMOX) (DAO) | Dao Dao1 | 3 | 1 | 0.046 | Controls the level of D-serine, a neuromodulator in the brain. Has high activity towards D-DOPA and contributes to dopamine synthesis. Possibly acts as a detoxifying agent which removes accumulated D-amino acids during aging. Does not act on acidic amino acids. |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q66HG6 | Carbonic anhydrase 5B, mitochondrial (Carbonate dehydratase VB) (Carbonic anhydrase VB) (CA-VB) | Ca5b Car5b | 1 | 3 | 0.046 | Reversible hydration of carbon dioxide. |
| **Immune-mediated** | | | | | | |
| D3ZJF8 | Fc fragment of IgG-binding protein | Fcgbp | 17 | 6 | <0.001 | Possibly involved in the maintenance of the mucosal structure as a gel-like component of the mucosa. May play an important role in immune protection and inflammation |
| P14740 | Dipeptidyl peptidase 4 (GP110 glycoprotein) (T-cell activation antigen CD26) | Dpp4 Cd26 | 9 | 3 | 0.004 | Cell surface glycoprotein receptor involved in the co-stimulation signal essential for T-cell receptor (TCR)-mediated T-cell activation. Induces T-cell proliferation and NF-kappa-B activation in a T-cell receptor/CD3-dependent manner. Regulates lymphocyte-epithelial cell adhesion. Possibly involved in the promotion of lymphatic endothelial cells adhesion, migration and tube formation. When overexpressed, enhances cell proliferation. Also regulates various physiological processes by cleaving peptides in the circulation, including many chemokines, mitogenic growth factors, neuropeptides and peptide hormones |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | Detected in the serum and the seminal fluid |
| Q5PQW8 | Guanylate binding protein 2 (Guanylate nucleotide binding protein 2) (Guanylate-binding protein 1) | Gbp2 | 4 | 1 | 0.049 | Hydrolyzes GTP to GMP in two cleavage reactions, with the major reaction product being GDP. Promotes oxidative killing and delivers antimicrobial peptides to autophagolysosomes, providing broad host protection against different pathogen classes |
| Q63663 | Guanylate-binding protein 1 (GTP-binding protein 2) (GBP-2) (Interferon-induced guanylate-binding protein 2) (p67) | | | | | |
| B2RZC6 | Ilf2 protein (Interleukin enhancer-binding factor 2) | Ilf2 | 3 | 0 | 0.008 | Immune response |
| **Multi-functional** | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| P18418 | Calreticulin (CALBP) (CRP55) (Calcium-binding protein 3) | Calr | 61 | 74 | 0.035572517069555 | Calcium-binding chaperone that promotes folding, oligomeric assembly and quality control in the endoplasmic reticulum (ER) via the calreticulin/calnexin cycle. This lectin interacts transiently with almost all of the monoglucosylated glycoproteins that are synthesized in the ER |
| | | | | | | Involved in maternal gene expression regulation. May participate in oocyte maturation via the regulation of calcium homeostasis |
| P46462 | Transitional endoplasmic reticulum ATPase (TER ATPase) (Valosin-containing protein) (VCP) | Vcp | 53 | 74 | <0.001 | Involved in the fragmentation and reassembly of Golgi stacks during and after mitosis. Is part of a protein complex that binds ubiquitinated proteins and exports misfolded proteins from the ER to the cytoplasm to be degraded by the proteasome. Involved in a stress-induced mechanism that selectively controls the translocation of stressed/damaged mitochondrial outer-membrane proteins to be degraded by proteasomes. Important for the clearance of ubiquitinated proteins via autophagy. May play a role in the ubiquitin-dependent sorting of membrane proteins to lysosomes where they undergo degradation. Important role in caveolin sorting within cells |
| | | | | | | Involved in the DNA damage response and is recruitment to double-strand breaks |
| | | | | | | Controls the steady-state expression of the IGF1R receptor |

| | | | | | | |
|---|---|---|---|---|---|---|
| P04785 | Protein disulfide-isomerase (PDI) (Cellular thyroid hormone-binding protein) (Prolyl 4-hydroxylase subunit beta) | P4hb Pdia1 | 37 | 72 | <0.001 | Catalyzes the formation, breakage and rearrangement of disulfide bonds. At the cell surface, appears to cleave disulfide bonds of proteins attached to the cell, thus causing structural modifications of proteins. Inside the cell, seems to form/rearrange disulfide bonds of new proteins. At high concentrations, functions as a chaperone that inhibits aggregation of misfolded proteins. At low concentrations, facilitates aggregation (anti-chaperone activity). Shows receptor activity, where activation increases disulfide reductase activity at the plasma membrane, altering the plasma membrane redox state and enhancing cell migration |
| | | | | | | May be involved with other chaperones in the structural modification of the TG precursor in hormone biogenesis |
| | | | | | | Also acts on structural subunits of various enzymes such as prolyl 4-hydroxylase and microsomal triacylglycerol transfer protein MTTP |
| O88767 | Protein/nucleic acid deglycase DJ-1 (Contraception-associated protein 1) (Protein CAP1) | Park7 Cap1 | 31 | 57 | <0.001 | Functions as a protein deglycase that glycates proteins. Deglycates cysteine, arginine and lysine residues in proteins, and thus reactivates these proteins by reversing glycation by glyoxals. Acts on early glycation intermediates to prevent the formation of advanced glycation end-products (AGE) which can cause irreversible damage. Also functions as a nucleotide |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (Fertility protein SP22) | | | | | deglycase that is able to repair glycated guanine in the free nucleotide pool (GTP, GDP, GMP, dGTP) and in DNA and RNA. Is therefore involved in a major nucleotide repair system named guanine glycation repair (GG repair), dedicated to reversing methylglyoxal and glyoxal damage via nucleotide sanitization and direct nucleic acid repair |
| | | | | | | Plays an important role in cell protection against oxidative stress and cell death acting as an oxidative stress sensor and redox-sensitive chaperone and protease |
| | | | | | | It is involved in neuroprotective mechanisms. Regulates astrocyte inflammatory responses |
| P10111 | Peptidyl-prolyl cis-trans isomerase A (PPIase A) (Cyclophilin A) (Cyclosporin A-binding protein) (Rotamase A) (p1B15) (p31) | Ppia | 35 | 45 | 0.040027964263290 5 | Exerts a strong chemotactic effect on leukocytes. Activates endothelial cells (ECs) in a proinflammatory manner by stimulating activation of NF-kappa-B and ERK, JNK and p38 MAP-kinases. In response to oxidative stress, initiates proapoptotic and antiapoptotic signaling in ECs via activation of NF-kappa-B and AKT1 and up-regulation of antiapoptotic protein BCL2 (By similarity). Lipid droplet organisation. Platelet activation and aggregation |

| | | | | | | |
|---|---|---|---|---|---|---|
| P38659 | Protein disulfide-isomerase A4 (Calcium-binding protein 2) (CaBP2) (Endoplasmic reticulum resident protein 70) (ERp70) | Pdia4 Cabp2 Erp70 | 22 | 32 | 0.0175722312589536 | Part of a large chaperone multiprotein complex. Involved in cell redox homeostasis; chaperone-mediated protein folding; response to endoplasmic reticulum stress |

**Table A.2:** Full table of significantly expressed spermatozoal proteins, divided into each of their functional categories. Annotated using the Uniprot protein database (https://www.uniprot.org/)

| Accession number | Protein name | Gene name | Control Mean | Diet Mean | Adjusted p value | Function |
|---|---|---|---|---|---|---|
| **Metabolic proteins** | | | | | | |
| P18163 | Acyl CoA Synthase (Long-chain-fatty-acid--CoA ligase 1) | Acsl1 Acs1 Acsl2 Facl2 | 87 | 79 | 0.049 | Initiates fatty acid utilization by producing acyl CoA synthase from fatty acids. Results in the production of free fatty acids. Activation of long-chain fatty acids for both synthesis of cellular lipids, and degradation via beta-oxidation. Activated by Adiponectin |
| Q68FY0 | Cytochrome b-c1 complex subunit 1, mitochondrial (Complex III subunit 1) (Core protein I) (Ubiquinol-cytochrome-c reductase complex core protein 1) | Uqcrc1 | 16 | 33 | 0.005 | This is a component of the ubiquinol-cytochrome c reductase complex, which is part of the mitochondrial respiratory chain. This protein may mediate formation of the complex between cytochromes c and c1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q66HF1 | NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial | Ndufs1 | 8 | 19 | 0.004 | Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed to belong to the minimal assembly required for catalysis. Complex I s in the transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone (By similarity). This is the largest subunit of complex I and it is a component of the iron-sulfur (IP) fragment of the enzyme. It may form part of the active site crevice where NADH is oxidized. |
| Q06437 | Pyruvate dehydrogenase E1 component subunit alpha, testis-specific form, mitochondrial (PDHE1-A type II) | Pdha2 | 6 | 18 | 0.001 | The pyruvate dehydrogenase complex catalyzes the overall conversion of pyruvate to acetyl-CoA and CO(2), and thereby links the glycolytic pathway to the tricarboxylic cycle.<br>Testis specific isoform |
| Q99NA5 | Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial | Idh3a | 6 | 15 | 0.013 | Catalytic subunit of the enzyme which catalyzes the decarboxylation of isocitrate (ICT) into alpha-ketoglutarate. The heterodimer composed of the alpha (IDH3A) and beta (IDH3B) subunits and the heterodimer composed of the alpha (IDH3A) and gamma (IDH3G) subunits, have considerable basal activity but the |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (Isocitric dehydrogenase subunit alpha) (NAD(+)-specific ICDH subunit alpha) | | | | | full activity of the heterotetramer (containing two subunits of IDH3A, one of IDH3B and one of IDH3G) requires the assembly and cooperative of both heterodimers. |
| Q920L2 | Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial (EC 1.3.5.1) (Flavoprotein subunit of complex II) (Fp) | Sdha | 4 | 12 | 0.019219187825555 9 | Flavoprotein (FP) subunit of succinate dehydrogenase (SDH) that is involved in complex II of the mitochondrial electron transport chain and is responsible for transferring electrons from succinate to ubiquinone (coenzyme Q). Can act as a tumor suppressor. |

| | | | | | | |
|---|---|---|---|---|---|---|
| P29410 | Adenylate kinase 2, mitochondrial (AK 2) (ATP-AMP transphosphorylase 2) (ATP:AMP phosphotransferase) (Adenylate monophosphate kinase) | Ak2 | 5 | 12 | 0.037 | Catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP. Plays an important role in cellular energy homeostasis and in adenine nucleotide metabolism. Adenylate kinase activity is critical for regulation of the phosphate utilization and the AMP de novo biosynthesis pathways. Plays a key role in hematopoiesis. |
| P18886 | Carnitine O-palmitoyltransferase 2, mitochondrial (EC 2.3.1.21) (Carnitine palmitoyltransferase II) (CPT II) | Cpt2 Cpt-2 | 3 | 10 | 0.042 | Involved in the pathway fatty acid beta-oxidation, which is part of Lipid metabolism. Fatty acid metabolism, lipid metabolism, transport. |

| | | | | | | |
|---|---|---|---|---|---|---|
| Q5XIH3 | NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial | Ndufv1 rCG_47623 | 3 | 9 | 0.013394238357001 2 | Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed to belong to the minimal assembly required for catalysis. Complex I s in the transfer of electrons from NADH to the respiratory chain. |
| Q6AYK3 | Inositol-3-phosphate synthase 1 (IPS 1) (EC 5.5.1.4) (Myo-inositol 1-phosphate synthase) (MI-1-P synthase) (MIP synthase) | Isyna1 Ino1 | 2 | 8 | 0.036581979503473 9 | Key enzyme in myo-inositol biosynthesis pathway that catalyzes the conversion of glucose 6-phosphate to 1-myo-inositol 1-phosphate in a NAD-dependent manner. Rate-limiting enzyme in the synthesis of all inositol-containing compounds. |

| | | | | | | |
|---|---|---|---|---|---|---|
| A0A0G2K502 | Malic enzyme | Me2 | 2 | 8 | 0.004176983831624 42 | Catalyze the oxidative decarboxylation of malate to produce pyruvate and carbon dioxide with the reduction of NAD(P) to NAD(P)H. ME2 is a mitochondrial isoform with a dual cofactor specificity for NAD and NADP, but has a higher affinity for NAD. Although the malic enzyme reaction is reversible, the flux of at least the cytosolic malic enzyme reaction is thought to be only in the direction of pyruvate formation in vivo |
| P00564 | Creatine kinase M-type (EC 2.7.3.2) (Creatine kinase M chain) (Creatine phosphokinase M-type) (CPK-M) (M-CK) | Ckm Ckmm | 0 | 5 | 0.000179723636398 417 | Reversibly catalyzes the transfer of phosphate between ATP and various phosphogens (e.g. creatine phosphate). Creatine kinase isoenzymes play a central role in energy transduction in tissues with large, fluctuating energy demands, such as skeletal muscle, heart, brain and spermatozoa. |

| | | | | | | |
|---|---|---|---|---|---|---|
| B2GV06 | Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial (EC 2.8.3.5) (3-oxoacid CoA-transferase 1) (Somatic-type succinyl-CoA:3-oxoacid CoA-transferase) (SCOT-s) | Oxct1 Oxct Scot | 0 | 4 | 0.010185455941172 5 | Key enzyme for ketone body catabolism. Transfers the CoA moiety from succinate to acetoacetate. Formation of the enzyme-CoA intermediate proceeds via an unstable anhydride species formed between the carboxylate groups of the enzyme and substrate (By similarity). {ECO:0000250\|UniProtKB:P55809}. |
| P13803 | Electron transfer flavoprotein subunit alpha, mitochondrial (Alpha-ETF) | Etfa | 0 | 3 | 0.027434121665561 5 | Heterodimeric electron transfer flavoprotein that accepts electrons from several mitochondrial dehydrogenases, including acyl-CoA dehydrogenases, glutaryl-CoA and sarcosine dehydrogenase. It transfers the electrons to the main mitochondrial respiratory chain via ETF-ubiquinone oxidoreductase (ETF dehydrogenase) (Probable). Required for normal mitochondrial fatty acid oxidation and normal amino acid metabolism. |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Structural proteins** | | | | | | |
| M0R8B6 | Tubulin beta chain | Tubb1 | 38 | 26 | 0.003 | Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain. |
| G3V885 | Myosin-6 | Myh6 | 1 | 25 | <0.001 | Actin filament binding, protein kinase binding |
| P08733 | Myosin regulatory light chain 2 (MLC-2) (MLC-2v) (Myosin light chain 2) | Myl2 Mlc2 | 5 | 22 | <0.001 | Contractile protein that plays a role in heart development. Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to actin binding sites to sustain thin filament activation as a means to fine-tune myofilament calcium sensitivity to force. During cardiogenesis plays an early role in cardiac contractility by promoting cardiac myofibril assembly. |
| P16409 | Myosin light chain 3 (Myosin alkali light chain 1, ventricular) (MLCIV) | Myl3 Mlc1v | 5 | 15 | 0.003 | Regulatory light chain of myosin. Does not bind calcium. |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (Myosin light chain 1, slow-twitch muscle B/ventricular isoform) (MLC1SB) (Ventricular myosin light chain 1) (rVMLC1) | | | | | |
| Q4PP99 | Cardiac troponin C (Troponin C1, slow skeletal and cardiac type) | Tnnc1 | 1 | 13 | <0.001 | Cytoskeletal protein. Troponin is the central regulatory protein of striated muscle contraction. Actin filament binding. |
| Q6AXV2 | Tektin-4 | Tekt4 Tek4 | 4 | 12 | 0.020 | Structural component of ciliary and flagellar microtubules. Forms filamentous polymers in the walls of ciliary and flagellar microtubules. |
| D4A0A0 | Dynein light chain roadblock | Dynlrb2 | 16 | 10 | 0.044 | Acts as one of several non-catalytic accessory components of the cytoplasmic dynein 1 complex that are thought to be involved in linking dynein to cargos and to adapter proteins that regulate |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | dynein . Cytoplasmic dynein 1 acts as a motor for the intracellular retrograde motility of vesicles and organelles along microtubules. |
| P51886 | Lumican (Keratan sulfate proteoglycan lumican) (KSPG lumican) | Lum Lcn Ldc | 0 | 5 | <0.001 | Extracellular matrix structural component. Collagen binding. |
| G3V8L9 | Caveolae-associated protein 1 (Polymerase I and transcript release factor (Predicted)) | Cavin1 Ptrf Ptrf_predicted rCG_32735 | 0 | 4 | 0.004 | Promotes ribosomal transcriptional activity in response to metabolic challenges in the adipocytes and plays an important role in the formation of the ribosomal transcriptional loop. Translocates to the cytoplasm from the caveolae upon insulin stimulation (By similarity). Colocalizes with calveolin-1 in lipid rafts in adipocytes. |
| P23693 | Troponin I, cardiac muscle (Cardiac troponin I) | Tnni3 Ctni Tni | 0 | 3 | 0.004 | Troponin I is the inhibitory subunit of troponin, the thin filament regulatory complex which confers calcium-sensitivity to striated muscle actomyosin ATPase activity. |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Reproduction specific** | | | | | | |
| Q6AYW7 | Capping protein (Actin filament) muscle Z-line, alpha 3 (F-actin-capping protein subunit alpha-3) | Capza3 | 1 | 5 | 0.011 | F-actin-capping proteins bind in a Ca(2+)-independent manner to the fast growing ends of actin filaments (barbed end) thereby blocking the exchange of subunits at these ends. Unlike other capping proteins (such as gelsolin and severin), these proteins do not sever actin filaments. |
| **Regulatory** | | | | | | |
| Q6AXN7 | 5'-nucleotidase, cytosolic IB | Nt5c1b | 170 | 157 | 0.003 | Adenosine metabolic process |
| P02091 | Hemoglobin subunit beta-1 (Beta-1-globin) (Hemoglobin beta chain, major-form) (Hemoglobin beta-1 chain) | Hbb | 70 | 62 | 0.049 | Involved in oxygen transport from the lung to the various peripheral tissues |
| P63039 | 60 kDa heat shock protein, mitochondrial | Hspd1 Hsp60 | 16 | 29 | 0.050 | Chaperonin implicated in mitochondrial protein import and macromolecular assembly. Together with Hsp10, facilitates the correct folding of imported proteins. May also prevent misfolding |

| | | | | | | |
|---|---|---|---|---|---|---|
| | (60 kDa chaperonin) (Chaperonin 60) (Heat shock protein 60) | | | | | and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix. |
| Q68FS4 | Cytosol aminopeptidase (Leucine aminopeptidase 3) (LAP-3) | Lap3 | 3 | 14 | <0.001 | Presumably involved in the processing and regular turnover of intracellular proteins. Catalyzes the removal of unsubstituted N-terminal amino acids from various peptides. |
| Q6PDU6 | Beta-glo | Hbb-b1 MGC72973 | 25 | 18 | 0.049 | Hemoglobin complex. Glutathione metabolic process, oxygen transport |
| D4A9P2 | Armadillo-like helical domain-containing 1 | Armh1 RGD1563714 | 4 | 11 | 0.046 | Not classified |
| Q0VGK3 | Glycerate kinase (EC 2.7.1.31) | Glyctk | 1 | 4 | 0.049444156941281 1 | Fructose catabolic process |

| | | | | | | |
|---|---|---|---|---|---|---|
| P97601 | Chaperonin 10 | Hspe1 CPN10 Hspd1 | 2 | 7 | 0.048851707654324 4 | Co-chaperonin implicated in mitochondrial protein import and macromolecular assembly. Together with Hsp60, facilitates the correct folding of imported proteins. May also prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix. |
| **Multifunctional** | | | | | | |
| Q6IMF3 | Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1) (K1) (Type-II keratin Kb1) | Krt1 Kb1 | 6 | 2 | 0.008 | May regulate the activity of kinases such as PKC and SRC via binding to integrin beta-1 (ITB1) and the receptor of activated protein C kinase 1 (RACK1). In complex with C1QBP is a high affinity receptor for kininogen-1/HMWK. |