Investigating physiological effects of weight loss on male fertility

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Thesis submitted for the degree of Doctor or Philosophy, Imperial College

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March 2022

Abstract

Infertility is an emotionally devastating condition for a couple. It is defined as the inability to conceive following 1 year of regular unprotected intercourse. Infertility affects 15% of couples with nearly 50% of cases due to poor sperm quality in the male partner. i.e. 'male factor infertility'. There are currently no approved pharmacological therapies to directly stimulate spermatogenesis; anti-oestrogens and aromatase inhibitors have limited effectiveness for the treatment of oligospermia and their usage is not supported by current clinical guidelines. Consequently, the only therapeutic option for male factor infertility is assisted reproductive technologies (ART), such as intra-cytoplasmic sperm injection (ICSI) which although effective are resource limiting and unaffordable for many couples worldwide. Therefore, there exists an important and unmet need to develop practical and cost-effective therapies for male factor infertility.

Over the last 50 years, whilst sperm quality has declined, obesity has doubled in prevalence. Evidence suggests an association between obesity and male infertility, which makes weight loss a plausible answer to this rising endemic problem. Currently, bariatric surgery is the most effective treatment for obesity leading to major weight loss. However, effects on semen parameters are controversial, with some studies suggesting that the acute starvation-like state induced by bariatric surgery paradoxically reduces sperm function. Recent observational reports have suggested that milder dietary weight loss is associated with improved semen quality and DNA fragmentation index (DFI) in men with obesity and infertility. Low energy diet (LED) is a safe, well-tolerated and established method of achieving modest weight loss which could therefore provide a novel, non-pharmacological therapy for men with obesity-related male factor infertility. However to date, there are currently no prospective randomised controlled studies investigating whether weight loss via LED can improve sperm quality in obese men. Additionally, it is unclear what level of weight loss would be ideal to optimise sperm quality in obese men.

This thesis outlines the first ever three randomised controlled studies investigating the physiological effects of weight loss by LED on sperm quality in obese fertile (study 1 and 2) and infertile men (study 3) respectively. I hypothesized that some, but not all, degrees of weight loss would significantly improve sperm concentration in men with obesity, with a potential threshold of weight loss in men leading to improvements

in sperm quality. I have measured novel molecular markers, such as seminal reactive oxygen species (ROS) and DFI, associated with male infertility in obese men undergoing weight loss.

Collectively, these results will extend our understanding of the physiological effects of weight loss on sperm quality in obese men. This could potentially lead to larger studies determining the effect of weight loss on live birth rates in couples affected by obesity-related male infertility.

Declaration of originality and contributors

Declaration of Originality: The described work in this thesis is my own. Any collaboration and assistance is outlined below. Contributors are within Section of Investigative Medicine & Endocrinology, Imperial College London unless stated otherwise. Academic collaborators included Prof Gary Frost, Prof Anthony Leeds and Prof Adrian Brown who provided nutrition advise on the LED for the 3 included studies. Semen analysis for all 3 studies was performed by Andrology Department, Hammersmith Hospital, Imperial College Healthcare NHS Trust. All serum samples were analysed by Department of Biochemistry, Hammersmith Hospital, Imperial College Healthcare NHS Trust. I was the principal recruiter and led on the 3 studies. I also analysed data for the studies presented in this thesis.

Chapter 2: The ethics of this study was designed by Drs Channa N Jayasena and Natasa Dimakopoulou. I co-led this study with Drs. Natasa Dimakopoulou. Dr Emad Sindi helped with participant screening visits. DNA fragmentation analysis using the COMET assay was carried out by Prof Sheena Lewis' team (Belfast). Cambridge Weight Plan Ltd, an academic collaborator, supplied the LED products for the study. I wrote the first draft of the manuscript (as joint first author) which is being submitted reporting the outcomes of the study.

Chapter 3: I led on this study with the supervision of Dr Channa N Jayasena. Drs. Thilipan Thaventhiran and Nikoleta Papanikolaou helped with recruitment and study visits. Drs Seraphina Luo and Sara Abou Sherif assisted with screening and scheduling participant visits. DNA fragmentation using the TUNEL assay was performed with the expertise of Ms Dalia Khalifa and Dr Emad Sindi. Cambridge Weight Plan supplied the LED products for the study. Manuscript for publication is being prepared (1st author).

Chapter 4: I led on this study with the supervision of Dr Channa N Jayasena. I performed all the recruitment and study visits with the assistance of Drs. Thilipan Thaventhiran, Nikoleta Papanikolaou, Olivia Holtermann Entwistle, Seraphina Luo and Sara Abou Sherif. I performed all reactive oxygen species using chemiluniscence assay. Lighterlife Ltd supplied the LED products for the study. Manuscript for publication is being prepared (1st author).

My PhD was funded by Imperial Healthcare Charity Fellowship and BRC.

Declaration of copyright

Acknowledgements

First and foremost, I would like to express my sincerest thanks to Dr. Channa N Jayasena for his continued support, guidance and encouragement throughout my PhD. It has been a huge privilege to work with my co-supervisors Prof Waljit Dhillo and Prof Suks Minhas.

I would also like to thank the wonderful staff of the Andrology Department at Hammersmith Hospital for their tireless assistance with my work. I am also extremely grateful to our academic collaborators for their kind help and expert advice.

I have been privileged to work with a number of excellent colleagues, and would like to specially thank Dr Anastasia Dimakopoulou, Dr Emad Sindi, Dr Thilipan Thaventhiran and Dr Nikoleta Papanikolaou for their continued dedication and hard work in helping with the clinical studies.

Thank you to all my friends who have been incredibly supportive and a sounding-board during these years.

This thesis is dedicated to my family, in particular my mum Bharti, for all the love, care and unwavering support.

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Abbreviations

ACE2	Angiotensin converting enzyme 2
ACS	Average Comet Score
AMH	Anti-Mullerian hormone
ANOVA	Analysis of Variance
ART	Assisted reproductive technology
AUC	Area Under the Curve
BMI	Body Mass index
BP	Blood pressure
CI	Confidence interval
CHH	Congenital hypogonadotropic hypogonadism
CRN	Clinical research network
CWP	Cambridge weight plan
DBP	Diastolic blood pressure
DFI	DNA fragmentation index
DHEAS	Dehydroepiandrosterone sulphate
DNA	Deoxyribonucleic acid
E2	Oestradiol
EDC	Endocrine Disrupting Chemical
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Oestrogen receptor
ESHRE	European Society of Human Reproduction and Embryology
FAI	Free Androgen Index
FSH	Follicle-stimulating hormone
FT	Free testosterone
GnRH	Gonadotropin releasing hormone
HbA1c	Glycated haemoglobin
HCG	Human Chorionic Gonadotropin
HCS	High Comet Score
HDL	High-density lipoprotein
HFD	High fat diet
HPT	Hypothalamic-pituitary-testicular
HTN	Hypertension
ICHNT	Imperial College Healthcare NHS Trust
ICRF	Imperial College Research Facility

ICSI	Intracytoplasmic Sperm Injection
IHH	Idiopathic Hypogonadotropic Hypogonadism
IIEF	International Index of Erectile dysfunction
INSL-3	Insulin-like Peptide 3
IQR	Inter-quartile Range
IVF	In vitro fertilisation
KISS1	Kisspeptin
KISS1R	Kisspeptin receptor
LBM	Lean body mass
LCS	Low Comet Score
LDL	Low-density lipoproteins
LED	Low Energy Diet
LH	Luteinizing hormone
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NIHR	National Institute for Health Research
NKB	Neurokinin B
NOA	Non obstructive azoospermia
OAT	Oligo-astheno-teratozoospermia
OHSS	Ovarian hyperstimulation syndrome
OR	Odds ratio
ORP	Oxidation reduction potential
PCR	Polymerase chain reaction
PI	Principal investigator
PPE	Personal Protective Equipment
RCT	Randomized controlled trial
RIA	Radioimmunoassay
RLU	Relative light years
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RPL	Recurrent pregnancy loss
RYGB	Roux-en-Y Gastric Bypass
SBP	Systolic blood pressure
SD	Standard deviation
SCSA	Sperm Chromatin Structure Assay
SEM	Standard error of mean

SHBG	Sex hormone binding globulin
SHIM	Sexual Health Inventory for Men Questionnaire
T2DM	Type 2 Diabetes Mellitus
TDR	Total diet replacement
TT	Total testosterone
TMC	Total Motile Count
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UCL	University College of London
VLED	Very Low Energy diet
WC	Waist circumference
WHO	World Health Organization

Chapter 1: General Introduction

1.1 Testicular functions

The testes are essential for male reproduction through their two prime functions, namely spermatogenesis and steroidogenesis. These testicular functions are dependent on the hypothalamic-pituitary-gonadal (HPG) axis.

1.2 Spermatogenesis

Spermatogenesis is a spatio-temporally coordinated process whereby male germ cells (spermatogonia) in the testes develop into mature spermatozoa through the processes of mitosis, meiosis and cell differentiation. It is a continuous process initiated at puberty and continues throughout life. The time taken for spermatogenesis is species-specific (Heller and Clermont, 1963), however, there is a large individual biological variability within species in the duration of spermatogenesis. The entire spermatogenic process to produce ejaculated mature spermatozoa in men takes 64 +/- 8 (range 42 to 76) days using a stable isotope-mass spectrometric method (Misell et al., 2006). This is an important consideration when assessing the therapeutic impact of any lifestyle/medical changes in subsequent semen analysis.

1.2.1 Seminiferous tubules

The male testis consists of lobules containing highly convoluted seminiferous tubules that are lined by Sertoli cells and supported by interstitial tissue that contains Leydig cells. In men, spermatogenesis occurs in the recesses of these Sertoli cells located along the entire length of the seminiferous tubules of the testes in a helical arrangement (Griswold, 2016). Several stages of spermatogenesis are represented in a single seminiferous tubule cross-section (Gilbert, 2006) [Figure 1.1] creating a ‘spermatogenic wave’ of continuous spermatozoa production (Hess and Franca, 2008). Spermatogenesis occurs in three distinct phases which are regulated by several cell types, hormones, genetic and epigenetic factors (Nishimura and L’Hernault, 2017):

a) Mitosis (Spermatocytogenesis): the differentiation of self-renewing **spermatogonial** stem cells into **primary spermatocytes** via mitotic cell division.

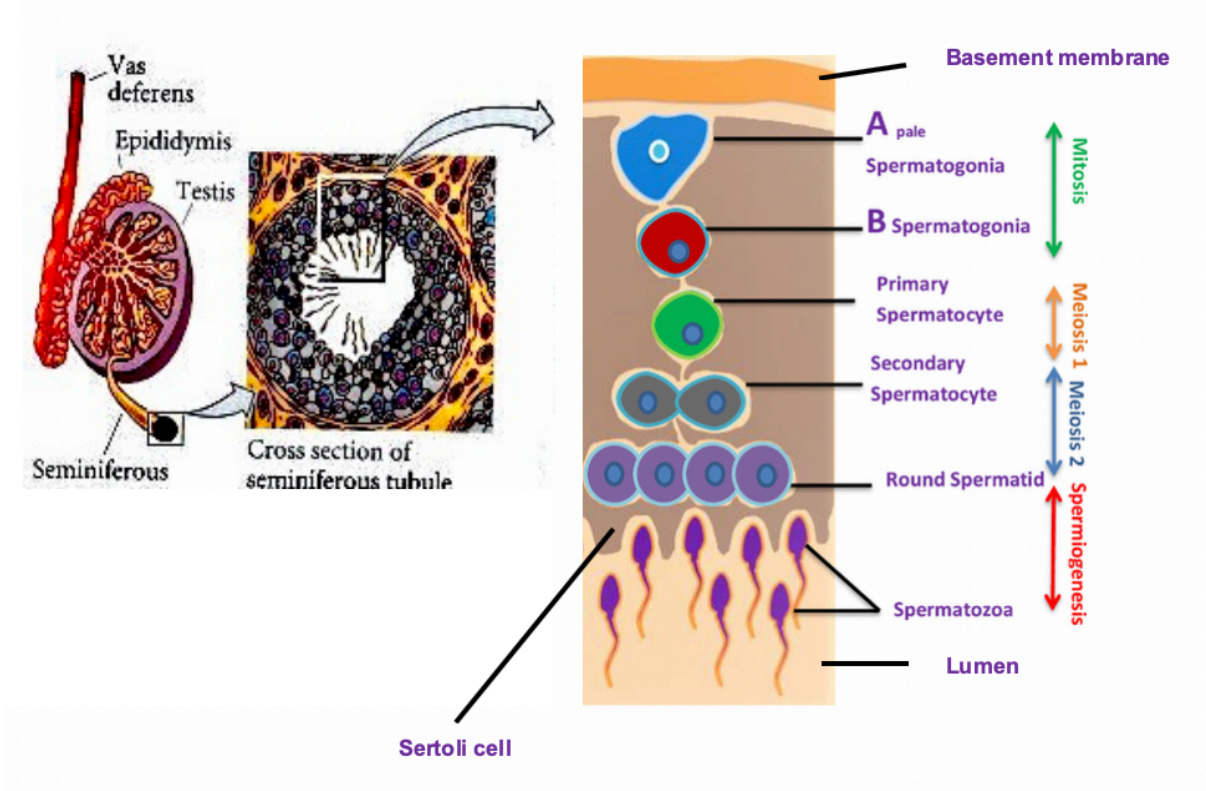
b) Meiosis: Diploid **primary spermatocytes** go through the first meiotic division to become **secondary spermatocytes,** which then undergo a second meiotic division to produce haploid **spermatids** that are connected to one another through their cytoplasmic bridges or syncytium.

c) Spermiogenesis: The final cytodifferentiation of spherical **spermatids** into elongated **spermatozoa.** As sperm cells mature, they progress toward the lumen of the seminiferous tubules.

Different metabolic reactions take place during the distinct sperm cell developmental stages for energy metabolism (Melendez-Hevia et al., 1996). Sertoli cells rely on β-oxidation of fatty acids for their internal energy consumption (Rato et al., 2014a). Conversely, germs cells rely on lactate generated from pyruvate to cover their energy requirements (Crisóstomo et al., 2017). Mature spermatozoa rely on glucose for energy production and partially on β-oxidation (Rato et al., 2014a). However it remains unknown how these reactions are regulated within the various testicular compartments.

Near the basement membrane of the seminiferous tubules, specialised adhesion junctions are formed between adjacent Sertoli cells that form the important blood-testis-barrier (BTB) which is essential for optimal spermatogenesis and protects the highly immunogenic spermatozoa (Ibtisham et al., 2017). These cellular interactions are essential to allow orientation of germ cells during differentiation as disorientation may induce germ cell apoptosis (Chang et al., 2011). Sertoli cells nourish and provide structural support to germ cells whilst Leydig cells synthesise steroid hormones (mainly testosterone through the process of steroidogenesis) in the interstitial compartment of the testes. These processes are highly dependent on the HPT axis (see section 1.3) (Matsumoto and Bremner, 1987).

Figure 1-1: Illustration of a section of the seminiferous tubule showing the relationship between Sertoli cells and spermatogenesis.

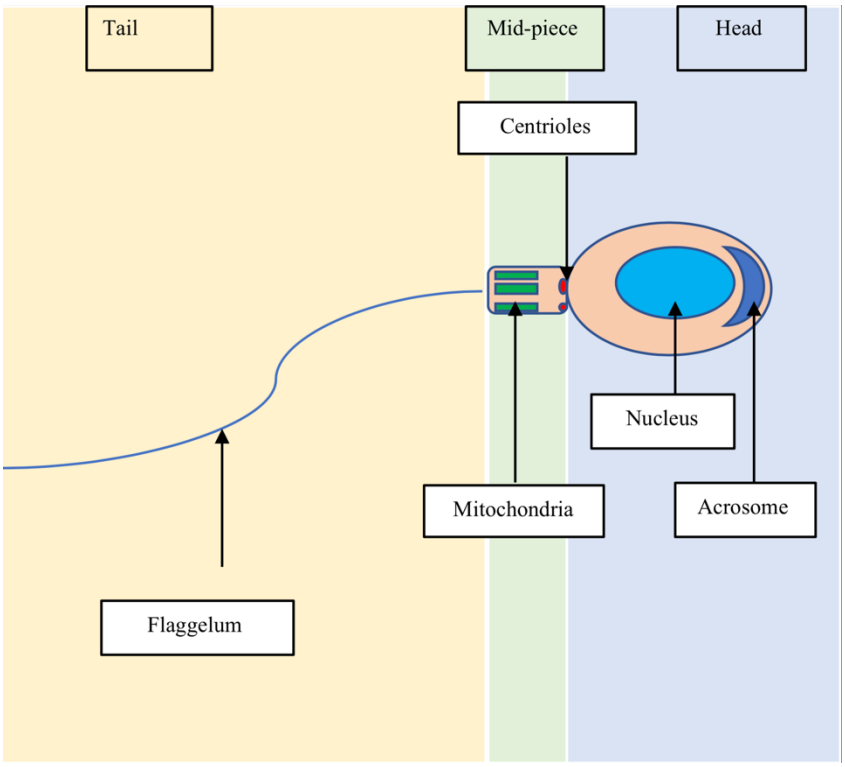


Diploid germ cells (blue colour) near the basal epithelium of the ST undergo mitosis to produce diploid primary spermatocytes (green colour). Primary spermatocytes undergo meiosis I, resulting in secondary spermatocytes (grey colour). Secondary spermatocytes undergo meiosis II, producing haploid round spermatids (purple colour), resulting in spermatozoa by spermiogenesis. Adapted from (Gilbert, 2006) and (Ibtisham et al., 2017).

1.2.2 Spermatozoa

Mammalian spermatozoa have a unique morphology to help migration within the female tract to fertilise an oocyte (female gamete) (García-Vázquez et al., 2016) [Figure 1.2]. The head contains the nucleus with genetic material and an acrosome which contains proteolytic enzymes which help it to penetrate the oocyte. The mid-piece or neck contains mitochondria for adenosine triphosphate (ATP)/energy production. The tail contains flagellum for forward motility. Furthermore, these spermatozoa undergo multiple steps to successfully fertilise the oocyte in female reproductive tract. Firstly, spermatozoa migrate from seminiferous tubules in the testis to the epididymis [Figure 1.1] whereby post-testicular *maturation* occurs. Matured spermatozoa are stored there until ejaculation. Secretions from male reproductive accessory glands, such as the seminal vesicles, are mixed with sperm to form 'seminal plasma'(Druart and Graaf, 2018). Upon entry into the female tract, sperm cells undergo rapid metabolic changes, collectively termed '*capacitation*', which prepare the sperm cells to reach and fertilise the oocyte.

Figure 1-2: Structure of a mature spermatozoon



1.3 Hormonal regulation of spermatogenesis

The hypothalamic-pituitary-testicular axis (HPT) regulates the testicular functions of steroidogenesis and spermatogenesis in both humans and animals (Corradi et al., 2016) [Figure 1.3]. George Harris' (Harris, G.W, 1955) first proposed the neural mechanisms controlling the pituitary-gonadal axis nearly 66 years ago. He proposed that 'nerve fibres from the hypothalamus liberate some humoral substance(s) into the capillaries of the primary plexus in the median eminence and that this substance is carried by the portal vessels to excite or inhibit the cells of the pars distalis.' After several years of research efforts, this hypothalamic humoral substance, gonadotropin-releasing hormone (GnRH), was isolated independently from bovine and ovine brain to regulate the synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) respectively (Amoss et al., 1971; Matsuo et al., 1971). In 1978, Knobil's laboratory demonstrated that intermittent GnRH stimulation of the pituitary was a prerequisite for the maintenance of physiological anterior pituitary LH and FSH secretion (Belchetz et al., 1978) (Knobil, 1980). Conversely, continuous GnRH treatment paradoxically inhibited gonadotropin release. Several recent studies have since confirmed that hypothalamic network of kisspeptin/neurokinin B (NKB)/dynorphin A (KNDy) co-expressing neurones mediates pulsatile secretion of GnRH into the hypophyseal-portal circulation (Lehman et al., 2010) (Figure 1.3). Hypothalamic pulsatile secretion of GnRH in turn stimulates, FSH and LH, from the anterior pituitary to stimulate the testis for spermatogenesis and testosterone production respectively. FSH and LH are heterodimers with structural similarities; each consists of α and β subunits with the α subunit identical in both hormones (Pierce and Parsons, 1981). The β subunit provides structural and biochemical specificity for receptor interaction (Matsuo et al., 1971). FSH stimulates Sertoli cell function and production of inhibin B which is a marker of spermatogenesis (Kathrins and Niederberger, 2016). LH acts on Leydig cells stimulating enzymatic conversion of precursor cholesterol to testosterone (TT) creating a high local concentration of testosterone in the testis compared to testosterone levels in the circulation (Smith and Walker, 2014). Testosterone and Inhibin B have negative feedback effects at pituitary and hypothalamic levels. Optimal spermatogenesis requires the action of both testosterone and FSH, with derangements at any of these steps could lead to male infertility (Clavijo and Hsiao, 2018).

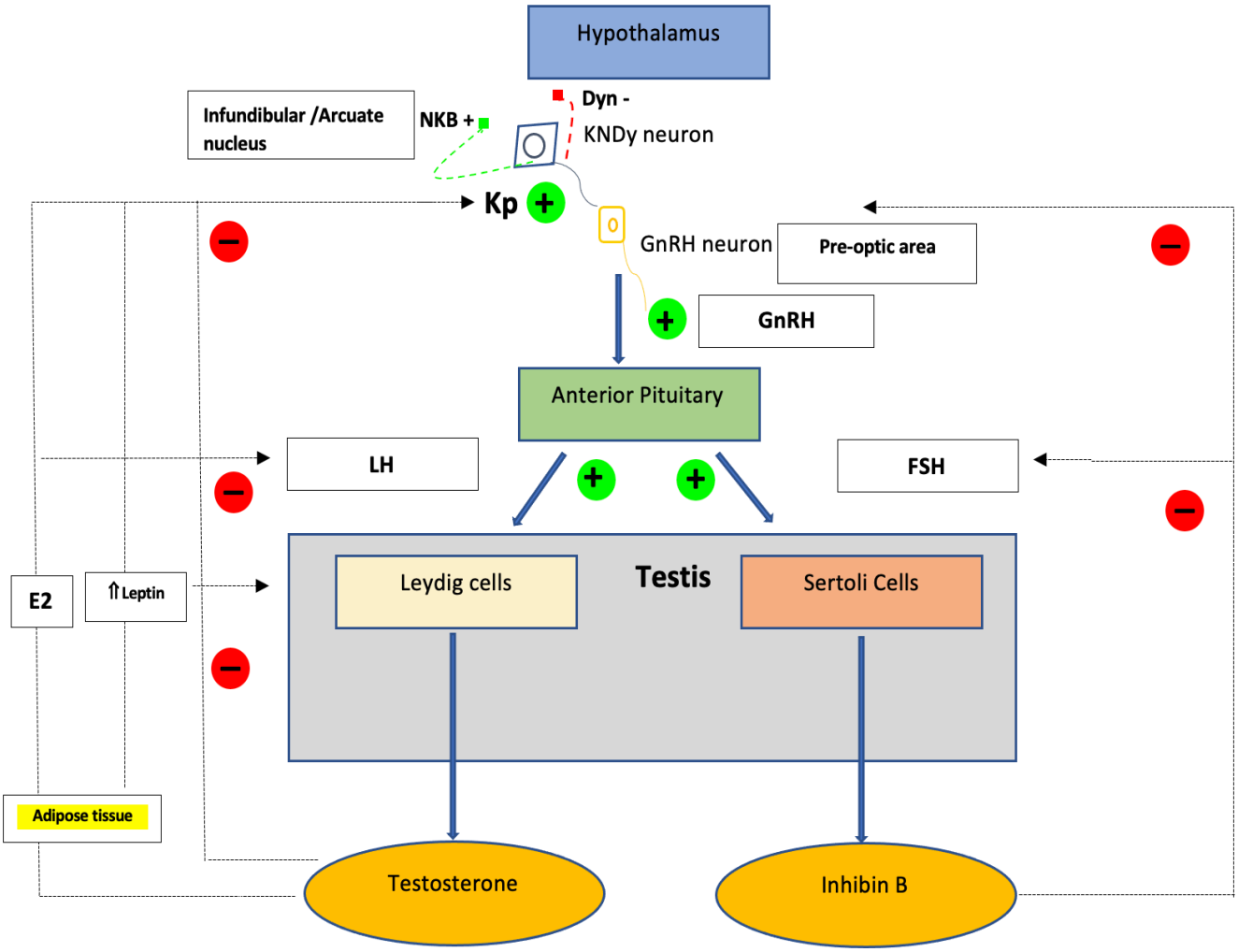
Multiple other hormones and their receptors (Foresta et al., 2008) are observed in testes and spermatozoa of animals and humans such as kisspeptin (Sharma et al., 2020b), leptin (Soyupek et al., 2005; Tena-Sempere and Barreiro, 2002), insulin (Schoeller et al., 2012), oestradiol and insulin like factor 3 (INSL-3) ((Cannarella et al., 2018; Ferlin et al., 2006) with postulated paracrine and endocrine roles in regulating testicular functions.

1.3.1 Kisspeptin & KNDy (kisspeptin/neurokinin B/dynorphin A) neurones

Despite the central role of GnRH neurones in HPT axis (Figure 1.3), they do not express receptors for leptin, insulin or oestradiol receptor alpha, ERα, required for feedback for gonadotropin secretion. Recent evidence suggests that the major hypothalamic network namely KNDy neurons in the infundibular (humans)/arcuate (rodent and ruminant) nucleus are major regulators of hypothalamic GnRH neuron activity (Lehman et al., 2010). These neurones are strongly conserved across a range of species from rodents to humans (Ohkura et al., 2009). With direct projections onto GnRH neurones, KNDy neurones incorporate sex steroid, environmental and metabolic cues (e.g. from leptin) to regulate GnRH secretion (Pinilla et al., 2012). KNDy neurones mediate a paracrine *stimulatory* role of NKB and *inhibitory* action of Dyn to coordinate the pulsatile release of kisspeptin, which in turn drives the pulsatile secretion of GnRH and LH (Figure 1.3) (Nagae et al., 2021). Therefore, sex steroid negative feedback on KNDy neurones leads to suppression of kisspeptin and NKB and stimulation of Dyn, which act synergistically to reduce the activity of GnRH neurones, and subsequent gonadotropin secretion (Yen et al., 1985). Perturbations in the hypothalamic *KISS1/KISS1R* system can lead to multiple reproductive disorders; inactivating mutations cause lack of pubertal maturation and hypogonadotropic hypogonadism, conversely activating mutations cause precocious puberty (Roux et al., 2003; Seminara et al., 2003).

There is also evidence of peripheral *KISS1*/*KISS1R* expression and peptide distribution in the cells of the testes of multiple animal species and humans. However, variability is observed in the testicular cell types expressing *KISS1/KISS1R*, with direct testicular action on steroidogenesis or spermatogenesis yet to be determined in both animals and humans (Sharma et al., 2020b).

Figure 1-3: Hypothalamic Pituitary Testicular Axis.



GnRH: Gonadotropin Releasing Hormone; FSH: Follicle stimulating hormone; LH: Luteinizing hormone; NKB: Neurokinin B; Dyn: Dynorphin A, Kp: kisspeptin; KNDy: kisspeptin, neurokinin B, dynorphin A neurones, E2 oestradiol

1.3.2 Leptin

Leptin is a key metabolic signal regulating reproduction. Leptin is a circulating glycoprotein encoded by the *ob* gene in adipose tissue such that circulating serum leptin levels correlate positively with adiposity. The central role of leptin at the hypothalamus has been studied such that congenital leptin deficiency (for example by loss of mutations in *ob* or *ObR* genes) is associated with early onset obesity, hyperphagia, and delayed onset of puberty due to hypogonadotropic hypogonadism (Farooqi et al., 2007; Guy-Grand et al., 1998). There is evidence of leptin 'cross-talk' with other central neuronal mediators involved in energy intake and reproductive function. In mice, *OB-R* has been co-localised with agouti-related peptide/neuropeptide Y (AgRP/NPY, orexigenic neuropeptides), proopiomelanocortin (POMC, anorexigenic neuropeptide), and *KISS1* neurones. This suggests an indirect action of leptin in regulating gonadotropin secretion by modulating KNDy neurones in the arcuate nucleus, with metabolic cues from the above orexigenic and anorexigenic neuropeptides (Wahab et al., 2018). Furthermore, animal studies have shown a local secretion of leptin at the pituitary with *Ob-R* also expressed in anterior pituitary suggestive of potential paracrine and autocrine actions of leptin in gonadotropin secretion (Jin et al., 2000). However, the exact neuronal mechanisms of leptin action at the hypothalamus and pituitary and its implications are yet to be unequivocally confirmed.

In addition to the central actions of leptin, there is recent evidence of leptin expression in seminiferous tubules and spermatozoa in humans, and leptin receptor has been isolated from Sertoli, Leydig and testicular germ cells in rodents (El-Hefnawy et al., 2000), and Leydig cells and ST in humans (Ishikawa et al., 2007) suggesting that leptin may also directly modulate testicular functions.

1.3.3 Insulin

Insulin secreted by pancreatic β-cells stimulates hypothalamo-pituitary function. Several studies demonstrate that insulin replacement increases pulsatile LH secretion in rodent models with diabetes (Dong et al., 1991). Conversely, knockout of the insulin receptor gene in the hypothalamus impairs LH production (Brüning et al., 2000). Insulin is also expressed in the testes and regulates Leydig cell function, promoting steroidogenesis during puberty. Similarly, insulin is important for Sertoli cell function, as it mediates glucose transport and lactate synthesis, which is an important substrate for germ cells and has an anti-apoptotic effect (Mita et al., 1985). Insulin is essential for sperm plasma membrane integrity and acrosome reaction required for

fertilisation (Silvestroni et al., 1992). An *in vitro* study by Lampiao et al observed that washed human spermatozoa, from normozoospermic donors, treated with insulin and leptin significantly increased sperm motility and acrosome reaction compared to non-treated spermatozoa (Lampiao and Du Plessis, 2008). Therefore, insulin increases LH secretion from the pituitary and has postulated local testicular functions in the development of germinal epithelium, motility of mature spermatozoa and acrosome reaction.

1.3.4 Insulin like factor 3

Fetal testis produces insulin-like factor 3 (INSL3) and anti-Müllerian hormone (AMH); AMH secreted from fetal Sertoli cells results in regression of Müllerian ducts which prevents formation of internal feminine genitalia, whilst INSL3 with testosterone controls the descent of the testis. Oligospermia due to cryptorchidism may be secondary to novel mutations in *INSL3* and leucine-rich repeat-containing G protein-coupled receptor 8 (*LGR8*) genes (Bogatcheva and Agoulnik, 2005).

1.3.5 Inhibin B

Inhibin B is a glycoprotein dimer, composed of α and β subunits. It is secreted by the testicular epithelium and reflects the proliferating activity of the Sertoli cells (Kathrins and Niederberger, 2016) Studies of the male rhesus monkey provided the most convincing evidence for a major role of testicular inhibin B in regulating the secretion of FSH by a direct negative feedback action at the level of the anterior pituitary (Majumdar et al., 1995). Further studies of the male adult rhesus monkey (macaca mulatta) demonstrate that in a physiological setting Sertoli cells are positively correlated with the levels of inhibin B number and are the major determinant of circulating concentrations of inhibin B (Ramaswamy et al., 1999).

1.3.6 Testosterone and its metabolites

Testosterone is secreted from the Leydig cells of the testis under the influence of LH. It circulates bound to carrier proteins: 65% to sex hormone binding globulin (SHBG) with high affinity and 33% to albumin with low affinity with 1 to 4% representing free unbound testosterone (FT). FT and albumin-bound testosterone is called ‘bioavailable’ testosterone (Corradi et al., 2016). Changes in these carrier proteins particularly SHBG due to certain conditions such as obesity can affect the level of TT and FT in men (Rastrelli et al., 2018).

Intra-testicular testosterone concentrations are more than 100-fold higher compared to the systemic circulation to enhance spermatogenesis (Kathrins and Niederberger, 2016).

Testosterone is converted to two other active metabolites, oestradiol, and dihydrotestosterone from conversion by the aromatase or 5 α-reductase enzymes respectively, which can exert negative feedback and modulate gonadotropin release (Figure 1.3).

1.3.7 Oestradiol

In men, the primary source of oestradiol is from the aromatization of testosterone by the action of the enzyme aromatase, mainly found in adipose tissues with only 10–25% localised in the testes (Boon et al., 2010; Simpson et al., 1994). Oestradiol acts on oestrogen receptors alpha and beta (*ERα* and *Erβ*) present in several hypothalamic nuclei and pituitary gonadotrophs, indicating that oestradiol regulates the hypothalamus-pituitary axis feedback (Figure 1.3)(Shughrue et al., 1997). However, several studies, involving a range of species and both sexes, have demonstrated that GnRH neurons do not express *Erα* which is the predominant receptor involved in mediating oestradiol suppression of gonadotropin release and gonadotropin subunit mRNA expression (Lindzey et al., 1998). Further research is needed to better clarify what the specific target cells for oestradiol action at the hypothalamic level are and what receptors are involved in men.

ERα and *ERβ* together with the membrane associated G-protein-coupled ER (*GPER*), and the enzyme aromatase have also been localised within the testis (Fietz et al., 2014). In consonance with localization studies, mice with targeted deletion of the aromatase gene, *ERα* and/or *ERβ* showed altered testicular morphology and derangements of spermatogenesis(Eddy et al., 1996; Fisher et al., 1998; Robertson et al., 1999). In addition, oestradiol is produced in immature germ cells, spermatozoa, epithelium of the efferent ductules, proximal epididymal duct and both Leydig and Sertoli cells (Lambard et al., 2004, 2003) whereby it regulates numerous aspects of spermatogenesis, including proliferation, differentiation, survival and apoptosis of germ cells, and regulation of Leydig cell function.

1.4 Sperm quality and function

Conventional semen analysis is the only routine diagnostic test for male infertility. It reflects the production of spermatozoa in the testes with measurements of sperm concentration, total sperm count, total and progressive motility, morphology and semen volume (Table 1.1) (Baskaran et al., 2020).

Examples of abnormalities related to semen analysis include azoospermia and oligozoospermia. Azoospermia is defined as absence of sperm in seminal plasma whilst oligozoospermia is a low sperm count (<15million sperms/ml of ejaculate). A reduced number of spermatozoa (low concentration/total count), predominately malformed spermatozoa (low morphology) or reduced and inefficient motility below the WHO reference ranges may be the cause for male factor infertility (Cooper, 2010). These significant sperm defects associated with male infertility are summarised in Table 1.1.

1.5 World Health Organization reference for semen analysis

Lower reference ranges for semen parameters were generated based on data from fertile men whose partners had time to pregnancy of within 1 year (Cooper, 2010) [Table 1.1]. The 5th centile is given as the lower reference limit published by the WHO to help standardise and validate the procedure for the examination of human semen. The reference ranges were generated based on data from semen samples from 4500 men from 14 different countries on 4 continents (Australasia, Americas, Europe and Asia), however data on ethnicity or race-specific ranges were not provided. WHO reference ranges generated from unselected men (with unknown fertility status) may have provided a more appropriate reference population for screening the male population without regard to prior fertility (Cooper et al., 2010).

Table 1-1 World Health Organization reference ranges for semen analysis and associated sperm abnormalities

SEMEN PARAMETER	REFERENCE RANGE	ABNORMALITY
Semen Volume	≥1.5 ml	
pH	≥7.2	
Sperm Concentration	≥15 million sperm/ml	**Azoospermia:** Absence of sperm in seminal plasma **Oligozoospermia:** <15 million spermatozoa/ml **Cryptozoospermia:** <1 million spermatozoa/ml
Total Sperm Count	≥39 million sperm/ejaculate	
Total Sperm Motility	≥40% motile sperm	**Asthenozoospermia:** <40% total motile spermatozoa or <32% progressive motile spermatozoa
Progressive Sperm Motility	≥32% progressively motile sperm	**Asthenozoospermia:** as above
Sperm Morphology	≥4% morphologically normal sperm	**Teratozoospermia:** <4% normal form/morphology
		Oligo-astheno-teratozoospermia (OAT syndrome): <15 million spermatozoa/ml, <32% progressive motile spermatozoa and <4% normal form

Adapted from review by (Cooper, 2010).

Semen analysis is used as a surrogate measure of male fertility (Cooper et al., 2010). The total number of spermatozoa per ejaculate and the sperm concentration are predictors of conception (Bonde et al., 1998) and related to important fertility outcomes such as time to pregnancy (Slama et al., 2002) and pregnancy rates (Zinaman et al., 2000). However, semen analysis has its limitations and may not provide adequate information about the defects of spermatogenesis (Holstein et al., 2003). It is also difficult to accurately assess sperm quality using a single semen sample due to marked biological variation, with standard deviations comparable to mean values (Castilla et al., 2006). In addition, the total number of spermatozoa in an ejaculate may vary depending on the time of abstinence, testicular volume, epididymal sperm reserve and ductal patency. Furthermore, the predictive value of semen analysis for natural conception and fertility rates are low in most settings, such that men with abnormal semen parameters by the WHO criteria (i.e. below 5th centile) may still have natural conception. In addition, these reference ranges are based from data within a discrete reference group, which might not be applicable to an individual patient. Furthermore, there have been limited studies investigating to what extent abnormalities in sperm function reduce fertility of the couple. Therefore, novel

molecular diagnostic tests for semen function have since been developed including oxidative stress via seminal reactive oxidative species (ROS) (Bisht et al., 2017; Tremellen, 2008) and DNA fragmentation index (DFI) (Zeqiraj et al., 2018), albeit these currently remain as research tools.

1.6 Seminal oxidative stress

Seminal reactive oxidative species (ROS) are released physiologically by immature or abnormal spermatozoa and leucocytes, as well as by-products of intracellular metabolic pathways and during ATP production from the sperm mitochondria (Kessopoulou et al., 1992). The fine balance of endogenous semen ROS and body's natural antioxidants is normally kept in close homeostasis. Small amounts of semen ROS are essential for optimum sperm function such as sperm maturation, movement, and fertilization of oocyte. Furthermore, the processes of capacitation and acrosome reaction are facilitated by ROS which trigger signalling cascades and result in membrane fluidity and sperm-oocyte fusion (Agarwal et al., 2018). However, a number of exogenous factors such as obesity, genito-urinary infections and varicocele may elevate semen ROS (Dutta et al., 2019; Pourmasumi et al., 2017). Spermatozoa are highly susceptible to oxidative damage (Gharagozloo et al., 2016) because their plasma membrane contains high amounts of polyunsaturated fatty acids (PUFAs) which contribute to membrane fluidity, and their cytoplasms contain low concentrations of scavenging enzymes (Sharma and Agarwal, 1996). As a result, high ROS may cause male infertility by adversely affecting the sperm membrane permeability, lipid peroxidation, motility, acrosome reaction, and subsequent sperm DNA fragmentation, resulting in defective paternal DNA passage to the offspring (Muratori et al., 2015). Therefore, measurement of ROS is a recently identified pathway for sperm damage and is a potential novel tool of added value in the investigation of male infertility. Between 30-40% of infertile men are estimated to have elevated seminal ROS with increasing evidence of its aetiological role in male infertility (Bisht et al., 2017; Tremellen, 2008). Previous studies have suggested that elevated semen ROS levels are associated with reduced sperm function in men with idiopathic infertility and recurrent miscarriage (Agarwal et al., 2008; Jayasena et al., 2019). 'Male oxidative stress infertility' or MOSI is a new proposed term by Agarwal et al to describe men with idiopathic infertility who have raised semen ROS (Agarwal et al., 2019a).

There are multiple methods of measuring ROS. One of the methods used in my host laboratory (used in study 3; chapter 4) is by a chemiluminescence assay whereby oxidation of luminol (5-amino-2, 3 dihydro-1, 4

phthalazinedione reagent; $C_8H_7N3O_2$) is measured by generation of light emission (45). Other direct ROS measurements can be performed with quantification of nitroblue tetrazolium (NBT) activity, measurement of cytochrome C reduction, flow cytometry with the use of fluorescent probes or electron paramagnetic resonance spectroscopy (Vessey et al., 2014), albeit these tests are expensive, time-consuming and require technical training.. A recent novel method based on galvanostatic measure of electron movement is the Male Infertility Oxidative System (MiOXSYS). It measures the seminal oxidation-reduction potential (sORP) suggestive of the overall balance between seminal oxidants and antioxidants (Dutta et al., 2019). Preliminary studies have correlated ORP to poor semen quality (Agarwal et al., 2016b).

1.7 DNA fragmentation

Sperm DNA fragmentation index (DFI) is the percentage of spermatozoa with fragmented or damaged DNA. It is being recognised as an important index of male infertility (Zeqiraj et al., 2018). Factors that raise semen ROS and reduce seminal antioxidants may also cause DNA fragmentation. Elevated DFI may affect fertility by hindering fertilization, early embryo development, implantation and pregnancy (Lewis et al., 2013). Studies evaluating the relationship between sperm DNA fragmentation and spontaneous and intrauterine insemination pregnancies found that men with DNA fragmentation < 30% were more likely to achieve a pregnancy or live birth (Spanò et al., 2000). Likewise, worse outcomes of ART (Bungum et al., 2004; Nicopoullos et al., 2019; Simon et al., 2019) were seen with DNA fragmentation >30%, including recurrent pregnancy loss (Tan et al., 2019).

There are multiple available assays for DNA fragmentation including sperm chromatin structure assay (SCSA), sperm chromatin dispersion (SCD), terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) and Comet assay (Agarwal et al., 2016a). There is large variability in these assays with a lack of consensus or external quality control for any method. Currently, there is insufficient evidence to support the routine use of ROS and DNA fragmentation in male factor infertility, as such, these are not currently recommended by clinical guidelines. The Single cell gel electrophoresis (SCGE) or Comet assay is a versatile, sensitive yet simple technique used to measure DNA damage. It is the best test to predict male infertility in receiver operating characteristic (ROC) curve analysis, followed by the TUNEL assay providing direct assessment of DNA fragmentation (García-Peiró et al., 2013). The COMET assay

consists of microscope slides where spermatozoa are lysed in agarose gel. DNA from lysed spermatozoa is decondensed in high salt to form supercoiled loops of intact DNA or less coiled strands of damaged DNA. The slides are then placed in an electrophoretic field. The extent of DNA migration depends directly on the DNA damage present in the cells such that broken DNA migrates away from the intact DNA to form a tail producing a comet like appearance on fluorescent microscopy. The intensity and length of the comet tail relative to the head reflects the number and degrees of DNA breaks (Lewis et al., 2013). This is the method used in Study 1, Chapter 2 to measure seminal DNA fragmentation. In comparison, TUNEL assay uses an enzyme to catalyse the attachment of fluorescent deoxynucleotides to 'nicks' or 3′- hydroxyl-termini 'free ends' of DNA breaks and quantifies this using flow cytometry (García-Peiró et al., 2013) (This is the method used in Study 2&3, Chapter 3,4). It is a highly sensitive and reproducible method albeit lacks strict inter-laboratory standardization.

It is suggested that use of antioxidants and lifestyle changes may reduce the risk of high ROS and sperm DFI to improve male infertility; however, there is paucity in the data with lack of good quality randomized controlled trials available. Men with idiopathic infertility and varicocele-associated infertility taking antioxidant therapy have an associated significant improvement in semen parameters and live birth rates (Cavallini et al., 2004; Imamovic Kumalic and Pinter, 2014). As such, commercial anti-oxidants such as L-carnitine and acetyl-L-carnitine (LAL) are readily available over-the counter for men to use as empirical therapies, and are often self-administered by infertile men to improve sperm quality and function (Balercia et al., 2005; Lenzi et al., 2004). However evidence underpinning their efficacy has been controversial (Raigani et al., 2014; Sigman et al., 2006). In addition, there are increasing concerns regarding indiscriminate anti-oxidant use causing reductive stress-mediated sperm damage (Henkel et al., 2019). We had carried out a prospective uncontrolled pilot study in 44 men attending the reproductive clinic at Hammersmith Hospital and investigated the effects of LAL therapy taken orally daily for 3 months in infertile men with abnormal baseline sperm quality (Vessey et al., 2021). Our results suggested that LAL therapy improved sperm quality (sperm concentration, total and progressive motility) and reduced ROS 5-fold in men who had a high baseline ROS prior to treatment, however, sperm quality did not change in men with normal baseline ROS levels (published paper included on list at end of thesis, under Publications).

1.8 Male infertility

Infertility is defined by the WHO as the inability to conceive after 1 year of regular (at least 2X per week) unprotected intercourse (Human Fertilisation and Embryology Authority, 2013). It is a common condition estimated to affect 10–15% of couples. Male factor infertility contributes to 50% of all cases of infertility (Practice Committee of the American Society for Reproductive Medicine, 2015; Sharlip et al., 2002), however, the true prevalence of male factor infertility may be underestimated as the current estimates are based on couples undergoing ART. Idiopathic male infertility, whereby no cause is found, affects 30-40% of couples (Practice Committee of the American Society for Reproductive Medicine, 2006), majority of which tend to be oligospermic, with novel monogenic genes linked in the etiopathogenesis and ongoing research efforts to identify novel genetic abnormalities. Non-obstructive azoospermia (NOA), i.e. no sperm in the ejaculate, is the most severe manifestation of testicular failure and affects 1% of all men (Jarow et al., 1989). Assessment of male fertility includes full medical history, physical examination, endocrine assessment and semen analysis. There are currently no approved pharmacological therapies to directly stimulate spermatogenesis. Consequently, couples with male factor infertility unable to conceive naturally, require to undergo ART such as *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) (Human Fertilisation and Embryology Authority, 2013). These require daily hormonal injections to stimulate several follicles to grow in the female partner. The eggs are collected surgically from the ovaries before being fertilised with sperm from the male partner, prior to incubation and re-implantation of embryos to the uterus. Although highly effective, ART is therefore invasive and confers potential health risks to the female partner such as ovarian hyperstimulation syndrome (Practice Committee of American Society for Reproductive Medicine, 2008). Furthermore, it is unaffordable for many patients and healthcare systems worldwide (Inhorn and Patrizio, 2015). In 2013, over 20,000 ICSI cycles were performed in the UK for male factor infertility, costing a total of £120M (Human Fertilisation and Embryology Authority, 2013). It is therefore critical that couples with male infertility are given effective lifestyle advice to optimise their own fertility using non-pharmacological approaches.

1.9 Causes of male infertility

A recent systematic review and meta-regression analysis of 42935 men across North America, Europe and Australasia reported a significant 50-60% decline in sperm counts (measured by sperm concentration and total sperm count) between 1973 and 2011 (Levine et al., 2017). This raises significant public health concerns urging the need for further research on the cause of this decline. Furthermore, male infertility is increasingly observed as a 'canary in the coal mine' for future male health conditions (Choy and Eisenberg, 2018), with an association with cardiovascular disease, testicular cancer, quality of life and increased-all cause mortality (Jensen et al., 2009).

Male infertility maybe the result of congenital or acquired conditions that may disrupt the HPT axis at any level, further subcategorised into three groups (Karavolos et al., 2013; Krausz, 2010; Stahl et al., 2012):

1) Pre-testicular: hypothalamo-pituitary causes of hypogonadotropic hypogonadism (HH). Biochemically, it is typically characterized by low or inappropriately normal gonadotropins in the context of low testosterone, and low testicular volume. Congenital GnRH deficiency represents genetic conditions with a failure of GnRH secretion, action, or impaired pituitary GnRH receptor function. Acquired causes (structural or functional) affect the HPT axis by either suppressing GnRH synthesis or secretion from hypothalamus or preventing GnRH from reaching the gonadotrophs by stalk injury or a pituitary defect. Obesity, chronic prescription opioid use, and anabolic-steroid withdrawal hypogonadism are commonly recognised causes of acquired HPT axis dysfunction (Carrageta et al., 2019).

2) Testicular: i.e. primary testicular dysfunction, resulting in deranged spermatogenesis and often accompanied by primary hypogonadism. Klinefelter's syndrome (Aksglaede et al., 2013) and uncorrected cryptorchidism (Toppari et al., 2007) are the two main congenital causes of primary hypogonadism, whilst acquired forms of hypergonadotropic hypogonadism include mumps orchitis and testicular cancer. Biochemically, it is typically characterized by high gonadotropins in the context of low testosterone, and typically low testicular volume.

3) Post-testicular: obstruction in the genital tract causing obstructive azoospermia (OA), or ejaculatory dysfunction (Jarvi et al., 2015) [Table 1.2]. These patients have intact spermatogenesis, intact biochemistry and testicular volume.

Treatment strategies for male factor infertility are guided by the underlying cause, however this can be challenging at times as many causes of male infertility remain idiopathic. In the context of acquired male infertility, worsening exposure to adverse lifestyle and environmental factors such as obesity are associated with male infertility. 'Lifestyle factor' refers to adverse health behaviours to appreciate the complexities of health behaviours as not solely a result of individual choices. Large cross-sectional studies suggest that adverse health behaviours such as excessive alcohol intake (R. A. Anderson et al., 1983; Thiel et al., 1983), smoking (Evans et al., 1981; Richthoff et al., 2008), recreational drugs (Bracken et al., 1990; Close et al., 1990) and obesity (Kort et al., 2006) are associated with reduced fertility in men (see section on Lifestyle factors), with obesity by far the most important cause. A prospective cross-sectional anonymous questionnaire-based study in 1149 male partners of couples investigated for infertility in our andrology centre (2nd author in the publication) at Hammersmith hospital showed that twenty-seven percent of all respondents reported a waist-circumference of above 36 inches (91cm), which is a validated measure of central adiposity/obesity as per national and international guidelines (Jayasena et al., 2020). Interestingly, about one quarter of our surveyed andrology population were unaware that obesity could reduce fertility and 72% of all respondents wanted further lifestyle education to improve their fertility. Furthermore, recent evidence suggests that amelioration of adverse lifestyle factors may improve markers of male fertility (Santos et al., 2011) and quality of life (Teskereci and Oncel, 2013) . Therefore, in the absence of approved pharmacological therapies to improve sperm quality in men with infertility, it is essential that men are aware of adverse lifestyle factors that impair sperm quality. Recently published European Society for Human Reproduction & Embryology (ESHRE) consensus guidelines recommended that clinicians should elicit a history of adverse lifestyle factors in all couples with male sub- or infertility (ESHRE Guideline Group on RPL et al., 2018). It is therefore important that research is carried out to assess the impact of lifestyle factor modification, e.g. weight loss in obese men, on male fertility, and further health promotion could be aimed at increasing awareness of these adverse lifestyle factors.

Table 1-2: Causes of male infertility

PRIMARY TESTICULAR FAILURE	
Congenital	Karyotype: Klinefelter's syndrome(47XXY) Y chromosome micro-deletions: partial and complete Numerical/structural chromosomal abnormalities: Robertsonian translocations/inversions Male XX syndrome Congenital cryptorchidism
Acquired	Infections e.g., mumps orchitis Testicular torsion, trauma or malignancy Large varicoceles Chemotherapy, pelvic irradiation or surgery Medications e.g., Cimetidine, Spironolactone Ageing Idiopathic
SECONDARY / HYPOGONADOTROPIC HYPOGONADISM	
Congenital	Congenital hypogonadotropic hypogonadism (normosmic or anosmic i.e. Kallmans syndrome)
Acquired	Structural hypothalamic/pituitary disease e.g. tumours/infiltrative Inflammatory disorder such as sarcoidosis Trauma Medications e.g. opioids, glucocorticoids, androgenic steroids Obesity Endocrine disrupting chemicals Idiopathic
OBSTRUCTIVE (DEFECTS IN SPERM TRANSPORT)	
Congenital	Cystic fibrosis resulting in congenital bilateral absence of vas deferens (CBAVD)
Acquired	Vasectomy Post surgical Post-inflammatory e.g. genito-urinary infections, epididymitis, prostatitis Sperm autoimmunity Functional (ejaculatory dysfunction) Medications e.g. alpha blockers, serotonin reuptake inhibitors

Adapted from (Wall and Jayasena, 2018).

1.10 Lifestyle factors affecting male fertility

Cigarette smoking: Rodent models have demonstrated that mutagenic components of cigarette smoking such as polycyclic aromatic hydrocarbons and nicotine can cause atrophy of seminiferous tubules, testis and reduce or block spermatogenesis(Mackenzie and Murray Angevine, 1981). Cigarette smoking is also suggested to cause oxidative stress induced increased sperm DFI (Hammadeh et al., 2010; Yousefniapasha et al., 2015). Smoking is associated with lower sperm motility and increased sperm morphological defects (Mitra et al., 2012), lower sperm concentration and fertility index in heavy smokers (over 20 cigarettes daily), compared with mild or non-smokers (Collodel et al., 2010; Reecha Sharma et al., 2016). However, definitions of smoking varies between studies and underlying mechanisms are not elucidated.

Recreational drug use: Recreational drug use, such as opioids and cannabis abuse, is correlated with high DNA fragmentation in sperm (Safarinejad et al., 2013) and reduced sperm parameters (Bracken et al., 1990). Cannabinoid receptors are found in testicular cells and spermatozoa (du Plessis et al., 2015). In contrast, a large birth cohort of young men observed that recreational drugs were not associated with any semen variables (Hart et al., 2015). Opioids are also known to cause secondary hypogonadism by inhibiting KNDy neuronal activity (Skorupskaite et al., 2014) but may also have direct testicular effects due to presence of endogenous opioid receptors through the testis (Subirán et al., 2011).

Alcohol: Mice model studies have shown an association between the amount and duration of ethanol exposure with sperm quality and fertilization ability (J. R. A. Anderson et al., 1983). Meta-analysis of 16395 men showed that alcohol intake was negatively associated with semen volume and morphology with a marked difference in daily versus occasional drinkers (Ricci et al., 2016). In addition, alcohol intake is associated with increased incidence of teratozoospermia, asthenozoospermia and oligozoospermia (Jensen et al., 2014). Furthermore, ethanol may be toxic to Leydig cells (Muthusami and Chinnaswamy, 2005).

Endocrine disrupting chemicals: The European Union Scientific Committee of Toxicity, Ecotoxicity and Environment define an endocrine disrupting chemical (EDC) as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)population.”(Birnbaum, 2013). Humans are exposed to these chemicals daily, as they are found ubiquitously in the environment and in everyday objects. A number of different types

of EDCs including bisphenol A, phthalates, pesticides and other environmental chemicals have been shown in both animal and human studies to impact upon male reproductive health. These chemicals may exert oestrogenic and/or antiandrogenic effects, or directly induce testicular toxicity by impairing Sertoli or Leydig cell function, increased oxidative stress, sperm DNA damage, or sperm epigenetic changes (Sidorkiewicz et al., 2017). Some studies suggests that EDCs such as Bisphenol A (BPA) inhibit ATP production (Hulak et al., 2013), perhaps by disrupting mitochondria (Rahman et al., 2017), impairing sperm motility. Furthermore, recent meta-analysis have suggested that EDCs not only have a deleterious effect on sperm quality but may also be associated with cryptorchidism, hypospadias and testis cancer; the so called 'testicular dysgenesis syndrome' (Skakkebaek et al., 2016, 2001). However, clinical evidence remains limited (Bonde et al., 2016). Inconsistencies observed may be due to differences in study populations, degree of exposure, synergistic effects from exposure to multiple EDCs, and residual confounding (e.g. concurrent adverse lifestyle factors).

Much of the current evidence comes from men presenting to infertility clinics and may not represent the effect of lifestyle factors on male fertility in the general population. In addition, almost all the studies focus on specific effects of 1 or at most 2 lifestyle factors under evaluation. However, exposure to these risk factors does not typically occur individually but simultaneously (Sharma et al., 2020a). Therefore, we may be underestimating the consequences of each adverse lifestyle exposure to male infertility in the general population.

In my clinical studies (chapter 2,3,4 of this thesis), we excluded men who were current smokers, recreational drug users or had excess alcohol intake to avoid confounders in the effects on semen parameters.

1.11 Obesity

Obesity is defined by the WHO as a body mass index (BMI) above 30kg/m^2 and an estimated 13% of world's adult population (11% of men and 15% of women) in 2016 were obese (W.H.O., 2000). The effects of obesity on reproductive function in women are extensively studied (Gesink Law et al., 2007; Jensen et al., 1999). However, the negative impact of obesity on male fertility has been less studied albeit the rising prevalence of obesity is a major factor postulated to contribute to oligozoospermia (Sermondade et al., 2013).

Rodent models of diet-induced obesity found that sperm of obese mice had lower sperm viability and motility, and higher incidence of head and tail defects, as well as increased oxidative stress and DNA damage (Bakos et al., 2011). Furthermore, when oocytes were fertilized with spermatozoa from obese male mice, they had delayed kinetic development and aberrant mitochondrial activity affecting their fertilisation capacity.

Data from three large epidemiological studies suggest that a BMI>25kg/m^2 in the male partner increases risk of infertility in couples (Nguyen et al., 2007; Ramlau-Hansen et al., 2010; Sallm n et al., 2006). Sallmen et al observed in 1329 couples that a 3-unit increase in male BMI was associated with infertility (OR =1.12; 95% CI =1.01-1.25). The association was similar in older and younger men suggesting that erectile dysfunction in older men does not explain the observation (Sallm n et al., 2006).

Another large retrospective Norwegian Mother and Child Cohort Study of 26303 planned pregnancies from 1999 to 2005 whereby infertility was described as time to pregnancy of >12 months or requiring infertility treatment, the OR for infertility was 1.20 for overweight men (BMI 25–29.9; 95% CI = 1.04–1.38) and 1.36 for obese men (BMI 30–34.9; 95% CI = 1.13–1.63) compared to a reference group of men with low-normal BMI (20.0–22.4). Body mass index was observed to have its maximum negative effect on fertility at the range of 32 to 43kg/m^2 with the effect plateauing above 43kg/m^2 (Nguyen et al., 2007). The retrospective nature of these studies, as well as the lack of timely assessment of the men's BMI points to some of its limitations.

Several studies have reported a negative correlation between BMI and weight with TT, FT, testosterone:oestradiol ratio, and SHBG (Osuna et al., 2006; Tsai et al., 2004) overriding the effects of ageing and other comorbidities (Tajar et al., 2010). In the large European Male Ageing Study (EMAS), a quarter (24.4%, BMI ≥30 kg/m^2) to a third (35.1%, waist circumference ≥102cm) of men were obese (Wu et al., 2010). Within these, 73% of men with reduced testosterone were overweight or obese, and TT and FT of men with a body mass index (BMI) ≥30 kg/m^2 was 5.1nmol/l (30%) and 53.7pmol/L (18%) lower respectively compared with normal weight (BMI <25 kg/m^2) men. Recent evidence has further highlighted the 'bidirectional' relationship between obesity and hypogonadism whereby low testosterone may further contribute to accumulation of fat tissue because of reduced lipolysis (Carrageta et al., 2019).

A large meta-analysis of 21 studies including 13077 men reported a 'J-shaped association' between male BMI and low sperm count compared with normal weight men; the OR (95% CI) for oligozoospermia or

azoospermia were: 1.11 (1.01–1.21) for overweight men, 1.28 (1.06–1.55) for obese men and 2.04 (1.59–2.62) for morbidly obese men (Sermondade et al., 2013). Jensen et al. studied 1558 young Danish men and found that overweight and obese men had mean sperm concentration lower than men with normal BMI (Jensen et al., 2004). Evidence on the relationship between obesity and sperm motility and morphology has been more conflicting. Jensen et al found no association between increasing BMI and sperm motility or morphology (Jensen et al., 2004) . In contrast, another study of couples attending infertility clinic observed that the BMI of male partners showed a negative correlation with their motile sperm count (Kort et al., 2006). A systematic review of 30 studies comprising 115158 participants reported that obese men had a higher percentage of sperm with DNA fragmentation, abnormal morphology, and low mitochondrial membrane potential and they were more likely to be infertile when compared with men with normal BMI (Campbell et al., 2015). The rate of live birth per cycle of ART was reduced (OR = 0.65, 95% CI 0.44–0.97) in obese men with a 10% increased risk of a non-viable pregnancy (Campbell et al., 2015). In addition, population-based studies have observed that obese fathers are more likely to father an obese child (Danielzik et al., 2002), corrected for age and gender, with a reported link between obesity and reduced sperm DNA integrity (Dupont et al., 2013). This highlights the potential benefit of paternal preconception advise in relation to obesity.

1.12 Potential mechanisms for obesity associated male infertility

Several mechanisms have been implicated to explain the negative effect of obesity on male reproduction (Neto et al., 2016), albeit not yet fully understood [Figure 1.4]. Obesity may adversely affect the hormonal milieu of the HPT axis, modify the micro-testicular environment with increased pro-inflammatory cytokine production causing increased ROS and DNA fragmentation (Du Plessis et al., 2010). Furthermore, emerging research in seminal fluid metabolomics, and epigenetic modifications due to obesity may offer novel mechanisms linking obesity with male infertility.

1.12.1 Changes in the hormonal levels regulating spermatogenesis

Oestradiol: Serum oestradiol levels are higher in obese and overweight men due to increased peripheral conversion of testosterone to oestradiol by the aromatase cytochrome P450 enzyme in the adipose tissue (de Boer et al., 2005; Schneider et al., 1979). High oestradiol levels by negative feedback on the hypothalamus

and anterior pituitary gland result in hypogonadotropic hypogonadism (Davidson et al., 2015) with reductions in LH, FSH, TT and testosterone/oestradiol ratio (Giagulli et al., 1994) (Figure 1.3). In addition, mean diurnal LH levels and mean diurnal LH pulse amplitude are significantly lower in obese men than controls (Vermeulen et al., 1993). However, it is mainly in animal models that the effects of elevated oestradiol levels in males have been evaluated. In an *in vivo* mouse model, oestradiol exposure resulted in premature capacitation of the cauda epididymal sperm, which could be correlated with decreased sperm viability in the reproductive tract of the female (Ded et al., 2013). Furthermore, chronic exposure to oestradiol 3-benzoate in rats has been reported to cause significant effects on the epididymis, testis, seminal vesicle and prostate including a decrease in weight of these sex organs, impaired spermatogenesis and a reduced number of germ cells secondary to an increase in germ cell apoptosis (M. C. Kaushik et al., 2010). Furthermore, oestrogen receptors are present in most cell types of the human testes including Leydig and Sertoli cells, suggestive of a direct impact on the testicular function (Cavaco et al., 2009). Oestrogen treatment has been shown to cause an increase in ERα expression and decrease in AR expression in the rat testis affecting both steroidogenesis and spermatogenesis (Mahesh C. Kaushik et al., 2010). Furthermore, histological analysis showed a loss of the apico-basal differentiation in Sertoli cells and dedifferentiation of Leydig cells with oestrogen treatment (Schulze, 1988).

Insulin: Hyperinsulinaemia secondary to insulin resistance of obesity leads to negative feedback at the hypothalamic (KNDy neurones) and pituitary level suppressing the HPT axis (Vermeulen et al., 1993). Peripheral insulin resistance additionally inhibits SHBG production by hepatocytes reducing the 'travel capacity' of testosterone (Nielsen et al., 2007; Tsai et al., 2004). Furthermore, insulin is described in human semen synthesized and secreted by ejaculated spermatozoa, with insulin receptors present on the plasma membrane of spermatozoa. The increased semen insulin possibly leads to insulin resistance within the spermatozoa leading to negative influence on the metabolic signalling pathways, causing mitochondrial dysfunction related to ROS overproduction. Defective Sertoli cell function with poor Leydig cell secretory activity result in immature sperm, prone to oxidation and local inflammatory response (Pitteloud et al., 2005). Increased ROS targets spermatozoa DNA integrity, leading to decreased sperm quality (Leisegang et al., 2014).

Leptin: Leptin is increased in overfeeding states due to resistance such that circulating serum leptin levels correlate positively with adiposity. Leptin resistance leads to defective hypothalamic signalling, reduced gonadotropin secretion and subsequent hypogonadotropic hypogonadism (Alves et al., 2016). In rodent models of diet-induced obesity (DIO), the DIO mice showed significant downregulation of the GnRH, *KISS1*, GPR54, and Ob-R genes and protein expression (Zhai et al., 2018). These suggest that downregulation of Ob-R and kisspeptin/GPR54 in the murine hypothalamus may contribute to male hypogonadotropic hypogonadism caused by high-fat diet-induced obesity (Zhai et al., 2018). Leptin resistance in obesity also results in high seminal leptin concentrations and increased number and volume of cytoplasmic lipid droplets in Leydig cells. Accumulation of lipid droplets triggers lipid peroxidation and germ cell apoptosis (Martins et al., 2015). Elevated levels (leptin resistance) may also have a direct inhibitory signal for testicular steroidogenesis (Tena-Sempere et al., 1999). The underlying molecular pathways are yet to be fully understood but reduction of several steroidogenic genes such as steroidogenic factor-1 (Nr5a1), steroidogenic acute regulatory protein (StAR) and cytochrome P450 cholesterol side-chain cleavage (CYP11A1) enzyme involved in testosterone synthesis may have a postulated role (Landry et al., 2017).

1.12.2 Physical effects of obesity

Heat stress: The process of spermatogenesis is highly heat sensitive. Increased scrotal fat disrupts thermoregulatory mechanisms in male and can lead to increased testicular temperatures (Shafik and Olfat, 1981) leading to increased oxidative stress, lower DNA integrity and increased germ cell apoptosis (Martins et al., 2018).

Endothelial dysfunction: Endothelial dysfunction in obesity reflects a decrease in nitric oxide (NO), which is associated with high cholesterol, insulin resistance and oxidative stress. NO is the primary neurotransmitter responsible for the relaxation of penile smooth muscles and when released at suboptimal levels, erections are difficult to achieve (Shamloul and Ghanem, 2013).

Obstructive sleep apnoea: Sleep fragmentation due to obstructive sleep apnoea, decreases LH production and further reduces circulating testosterone levels contributing to the general pro-inflammatory state (Craig et al., 2017). The exact pathological mechanism is not well established however chronic hypoxia has been proposed as the mechanism disrupting the nocturnal testosterone rhythm (Luboshitzky et al., 2002). Moreover, the

adjusted mean (corrected for age and BMI) total testosterone is reduced proportionally to the severity of the sleep apnoea.

Other co-mechanisms: Obesity is also associated with reduced coital frequency in couples. Animal studies involving obese rodents, demonstrate lack of sexual behaviour with low mating rates (Crean and Senior, 2019). A higher BMI is also associated with a greater impairment in sexual quality of life (Kolotkin et al., 2006). Furthermore, other adverse lifestyle factors involved in the pathogenesis of obesity such as sedentary lifestyle, smoking and alcohol may directly or indirectly impact spermatogenesis (Rato et al., 2014a) (see section on Lifestyle Factors).

1.12.3 Oxidative stress and DNA fragmentation associated with obesity

Obesity is a chronic inflammatory state whereby production of cytokines and interleukins is increased at both systemic and seminal levels (Oliveira et al., 2017). High calorie diets increase body weight, glucose and lipid levels with subsequent rise in the metabolic rate to sustain the body energy expenditure (Oliveira et al., 2017) Production of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukins (IL-1, IL-6 and IL-18) increases in parallel to the metabolic rate in obesity and induces oxidative stress suppressing the HPT axis and spermatogenesis both at the level of the hypothalamus and testis. Furthermore, cytokines such as TNF- α and IL-1 cause direct damage to the assembly of junctional proteins supporting the network of Sertoli cells with significant impairments of the seminiferous epithelium and blood-testis barrier. Therefore during spermatogenesis damage to the blood-testis barrier (BTB) integrity may be one of the crucial underlying factors accounting for decreased fertility (Fan et al., 2015).

In addition, these pro-inflammatory cytokines inhibit LH function at the Leydig cells leading to further low testosterone and poor sperm quality (Liu and Ding, 2017a). Therefore, obesity results in a low-grade systemic as well as testicular inflammatory state with high levels of ROS.

Sperm DNA fragmentation in obesity is attributed to high ROS production which surpasses seminal antioxidant capacity and impairs sperm quality leading to infertility (Lewis et al., 2013). Studies have shown that oxidative stress increases with an increase in BMI, partly due to an increase in seminal macrophage activation. However the exact mechanisms by which ROS cause DNA damage are not well established. One of these mechanisms postulated is through the production of lipid degradation by-products especially

malonaldehyde which either causes oxidation of DNA bases (mainly guanosine) into 8′-hydroxyguanosine (which is promutagenic) or through direct interaction with the DNA strand leading to non-specific single- and double-strand breaks (Niederberger, 2012).

1.12.4 Epigenetics

Epigenetics refers to the information in the genome over and above that contained in the DNA sequence that can be inheritable to offspring. These changes may include methylation, carboxylation and hydroxylation of DNA nucleotides, and histone modifications (Gunes et al., 2016). Recent research in the field of epigenetics has suggested that paternal obesity can affect offspring metabolic and reproductive phenotypes by the epigenetic reprogramming of spermatogonial stem cells. High fat diet induced histone acetylation of late spermatids resulted in increased levels of DNA damage (Davidson et al., 2015). Similarly, Donkin et al demonstrated that the expression level of specific mitochondrial RNAs, and small nuclear RNA (snRNA) fragments, was altered in the spermatozoa of men with obesity (Donkin et al., 2016). They suggested that this altered expression modulated the expression of genes involved in behaviour and food intake and could participate in predisposing the offspring to obesity.

1.12.5 Metabolomics

Seminal fluid is a complex biological fluid containing a variety of organic species like low molecular weight compounds, peptides, hormones, free amino acids, proteins, and high levels of inorganic ions like Zn2+,Mg2+,Ca2+,K+, and Na+ (Jodar et al., 2016; Sørensen et al., 1999). Seminal plasma is also a favourable non-invasive material which can allow metabolic quantification to understand dysregulated metabolism in the diagnosis of obesity associated male infertility (Sørensen et al., 1999). For example, citrate, lactate, glycerophosphocholine (GPC), glycerophospho-ethanolamine, and other metabolites associated with oxidative stress are observed to be related to male factor infertility.

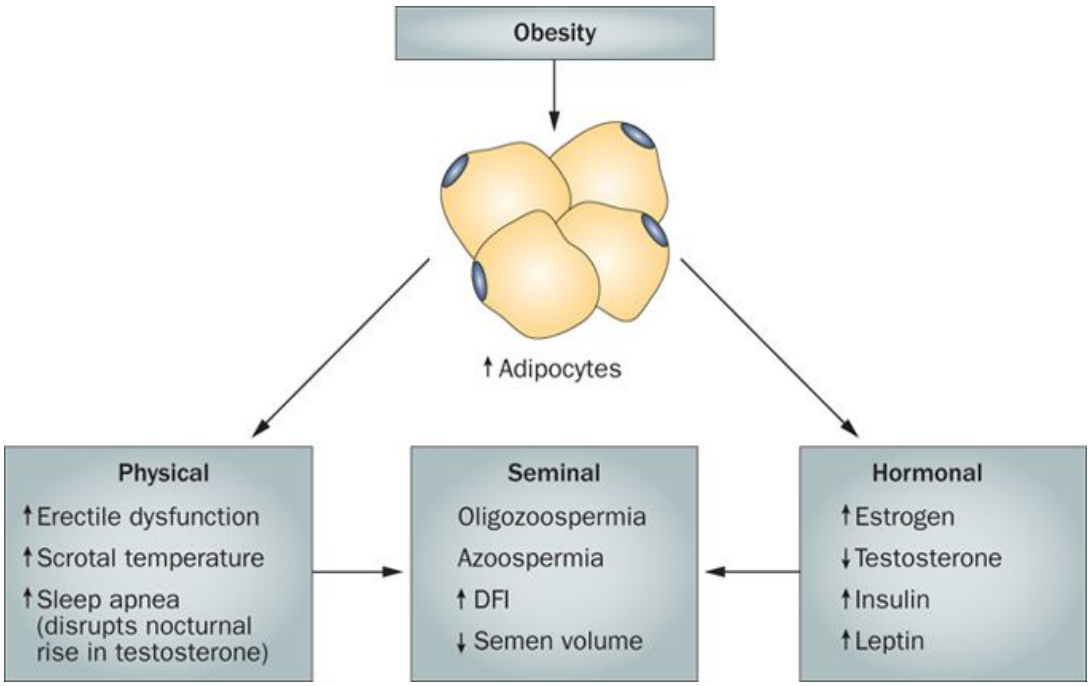
Furthermore, previous observational studies showed that metabolomics analysis can be used to differentiate males with low sperm concentration (Courant et al., 2013) or asthenozoospermia (Gilany et al., 2014) from normozoospermic men. A variety of techniques such as mass spectrometry, nuclear magnetic resonance

spectroscopy, and Fourier transform infrared spectroscopy are available in metabolomics (Dunn and Ellis, 2005) albeit expensive, time consuming and not as widely available.

Mice fed high fat diet verses normal diet for 10 weeks had abnormalities at the metabolomic, proteomic, and transcriptomic levels compared with mice with non-obese diet (Binder et al., 2015). In this study epididymal sperm and seminal vesicle fluid were collected from non-obese and obese mice. Transcriptome analysis identified significantly higher levels of cytochrome *c* oxidase subunit IV isoform 1 (*Cox4i1*) mRNA in sperm from obese males compared with non-obese. Nuclear encoded *Cox4i1* is part of the terminal enzyme of the mitochondrial respiratory electron transport chain and the protein localizes to the inner mitochondrial membrane, therefore affects mitochondrial potential and energy metabolism in the spermatozoa. In addition, seminal vesicle fluid from obese males also differed significantly from non-obese in both protein and metabolite composition. Fructose, a major glycolysable substrate of seminal plasma was significantly increased in the seminal vesicle fluid of obese male mice compared with non-obese mice. Similar to obese human males fructose is widely accepted as a marker of seminal vesicle function (Ndovi et al., 2006; Raj et al., 2014). Taurine, widely recognized for its antioxidant capabilities, was also significantly increased in the seminal vesicle fluid of obese mice, compared with normal mice, suggestive of high ROS levels in obese mice requiring upregulation of seminal antioxidants (Holmes et al., 1992). Similarly, a recent study investigated the impact of high-fat feeding male mice (F_0) on the testicular metabolome and function of their sons (F_1) and grandsons (F_2) (Crisóstomo et al., 2021). Testicular content of metabolites related to insulin resistance, cell membrane remodelling, nutritional support and antioxidative stress (leucine, acetate, glycine, glutamine, inosine) were altered in sons and grandsons of mice fed with HFD, compared with descendants of chow-fed mice (Crisóstomo et al., 2021).

In summary, the -omics (metabolomic, proteomic, transcriptomics), are an area of novel interest in male infertility, particularly in cases of unexplained male infertility. Future research in this area could offer a better understanding of underlying mechanisms postulated to affect the dysregulated metabolism of obesity associated male infertility and may explain how sperm function may change with weight loss, and the observed transgenerational effects of obesity.

Figure 1-4: Potential mechanisms of obesity associated male infertility



There are several mechanisms involved in obesity-induced male infertility. Firstly, hormonal mechanisms include hypogonadotropic hypogonadism, high oestradiol levels and leptin as well as insulin resistance. Secondly, there are local as well as other physical mechanisms implicated such as inflammatory response, heat stress, erectile dysfunction and obstructive sleep apnoea related to obesity. Finally, obesity-induced infertility has been linked to seminal oxidative stress, sperm DNA damage and epigenetic changes Source: Adapted from (Craig et al., 2017; Du Plessis et al., 2010) [DFI, DNA fragmentation index.]

1.13 Effect of weight loss on male fertility

The consistent findings of the negative impact of obesity on male fertility are suggestive of weight loss as a logical potential therapy for male infertility. Therefore, it is important to review the current literature on both surgical and lifestyle methods of weight loss and their impact on male fertility.

1.14 Bariatric surgery

The first successful bariatric bypass was conducted in the year 1966 when it was noted that patients with cancer who underwent sub-total gastrectomy lost considerable amount of weight (Mason and Ito, 1967). In 1994 the first laparoscopic gastric bypass was performed by an American surgeon and since then it has become a mainstream treatment for obesity. It is the most effective method of weight loss associated with improved quality of life and comorbidities (Karlsson et al., 2007). It is also associated with an increase in TT, FT, and gonadotropins with reduction in oestradiol levels reflecting reactivation of the HPT axis. However, there remains a controversy regarding the effect of excess weight loss by bariatric surgery on semen parameters with some studies suggesting that the acute starvation-like state induced by bariatric surgery paradoxically reduces sperm quality and function (Frega et al., 2005; Legro et al., 2014). In contrast, there has been extensive research done on women, who undergo majority of bariatric surgeries, with proven improvements in ovulatory cycles and pregnancy outcomes. A recent meta-analysis (Wei et al., 2018) reported no change in semen concentration and progressive motility after bariatric surgery. Semen morphology showed a slight but statistically significant increase after sleeve gastrectomy.

The prospective studies investigating the effect of bariatric surgery on semen parameters till date are summarised in the table below. [see Table 1.3] These studies are limited due to variation in morphological analysis, geographical differences, small sample size, patient selection (infertility clinics vs obesity clinics), length of follow-up and differing types of bariatric surgery.

Table 1-3: Prospective studies showing the effect of bariatric surgery on semen parameters.

Author (Year)	Study Design	Country	Source of patients	Length of Follow-up	Type of surgery	Sample size	Outcome measures	Limitations	Results
(Samavat et al., 2018)	Prospective case-control study	Italy	Obesity clinic	6 months	Gastric bypass	23	Sperm motility, morphology, number, volume, DNA fragmentation	No power calculation	Increased sperm count, progressive motility and ejaculate volume not reaching statistical significance. Reduction in DNA frag. Significant improvement in sex hormones
(Reis et al., 2012)	Prospective case-control study	Brazil	Obesity clinic	24 months	Gastric bypass	10	Semen volume, pH, motility, concentration, leukocytes, vitality, vitality, normal morphology	No power calculation	Significant increase in IIEF-5 score (quality of sexual function questionnaire), total and free testosterone levels but did not affect sperm quality
(Bardisi et al., 2016)	Prospective cohort study	Qatar	Infertility clinic	12 months	Sleeve gastrectomy	46	Semen volume, concentration, motility, normal morphology	No power calculation, odds ratio not provided, no control group	Semen quality was not affected by surgical weight loss except for subgroups of men with pre-existing azoospermia or oligospermia
(Legro et al., 2014)	Prospective cohort study	USA	Not available	12 months	Gastric bypass	6	Semen volume, concentration, motility, normal morphology	Odds ratio not provided, no control group designed	No improvement in semen parameters of obese men that underwent gastric bypass (reduced first 6 months then reached back to baseline). Increase in IEEF score, total testosterone and urinary testosterone.

Adapted from (Wei et al., 2018)

Case series have suggested that bariatric surgery may reduce fertility in men. A series of six previously fertile men with a weight loss of 60-80kg (76.8 +/-12.3 kg, mean +/-SD) with a mean follow-up time between the Roux-en-Y gastric bypass operation to study visit of 16.8+/- 3.9 months (mean +/-SD) experienced azoospermia leading to secondary infertility (Frega et al., 2005). The authors concluded a complete spermatogenic arrest on testicular biopsy with normal sex hormones suggestive of a 'developmental block of spermatogenesis at a point unrelated to the action of sex hormones on the testis'(Frega et al., 2005). Similarly, another case series of 3 men reported worsening semen parameters after bariatric surgery (Sermondade et al., 2012).

Three hypothetical mechanisms have been proposed for this negative semen profile observed:

- Rapid weight loss post-bariatric surgery leads to a starvation-like, 'under-nutrition' state with deficiencies in key nutrients such as iron, calcium, vitamins B1, B9 and B12 which are essential for spermatogenesis, despite vitamin and mineral supplementation (Coupaye et al., 2009). Low levels of trace elements has a negative impact on regulation of spermatogenesis and germ cell division.
- Rapid weight loss may interrupt the normal pulsatile GnRH release from the hypothalamus leading to disruption in HPT axis (Tsutsumi and Webster, 2009).
- Surgery may cause a release of toxic liposoluble substances and oestrogens stored in the adipose tissue leading to endocrine-disrupting effects (Magnusdottir et al., 2005). Oestrogen metabolising enzymes become saturated and persistently high oestrogen levels despite weight loss exert negative feedback on the HPG axis (Calderón et al., 2019).

Recent work in both clinical studies and animal models supports bile acids (BAs) as key mediators of lipid and glucose homeostasis (Sèdes et al., 2017). Increased bile acids post bariatric surgery act via the nuclear Farnesoid-X-Receptor alpha (FXRa; NR1H4) (Makishima et al., 1999; Parks et al., 1999) and the membrane receptor TGR5 (GPBAR1, G protein-coupled bile acid receptor) (Maruyama et al., 2002) to support fat mass loss and related metabolic benefits of bariatric surgery (Wang et al., 2019). However, preliminary studies in animal models suggests that bile acid signalling could play a role in testicular pathophysiology as both FXRa and TGR5 are expressed in the testis (Volle et al., 2007). Elevated plasma BA levels in mice fed with dietary BA supplementation (cholic acid) for 4 months led to germ cell sloughing and blood-testis barrier rupture, as well as apoptosis of spermatids (Baptissart et al., 2014). Similarly, a recent study showed that in-vivo

testicular testosterone synthesis was repressed by a synthetic agonist of the nuclear bile acid receptor, FXRa, with decreased expression of genes encoding for key enzymes of steroidogenesis namely, Star, Cyp11a1 and 3b-Hsd (Volle et al., 2007). These data therefore identify the testis as a new target of BA's with deleterious testicular effects (Sèdes et al., 2017). Therefore, one may hypothesise that the increased BA levels observed post bariatric surgery, due to increased intestinal reabsorption, with metabolic benefits may underlie the adverse semen parameters observed post surgery. However, no study has directly looked at this association to date. Studies investigating direct effects of high concentration of BA's post bariatric surgery on semen parameters would be useful to elucidate a link between these.

Recently, Lee et al carried out a meta-analysis of 28 cohort studies with 1022 patients, median follow up of 12 months, and concluded that sustained weight loss with bariatric surgery had a significant effect on increasing male sex hormones (testosterone, SHBG, LH, and FSH, with decrease in oestradiol) however no change in semen parameters (only 5/28 studies reported on semen parameters) (Lee et al., 2019).

In summary, there is heterogeneity in data with some prospective studies suggestive of an increase in sperm parameters whilst others suggesting a decrease or no effect on semen parameters. Furthermore, longer term effects of bariatric surgery on male fertility remain unknown. Taking into account the surgical risks, it is important to investigate non-surgical approaches of weight loss to target obesity-associated male factor infertility.

1.15 Lifestyle-mediated weight loss and male fertility

Weight loss by lifestyle changes are considered valuable in restoring hormone profiles and fertility especially in the setting of female fertility clinics. However, there have been limited studies investigating this in men.

Over the last fifty years dietetic patterns have changed dramatically to reflect 'westernisation' with higher intake of processed food and fats with less seafood, vegetables and whole grains. Consequently, western diets are associated with linear decline of sperm concentration and sperm morphology (Hayden et al., 2018; Liu et al., 2015). A recent systematic review of 33 observation studies with 8477 healthy men and 1204 men recruited from fertility clinics suggested that diets consisting of fruit and vegetables, for their contents in vitamins, and fish or low-fat dairy products as the main source of proteins, are associated with better semen

quality compared with 'Western' diet (Ricci et al., 2018). However, the underlying mechanisms for these observed improvements are not known. Similarly, a cross-sectional study conducted in 189 healthy young men reported that the 'prudent' diet (characterized by a high intake of fish, chicken, fruit, vegetables, legumes and whole grains) was significantly associated with higher progressive sperm motility compared with the 'Western' diet (characterized by high intake of red and processed meat, refined grains, pizza, snacks, high-energy drinks and sweets) (Gaskins et al., 2012). Furthermore, Vujkovic *et al*. (Vujkovic et al., 2009) observed that a health-conscious diet, including fruits, vegetables, fish, and whole grains, in men from a fertility clinic, was inversely associated with sperm DFI. However, evidence is not entirely consistent, and different findings were observed among studies with notable disparity in study design, trial quality, and populations studied (Giahi et al., 2016).

Diet programmes including low energy diets (LED) aim to generate an energy deficit below the estimated daily energy requirements leading to weight loss. A recent animal study reported that simple diet and exercise interventions can be used to reverse the detrimental effects of obesity on sperm function (Palmer et al., 2012). Observational studies have shown physically active men have healthier sperm (Vaamonde et al., 2012). Garcia et al randomly allocated sedentary men to 16-week aerobic exercise programme (Rosety et al., 2017). Sperm count, motility, morphology were significantly increased compared to controls. Exercise also improved total testosterone. Oxidative stress or DNA fragmentation were not measured. Tartibian and Maleki observed that sperm from recreationally active men had less oxidative stress-induced DNA damage (Tartibian and Maleki, 2012).

During a 9-week very low-calorie diet (VLCD) programme, 58 abdominally obese men lost on average 16.3+/-4.5kg and maintained 14.3+/-9.1 kg weight loss after a 12-month maintenance period. The study reported a significant increase in free testosterone and SHBG at 12 months compared to baseline (Niskanen et al., 2004). Similarly, 6 sub-fertile obese men who underwent several months of an unreported personalised healthy lifestyle programme consisting of a 'balanced diet' and regular exercise, lost abdominal weight with significant improvement in sperm DNA fragmentation, lipid profile, testosterone: oestrogen ratio and successful pregnancy outcomes with live births. No significant difference in other basic sperm parameters was seen (Faure et al., 2014).

Hakonsen et al investigated 43 obese men (BMI 33-61kg/m^2) who underwent a 14-week residential weight-loss programme (3.5-25.4kg weight loss). The subgroup with the largest weight loss had an increase in total sperm count, semen volume, testosterone and SHBG (Håkonsen et al., 2011). Recently, an Indian group studied 105 men through a 12-week weight loss programme consisting of 'healthy diet and exercise' and reported a higher mean DNA fragmentation index before weight loss (20.2%) vs after weight loss (17.5%) p=<0.01. Significant improvement in sperm morphology and progressive motility post weight loss were also observed (Mir et al., 2018). In all these studies, the weight loss programmes are not described in detail and furthermore, the studies did not have control groups.

Table 1-4: Summary of studies showing the effect of dietary weight loss on semen parameters.

Study	Male, n	Study population	Results/ Conclusion	Comments
(Håkonsen et al., 2011)	43	Initial cross-sectional study during a residential weight loss program and subsequent longitudinal study of 27 men in a 14-week diet programme	15% median weight loss with increase in total sperm count, semen volume, TT, SHBG with no improvement in DFI	No control group
(Faure et al., 2014)	6	Case series on 3-8 weeks diet and exercise. Sub-cohort from the ALIFERT study	3.9% BMI reduction, significant improvement in DFI, TGL, T/E ratio, 1 live birth per couple	Case series with control group (n=7) that had no hormonal or seminal parameters examined
(Belan et al., 2015)	52	Controlled prospective cohort study nested in a randomized- controlled study for couples attending a Canadian Fertility Academic Clinic. Follow up over 12.7 months	Male partners with improved weight and lifestyle increase the odds of their couple to conceive	Nested study, flawed control selection. No hormonal parameters checked.
(Mir et al., 2018)	105	Prospective cohort study from the Infertility department or weight loss centres in Bangalore, on 12-week 'healthy diet'. Follow up 6 to 12 months	7.9% BMI reduction, significant improvement in mean DFI, sperm morphology and PM.	No control group. No hormonal parameters measured.

TT; total testosterone, DFI; DNA fragmentation index, BMI; body mass index, TGL; triglycerides, T/E ratio; Testosterone over oestradiol ratio, PM progressive motility

1.16 Low energy formula diets

Energy balance with net energy deficit whereby caloric expenditure exceeds caloric intake is an established method to achieve weight loss (NICE (National Institute for Health and Care Excellence), 2014). There are hundreds of diets available or advertised for weight loss which would be beyond the scope of this thesis, ranging from healthy eating/balanced diet, high-protein, low-fat, atkins, detox and Mediterranean diets. Formula very-low/low energy diets (VLED/LED) have become popular as meal replacement products to aid weight loss. Therefore I will focus my literature review on commercially available formula meal replacement diets as it was our dietary intervention for the 3 studies included in this thesis (Chapter 2, Study1; Chapter 3, Study 2; Chapter 4, Study 3).

VLEDs (<800kcal/day; <3347kJ) and LEDs (800-1200kcal/day, 3351–5021 kJ) are specially formulated, widely available food products, usually in the form of liquid soups, shakes and bars, and have been available in the UK for about 35 years (Leeds, 2014). LED provides between 800 and 1200 kcal/day and can either be a total diet replacement (TDR) or the formula products can be incorporated into modified conventional meals as a partial diet replacement at the higher energy levels (Brown and Leeds, 2019). Initial VLEDs in the 1970s were associated with multiple nutrient deficiencies and inadequate amount of protein, however the recent standardised formula diets provide high protein and full 100% dietary reference value (DRV) for vitamins and minerals in a defined number of daily portions (3-4 per day). Therefore, VLEDs and LEDs aim to offer rapid, short to medium term weight loss option in the obese population that repletes nutrient levels rather than further depletes them.

Formula LEDs are effective for weight loss in the short term (up to 20 weeks) (Christensen et al., 2011; Mulholland et al., 2012) and new evidence demonstrates long-term weight loss maintenance (up to 4 years) of approximately 10% (Lean et al., 2018). NICE guidelines recommend a maximum of 12 weeks of TDR at present, although there is limited evidence for this time limit (NICE (National Institute for Health and Care Excellence), 2014).

Majority of research on formula diets is in people with BMI between 30 and 40kg/m^2, with limited evidence on populations above 40kg/m^2. TDR phase normally lasts 8-20 weeks and is reported to achieve weight reductions of between 10 to 16 kg or 10% and 15% of bodyweight (Johansson et al., 2014; Lean et al., 2018;

Leeds, 2014), with associated improvements in a number of obesity-related co-morbidities including T2DM, obstructive sleep apnoea and osteoarthritis (Brown and Leeds, 2019). Other studies have shown more variability in the weight loss achieved. Frost et al prescribed 1100–1600 kcal/day in obese patients and achieved mean weight loss of 2.9-3.3 kg over a period of 12 weeks (Frost et al., 2007). A review confirmed that LED from 25 days to 9 months are associated with a mean weight loss of 13.6kg (±5.5) and significant improvement in total cholesterol as well as fasting glucose (Mulholland et al., 2012). VLED for nine weeks is sufficient to increase SHBG and free testosterone at statistically significant levels (Niskanen et al., 2004). In addition, mild intensity aerobic activity for 150 min/weekly and energy deficit of 170-250kcal/day to achieve 10% weight loss increases testosterone, normalises oestradiol levels and reverses obesity related hypogonadism without having to resort to bariatric surgery (Lorenzo et al., 2018). Recent evidence indicates that using a formula LED with a weight loss maintenance programme can help people with overweight or obesity and T2DM achieve remission (Lean et al., 2018). Overall, the effects of LEDs on metabolic parameters and hypogonadism are well described in the literature but data on semen parameters are limited.

VLEDs should produce greater weight loss than LED because of greater energy restriction but this has not been shown to be the case over the long term (Christensen et al., 2011; Norris et al., 2005; Saris, 2001). In a meta-analysis of six randomised controlled trials, initial short-term weight loss was significantly greater following VLEDs compared to LEDs (16.1% vs. 9.7% of initial weight) (Tsai and Wadden, 2006). However, this difference was not sustained over time with greater weight regain reported in VLED group compared to LED group. The lack of short-term difference was further demonstrated by a randomised controlled trial (Christensen et al., 2011) whereby at both 8 and 16 weeks, there was no significant difference in weight loss between participants who used a VLED (420–554 kcal/day) and those who used an LED (810 kcal/day). Furthermore, Christenesen et al (Christensen et al., 2011) reported a significantly higher loss of lean tissue together with more frequently reported side effects and non-adherence in the VLED group compared with LED group. This favoured the choice of LED over VLED for our weight loss intervention in our 3 studies on men with obesity. In conclusion, weight loss via LED provides a flexible model to investigate semen parameters during different levels of energy restriction.

Commercially available LEDs

There are various commercially available LEDs, each with micro and macronutrients as regulated by the EU directive, and European Food Safety Authority (EFSA) ("Dietary reference values | EFSA," n.d.) with some slight variations between brands. Each meal replacement product contains protein, carbohydrates and fat fortified with vitamins and minerals.

We used Cambridge Weight Plan (CWP) meal replacement products for Study 1 and 2 (https://www.one2onediet.com/) and LighterLife products (https://www.lighterlife.com/) for Study 3 as we had an academic collaboration with the respective groups. The tables below provide macro-nutritional content of CPW (Table 1.5) and Lighter Life products (Table 1.6). Four products of CWP would, for example provide 806kcal/day with 18.2g of fat (10.5%), 95.9g of carbohydrates (55%), 60.1g of protein (34.5%), 11.1g of fibre and 113%DRV of micronutrients. In comparison, 4 products of LighterLife would provide 611kcal/day with 18.6g of fat (15%), 54.8g of carbohydrates (43%), 52.8g of protein (42%), 12.4g of fibre and 100-133% DRV micronutrients. In order to match the calories in 4 products of CWP (806kcal/day) to 4 products of Lighterlife (611kcal/day), with input from our team of academic nutritionists (Prof Brown, UCL and Prof Leeds, University of Denmark), we added 400mls of semi-skimmed milk to the dietary intervention of LED, as 400mls of semi-skimmed milk has 206kcal, with 8.13g fat, 19.31g carbohydrates, and 13.61g protein, making the macro and micronutrient composition similar (see Chapter 4, Study 3 for more details).

Table 1-5: Macro-nutrient content of CPW products

	Energy (kcal)	Saturated fats (g)	Unsaturated fats (g)	Salt (g)	Sugars (g)	Carbohydrates (g)	Fibre (g)	Protein (g)
Bar	206	2.5	4.4	0.4	16.0	22.30	3.1	13.4
Shake	200	0.8	3.1	1.0	3.0	22.3	2.6	17.6
Soup	200	0.6	3.0	1.7	9.5	23.7	2.7	16.6
Porridge	200	1.0	2.8	0.0	7.50	27.6	2.7	12.50
TDR with 4 products/day	806	4.9	13.3	3.1	36	95.9	11.1	60.1

Ingredient and nutritional information. TDR, total diet replacement

Table 1-6: Macro-nutrient content of Lighter life products

	Energy (kcal)	Saturated fats (g)	Unsaturated fats (g)	Salt (g)	Sugars (g)	Carbohydrates (g)	Fibre (g)	Protein (g)
Bar	155	1.9	2.6	0.27	9.5	16.9	3.8	12.7
Shake	153	1.88	2.72	1.4	11.9	12.7	3.6	13.4
Soup	150	1.6	3.4	1.47	4.8	12.7	2.5	12.5
Porridge	153	1.44	3.06	1.3	5.1	12.5	2.5	14.2
TDR with 4 products/day	611	6.82	11.78	4.4	31.3	54.8	12.4	52.8

Ingredient and nutritional information. TDR, total diet replacement

Attrition and side effects

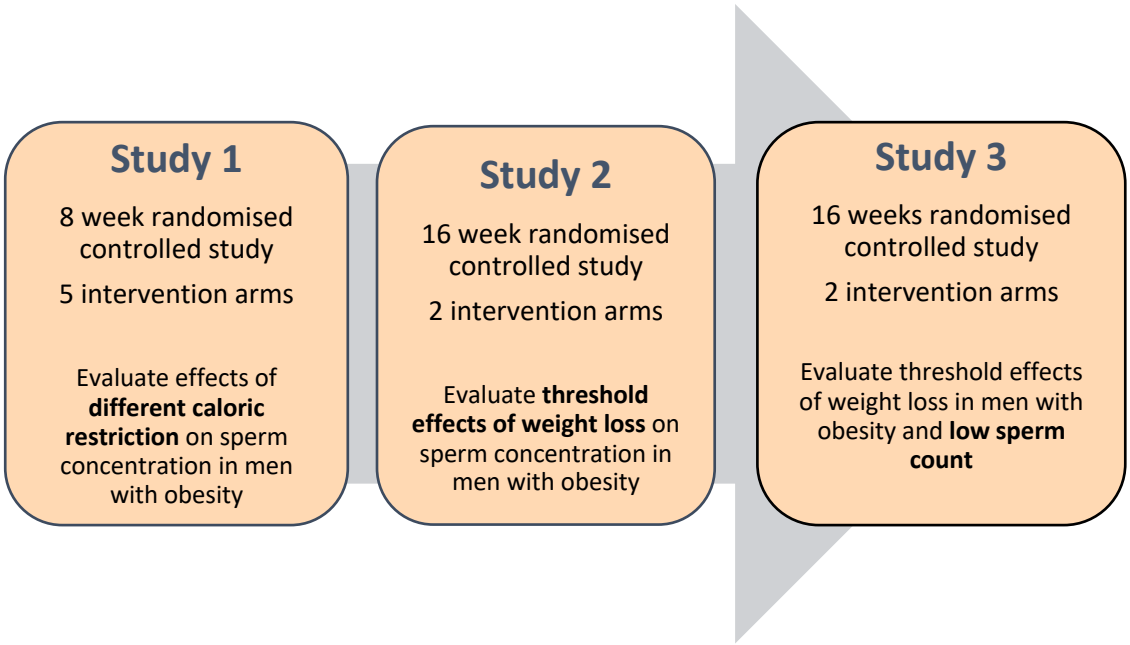
LED meal replacement products are safe, convenient and an effective weight loss tool. They help remove food-related daily-decision making process for the individuals. However, the recognised challenge of formula diets or any diet for that matter is weight maintenance by engaging people with long-term behavioural change strategies (Leeds, 2014). Weight regain following weight loss has its potential psychological effects. The exact mechanisms that drive weight regain following weight loss remain poorly understood but include physiological, psychological and biological factors. These could include higher hunger levels due to higher circulating levels of ghrelin (hunger hormone), and lower levels of satiety hormones such as leptin, peptide YY (PYY), and amylin (Purcell et al., 2014). However, provided initial weight loss is delivered in parallel with an education programme about nutrition, lifestyle and psychological support for long-term maintenance, subsequent weight maintenance after VLCDs and LCDs has been shown to be possible ((Christensen et al., 2017; Johansson et al., 2014; Mulholland et al., 2012). In addition, food reintroduction with extended use of LED meal replacements (1 per day) and high protein diets were associated with improved weight loss maintenance (Christensen et al., 2017; Johansson et al., 2014). Furthermore, recent evidence suggests a positive correlation between larger initial weight loss and long-term weight maintenance up to 4 years (Rössner et al., 2000; Wadden et al., 2011).

Side -effects are usually mild and self-limiting. During the initial stages of rapid weight loss, individuals may experience side effects such as hair loss, fatigue, dizziness, constipation, acute gout, cold intolerance, headaches, muscle cramps and gallstones (Saris, 2001; Wadden and Stunkard, 1986). Raised uric acid following significant weight loss may precipitate acute gallstone and gout (Christensen et al., 2011; Johansson et al., 2011). Therefore, gout and gallstone disease were a relative contraindication to being included in our studies. Lastly, as a consequence of energy restriction, formula LEDs can be associated with reductions in lean body mass (LBM) (Snel et al., 2012). However resistance exercise training (RET) may significantly improve LBM retention during LEDs, although the precise effect and magnitude are not yet elucidated. There are also inadequate evidence to discern the effect of additional dietary protein during LEDs with exercise to maintain LBM.

1.17 Rationale

In summary, spermatogenesis and steroidogenesis are the main functions of the testis. These are intricate processes under multiple levels of regulation dependent on the HPT axis. Semen analysis is the hallmark diagnostic test for male factor infertility worldwide, albeit not without its limitations, such that novel molecular markers such as ROS and DFI have since been developed as part of the work up of male factor infertility. Studies suggest that obesity is an increasingly recognised cause of male factor infertility associated with poor sperm quality, function and adverse reproductive hormone profile. Therefore it is important to consider weight loss as a possible solution to obesity associated male factor infertility especially in the context of lack of pharmacological treatments available. Bariatric surgery is the most effective method of weight loss however its effect on semen parameters is controversial, with many case series suggesting a deleterious impact on semen quality urging men who undergo bariatric surgery to consider sperm cryopreservation prior to surgery for future fertility options. Therefore, non-surgical approaches to weight loss by dietary methods such as LED offer a promising solution to male factor infertility, that needs further research with limited current available evidence, and no randomised controlled studies to date. I therefore carried out three randomised controlled studies to investigate the physiological effects of dietary weight loss on sperm quality in men with obesity (Figure 1.5).

Figure 1-5: Summary overview of the 3 studies included in this thesis



1.18 Aims of the thesis

1) To investigate the physiological effects of different levels of energy intake and weight loss on sperm quality to determine a potential threshold of weight loss in men with obesity (without known infertility) (Study 1, Chapter 2).

2) To investigate the effects of a fixed weight loss target (from Study1) observed to have optimum effects on sperm concentration verses a standard NHS advice arm in men with obesity without known infertility (Study 2, Chapter 3).

3) To determine if weight loss by LED improves sperm quality in men with obesity and **known oligospermia** (Study 3, Chapter 4).

Chapter 2: Effects of caloric restriction on sperm concentration in men with obesity

2.1 Introduction

Obesity is implicated as a potential cause of male infertility (Du Plessis et al., 2010; Hammoud et al., 2008; Ramlau-Hansen et al., 2007; Sallm n et al., 2006). Epidemiological data suggests that men with obesity have reduced sperm quality versus lean men however interventional data is lacking (Campbell et al., 2015; Punab et al., 2017; Sermondade et al., 2013). It has also been suggested that obesity could affect the epigenetic content of sperm or the metabolomic content of seminal fluid and thus impact on the reproductive and metabolic health of offspring and grand-offspring (Binder et al., 2015; Crisóstomo et al., 2021; Davidson et al., 2015; Donkin et al., 2016) (See Chapter 1 for details). Yet, the relationship between weight loss and semen quality is not fully understood in men with obesity.

Bariatric surgery is currently the most effective treatment for obesity with weight reductions of 20–35% reported (Miras and le Roux, 2013) two years after surgery and a steeper increase in testosterone levels than dietary weight loss (Corona et al., 2013a). However, effects on semen parameters in men are controversial (Lee et al., 2019; Wei et al., 2018). Some studies have suggested that the acute starvation-like state induced by surgery reduced semen quality, making this an inappropriate treatment option for obesity associated male infertility. Legro et al also observed that bariatric surgery reduces sperm concentration by 65% within the first month after bariatric surgery followed by a mild non-significant increase from baseline 12 months after surgery (Legro et al., 2014). Furthermore, a recent meta-analysis suggested that men undergoing bariatric surgery have no significant change in their sperm concentration or motility even 24 months post-surgery (Wei et al., 2018). Consistent with these human studies, no difference in sperm concentration was observed in male high-fat-diet-induced obese Sprague–Dawley rats 8 weeks after sleeve gastrectomy (Xiang et al., 2018).

Interestingly, recent evidence suggests that moderate weight loss following diet programmes with or without exercise could improve sperm quality and function. In male mice fed high fat diet, exercise or a combination of diet and exercise increases sperm motility, reduces sperm mitochondrial ROS and improves sperm DNA fragmentation in comparison to mice continuously fed a high fat diet (Palmer et al., 2012). Similarly, non-randomised uncontrolled prospective studies investigating the effect of weight loss via diet on semen parameters in men reported improvements in total sperm count (Håkonsen et al., 2011), morphology (Mir et al., 2018) and sperm DNA fragmentation (Faure et al., 2014), as well as an increase in the likelihood of

successful conception (Belan et al., 2015; Faure et al., 2014). We therefore decided to use LED as our weight loss intervention for Study 1 either as total diet replacement (TDR) or in combination with healthy meals to provide varying levels of caloric restriction to achieve weight loss. LED provides net energy deficit to achieve modest weight loss. It is also a convenient, widely-available and safe method to achieve modest weight loss in the short-term. However, to date, there are currently no prospective randomised controlled studies investigating whether weight loss via LED can improve sperm quality in men with obesity. Additionally, it is unclear what level of weight loss would be ideal to optimise sperm quality in obese men. We therefore conducted the first preliminary study investigating the physiological effects of different levels of energy intake on sperm quality in men with obesity.

Multiple studies have determined that sperm concentration is one of the best predictors of conception (Bonde et al., 1998) and is associated with important fertility outcomes such as time to pregnancy (Slama et al., 2002) and pregnancy rates (Zinaman et al., 2000). Therefore, we chose change in sperm concentration as our primary outcome, with change in other sperm parameters as secondary outcomes. Sperm DNA fragmentation is a recently identified marker of sperm damage which is associated with reduced fertility outcomes (Bungum et al., 2004; Spanò et al., 2000; Zeqiraj et al., 2018). We, therefore, also measured sperm DNA fragmentation pre and post intervention as an exploratory secondary outcome using the validated COMET assay (Lewis and Agbaje, 2008) (see methodology for details).

The study intervention period of 8 weeks was decided in collaboration with our nutrition team as a previous meta-analysis reported a pooled mean weight loss of -12.3kg (approximately 10%) over a median 8-week (range 3-16 weeks) LED/VLED dietary intervention (Johansson et al., 2014). We opted for LED (800-1200kcal/day) over VLED (<800kcal/day) as evidence shows comparable mean weight loss with either option, but better adherence, less side effects and less weight regain post LED vs VLED (Christensen et al., 2011) (see Chapter 1 on LED for details). We performed serial measurements (weekly to 2 weekly) of clinical, biochemical and andrological parameters during the study period to allow us to assess time course of response in our primary or secondary outcomes in men with obesity.

2.2 Hypothesis, aims and objectives

Hypothesis

Caloric restriction with LED would significantly improve sperm concentration in men with obesity.

Aims

To determine the following:

(i) Effects of different levels of caloric restriction on sperm parameters in men with obesity

(ii) Optimum level of weight loss by caloric restriction to improve semen sperm parameters in men with obesity

(iii) Effects of different levels of caloric restriction and weight loss on sperm DNA fragmentation, reproductive hormones and metabolic profile in men with obesity

Objectives

Primary objective:

1. Investigate the effects of weight loss by caloric restriction on **sperm concentration** in men with obesity.

Secondary objectives

1. Investigate the effects of weight loss on other sperm parameters (total motility, progressive motility, semen volume, morphology, TMC) and sperm DNA fragmentation in men with obesity.

2. Investigate the effects of weight loss on reproductive hormone parameters (serum total and calculated free testosterone, oestradiol, SHBG, LH and FSH) and metabolic parameters including weight loss, HbA1c, fasting glucose and lipid profile.

2.3 Methods

2.3.1 Ethical approval and study design

Ethical Approval

Ethical approval was granted by the London-Queen Square Research Ethics Committee (18/LO/0376).

Study Design

Participants were randomised to receive one of the five different levels of caloric intake per day (800kcal, 1000kcal, 1500kcal, standard NHS advise ('healthy plate'), and control (observation only) arm during an 8-week study protocol. Participants randomised to one of the energy restricted groups (800kcal, 1000kcal, 1500kcal) received LED using the Cambridge Weight Plan products (approximately 200kcal per product). These were used alone or in combination with normal meals to achieve the desired level of caloric intake. The NHS diet is based on the NHS advise on a healthy plate, whilst the control group carried on with their regular unrestricted Western diet (The British Dietetic Association, Controlling your portions 2015).

2.3.2 Participant recruitment

Written informed consent was obtained from all subjects. This study was performed in accordance with the Declaration of Helsinki. Participants were recruited through local posters, Imperial College web adverts, and healthy volunteer database from the Imperial Clinical Research Facility (ICRF). Recruitment was additionally supported by the National institute for Health Research Clinical research network (NIHR CRN, https://www.nihr.ac.uk/explore-nihr/support/clinical-research-network.htm) at North West London.

Responders to adverts were invited for a screening visit in the ICRF. The screening visit ensured patient fulfilled the inclusion and exclusion criteria by evaluating with a detailed medical history, clinical examination, blood tests and semen analysis. Full medical history included past cancer or mumps diagnosis, testicular surgery or trauma, systemic immunological disease, chronic cardiac, renal or liver disease, acute systemic illness, sexually transmitted disease, smoking or recreational drug use within the previous year, alcohol intake >30 units per week, sexually transmitted disease (STD) within previous year, medications likely to affect sperm function such as anabolic steroids, opiate analgesia and calcium channel blocker

(Mortimer et al., 2013). Testicular examination allowed screening for clinical conditions directly linked to impaired fertility such as undescended testis, testicular tumours, surgery or varicocele. Testicular volume was estimated using a Prader orchidometer. Screening baseline blood tests performed were as follows: LH; FSH; oestradiol; testosterone; SHBG; prolactin; fasting glucose; HbA1c and fasting lipids. Participants were asked to provide a semen sample in a designated private room in the Andrology Department of Hammersmith Hospital. Semen analysis was carried out using the WHO criteria following 2-7 days of sexual abstinence.

The inclusion and exclusion criteria are listed as below.

Inclusion criteria:

- Men aged 18 – 60 years old
- BMI ≥30 kg/m^2
- No known infertility
- Normal baseline semen parameters as per WHO criteria
- Stable weight over the previous 6 months duration

Exclusion criteria:

- Acute illness
- History of undescended testes, testicular surgery or mumps infection
- Hormonal therapy such as testosterone or selective oestrogen receptor modulators
- History of systemic cytotoxic therapy or pelvic radiotherapy
- Chronic systemic disease, such as cardiac, renal or liver failure
- Smoking
- Excessive ethanol intake (>30 units per week)
- Recreational drug use
- Impaired ability to provide full consent to take part in the study
- An occupation requiring strenuous physical exercise whereby caloric restriction would not be advisable.

2.3.3 Protocol

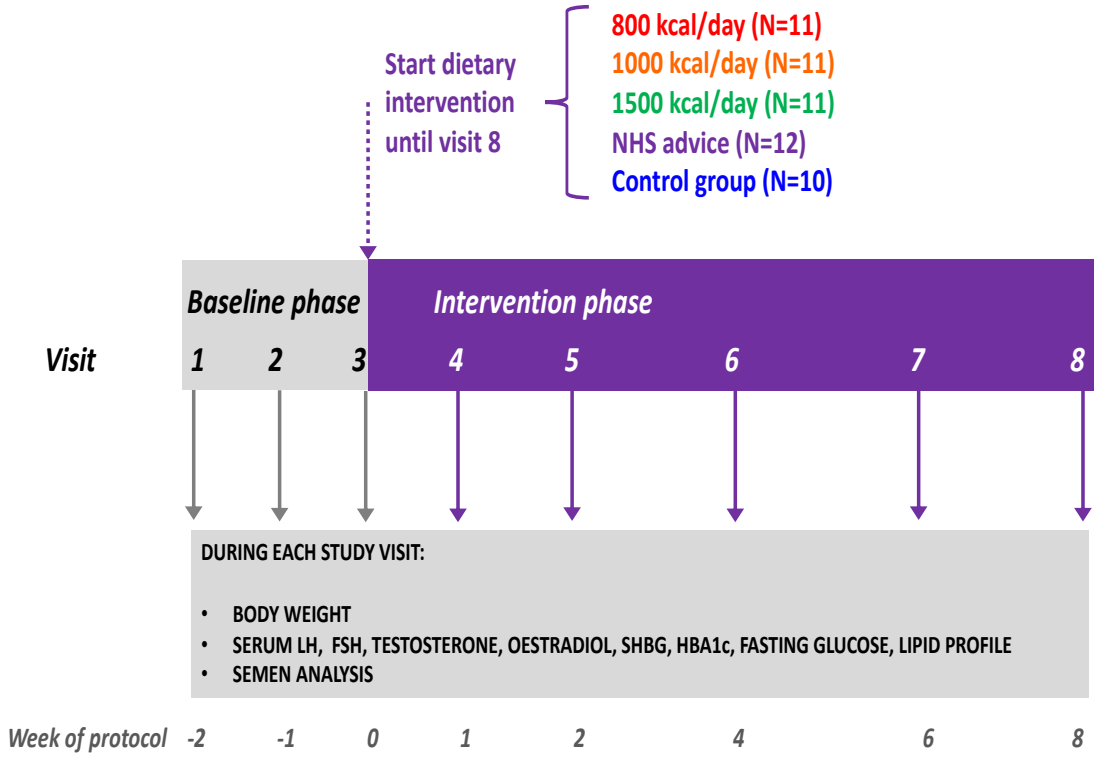
A randomized, controlled open label study was performed (see Protocol Summary, Figure 2.1).

Baseline period: The initial 2-week period (weeks -2 to 0, visits 1-3) allowed the measurement of baseline values of reproductive hormones, metabolic profile, semen analysis and the acclimatisation of participants to study conditions.

Intervention period: During week 0 (visit 3) of the protocol, participants were initiated on one of the five randomised study groups (800kCal/day, 1000kCal/day, 1500kCal/day, standard NHS advise ('healthy plate' 1800-2200kCal/day), or Western (control) diet for a 8-week intervention period involving further 5 visits. Dr. Jayasena, PI of the study, performed randomisation of the diet groups with stratification into each group at a ratio of 1:1. (random.org). Dietary intervention was carried out between weeks 0-8. Serial measurements of body weight, reproductive hormones, metabolic profile and semen analysis were carried out at each of the 8 study visits.

The study protocol for study 1 is summarised in Figure 2.1.

Figure 2-1: Summary of study protocol.



Fifty-five participants completed the study with 11 participants in 800kcal/day, 1000kCal/day, 1500kcal/day, 12 participants in NHS arm, and 10 participants in the control arm (observation only). LH, Luteinizing hormone; FSH, Follicle Stimulating Hormone; E2, Oestradiol; SHBG, Sex Hormone Binding Globulin

2.3.4 Diet groups

Cambridge Weight Plan products consisted of soups, shakes and bars of approximately 200kcal per product (Table 1.5, chapter 1). These LED products contained an array of micro and macronutrients (Table 1.5). These were used alone or in combination with normal meals to achieve the desired level of caloric intake as follows: 1) 800kcal/day diet consisted of 4 CWP products per day; 2)1000kcal/day: 4 CWP products and a healthy balanced meal of 200kcal; 3)1500kcal/day: 1 CWP product and 3 healthy meals of 1000kcal in total; 4) NHS diet was based on the standard NHS advise consisting of a balanced diet (1800-2200kcal/day) ("The

Eatwell Guide," 2018) whereby energy intake for weight loss was based on the Mifflin-St. Joer equations (Mifflin et al., 1990) and physical activity levels; 5) control group carried on with their regular unrestricted Western diet. All participants were advised to drink at least 2.5litres of water per day. In addition participants on TDR (800kcal/day) were provided with additional fibre supplements in the form of ispaghula husk sachets, as when required, to prevent constipation which can be one of the side effects of LED. These diets were planned with the expertise of our nutrition team (Profs Frost (Imperial College, Brown (University College London) and Leeds (University of Denmark).

2.3.5 Semen sampling

Participants were asked to provide a semen sample in a designated private room in the Andrology Department of Hammersmith Hospital according to WHO 2010 guidelines (Cooper, 2010) and UKNEQAS (UK National External Quality Assessment Service) accreditation. All semen samples were produced on site, to prevent sample degradation during transport, following 2-7 days of sexual abstinence. Samples were incubated at 36±1°C for liquefaction up to 60 minutes prior to analysis. All samples were manually analysed by experienced, Health and Care Professions Council (HCPC) registered biomedical scientists. Sperm morphology was examined on Papanicolaou pre-stained slides, using Kruger strict criteria. Sperm motility was determined as the percentage of progressive motile, non-progressive motile, and immotile spermatozoa by scoring at least 200 spermatozoa/slide (Cooper, 2010). Reference ranges for semen analyses were as follows: ≥15million/mL, sperm concentration; ≥1.5mL, volume; ≥40%, total motility; ≥32% progressive motility; ≥4%, normal morphology; ≥20million, total motile count. Total motile sperm count (TMC) was calculated using the formula: sperm concentration (million/ml) x percentage total motility (%) x semen volume (ml). Lastly, 1ml of fresh liquefied semen was stored at -20^{O}C for later DNA fragmentation analysis.

2.3.6 Sperm DNA fragmentation

DNA fragmentation index was calculated on the semen samples using the COMET assay (Lewis and Agbaje, 2008). Stored frozen semen was carefully thawed for the analysis to take place. The Comet assay is a versatile, sensitive yet simple and economical technique used to measure DNA damage. It consists of single-cell gel electrophoresis for measuring DNA strand breaks in individual sperm. Cells are embedded in agarose

on glass slides followed by lysis of the cell membranes after which damaged DNA strands are electrophoresed away from the nucleus towards the anode and deposited to one side giving the appearance of a comet tail. The extent of DNA migration depends directly on the DNA damage present in the cells such that damaged DNA migrates away from the intact DNA to form a tail producing a comet like appearance. Therefore, DNA fragmentation index can be measured by assessing the relative fluorescence of the damaged DNA in the tail compared with its intact DNA head, using specific image analysis software package (Simon and Carrell, 2013).

2.3.7 Measurement of reproductive hormones and metabolic profile:

Morning fasting blood samples were performed at each visit for measurements of LH, FSH, oestradiol, testosterone, SHBG, fasting glucose, HbA1c and fasting lipids. These were analysed in the clinical biochemistry department of Charing Cross Hospital, using Abbott ARCHITECT, an automated immunoassay platform under UKNEQAS accreditation. Hexokinase method was used for fasting serum glucose analysis. The Tosoh G8 Analyser was used for HbA1c testing and was performed by the clinical biochemistry department of Charing Cross Hospital. Reference ranges for males were as follows: LH, 2-12iu/L; FSH, 1.7-8iu/L; oestradiol <190pmol/L; SHBG 15-55 nmol/L; testosterone, 10-30nmol/L; fasting glucose, <7mmol/L; HbA1c, <48mmol/mol; total cholesterol <5mmol/l; LDL, <3mmol/l; HDL, >1mmol/l; triglycerides, <1.7mmol/l.

Inter-assay coefficients of variation were as follows: LH, 4.7%; FSH, 2.7%; oestradiol, 7.7%; SHBG, 6.4%; testosterone 8.1%; fasting glucose 1.8%; HbA1c 2.6%, total cholesterol 1.4%; HDL 2.1%, Limits of quantification for each assay were as follows: oestradiol 88pmol/l; FSH 0.11mIU/ml; LH 0.12mIU/ml; SHBG 4.5nmol/l; testosterone 0.06nmol/L; fasting glucose 0.12mmol/L; HbA1c 20mmol/mol, total cholesterol 0.16mmol/L; HDL 0.13mmol/L. LDL is not measured but calculated from the Friedewald equation: LDL=total cholesterol –(triglyceride/5)-HDL.

2.3.8 Inhibin B enzyme-linked immunosorbent assay (ELISA)

Blood samples for serum analysis were collected in plain serum vacutainer tubes (Beckton Dickson, Franklin Lakes, NJ, USA). Samples were allowed to clot prior to centrifugation and separation of serum. Samples

were centrifuged at room temperature using a Hettich EBA 20 machine (Hettich International, Tuttlingen, Germany) for 15minutes at 3000rpm, and then separated. Serum samples were stored at -20^{O}C until analysis. Measurement of serum inhibin B was performed on the stored serum at the end of the study using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) manufactured by Oxford Bio-Innovation (Oxford, United Kingdom) (Debieve et al., 2000; Mitchell et al., 2010). The intraassay and interassay variations were <10%. See Appendix 1: Inhibin B ELISA protocol

2.3.9 Other measurements

Anthropometric measurements included blood pressure (BP), weight and waist circumference (WC) carried out at each of the 8 visits.

2.3.10 Statistical analysis

Power calculation: This was performed in collaboration with Dr. Les Huson, Senior Statistician, NIHR Imperial Clinical Research Facility. A pilot study was performed in five men with obesity attending fertility clinic due to poor sperm quality, who were not taking any hormonal therapies (data not shown). During a 4-week formula LED with 800kcal daily energy intake, a 6-fold increase in median sperm concentration was observed. Sample size calculation was based on the methodology of Pinheiro et al. (Pinheiro et al., 2006), and these pilot data. We estimated that 12 subjects in each of the dietary groups will give greater than 90% power to detect a statistically significant linear trend in increased sperm concentration across the groups (alpha=0.05, two-sided) (Pinheiro et al., 2006). This calculation assumes that the between-patient standard deviation in sperm concentration is 6 mill/ml, which is a conservative estimate based on doubling the standard deviation observed in a sample of data from 1000 patients treated in the Andrology Department at Hammersmith Hospital. Furthermore, sample size of 12 per group matches the recommendation made by (Julious, 2005) for pilot and exploratory studies in cases where there is uncertainty about effect sizes.

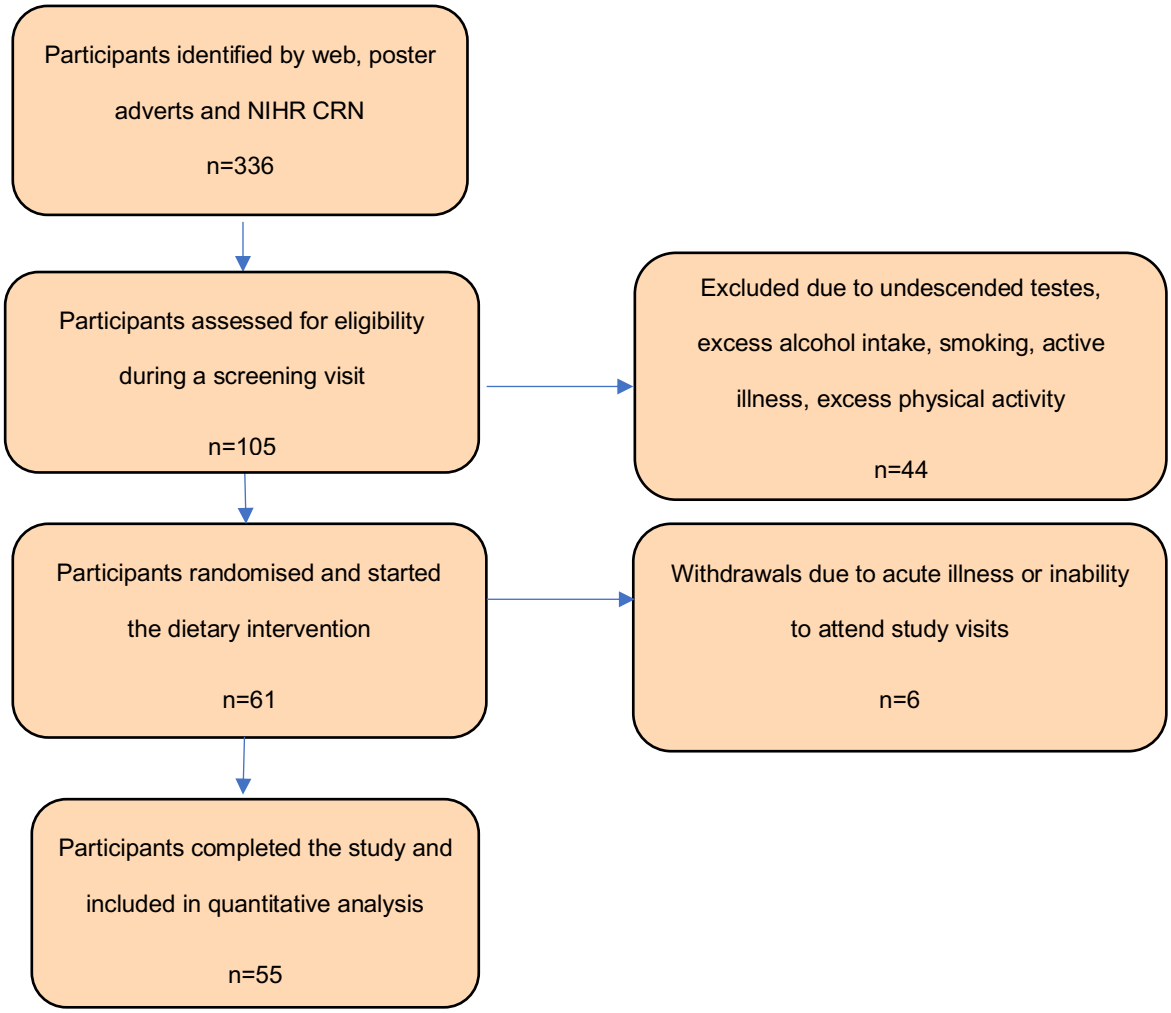
Data analysis: Data analysis was performed using GraphPad Prism v.8 (Graphpad Software Inc, La Jolla, CA, USA). Quantitative data was assessed for normality using D'Agostino-Pearson normality test followed by the appropriate analysis of variance (ANOVA). Comparison of means between the 5 intervention groups were compared using one-way ANOVA, with Tukey's multiple comparison test. Time profiles for semen

parameters during different time points were analysed using repeated measures two-way ANOVA with Geisser-Greenhouse post-hoc correction. Group comparisons with respect to categorical variables were performed using Chi-Squared test. In all cases, $P < 0.05$ was considered statistically significant. Data are presented as mean ± standard error of mean (SEM).

2.4 Results

We screened one hundred and six participants at a screening visit. Out of these, forty-three participants were excluded according to exclusion criteria above (see methods section). Sixty-one participants were included in the study. Six participants withdrew due to acute illness likely to affect the results of the study or inability to attend study visits. Fifty-five participants completed the study (Figure 2.2).

Figure 2-2: Patient flow diagram



NIHR: National Institute for Health and Research; CRN: Clinical Research Network

2.4.1 Baseline characteristics observed in men undergoing dietary intervention

Baseline clinical characteristics, metabolic and reproductive hormone profile, and semen parameters of male participants with obesity are summarised in Table 2.1. No significant differences in any of these baseline parameters were observed among any of the five randomised treatment groups.

Table 2-1: Baseline characteristics of male participants with obesity.

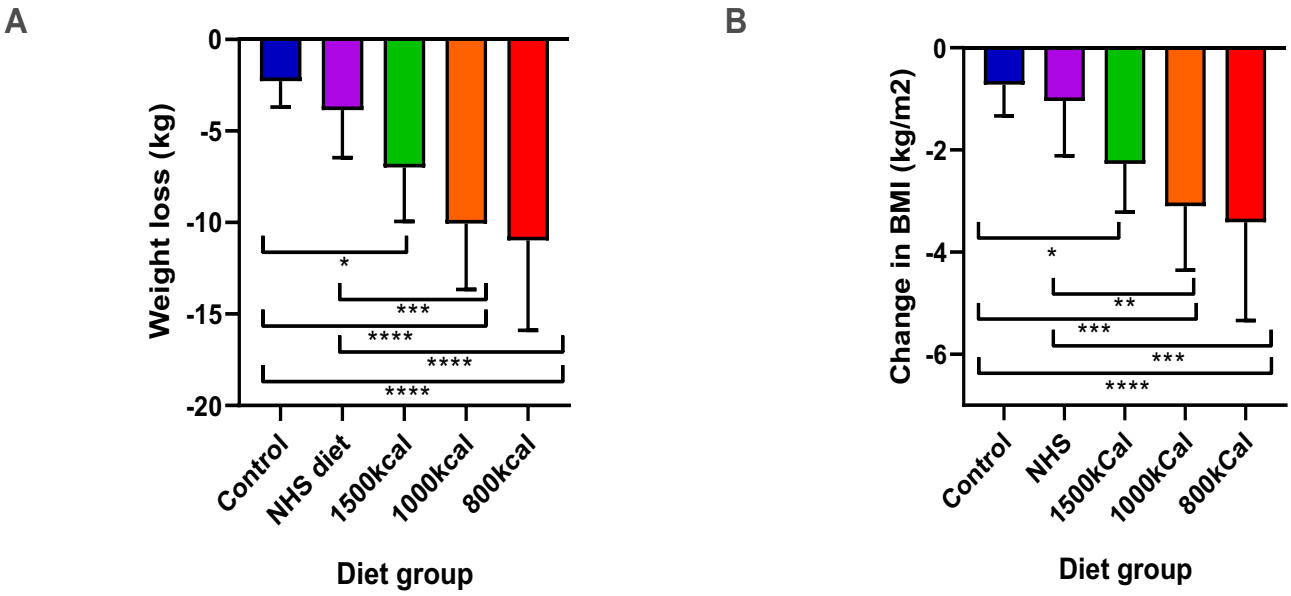
Characteristic (units)	Reference range	Dietary intervention group					p-value
		Control n=10	NHS n=12	1500kcal n=11	1000kcal n=11	800kcal n=11	
Age (years)	n/a	32.9 ± 2.7	40.5 ± 3.5	40.6 ± 2.5	39.6 ± 2.6	40.1 ± 3.0	0.33
Weight (kg)	n/a	105.3±3.8	110.9 ± 7.0	103.4 ± 3.0	107.8 ± 2.2	105.6 ± 3.7	0.78
BMI (kg/m^2)	n/a	35.0 ± 3.3	35.7 ± 5.0	33.3 ± 2.9	33.8 ± 3.2	34.6 ± 3.2	0.57
Waist circumference (cm)	n/a	114.9 ± 2.7	117.5 ± 4.4	117.0 ± 3.1	118.1 ± 3.9	122.5 ± 3.2	0.66
Ethnicity (White: non-White)	n/a	4:6	6:6	4:7	5:6	3:8	0.84
Testicular volume (ml)	n/a	20.3±1.7	17.8±1.7	19.2±1.3	17.4±1.3	18.5±1.8	0.19
Fasting blood glucose (mmol/l)	<7	5.5±0.3	5.7±0.4	5.3±0.2	5.0±0.1	5.6±0.4	0.43
HbA1c (mmol/mol)	<48	38.4±2	34.4±1.7	39.7±1.6	35.6±1.6	38.7±2	0.18
Total Cholesterol (mmol/l)	<5	5.3±0.3	4.9±0.3	5.1±0.2	5.2±0.3	5.2±0.2	0.81
LDL (mmol/l)	<3	3.2±0.3	3.1±0.3	3.5±0.2	3.5±0.2	3.7±0.2	0.41
HDL (mmol/l)	>1	1.0±0.1	1.2±0.1	1.1±0.1	1.1±0.1	0.9±0.0	0.08
Triglycerides (mmol/l)	<1.7	2.2±0.6	1.0±0.2	1.2±0.1	1.3±0.2	1.4±0.2	0.07
LH (units/l)	2-12	2.8± 0.3	3.3± 0.3	3.3± 0.2	4.3± 0.7	3.3± 0.3	0.10
FSH (units/l)	1.7-8	3.7± 0.7	3.5± 0.4	4.1± 0.6	5.4± 0.8	3.1± 0.6	0.10
Testosterone (nmol/l)	10-30	14.1± 1.9	12.5± 1.5	14.9± 2.0	14.3± 1.6	11.9± 1.4	0.67
SHBG (nmol/l)	15-55	22.8±3.8	26.6±2.8	23.7±2.4	26.3±2.7	26.0±3.4	0.87
Oestradiol (pmol/l)	<190	114.3± 5.9	103.7± 2.3	105.6± 3.2	117.5± 8.8	116.2± 6.4	0.21
Sperm concentration (million/ml)	≥15	63.6±18.0	73.9 ± 18.9	73.4 ± 18.0	73.9 ± 18.2	90.2 ± 27.7	0.93
Total motility (%)	≥40	64.0 ± 3.6	53.3 ± 3.5	61.1 ± 3.8	60.8 ± 2.4	62.6 ± 2.1	0.14
Progressive motility (%)	≥32	54.7 ± 5.9	46.5 ± 3.6	55.8 ± 4.9	55.5 ± 2.9	57.0 ± 2.4	0.35
Sperm morphology (%)	≥4	2.3 ± 0.7	3.3 ± 0.5	2.7 ± 0.7	2.8 ± 0.7	2.7± 0.5	0.89
Sperm volume (ml)	≥1.5	3.1± 0.6	3.2± 0.5	3.4± 0.4	3.4± 0.5	3.4± 0.9	0.90
Total motile count (million/ejaculate)	≥39	123.9±47.6	107.0±27.0	141.4±30.8	121.9±29.8	163.8±49.2	0.84

Data presented as mean+/-SEM; p-value calculated by one-way ANOVA, except for ethnicity which is expressed as %, with p-value calculated by chi-square test. BMI, body mass index; HbA1c, glycated haemoglobin; LDL, low density lipoprotein; HDL, high density lipoprotein; LH, luteinizing hormone; FSH, follicle stimulating hormone; SHBG, sex-hormone binding-globulin.

2.4.2 Weight loss observed in men undergoing dietary intervention

As expected, LED dietary interventions resulted in significantly greater weight loss when compared with either the NHS diet or the control group, with the greatest weight loss in the 800kcal/day group (mean change in weight in kg: -2.3 ± 0.5, control; - 3.9 ± 0.7, NHS; -7.0 ± 0.9, 1500kcal/day; -10.1 ± 1.1, 1000kcal/day; -11.0 ± 1.5, 800kcal/day, $P<0.0001$ vs. NHS, $P<0.0001$ vs. control) (Figure 2.3A). A similar trend was observed with change in BMI (Figure 2.3B), with the greatest decline in BMI in the 800kcal/day group (mean change in BMI in kg/m^2: -0.7 ± 0.2, control; - 1.0 ± 0.3, NHS; -2.3 ± 0.3, 1500kcal/day; -3.1 ± 0.3, 1000kcal/day; -3.4 ± 0.6, 800kcal/day, $P<0.001$ vs. NHS, $P<0.0001$ vs. control).

Figure 2-3: Bar graphs of changes in weight and BMI by dietary intervention groups



A-B: Bar graphs of the overall mean change in weight (A) and BMI (B) at the end of the dietary intervention period (mean level during visit V8) when compared with baseline values (mean of visits V1 to V3). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. **Blue,** Control; **Purple,** NHS; **Green,** 1500kCal; **Orange,** 1000kCal; **Red,** 800kCal.

2.4.3 Effects of dietary intervention on male metabolic profile

Table 2.2 summarises the changes in metabolic parameters from baseline to end of study period. Waist circumference (WC) and fasting glucose significantly reduced in the 800kcal group verses the NHS and control groups (waist circumference reduction in cm: 11.5 ± 1.9, 800kcal; 4.2 ± 1.5, NHS, P<0.05 vs. 800kcal; 2.2 ± 1.0, control; P<0.05 vs. 800kcal). Similarly, in the 800kcal/day group a significant reduction in total cholesterol was also observed in comparison to NHS (total cholesterol reduction in mmol/L: 1.2 ± 0.3, 800kcal; 0.5 ± 0.1, NHS, P<0.05 vs. 800kcal; 0.4 ± 0.1, control, P<0.05 vs. 800kcal).

Table 2-2: Change in metabolic parameters.

Characteristic (units)	Reference range	Dietary intervention group				
		Control n=10	NHS n=12	1500kcal n=11	1000kcal n=11	800kcal n=11
Waist circumference (cm)		-2.2 ± 1.0	-4.2 ± 1.5	-9.1 ± 2.2	-8.8 ± 2.3	-11.5 ± 1.9 $^{\alpha, \beta}$
Fasting glucose (mmol/L)	<7	- 0.5 ± 0.1	-0.8 ± 0.1	-0.6 ± 0.1	-0.7 ± 0.1	-1.3 ± 0.3 $^{\alpha\alpha,\beta\beta}$
HbA1c (mmol/mol)	<48	-1.5 ± 0.4	-1.4 ± 0.3	-2.5 ± 0.8	-3.4 ± 1.1	-3.7 ± 0.1
Total Cholesterol (mmol/l)	<5	-0.4 ± 0.1	-0.5 ± 0.1	-0.8 ± 0.1	-1.5 ± 0.2 $^{\alpha\alpha\alpha\alpha, \beta\beta\beta\beta}$	-1.2 ± 0.3 $^{\alpha\alpha\alpha, \beta\beta\beta}$
LDL (mmol/l)	<3	-0.43 ± 0.1	-0.33 ± 0.1	-0.80 ± 0.2	-1.09 ± 0.2 $^{\alpha\alpha\alpha\alpha\alpha}$	-0.91 ± 0.3
HDL (mmol/l)	>1	0.13 ± 0.0	0.13 ± 0.0	0.03 ± 0.0	-0.04 ± 0.0	0.59 ± 0.3
Triglycerides (mmol/L)	<1.7	-0.4 ± 0.1	-0.5 ± 0.2	-0.4 ± 0.1	-0.6 ± 0.1	-0.7 ± 0.2

Mean waist circumference, fasting glucose, HbA1c and fasting lipid profile change from baseline to the end of study period. Baseline study period refers to the mean of the first three visits (pre-start of diet). α = P≤0.05 800kcal vs NHS; $\alpha\alpha$ = P≤0.05 800kcal vs NHS; $\alpha\alpha\alpha$ = P≤0.05 800kcal vs NHS; $\alpha\alpha\alpha\alpha$ = P≤0.01 1000kCal Vs NHS; $\alpha\alpha\alpha\alpha\alpha$= P≤0.05 1000kcal vs NHS. β = P≤0.05 800kcal vs Control; $\beta\beta$ = P≤0.05 800kcal vs control; $\beta\beta\beta$ = P≤0.05 800kcal vs Control; $\beta\beta\beta\beta$ = P≤0.01 1000kcal vs Control

2.4.4 Effects of dietary intervention on reproductive hormones

During the intervention period, serum reproductive hormones (LH, FSH, total and calculated free testosterone, oestradiol, and SHBG) were not significantly different from baseline in any of the dietary intervention groups (Table 2.3).

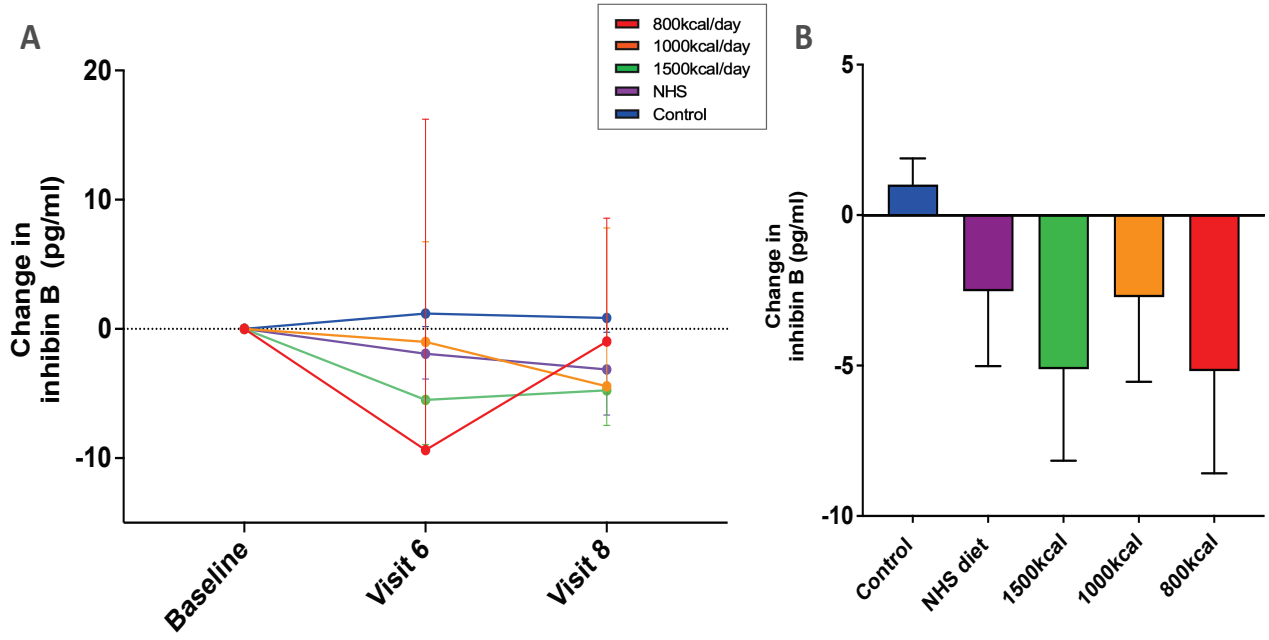
Table 2-3: Changes in reproductive endocrine hormones from baseline amongst the five dietary intervention groups.

Characteristic (units)	Reference range	Dietary intervention group				
		Control n=10	NHS n=12	1500kcal n=11	1000kcal n=11	800kcal n=11
LH (units/l)	2-12	+1.2 ± 0.4	+1.2 ± 0.2	+0.9 ± 0.3	+1.5 ± 0.5	+1.3 ± 0.3
FSH (units/l)	1.7-8	+0.4 ± 0.1	+0.4 ± 0.1	+0.1 ± 0.1	+0.2 ± 0.3	+0.2 ± 0.2
Testosterone (nmol/l)	10-30	+2.5 ± 0.8	+2.9 ± 0.9	+3.4 ± 0.8	+2.8 ± 0.6	+4.4 ± 0.7
Oestradiol (pmol/l)	<190	-8.5 ± 5.3	-6.1 ± 1.9	-3.1 ± 1.7	-13.3 ± 5.5	-5.2 ± 4.7
SHBG (nmol/l)	15-55	+4±1	+7±2	+5±1	+9±2	+10±2
Calculated free testosterone[§] (nmol/L)		+0.055±0.03	+0.073±0.03	+0.067±0.02	+0.026±0.01	+0.076±0.01

Mean change in reproductive hormone levels from baseline phase (mean of V1-V3) to end of dietary intervention. [§] § Free testosterone was calculated using the free and bioavailable testosterone calculator based on the Vermeulen formula (http://www.pctag.uk/testosterone-calculator*).*

Inhibin B: FSH stimulates inhibin B from Sertoli cells, as a marker of spermatogenesis (Pierik et al., 1998). We measured the inhibin B at baseline (visit 3, pre-start of diet), visit 6 (1 month post-diet start), and visit 8 (end of diet), using a commercial ELISA kit (Appendix 1). Similar to FSH, no significant changes were observed in inhibin B at visit 6 or 8 compared with baseline levels in any of the dietary intervention groups (Figure 2.4).

Figure 2-4: Time profiles and bar graphs of changes in Inhibin B in men with obesity during 8 week dietary intervention.

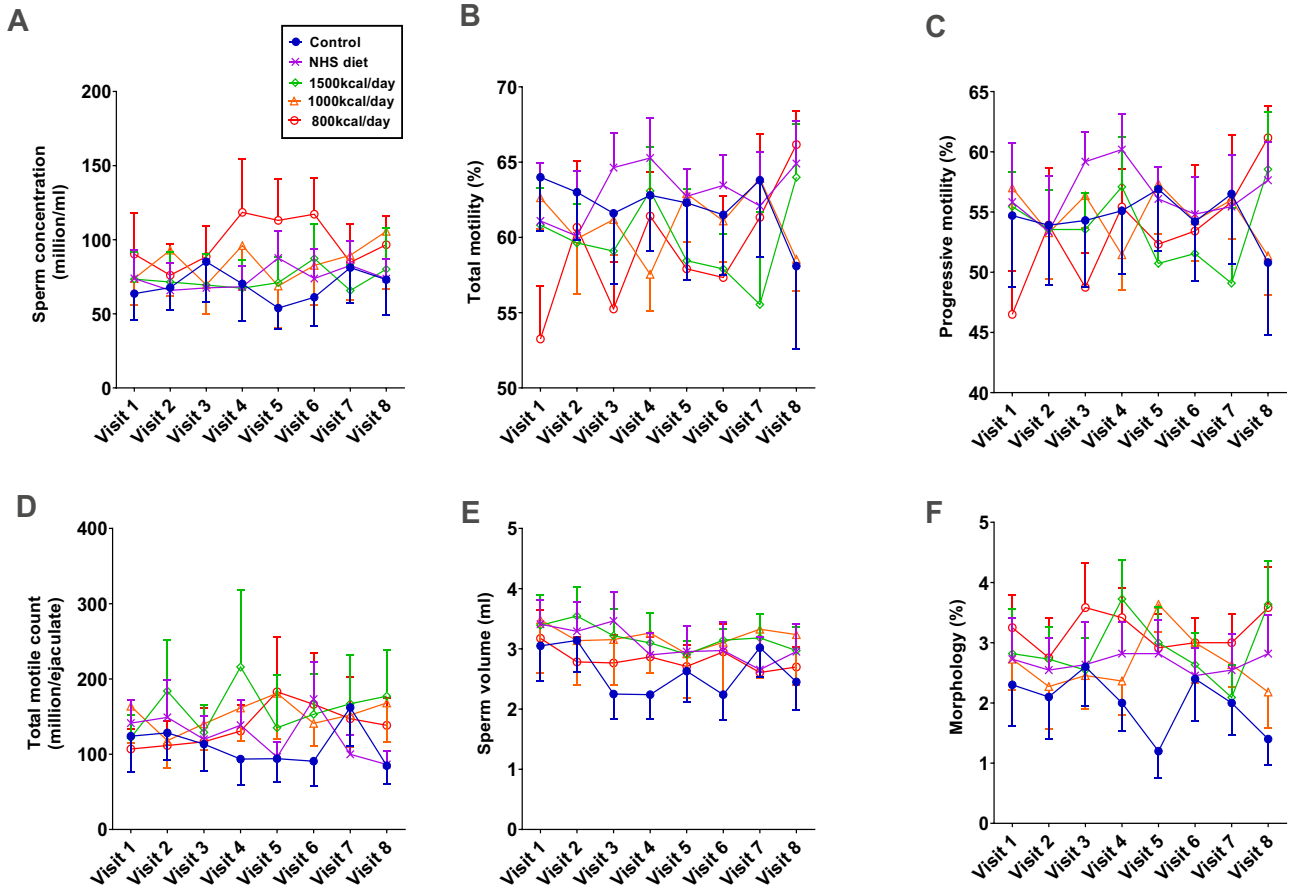


A: Time profile of change in mean inhibin B (+/-SD) at 1 month of dietary intervention (visit 6)and end (visit 8) of dietary intervention period compared with baseline (visit 3). **B:** Bar graphs of overall mean change +/-SD in Inhibin B level (mean of visits V6 and V8) when compared with a baseline visit (visit V3). SD: standard deviation.

2.4.5 Effects of dietary intervention on sperm quality

Semen parameters are subject to large biological variation within individuals with standard deviations comparable to mean levels. Therefore to compare multiple time points at each of the 8 study visits, we used a repeated measures two-way ANOVA to illustrate time profiles of semen parameters in the five different intervention groups in men with obesity (Figure 2.5).

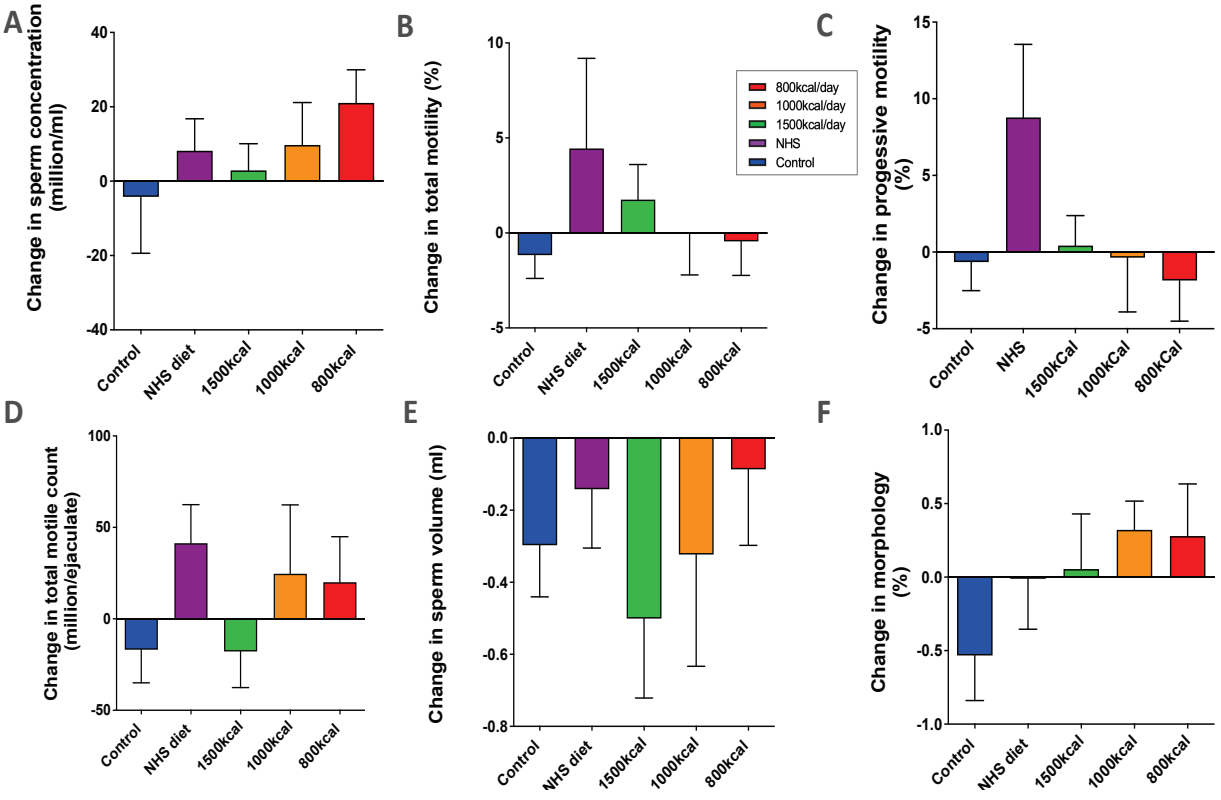
Figure 2-5: Time profiles of changes in semen parameters in men with obesity randomised to five different dietary interventions.



A-F: Time profiles of change in mean (+/-SD) sperm concentration (A), total motility (B), progressive motility (C), total motile count (D), sperm volume (E) and morphology (F) during each timepoint of the study period (visits V1 -V8) for the five dietary intervention groups. **Blue,** Control; **Purple,** NHS; **Green,** 1500kCal; **Orange,** 1000kCal; **Red,** 800kCal. SD: standard deviation

Furthermore, to allow for this intra-individual variability in semen analysis, we used mean values of each semen parameter during multiple visits for further analysis; for example, baseline study period was the average measurement of the 1st three visits (pre-start of diet). We then compared the overall mean changes in semen parameters during the dietary intervention period (mean level during visits V4 to V8) when compared with baseline values (mean of visits V1 to V3) during each dietary intervention (Figure 2.6). An increase in mean sperm concentration was observed during 800kcal/day diet when compared with baseline levels but this change was not significantly different from any other diet group (change in sperm concentration in mill/ml: -4.2 ± 15.2, control; +8.2 ± 8.6, NHS; +2.9 ± 7.2, 1500kcal/day; +9.7 ± 11.4, 1000kcal/day; +21.1 ± 8.9, 800kcal/day, $P>0.05$). No significant changes in any other semen quality parameters were observed with any of the dietary intervention groups.

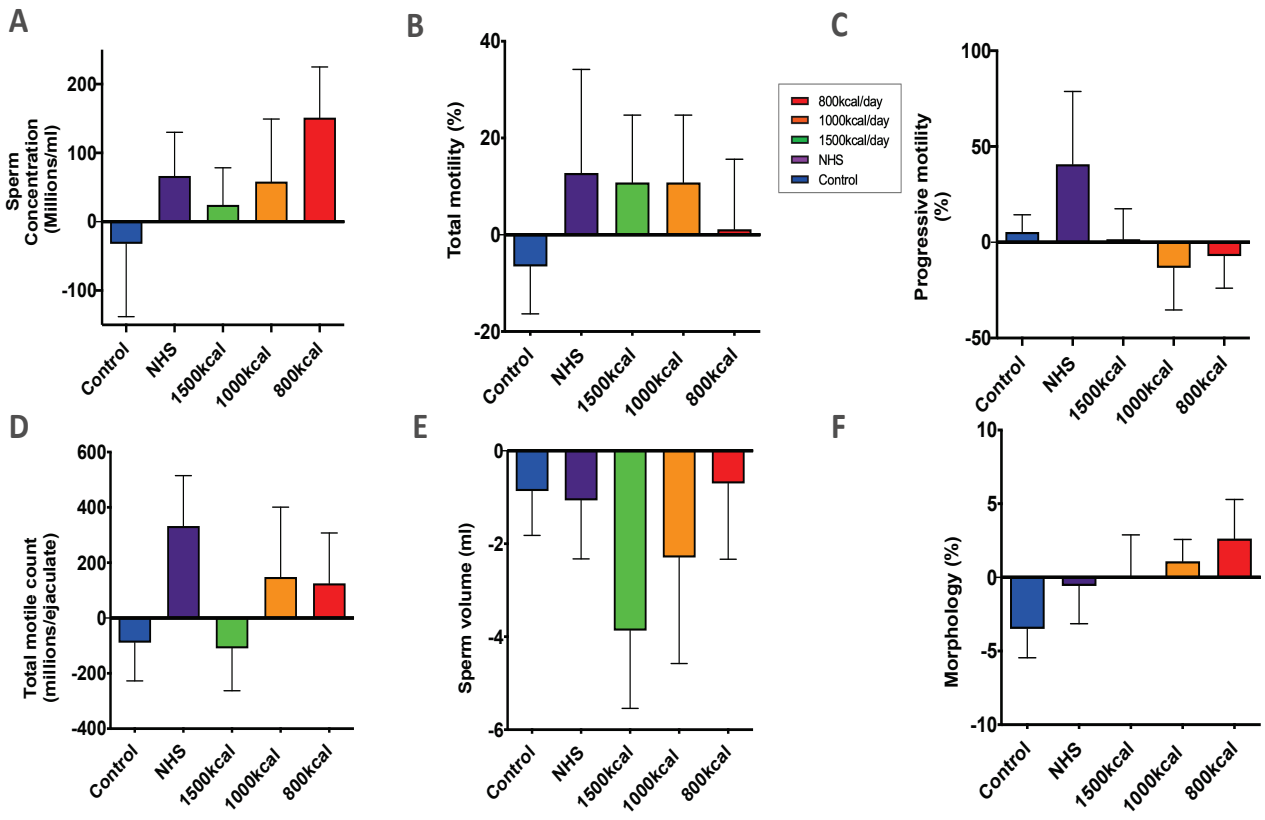
Figure 2-6: Bar graphs of changes in semen parameters in men with obesity randomised to five different dietary intervention groups.



A-F: Bar graphs of overall mean change in sperm concentration (A), total motility (B), progressive motility (C), total motile count (D), sperm volume (E) and morphology (F) during the dietary intervention period (mean level during visits V4 to V8) when compared with baseline values (mean of visits V1 to V3).

I also analysed the data using cumulative area under the curve (AUC) for mean changes in semen parameters from baseline in each of the 5 intervention groups. No significant differences in AUC change from baseline of any semen parameter were observed during the study (Figure 2.7).

Figure 2-7: Area under the curve of sperm parameters during the study period for each of the diet groups.



The bar graphs present area under the curve (mean increment against time) for sperm concentration (A), total motility (B), progressive motility (C), total motile count (D), sperm volume (E) and morphology (F) for each of the five diet groups. AUC, Area under the curve.

Sperm DNA fragmentation index: Sperm DNA fragmentation is a recently identified marker of sperm damage which is associated with reduced fertility outcomes (Lewis and Agbaje, 2008). COMET assay was used to measure DNA fragmentation in the semen samples (see section on Methods) at baseline (i.e. visit 3, pre-start of diet) and V7 (end of diet period). DNA fragmentation scores were reported in three COMET parameters as follows:

- The Average Comet Score (ACS) – the average % DNA damage per sample
- The Low Comet Score (LCS) – % of sperm cells which are classified as normal
- The High Comet Score (HCS) - % of sperm cells which are classified as particularly bad (~>50% damage)

Baseline COMET parameters in male participants with obesity are summarised in Table 2.4. The baseline mean ACS and HCS scores were higher than reported reference ranges in the fertile population (Nicopoullos et al., 2019), suggestive of high baseline mean DNA damage (ACS), exceptionally high baseline numbers of sperm with damaged DNA (HCS), and very low numbers of sperm with high quality DNA (LCS) in men with obesity (Table 2.4). Furthermore, a significant difference was noted in baseline mean ACS and LCS scores between the 1500kcal/day group vs control group (ACS mean difference, CI in %: 10.5, 0.2 to 20.7, p-value=0.04 1500 vs control; LCS mean difference -18.5, 34.9 to -2.1, p-value 0.0196, 1500 vs control). Therefore the groups were not similar at baseline in terms of their sperm DNA fragmentation scores with the 1500kcal group having worse DNA fragmentation than the control group.

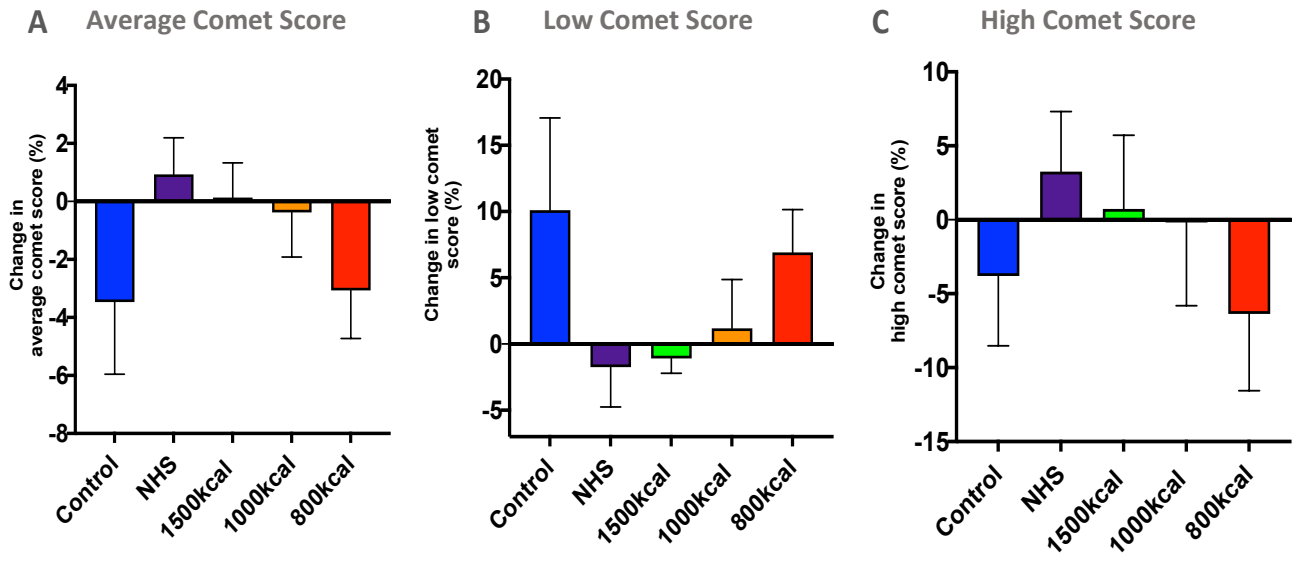
Table 2-4: Baseline DNA fragmentation as measured by COMET assay in male participants with obesity.

COMET score (units)	Fertile range**	Dietary intervention group					p-value
		Control n=10	NHS n=12	1500kcal n=11	1000kcal n=11	800kcal n=11	
Average Comet Score (ACS) %	0-26%	42.4 ± 2.6	46.6 ± 2.8	52.9 ± 2.0	46.5 ± 2.5	50.5 ± 2.5	0.054
Low Comet Score (LCS) %	74-100%	24.4 ± 6.2	16.8 ± 4.7	5.9 ± 1.7	15.3 ± 3.4	9.6 ± 2.7	0.03*
High Comet Score (HCS) %	0-4%	33.5 ± 7.5	42.2 ± 8.8	63.9 ± 6.7	41.0 ± 9.1	53.6 ± 8.4	0.098

Frozen semen used from visit 3 (pre-start of diet). Data presented as mean+/-SEM; p-value calculated by one-way ANOVA. ** values based on sensitivity/specificity data from fertile vs infertile men (Nicopoullos et al., 2019).

I thereafter analysed the incremental change in these COMET DNA fragmentation scores between the start (V3) and the end of study period (V7) (Figure 2.8). There were no significant changes observed in either ACS, LCS or HCS in any of the dietary intervention groups (Figure 2.8) in men with obesity.

Figure 2-8: Bar graphs of changes in DNA fragmentation indices in men with obesity randomised to five different dietary intervention groups.

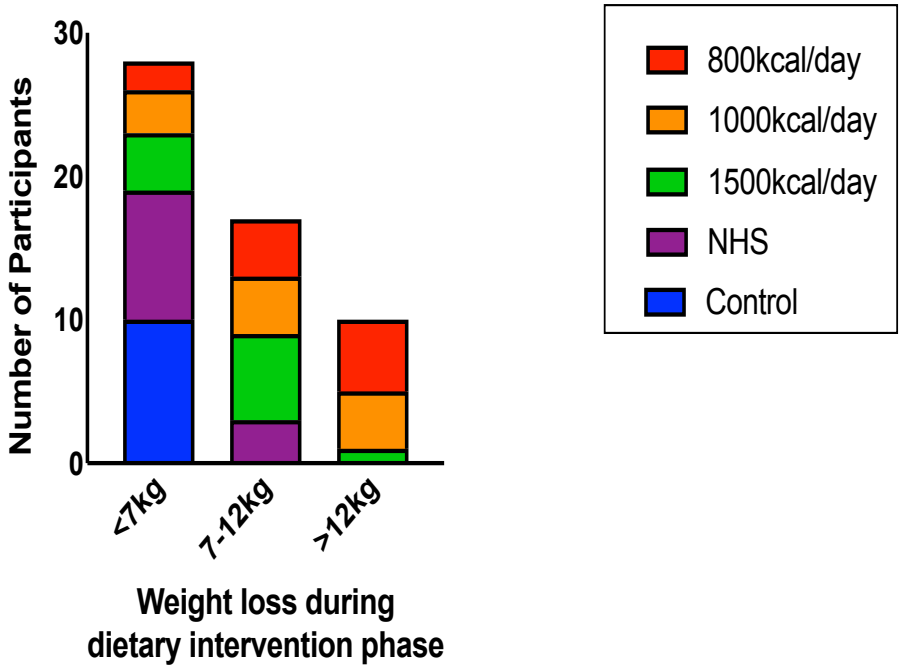


A-C: Bar graphs of overall mean change in Average Comet Score (A), Low Comet Score (B), and High Comet Score (C) at the end of the dietary intervention period (visit V7) when compared with a baseline visit (visit V3).

2.4.6 Classification by weight loss in men with obesity undergoing dietary intervention

The rationale of our study protocol with multiple dietary intervention groups was to achieve varying degrees of weight loss to investigate threshold-dependent effects of weight loss on sperm parameters. However, there was a large variation in the weight loss achieved within each dietary group suggestive of variable adherence in participants to the dietary protocols (Figure 2.9). Therefore, we did a post-hoc analysis of our data based on weight loss and investigated the effects of three levels of weight loss categories on sperm quality, DNA fragmentation, reproductive hormones including inhibin B and metabolic profile as detailed below.

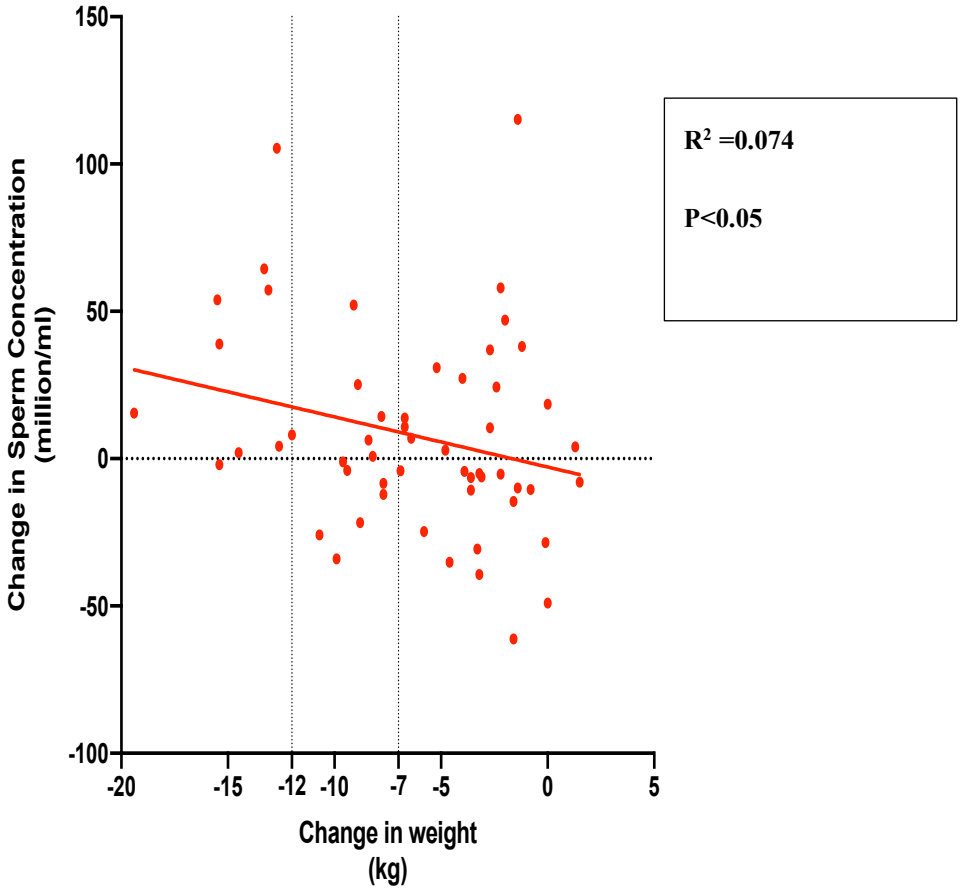
Figure 2-9: Classification by degree of weight loss achieved in men with obesity after 8 week dietary intervention.



Bar graph of number of men classified by degree of weight loss achieved from baseline (mean of visits V1 to V3) after 8 weeks of dietary intervention (<7kg; 7-12kg;>12kg). **Blue,** Control; **Purple,** NHS; **Green,** 1500kCal; **Orange,** 1000kCal; **Red,** 800kCal.

A scatter graph of change in mean sperm concentration against degree of weight loss from baseline observed an arbitrary threshold of weight loss of >12kg resulting in improvement in sperm concentration in our study cohort (Figure 2.10). To further investigate the effects of weight loss on sperm concentration, we compared the results of participants losing >12kg (n=10) with those losing 7-12kg (n=17) or <7kg (n=28) from baseline to the end of study period. We have henceforth presented the results with line graphs illustrating time profiles of mean changes in metabolic profile reproductive hormones and sperm parameters at different study time points, and overall mean changes from baseline to during study period using bar graphs in each of the three weight loss categories (>12kg, 7-12kg, <7kg).

Figure 2-10: Scatter graph of change in sperm concentration by change in weight from baseline to end of study period.

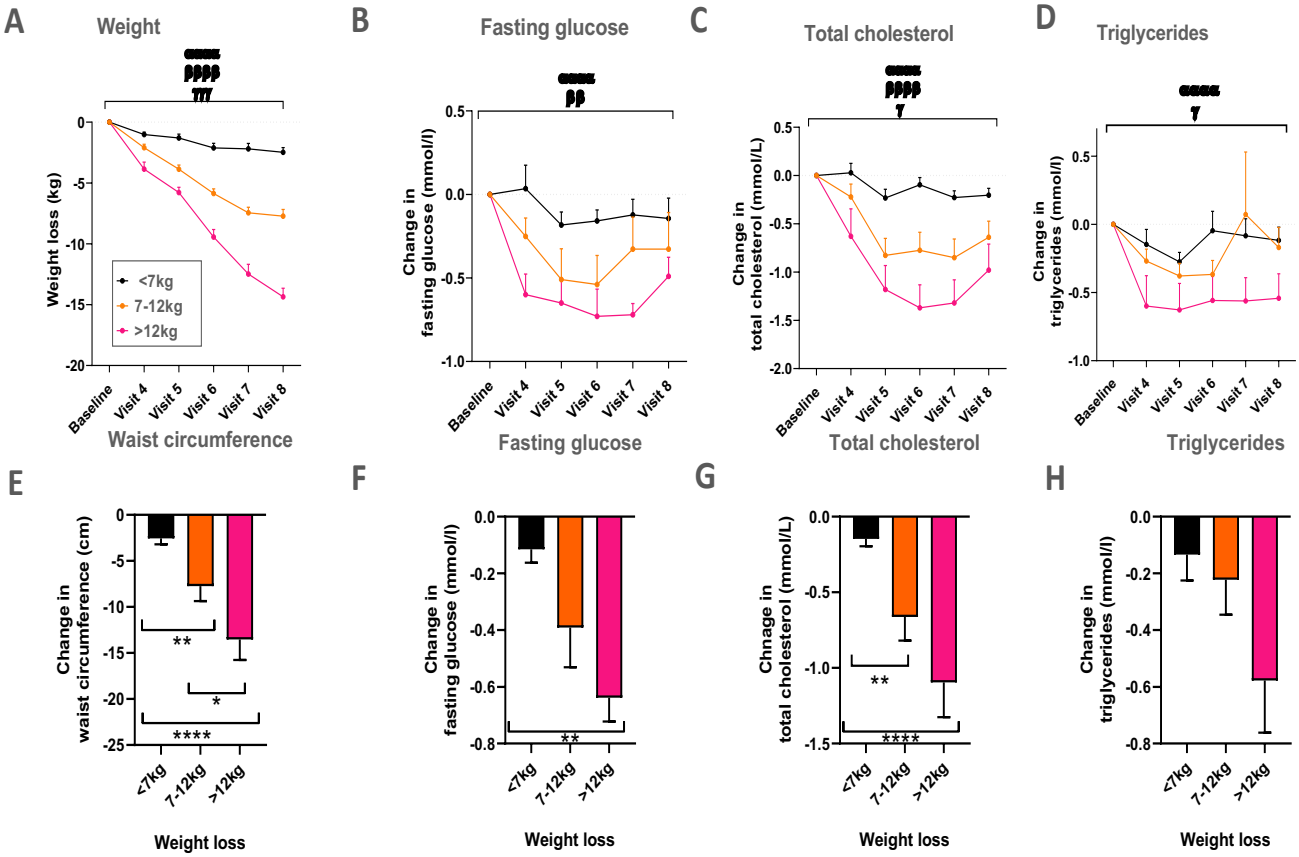


The dotted lines on the X axis highlight the threshold of weight loss at 7kg and 12kg used to define the three weight loss categories.

2.4.7 Effects of weight loss on metabolic profile

In men with obesity losing >12kg, mean waist circumference was significantly lower during the dietary intervention phase when compared with either <7kg or 7-12kg groups (change in waist circumference in cm: -2.5 ± 0.6, <7kg; -7.7 ± 1.6, 7-12kg; -13.5 ± 2.2, >12kg, P<0.0001 vs. <7kg, P<0.05 vs. 7-12kg) (Figure 2.11A). Mean fasting glucose was significantly lower in men with obesity losing >12kg weight when compared with <7kg but not 7-12kg group (change in fasting glucose in mmol/l: -0.1 ± 0.0, <7kg; -0.4 ± 0.1, 7-12kg; -0.6 ± 0.1, >12kg, P<0.01 vs. <7kg; P=ns vs 7-12kg) (Figure 2.11B). Furthermore, mean fasting total cholesterol was significantly lower in men with obesity losing >12kg weight when compared with either <7kg or 7-12kg groups (change in total cholesterol in mmol/l: -0.1 ± 0.0, <7kg; -0.7 ± 0.2, 7-12kg; -1.1 ± 0.2, >12kg, P<0.0001 vs. <7kg, P<0.01 vs. 7-12kg) (Figure 2.11C). No significant changes in serum triglycerides were observed in any of the weight loss groups (Figure 2.11D).

Figure 2-11: Time profiles of changes in metabolic parameters in men with obesity classified by degree of weight loss during 8 week dietary intervention.

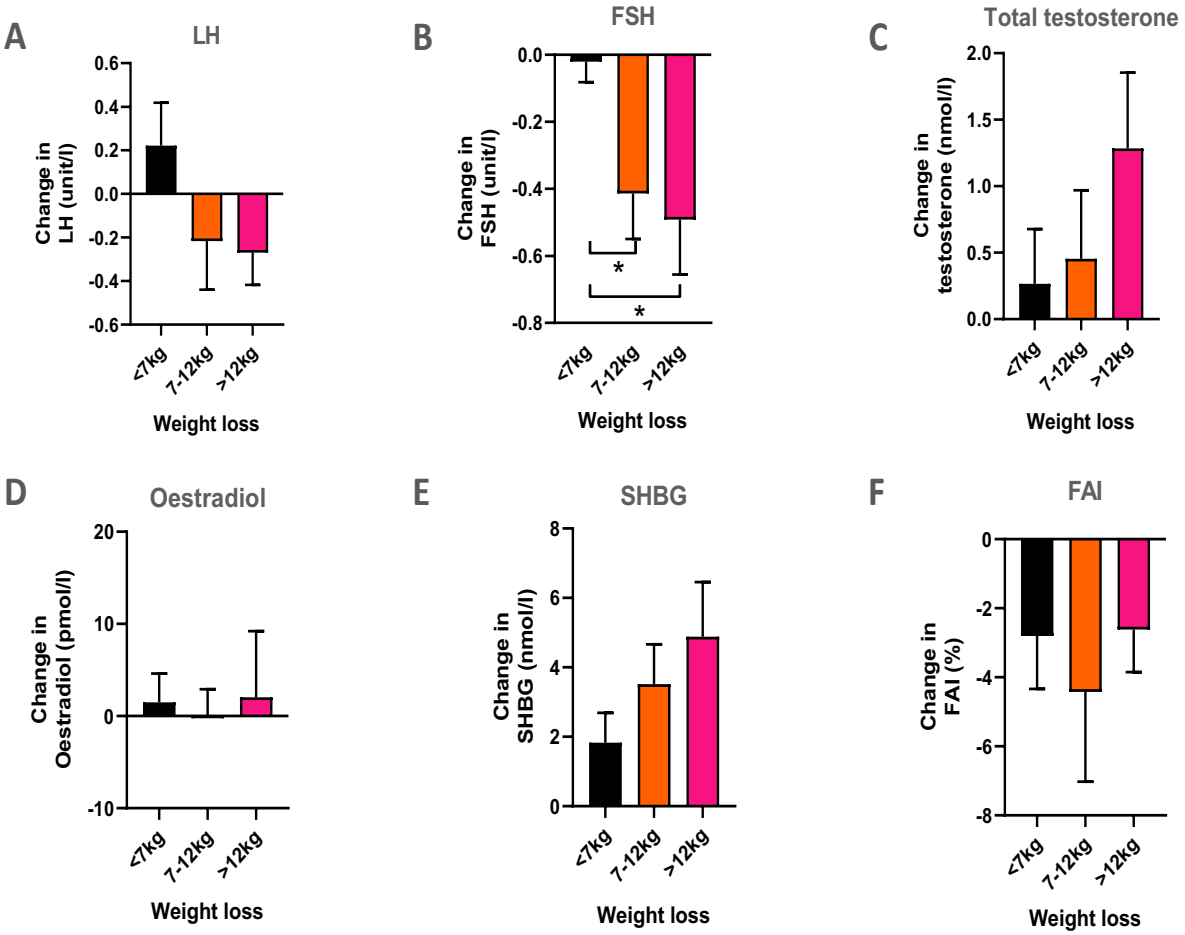


A-D: Time profiles of change in weight (A), fasting glucose (B), total cholesterol (C) and triglycerides (D) during the dietary intervention period (mean of visits V4 to V8) when compared with baseline values (mean of visits V1 to V3). αααα P<0.0001, >12kg vs. <7kg; ββ P<0.01, <7kg vs. 7-12kg; ββββ P<0.0001, <7kg vs. 7-12kg; γ P<0.05, >12kg vs. 7-12kg; γγγ P<0.001, >12kg vs. 7-12kg. **E-H:** Bar graphs of the overall mean change in waist circumference (E), fasting glucose (F), total cholesterol (G) and triglycerides (H) during the dietary intervention period (mean level during visits V4 to V8) when compared with baseline values (mean of visits V1 to V3). *, P<0.05;**, P<0.01; ****, P<0.0001. **Black,** <7kg; **Orange,** 7-12kg; **Pink,** >12kg.

2.4.8 Effects of weight loss on reproductive endocrine profile

Mean changes in reproductive endocrine hormones (Figure 2.12 A-F): Serum FSH was reduced in both 7-12 and >12kg weight loss groups when compared with the <7kg group (change in FSH in unit/l: -0.02 ± 0.1, <7kg; -0.4 ± 0.1, 7-12kg; -0.5 ± 0.2, >12kg, $P<0.05$ vs. <7kg) (Figure 2.12B). Total testosterone and SHBG increased in the >12kg group compared with both 7-12 and <7kg weight loss groups however the changes were not statistically significant (Figure 2.12C,E). No significant changes in serum LH, total testosterone, oestradiol, SHBG and FAI were observed (Figure 2.12A,C,D,E,F).

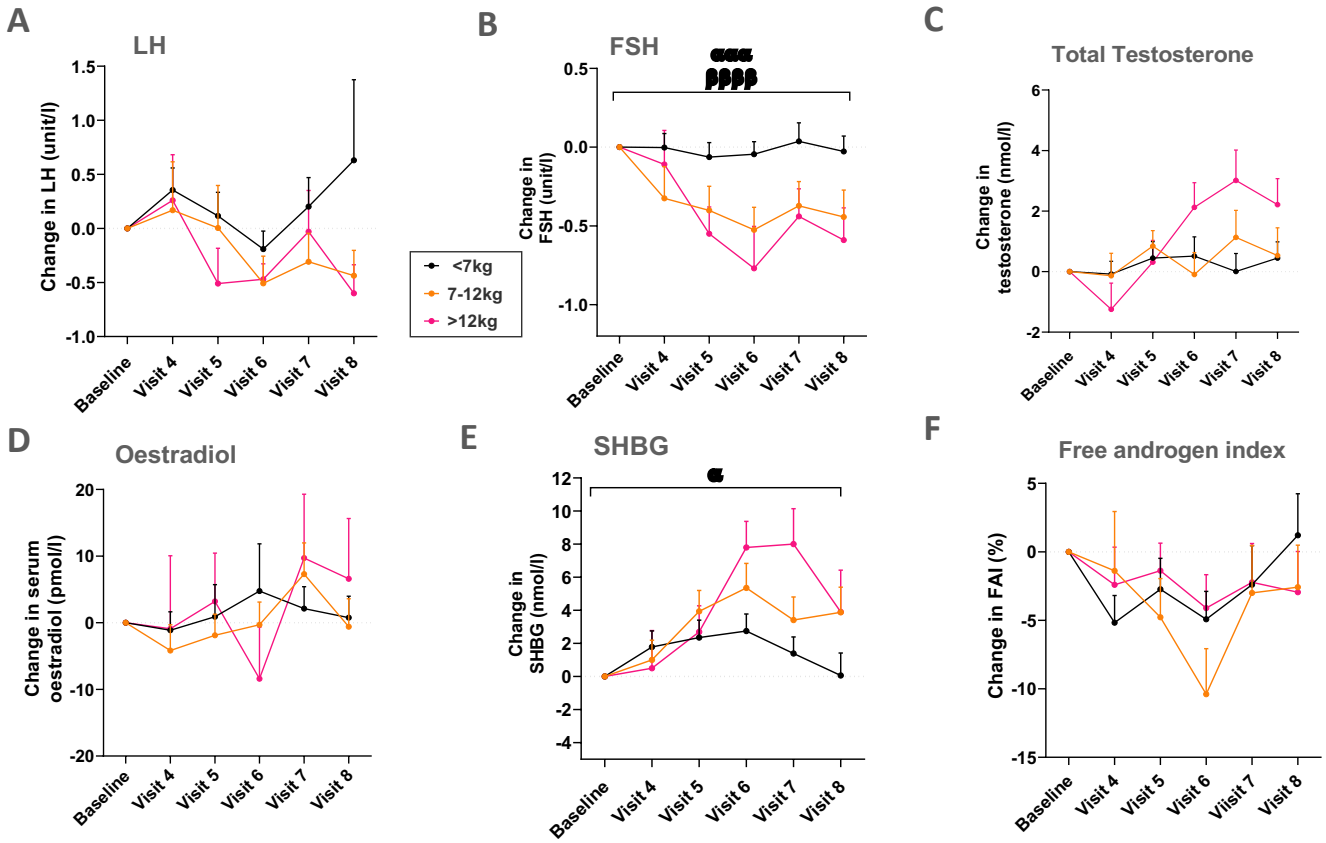
Figure 2-12: Bar graphs of changes in reproductive hormones by weight loss.



These bar graphs present mean changes in LH (A), FSH (B), testosterone (C), oestradiol (D), SHBG (E), FAI (F) from baseline to during and end of study period by weight loss. **Black,** <7kg; **Orange,** 7-12kg; **Pink,** >12kg. *<7kg vs. >12kg p= 0.0156 *<7kg vs. 7-12kg p= 0.0157

Time profiles of reproductive endocrine hormones (Figure 2.13A-F)): Time profiles of changes in serum reproductive hormones from baseline levels (mean of visits V1-V3) were analysed in men with obesity within each weight loss group (<7, 7-12 and >12kg). Serum FSH was reduced from baseline in men losing 7-12kg or >12kg, but not <7kg ($P<0.001$ vs. 7-12kg or >12kg) during the dietary intervention period. The maximal reduction in FSH within the >12kg group was -0.8 +/- 0.3 unit/l; this was observed during visit 6 (after 1 month of dietary intervention), which coincided with maximal increments in sperm concentration observed during dietary intervention (Figure 2.13B). Serum total testosterone levels were higher than baseline during visits 5-8 in men with obesity losing >12kg weight; however, no significant differences were observed when compared with either <7kg or 7-12kg weight loss groups (Figure 2.13C). SHBG is a circulating binding protein for testosterone. Serum SHBG levels increased from baseline in all weight loss groups but were highest in the >12kg group ($P<0.05$ vs. <7kg) (Figure 2.13E). However, no significant changes in time profiles of LH, oestradiol or FAI were observed (Figure 2.13A,D,F).

Figure 2-13: Time profiles of changes in reproductive endocrine hormones in men with obesity classified by degree of weight loss during 8 week dietary intervention.



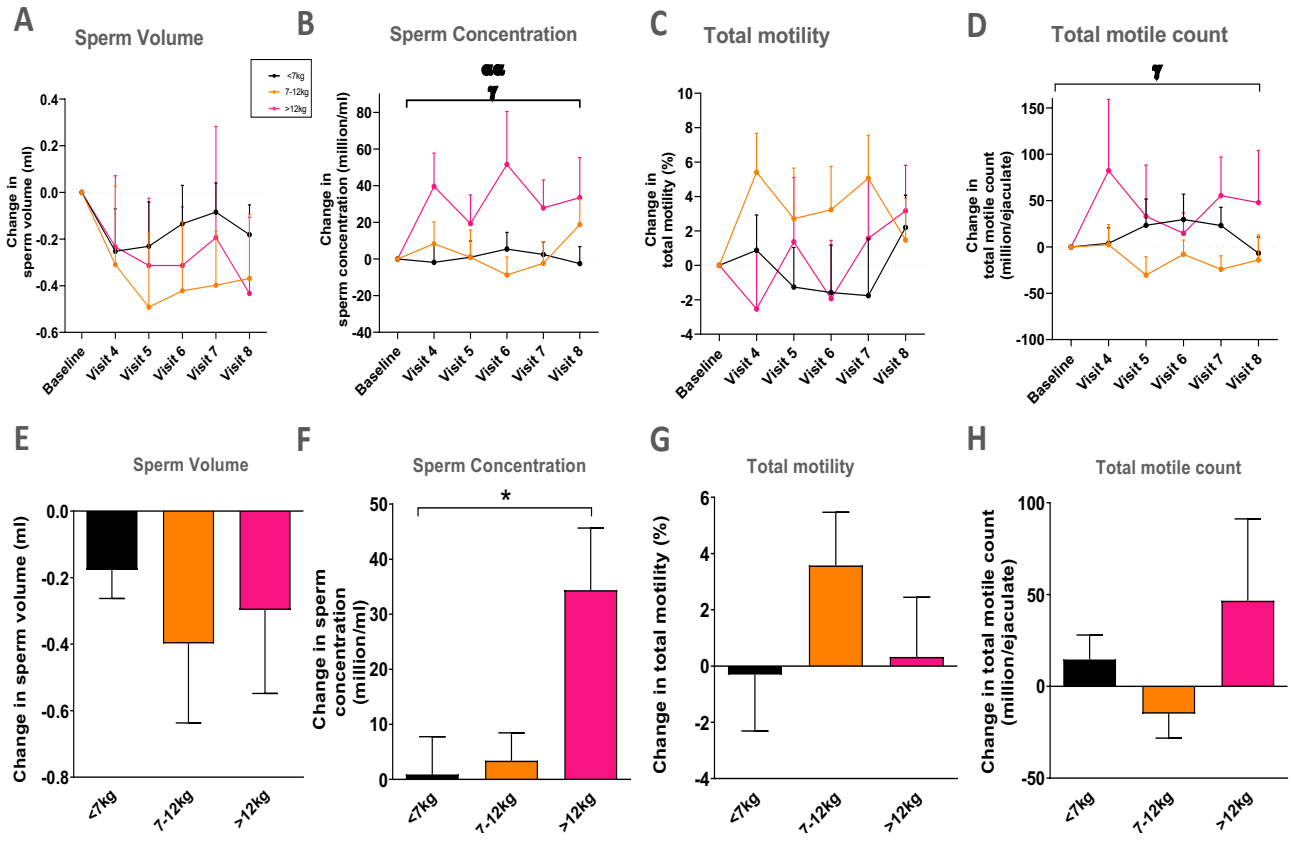
A-F: Time profiles of change in LH (A), FSH (B), testosterone (C), oestradiol (D), SHBG (E) and FAI (F) during the dietary intervention period (mean of visits V4 to V8) when compared with baseline values (mean of visits V1 to V3). **Black,** <7kg; **Orange,** 7-12kg; **Pink,** >12kg. α P<0.05, >12kg vs. <7kg; ααα P<0.001, >12kg vs. <7kg; ββββ P<0.0001, <7kg vs. 7-12kg.

2.4.9 Effects of weight loss on sperm quality

In men with obesity losing >12kg, mean change in sperm concentration was higher than baseline levels during each study visit within the dietary intervention phase (visits V4-V8); maximal increase in sperm concentration from baseline was 51.6+/-28.8 mill/ml, which was observed during visit 6 (after 1 month of dietary intervention) (Figure 2.14B). By comparison, sperm concentration changed minimally in men with obesity in the <7kg ($P<0.01$ vs. >12kg) and 7-12kg ($P<0.05$ vs. >12kg) groups. Overall, the mean change in sperm concentration was significantly elevated in men losing >12kg when compared with <7kg (mean change in sperm concentration in mill/ml: +0.9±6.9, <7kg; +3.4±5.16, 7-12kg; +34.4±11.3, >12kg, $P<0.05$ vs. <7kg) (Figure 2.14F).

In men with obesity losing >12kg, total motile count (TMC) was higher than baseline (mean of visits V1-V3) during each timepoint within the dietary intervention phase (visits V4-V8), which was significantly greater when compared with changes in TMC in the 7-12kg ($P<0.05$ vs. >12kg) group (Figure 2.14D). There was no significant change noted in the other sperm parameters.

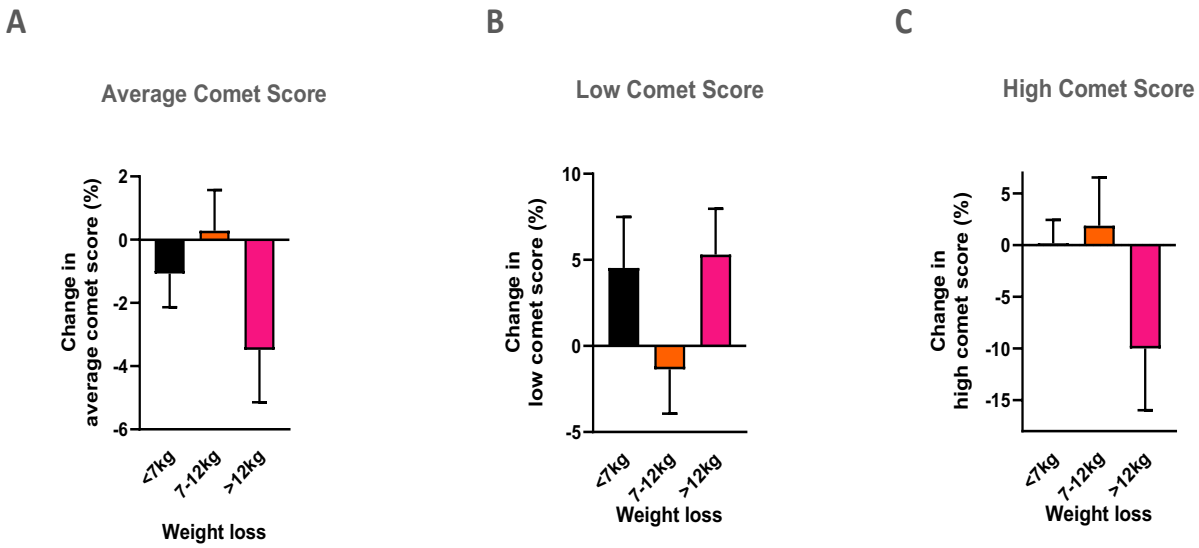
Figure 2-14: Time profiles and bar graphs of changes in semen parameters in men with obesity classified by degree of weight loss



A-D: Time profiles of change in in semen volume (A), sperm concentration (B), total motility (C) and total motile count (D) during the dietary intervention period when compared with baseline values (mean of visits V1 to V3). αα P<0.01, >12kg vs. <7kg; γ P<0.05, >12kg vs. 7-12kg. **E-H:** Bar graphs of overall mean change in semen volume (E), sperm concentration (F), total motility (G) and total motile count (H) during the dietary intervention period when compared with baseline values (mean of visits V1 to V3). *, P<0.05. Black: <7kg; Orange: 7-12kg; Pink: >12kg.

Sperm DNA fragmentation index: During the dietary intervention period, ACS and HCS reduced in the >12kg weight loss group, but these responses were not significantly different from either 7-12 or 7kg weight loss groups (change in ACS in %: -1.1 ± 1.1, <7kg; +0.3 ± 1.3, 7-12kg; -3.5 ± 1.7 >12kg; P=ns vs. <7kg or 7-12kg; change in HCS in %: +0.2 ± 2.3, <7kg; +1.9 ± 4.7, 7-12kg; -10 ± 6.0 >12kg; P=ns vs. <7kg or 7-12kg). (Figure 2.15).

Figure 2-15: Bar graphs of changes in DNA fragmentation indices in men with obesity classified by degree of weight loss during 8 week dietary intervention.

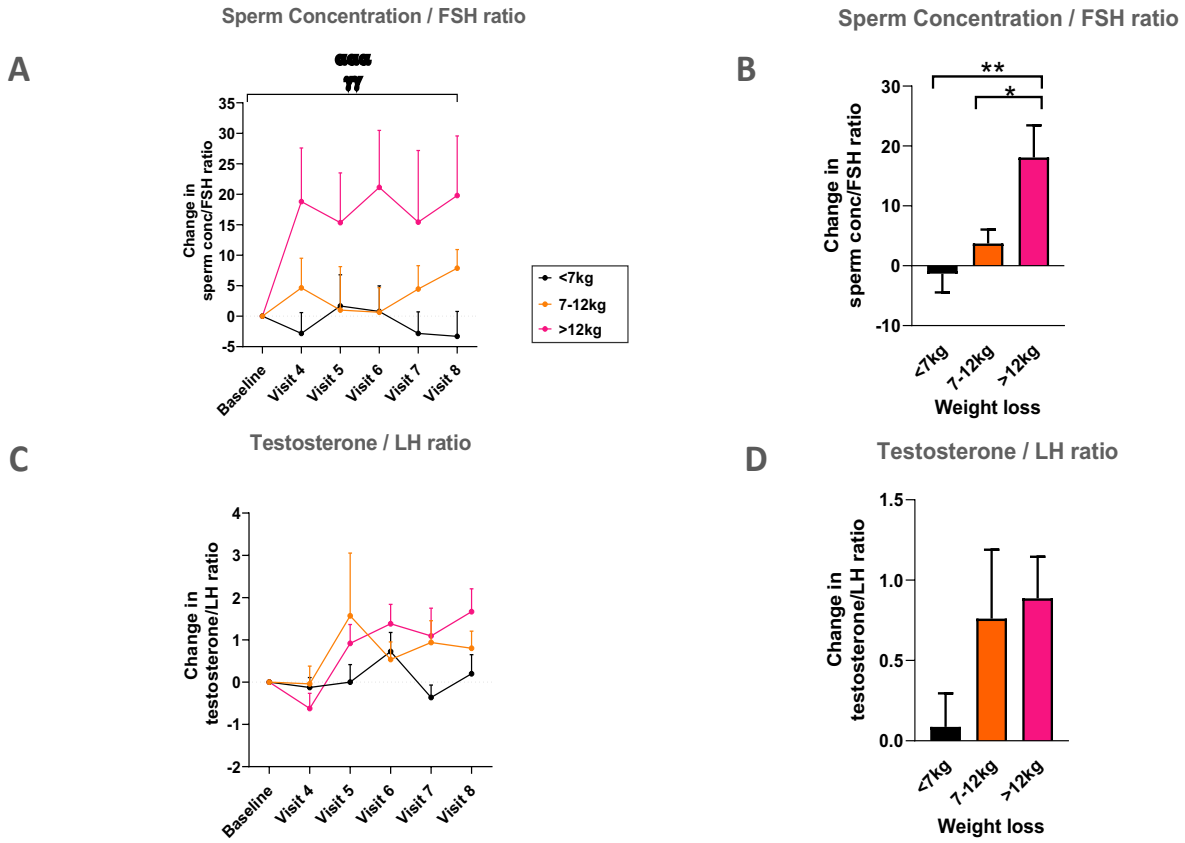


A-C: Bar graphs of overall mean change in Average Comet Score (A), Low Comet Score (B) High Comet Score (C) at the end of the dietary intervention period (visit 7) when compared with a baseline visit (visit 2). Black: <7kg; Orange: 7-12kg; Pink: >12kg.

2.4.10 Effectiveness of gonadotropins to stimulate testicular function

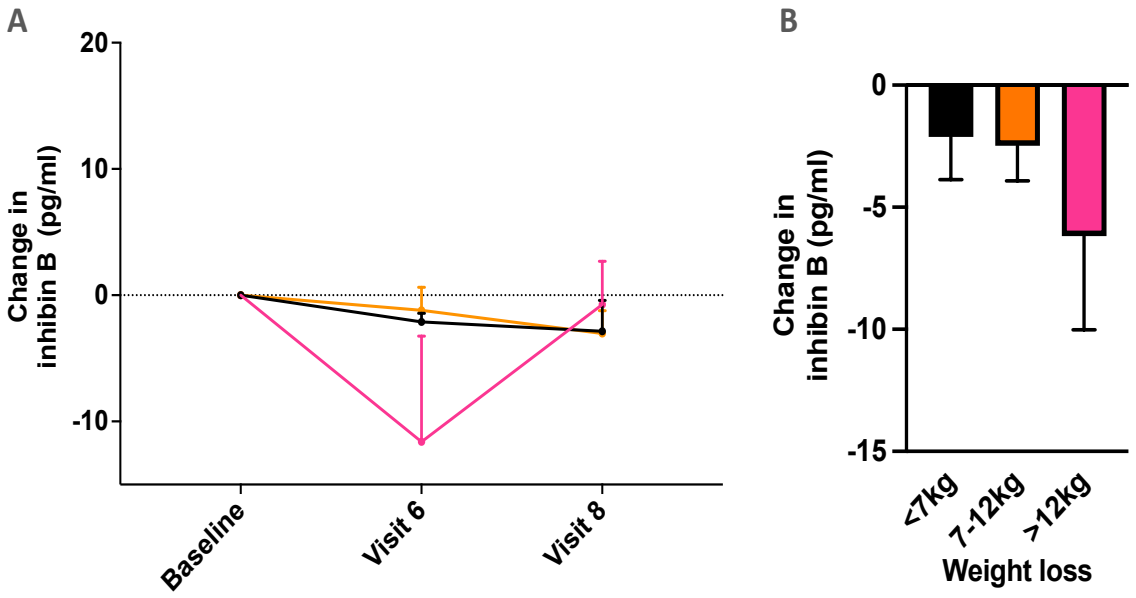
Time profiles and mean changes in testicular function (Figure 2.16A-D, Figure 2.17A,B): Having observed that sperm concentration was increased in men with obesity losing >12kg weight, we investigated why this occurred. The pituitary hormone, FSH, is required for stimulation of spermatogenesis within the testes. Time profiles of changes in testosterone/LH and sperm concentration/FSH ratio during the dietary intervention period (mean of visits V4 to V8) when compared with baseline levels (mean of visits V1-V3) were analysed in men with obesity. Similarly, time profiles and overall mean changes in inhibin B levels were analysed at visit 3 i.e. pre-start of diet, visit 6 (1 month post-diet start), and visit 8 (end of diet) (Figure 2.17A,B). In men losing >12kg, sperm concentration/FSH ratio increased throughout the dietary intervention period but changed minimally in the <7kg ($P<0.001$ vs. >12kg) and 7-12kg ($P<0.05$ vs. >12kg) weight loss groups (Figure 2.16A). The mean change in sperm concentration/FSH ratio was significantly greater in men with obesity losing >12kg weight when compared with either <7kg or 7-12kg groups (change in sperm concentration/FSH ratio: -1.3 ± 3.1, <7kg; +3.7 ± 2.3, 7-12kg; +18.1 ± 5.4, >12kg, $P<0.01$ vs. <7kg; $P<0.05$ vs. 7-12kg) (Figure 2.16B). The pituitary hormone, LH, is required for stimulation of testicular steroidogenesis within the testes, the principle marker of which is serum testosterone. Increments in the testosterone/LH ratio were observed in the 7-12kg and >12kg weight loss groups, but these were non-significant (Figure 2.16C-D). No significant changes in inhibin B were observed in either of the weight loss categories (Figure 2.17A,B), however similar to FSH reduction (Figure 2.12B), the inhibin B levels were the lowest in >12kg group compared to the 7-12kg and <7kg group, albeit this change was not statistically significant.

Figure 2-16: Time profiles and bar graphs of changes in ratios of sperm concentration/FSH and total testosterone/LH in men with obesity classified by degree of weight loss during 8 week dietary intervention.



A,C: Time profiles of change in ratios of sperm concentration/FSH (A) and total testosterone/LH (C) during the dietary intervention period (mean of visits V4 to V8) when compared with baseline values (mean of visits V1 to V3). ααα P<0.001, >12kg vs. <7kg; γγ P<0.01, >12kg vs. 7-12kg. **B,D:** Bar graphs of the overall mean change in ratios of sperm concentration/FSH (B) and total testosterone/LH (D) during the dietary intervention period (mean level during visits V4 to V8) when compared with baseline values (mean of visits V1 to V3). *, P<0.05; **, P<0.01. **Black,** <7kg; **Orange,** 7-12kg; **Pink,** >12kg.

Figure 2-17: Time profiles and bar graphs of changes in Inhibin B in men with obesity classified by degree of weight loss during 8 week dietary intervention.



A: Time profile of change in inhibin B at 1 month of dietary intervention (visit 6) and end (visit 8) of dietary intervention period compared with baseline (visit 3). **B:** Bar graphs of overall mean change in inhibin B levels (mean of visits V6 and V8) when compared with a baseline visit (visit V3). **Black,** <7kg; **Orange,** 7-12kg; **Pink,** >12kg.

2.5 Discussion

We carried out the first ever randomised study investigating the effects of different levels of energy intake on semen quality in men with obesity. We failed to observe any significant changes in our primary analysis with randomisation by dietary intervention groups. Therefore we carried out a post-hoc analysis by weight loss categories suggesting potentially improved sperm concentration in men with obesity losing 12kg or more in body weight using LED, with reduction in sperm DNA fragmentation index. LED followed by effective behavioural change has recently been shown to deliver diabetes remission in non-insulin treated Type 2 diabetes with a threshold of 10kg weight loss and maintenance at two years associated with 63% diabetes remission (Lean et al., 2019). Future studies are needed to confirm our findings and investigate the mechanisms for these observed changes in sperm quality.

Obesity has a negative impact on male reproductive potential; however, the effects of weight loss on sperm quality in obese men remains unclear. We had collaborated with Prof Frost (University of Denmark) and Prof Leeds (University College of London) to plan the LED interventions for all three studies (Chapter 2,3,and 4). Our dietary interventions were well tolerated by our obese male participants with no adverse events. Our 800kcal/day dietary intervention led to significant reduction in weight, BMI and waist circumference (WC) compared to the control and NHS diet groups. A mean weight loss of 11kg was achieved in our cohort of obese men on 800kcal/day LED, however the weight loss ranged from a minimum of 0.7kg to a maximum of 19.5kg showing variability in weight loss potentially due to poor adherence to dietary protocols in some men, which is a known disadvantage of dietary weight loss methods. Men had smaller reductions in weight during the study than anticipated; approximately half of participants (28/55) lost <7kg weight, and only 10 participants lost >12kg. Weight loss by diet is challenging in those with obesity. Previous studies have reported slowing down of weight loss over time, possibly due to reduction in resting metabolic rate and reduced compliance to the formula diet (Hall et al., 2011; MacLean et al., 2015; Vink et al., 2016). Furthermore, individual variability in the total weight loss achieved is well reported in the literature, with men and those with a higher initial bodyweight typically losing more weight (Bischoff et al., 2012; Lean, 2011; MacLean et al., 2015). Several studies have reported that following the use of formula diets, the ratio of lean to fat mass loss is approximately 25:75 with both resistance and aerobic exercise shown to limit lean

tissue loss after formula LED (Chaston et al., 2007; Snel et al., 2012). We did not measure body composition in this study, which is a limitation, as we are unable to discriminate between lean and fat mass loss in the weight loss achieved. All participants were advised to do 30 minutes of resistance training 3 times a week during the study protocol to limit lean mass loss. However, as this was not quantified during the study, it is difficult to ascertain whether all men adhered to the recommended amount of exercise.

Waist circumference is a better indicator of visceral obesity with high levels conferring an increased cardio-metabolic risk. Previous studies have shown a significant association between WC and levels of total cholesterol and fasting glucose in men (Handjieva-Darlenska et al., 2010; Stelmach-Mardas and Walkowiak, 2016). Hence, it is not surprising that significant reductions in BMI and WC in our 800kcal/day cohort led to significant reductions in total cholesterol and fasting glucose. Similarly, significant improvements in waist circumference, fasting glucose and total cholesterol were observed in the >12kg weight loss group compared to the lesser weight loss categories. However, the interesting observation was that the significant reductions in these metabolic profiles mirrored the significant increment in mean sperm concentration in men losing >12kg weight. Germ cells rely on glycolysis and Sertoli cells rely on β-oxidation of fatty acids and for their energy requirements (Crisóstomo et al., 2017; Martins et al., 2015). Hence optimisation of the metabolic parameters, including total cholesterol and fasting glucose in obese men could have led to optimal testicular energy metabolism. This may suggest potential metabolic benefits of weight loss also at the testicular spermatogenesis level such as reduced sperm apoptosis and improved sperm survival.

Despite the observed beneficial cardio-metabolic effects of weight loss, the reproductive hormone profile of our participants did not significantly change by dietary groups or weight loss categories. We noted the highest decline in LH and FSH in the >12kg group compared to the lesser weight loss groups. It is plausible that higher weight loss may have suppressed the gonadotropins. In contrast, the highest increments in SHBG and mean total testosterone (albeit non-significant) were observed in the >12kg weight loss group. It is therefore plausible that Leydig cells are potentially more sensitive to LH leading to an increase in testosterone in the >12kg group. Corona et al. also noted a greater increase in testosterone in obese men losing 10% to 32% weight by either LED or bariatric surgery (Corona et al., 2013a). Similarly, LED for nine weeks has been sufficient to increase free testosterone and SHBG at statistically significant levels (Niskanen et al., 2004). Other studies have also shown a significant increase in total testosterone, SHBG and testosterone/oestradiol

ratio and lower oestradiol in obese men who underwent lifestyle associated weight loss (Faure et al., 2014; Håkonsen et al., 2011). However, the weight loss interventions in many of these previous studies were carried out for much longer than our 8 weeks of intervention, included both diet and exercise programs, and subsequently led to overall higher mean weight loss than our study, which may explain some of the significant differences in reproductive hormones observed. Furthermore, the mean BMI of our participants did not drop below 30kg/m^2 at the end of the study. Hence, most of the men in each of the diet groups remained within the 'obese' category after 8 weeks of intervention which may further explain the non-significant findings. Furthermore an 8 week protocol may have been too short to cover an entire cycle of spermatogenesis, and perhaps a longer protocol would have resulted in more positive findings.

The pituitary gonadotropin, FSH, stimulates Sertoli cell function and spermatogenesis within the seminiferous tubules of the testes (Kathrins and Niederberger, 2016). We were therefore interested to observe that increments in mean sperm concentration were accompanied by simultaneous reductions in serum FSH in men with obesity losing >12kg weight. In addition, men with obesity losing >12kg weight had significantly greater reductions in FSH secretion when compared with men with obesity losing either 7-12kg or <7kg. Furthermore, the ratio of sperm concentration to serum FSH was markedly elevated by >12kg weight loss, when compared with lesser weight loss. Our data therefore suggest that weight loss >12kg may stimulate spermatogenesis which leads to a subsequent reduction in requirement for FSH stimulation of testicular function. However, we would have expected the inhibin levels to be raised to support this hypothesis. Inhibin B is a marker of spermatogenesis and we did not observe any significant changes in the >12kg weight loss group compared the other two groups. If anything, inhibin B declined the most in the 800kcal/day and the >12kg weight loss group compared to the other groups. One may postulate that weight loss led to suppression of the gonadotropins as previously reported during acute fasting/energy deficient states (Martin et al., 2008). Another alternative explanation is a potential direct effect of weight loss on the testis such that Leydig cells and Sertoli cells are more sensitive to gonadotropins, which may explain the observed increase in testosterone and sperm concentration respectively in the >12kg group compared to the other two groups.

No prior randomised controlled study has been performed investigating the effects of weight loss on sperm quality in men with obesity. Accordingly, current clinical guidelines for the management of infertility do not recommend weight loss for male partners with obesity ("Fertility overview," 2016). The current randomised,

controlled interventional study observed a mean increment of +34.4 ± 11.3 million sperm per ml in in men that lost >12kg of weight, which is approximately double the level of sperm concentration required to achieve the lower limit of WHO reference range for fertility (Cooper, 2010). Our findings are in line with a study carried out by Hakonsen et al (Håkonsen et al., 2011) whereby a group of 43 obese men underwent a 14 week 'residential weight loss programme'. The subgroup with the largest weight loss (17.2 to 25.4% of weight loss) had an increase in total sperm count. However, in contrast to our findings, they also observed increased semen volume, testosterone and SHBG in their subgroup. Their intervention period was longer and involved both diet and exercise, and the semen was only checked twice (start and end of study). The rest of the semen parameters in our study did not show any significant changes. Interestingly, mean sperm volume decreased in all our diet groups including the control group, and this may be due to the frequent semen production required for the study. The reduced semen volume may suggest reduced seminal plasma proteins from the male accessory glands which are important for the functionality of spermatozoa including motility, which may partly explain the reduced motility noted in some of our diet groups. We noted a non-significant increase in sperm morphology in >12kg weight loss group. Similarly, ten per cent of BMI reduction has been reported to increase the percentage of normal sperm morphology after a 12-week diet period and follow-up at 6 months (Mir et al., 2018). Unlike our study, these studies did not have a control group for comparison and did not describe the weight loss programme in detail.

Obesity has been associated with elevated levels of inflammatory adipocytokines, such as TNF-α and IL-1, IL-6, and IL-18, that have been reported to cause sperm cell apoptosis (Oliveira et al., 2017). A study by Zhu et al investigated 54 infertile men and observed that sperm DFI and sperm apoptosis were significantly increased in overweight and obese men compared with men with normal BMI (Zhu et al., 2021). They used antibody microarray technology to identify protein markers associated with sperm apoptosis and sperm damage, and showed that Fas/Fasl, Bcl-2/Bax, caspase-3, caspase-8, p53, p21, TNF-α, TNF-β, sTNF-R1, and sTNF-R2 were all significantly upregulated in line with increasing BMI in men. Future studies are needed to study these biomarkers in response to sperm apoptosis with weight loss in men with obesity. The Comet assay measures DNA strand breaks and is associated with all the fertility checkpoints from fertilization to embryo quality, clinical pregnancy and miscarriage as well as live birth outcomes (Lewis and Agbaje, 2008). We observed a decrease in ACS and HCS in men with >12kg weight loss compared with the other two weight

loss groups which is consistent with findings from other studies that have reported improved DNA fragmentation indices with weight loss. Faure et al (Faure et al., 2014), reported improvement in DFI in men with 3.9% reduction in BMI. Therefore, one may postulate that reduced adipokines with weight loss perhaps led to reduced sperm apoptosis, decreased DFI (as observed with COMET), and therefore increased sperm concentration.

Interventional studies of sperm function are hindered by large, biological variation observed in semen parameters of all men (Schwartz et al., 1979; "WHO laboratory manual for the Examination and processing of human semen," 2010). An advantage of the current study was to perform serial measurements of clinical, biochemical and andrological parameters prior to and during the randomised dietary interventions. This approach allowed us to assess the time-course of response, and to assess the cumulative effects of dietary intervention on sperm function in men with obesity. A complete cycle of spermatogenesis in men takes 42 to 76 days (Misell et al., 2006), so it is possible that the 8-week intervention period in the current study may underestimate the effectiveness of weight loss to improve sperm function in men with obesity. The short intervention period may explain the lack of observed significant changes in sperm DNA fragmentation in our study. In addition, lack of data on body composition measurements of our participants, as well as monitoring of physical activity levels are further accepted limitations. BMI is the not best indicator of adiposity with its inability to distinguish body fat composition. Perhaps using body composition measurements may have provided more information. Secondly our control group also lost some weight with large variability in weight loss achieved within each dietary group which may have underestimated the difference seen between the groups, and therefore led to the post-hoc analysis by weight loss categories. Lastly, we did not measure or adjust for the confounding variable of physical activity in our participants. One may observe that men who volunteer to take part in weight loss research may be a 'healthier' cohort of obese men who are keen to make better lifestyle choices and may have also attempted previous weight loss strategies. According to literature 30% of obese men suffer from oligospermia (Kumar and Singh, 2015), however the mean baseline sperm concentration in our entire study population was within normal WHO reference range.

In conclusion, we failed to observe any significant improvements in sperm concentration in obese men randomized to different calorie intakes over an 8-week period. However, post-hoc analysis suggested that >12kg weight loss significantly improved sperm concentration in men with obesity. This is in contrast to

bariatric surgery whereby sperm count has been noted to acutely decrease within the first few months of surgery with no change in sperm quality noted at 24 months (Wei et al., 2018). Further studies are warranted to confirm these findings and investigate the longer-term effects of dietary weight loss on male reproductive function. Therefore, to further investigate the effects of weight loss on male fertility, we next randomised men to the weight loss observed to have optimum effects on sperm concentration (>12kg) verses NHS advice over a longer intervention period of 16 weeks, taking into consideration some of the limitations from this study by measuring body composition, exercise logs, and covering the entire one cycle of spermatogenesis (Chapter 3, Study 2).

Chapter 3: Investigating the effects of a fixed weight loss target on sperm concentration in men with obesity

3.1 Introduction

Post-hoc analysis of Study 1 (chapter 2) suggested that >12kg of weight loss may significantly improve metabolic profile and sperm concentration in men with obesity compared to men losing <12kg of weight. Therefore, we performed the current study (Study 2) to investigate prospectively if a weight loss threshold of >12kg was sufficient to improve sperm concentration in obese men with normal fertility.

A complete cycle of spermatogenesis in men takes 64 +/- 8 (range 42 to 76) days (Heller and Clermont, 1963; Misell et al., 2006). One of the main limitations of Study 1 was its duration of 8 weeks therefore we wanted to extend observations in the current study to cover an entire cycle of spermatogenesis. Twelve weeks of TDR with LED has been used safely previously (Johansson et al., 2014; Lean et al., 2018; Leeds, 2014). Furthermore, 12 weeks is the recommended maximum length of TDR by current NICE guidelines (NICE (National Institute for Health and Care Excellence), 2014). In addition, gradual reintroduction of food following a LED has been reported to assist with weight loss maintenance (Brown and Leeds, 2019; Leeds, 2014). For example, reintroducing food over a 6-week period resulted in significantly less weight regain at 52 weeks compared with a reintroduction period of 1 week (3.9 kg vs. 8.2 kg, respectively) (Gripeteg et al., 2010). Therefore, with the expertise of our nutrition team (Prof Leeds, University of Denmark and Prof Brown, University College London), we chose a 16 week study duration composed of 12 weeks of TDR phase with subsequent 4 weeks of gradual food reintroduction. We also measured body composition parameters in our current study (unlike Study 1) to further distinguish weight loss into fat and lean mass.

The Eatwell guide is a government based policy (Public Health England) tool to define recommendations on eating healthily using different macronutrients in a nutritionally balanced diet, with the appropriate proportions required to achieve a healthy balanced diet ("The Eatwell Guide," 2018; Traill et al., 2013). Healthy eating diets may provide 500-600 kcal/day energy deficit compared to a control Western Diet and can lead to weight loss of around 5kg in 12 weeks (Jolly et al., 2011). Therefore this NHS advice on 'healthy eating' or 'eatwell guide' was used as our control intervention as it was felt that obese men would not participate in a control arm without any perceived benefit (Leslie et al., 2014; Micha et al., 2017). Furthermore, both the ethics committee and the study team felt it would be unethical to provide no healthy eating advise to obese men.

Similar to Study 1, we chose change in sperm concentration as our primary outcome as sperm concentration is the most representative seminal parameter of testicular output. Furthermore, multiple studies determine sperm concentration and total motile sperm count as the best predictors of conception (Bonde et al., 1998), with an association with important fertility outcomes such as time to pregnancy (Slama et al., 2002) and pregnancy rates (Zinaman et al., 2000). We measured sperm DNA fragmentation pre and post intervention as an exploratory secondary outcome using a validated in-house TUNEL assay (see methodology for details). The extent of sperm DNA fragmentation, measured by the TUNEL assay, is one of the determinants of male fertility associated with spontaneous pregnancy and ART outcomes (Bungum et al., 2004; Duran et al., 2002; Nicopoullos et al., 2019; Simon et al., 2019).

3.2 Hypothesis and aims

Hypothesis

Threshold of weight loss of >12kg by caloric restriction with LED would significantly improve sperm concentration in men with obesity compared with standard NHS advice.

Aims

To determine the following:

(i) Effects of weight loss (aiming for >12kg) by LED on sperm parameters compared with NHS advice in men with obesity.

(ii) Effects of weight loss (aiming for >12kg) by LED on sperm DNA fragmentation, reproductive hormones and metabolic profile compared with NHS advice in men with obesity.

Objectives

Primary objective:

- Investigate the effects of weight loss by LED on sperm concentration in men with obesity.

Secondary objectives

- Investigate the effects of weight loss on other sperm parameters (total motility, progressive motility, semen volume, morphology, TMC) and sperm DNA fragmentation in men with obesity.
- Investigate the effects of weight loss on reproductive hormone parameters (serum total and calculated free testosterone, oestradiol, SHBG, LH and FSH) and metabolic parameters including waist circumference, blood pressure, HbA1c, fasting glucose and lipid profile.

3.3 Methods

3.3.1 Ethical approval and study design

Ethical Approval

Ethical approval was same as Study 1.

Study Design

Participants were randomised to receive either 800kcal/day or standard NHS advise ('healthy plate') during a 16-week study protocol. Participants randomised to 800kcal/day received LED using the Cambridge Weight plan products (approximately 200kcal per product) aiming for >12kg of weight loss. The NHS diet is based on the standard NHS advise on a healthy balanced plate ("The Eatwell Guide," 2018).

3.3.2 Participant recruitment

The same recruitment methods, inclusion and exclusion criteria were used as detailed in section 2.3.2 in chapter 2 (study1).

Written informed consent was obtained from all subjects. This study was performed in accordance with the Declaration of Helsinki. Twenty-four men aged 18-60 years with BMI≥30kg/m^2 and no infertility completed the study.

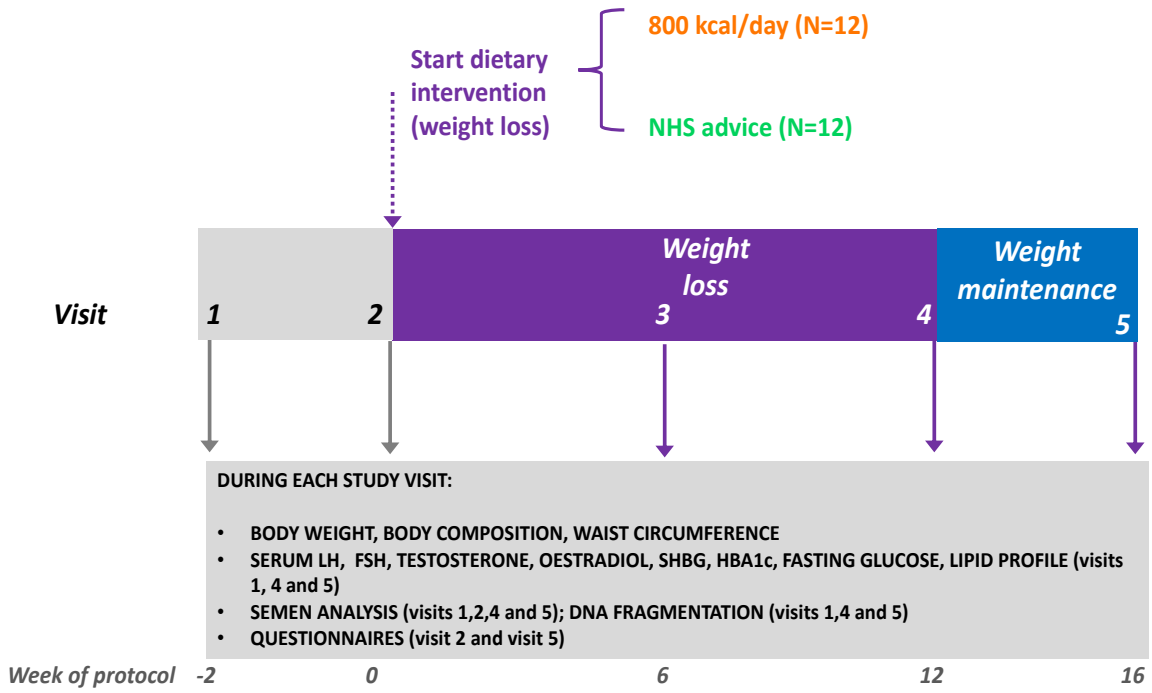
3.3.3 Protocol

A randomized, controlled study was performed.

The protocol consisted of two phases: 1) 800kcal/day weight loss phase of 12 weeks aiming for at least >12kg weight loss, and 2) 4 weeks of weight maintenance phase with gradual food reintroduction . The control arm consisted of NHS healthy eating advice for the entire 16 weeks of the protocol.

The study protocol for study 2 is summarised in Figure 3.1.

Figure 3-1: Summary of study protocol



Twenty-four participants completed the study with 12 participants in 800kcal/day and 12 in NHS arm. LH, luteinizing hormone; FSH, follicle stimulating hormone; SHBG, Sex Hormone Binding Globulin

Baseline period: The initial 2-week period (weeks -2 to 0, visits 1-2) allowed the measurement of baseline values of body composition, testicular volume, reproductive hormones, metabolic profile, semen analysis and the acclimatisation of participants to study conditions.

Intervention period: During week 0 (visit 2) of the protocol, participants were initiated on one of the two randomised study groups 800kcal/day or standard NHS advise (control) for a 16-week intervention period involving further 3 visits (weeks 6, 12 and 16). Dr. Jayasena, PI of the study, performed randomisation with stratification into each group at a ratio of 1:1. (random.org).

Serial measurements of body weight, waist circumference, body composition, blood pressure and semen analysis were carried out at visits 1, 2, 4 and 5. Semen was stored at -20^{O}C for DNA fragmentation analysis at V1 and V5. Fasting serum reproductive hormones and metabolic profile were measured at visits 1, 4 and

5. Validated questionnaires for erectile dysfunction (SHIM) (Rosen et al., 1999) and quality of life (SF-36) (Ware and Gandek, 1998) were done at the start and end of study.

3.3.4 Diet groups

Cambridge Weight Plan products (https://www.one2onediet.com/) consisted of soups, shakes and bars of approximately 200kcal per product (Table 1.5, chapter 1). These LED products contained an array of micro and macronutrients (Table 1.5). The 800kcal/day arm consisted of 4 CWP products per day for 12 weeks (visit 0 to visit 4). The weight maintenance phase for 4 weeks (visit 4 to visit 5) involved gradual food reintroduction by replacing one CWP product every 1-2 weeks with healthy meals (low carb, high protein) based on individual diet preferences (see details of *food reintroduction phase* below) in order to maintain the weight loss achieved at week 12.

NHS diet (control arm) was based on the standard NHS advise consisting of a balanced diet (1800-2200kcal/day) with general advice to reduce portion size, eating more vegetables and fruits, less saturated fats and carbohydrates. Energy intake for weight loss for NHS arm was based on the Mifflin-St. Joer equations (Mifflin et al., 1990) and physical activity levels.

All participants were advised to drink at least 2.5litres of water per day. In addition participants on TDR (800kcal/day) were provided with additional fibre supplements in the form of ispaghula husk sachets, as when required, to prevent constipation which can be one of the side effects of LED. All men were advised to limit physical activity to resistance training of 30minutes three times a week, and to keep an exercise log throughout the study. These diets were planned with the expertise of our nutrition team (Profs Brown, UCL and Prof Leeds, University of Denmark). Compliance was checked at each visit according to both product usage and self-reporting.

Food reintroduction phase at 12 weeks for 4 weeks of weight maintenance is summarised as follows:

- **STEP 1 for 1 week (intake of approx. 1000kcal/day) from week 12 to 13 of study:**
  - Take one CWP product off
  - Continue 3 products (600kcal/day)
  - Add 1 meal (aim approx. 400 kcal/day): healthy, low carb meal

- Examples include:
  - **Breakfast:** eggs (2-3), scrambled or omelette, may add spinach/tomatoes OR high protein yoghurt with blackberries/blueberries
  - **Lunch:** salad with protein or steamed vegetables with protein. Examples of protein: beans/pulses/tofu (approx. 150g), or chicken breast/fish fillet/salmon (palm size, approx. 4 ounces/125g)
  - **Dinner:** protein with steamed vegetables/salad (similar to lunch). Add herbs/spices

❖ **STEP 2** for next 2 weeks (intake of approx. 1200kcal/day) from weeks 13 to 15 of study:

- Take one further CWP product off
- Continue 2 products (400kcal/day) and 2 meals (approx. 800kcal/day)
- Meal options as above

❖ **STEP 3** for last 1 week, from week 15 to 16 (intake of approx. 1200 kcal/day)

- 2 meals (approx. 1000 kcal/day) and 1 CWP product (200 kcal/day)
- Meals can include bit of carb: e.g. rice crackers/quinoa/couscous/porridge. Fruits e.g. apples/strawberries/blueberries

3.3.5 Semen sampling

Semen sampling and analysis methods were identical to methods described in Chapter 2 (section 2.3.5).

3.3.6 DNA fragmentation

Sperm DNA fragmentation index (DFI) is the percentage of spermatozoa with fragmented or damaged DNA. One ml of fresh liquefied semen was stored at -20^{O}C for DNA fragmentation analysis at visits 1 and 5. An in-house validated TUNEL assay at my host laboratory at Imperial College was used for DFI measurements for Study 2 and 3. The principles and procedures of measuring sperm DNA damage by TUNEL have been described previously (Rakesh Sharma et al., 2016, 2016; Zini and Agarwal, 2011) (See Appendix 2).

TUNEL assay directly measures single and double strand DNA breaks using the enzyme terminal deoxynucleotidyl transferase (TdT) to catalyse the attachment of florescent labels or deoxyribonucleotides to the 3'-hydroxyl 'free ends' of single and double DNA breaks ('nicks') (García-Peiró et al., 2013). The

fluorescence, which is proportional to the number of strand breaks, is then quantified using flow cytometry (BD Accuri C6 Flow Cytometer). The more DNA strand break sites are present, the more labels are incorporated within a cell. Reference values: A cut off of 17% with greater than 95% specificity was validated in-house in our laboratory to differentiate infertile men with DNA damage from healthy men.

3.3.7 Measurement of reproductive hormones and metabolic profile

Blood collection, processing and analysis of serum were identical to methods described in Chapter 2 (section 2.3.7).

3.3.8 Other measurements

Baseline data collection included detailed history including age, past medical history, social history, alcohol and smoking. Anthropometric measurements included blood pressure (BP), weight, waist circumference (WC), body mass index and body composition which were measured at each visit. Body composition was measured using bioelectrical impedance analysis using the Tenata MC-780MA P (EU) for the following parameters: weight, BMI, fat mass (% and kg), lean mass (% and kg), water (% and kg), eBMR (estimated basal metabolic rate in kcal).

3.3.9 Questionnaires

Erectile dysfunction (ED) was assessed using the validated Sexual Health Inventory for Men (SHIM) questionnaire scores (Rosen et al., 1999) (1-2 Severe ED; 8-11 Moderate ED; 12-16 Mild to Mod ED; 17-21 Mild ED; 22-25 No signs of ED). Health related quality of life was assessed using the validated 36- item Short Form Health Survey (SH-36) questionnaire (Ware and Gandek, 1998). The SF-36 questionnaire consists of eight scales yielding two summary measures: physical and mental health. The physical health measure includes four scales of physical functioning (10 items), role-physical (4 items), bodily pain (2 items), and general health (5 items). The mental health measure is composed of vitality (4 items), social functioning (2 items), role-emotional (3 items), and mental health (5 items). A final item, termed *self-reported health transition*, is answered by the client but is not included in the scoring process.

Higher scores indicate better health status. These questionnaires were done at the start (visit 1) and end of the study (visit 5).

3.3.10 COVID precautions

Study 2 and Study 3 was carried out during the COVID pandemic, therefore we had to carry out COVID-specific risk and feasibility assessments to ensure we followed appropriate precautions for all study visits. These were approved by the research management team at ICHNT.

The risk assessment are summarized in Appendix 3.

3.3.11 Statistical analysis

Sample size of 12 per group matches the recommendation made by Julious (Julious, 2005) for pilot and exploratory studies in cases where there is uncertainty about effect sizes. We anticipated a 20% drop out rate and therefore aimed to recruit 15 men per group.

Statistical analysis was performed using GraphPad Prism v.9 (Graphpad Software Inc, La Jolla, CA, USA). Quantitative data was assessed using normal testing using the Shapiro Wilk Normality test. Data are presented as mean ± SD if normally distributed and median (IQR) if non-normally distributed. Comparison between the groups (means; if normally distributed and medians if non normally distributed) were calculated as independent samples T test for normally distributed data; non normally distributed data was compared using a Mann Whitney U Test. Categorical data was compared using a Chi Squared Fischer's Exact test. In all cases, $P<0.05$ was considered statistically significant.

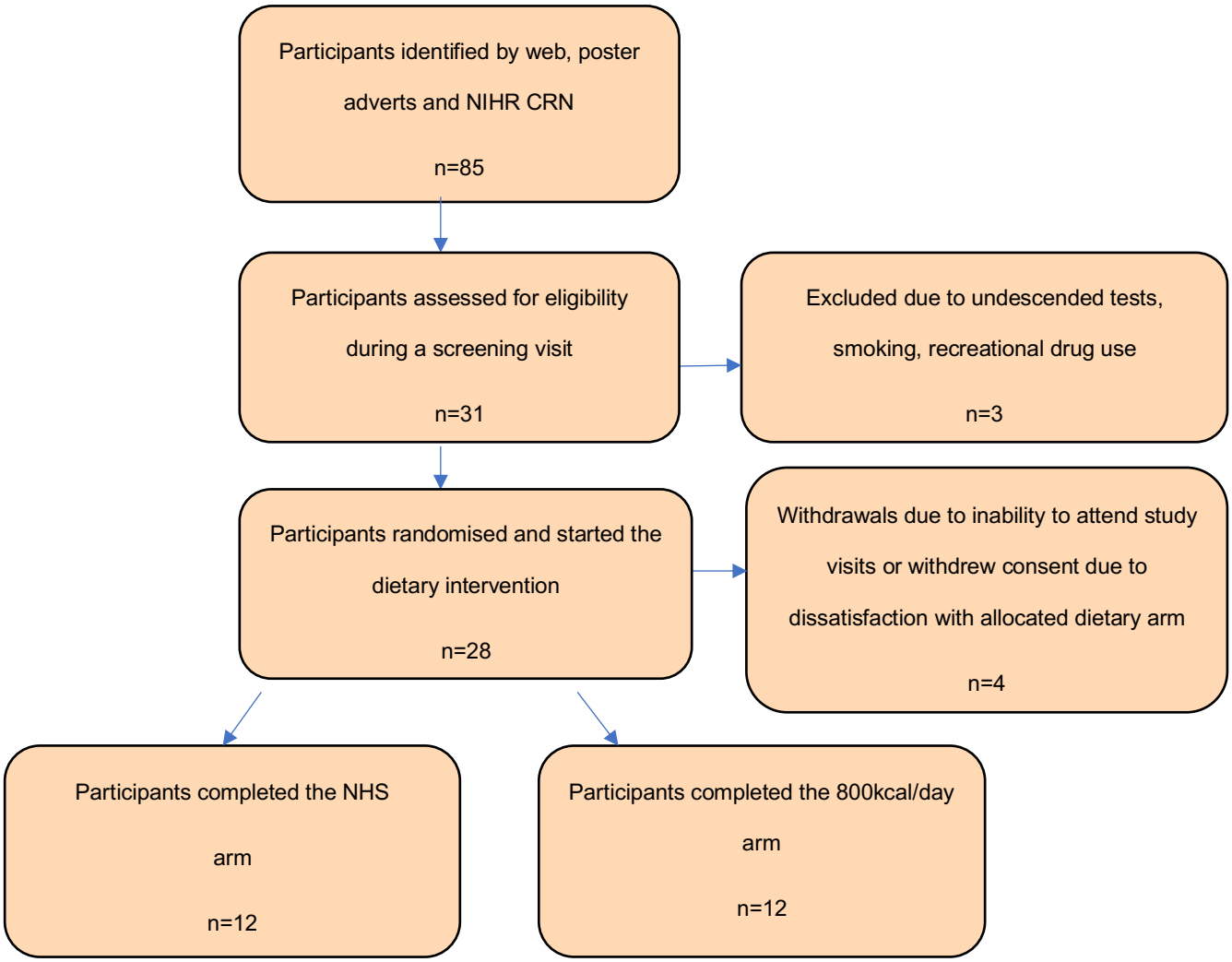
3.4 Results

We screened thirty-one participants at a screening visit. Out of these, three participants were excluded according to study inclusion and exclusion criteria (see methods section in Chapter 2, section 2.3.2). Twenty-eight participants were included in the study. Four participants withdrew due to inability to attend study visits or withdrew their consent. Twenty-four participants (12 in each arm) completed the study (Figure 3.2).

None of the participants had COVID symptoms or confirmed COVID during the current study. All participants received their 1st COVID vaccine (Pfizer or Astrazeneca) during the study period.

Figure 3-2: Patient flow diagram

NIHR: National Institute for Health and Research; CRN: Clinical Research Network



3.4.1 Baseline characteristics of participants recruited to the study

Baseline (at visit 1) demographics (Table 3.1), body composition, metabolic and reproductive hormone profiles (Table 3.2) are summarised below. No significant differences in any of these baseline parameters were observed among the two dietary groups (NHS vs LED) (Table 3.1 and Table 3.2).

For baseline semen parameters (Table 3.3), we used the means of visit 1 and 2 (pre-start of diet) to represent baseline values, and for all analysis of change. At baseline, there was a significant difference in total motile count (TMC) between NHS arm vs LED arm (91.3 vs 50.4million/ejaculate respectively), with no other significant differences observed in the other semen parameters.

Table 3-1: Baseline demographics of participants according to diet groups; NHS vs LED.

Demographics	Dietary intervention group				P value
	NHS (N = 12)		LED (N= 12)		
Age (years)	39.4 ± 6.4		40.2 ±9.6		0.84
Ethnicity	White	9	White	9	0.52
	Asian	3	Asian	1	
	Others	0	Others	2	
Occupation (Based on ISCO-08 classification)	Managers	1	Managers	2	0.33
	Professionals	5	Professionals	6	
	Technicians and Associate Professionals	1	Technicians and Associate Professionals	1	
	Clerical Support Workers	1	Clerical Support Workers	1	
	Service and Sales Workers	4	Service and Sales Workers	2	
Current partner	Yes	10	Yes	11	1.00
	No	2	No	1	
Partner's Age (years)	N= Median (IQR)	10 35.0 (12)	N= Median (IQR)	11 38.0 (23)	0.26
Children	Yes	3	Yes	6	0.40
	No	9	No	6	
Nutritional Supplements	Yes	6	Yes	4	0.68
	No	6	No	8	
Smoker	Non smoker	10	Non smoker	11	1.00
	Ex-smoker	2	Ex-smoker	1	
ETOH intake	Yes	8	Yes	7	1.00
	No	4	No	5	
Average ETOH (units/week)	N= Median (IQR)	8 3.0 (2.5)	N= Median (IQR)	7 4.0 (4.0)	0.44
Diabetes	Yes	1	Yes	1	1.00
	No	11	No	11	
HTN	Yes	0	Yes	1	1.00
	No	12	No	11	
Hypercholesterolaemia	Yes	2	Yes	4	0.64
	No	10	No	8	
Erectile dysfunction	Yes	2	Yes	2	1.00
	No	10	No	10	

Data presented at mean ± SD if normally distributed and median (IQR) if non normally distributed. P value calculated by independent samples T test for normally distributed data; non normally distributed data was compared using a Mann Whitney U Test. All categorical data (such as ethnicity, occupation) calculated by chi-square test. HTN, hypertension; ETOH, alcohol; ISCO-08, International standard of classification of occupations; LED: Low energy diet.

Table 3-2:Baseline anthropometric, metabolic and reproductive hormone profiles of participants according to diet groups; NHS vs LED.

Parameters (units)	Reference range (if applicable)	Dietary intervention group		P value
		NHS (N = 12)	LED (N = 12)	
BEDSIDE				
Weight (kg)		114.1 ± 11.0	109.5 ± 10.8	0.31
BMI (kg/m^2)		36.2 ± 2.4	34.8 ± 3.8	0.29
SBP (mmHg)		125 ± 8	124 ± 14.0	0.69
DBP (mmHg)		79.1 ± 9.3	77.8 ± 10.1	0.76
BODY COMPOSITION				
Waist circumference (cm)		119.5 ± 9.1	117.3 ± 7.6	0.54
Fat Mass (%)		33.5 ± 2.9	32.2 ± 4.2	0.36
Fat Mass (kg)		38.1 ± 6.0	35.7 ± 7.9	0.43
Lean Mass (%)		63.2 ± 2.7	64.5 ± 4.1	0.36
Lean Mass (kg)		71.2 ± 5.7	70.7 ± 5.8	0.83
Water weight (%)		46.7 ± 2.7	48.1 ± 3.0	0.24
Water weight (kg)		53.2± 4.5	52.8 ± 4.2	0.81
Visceral fat (%)		16.3 ± 2.3	15.4 ± 4.0	0.54
eBMR (kcal)		2273.1 ± 196.3	2249.7 ± 208.9	0.78
METABOLIC PROFILE				
Fasting glucose (mmol/L)	<7mmol/L	4.9 ± 2.2	5.1 ± 0.7	0.77
HbA1c (mmol/mol)	<48mmol/mol	39.1 ± 10.3	38.6 ± 7.6	0.89
Total cholesterol (mmol/L)	<5mmol/L	5.2 ± 1.1	5.6 ± 1.2	0.44
LDL (mmol/L)	<3mmol/L	3.0 ± 1.3	3.1 ± 1.1	0.85
HDL (mmol/L)	>1mmol/L	1.1 ± 0.2	1.1 ± 0.1	0.56
Triglycerides (mmol/L)	<1.7mmol/L	1.9 ± 1.4	2.0 ± 1.4	0.97
Prolactin (mIU/L)	60-300mIU/L	176.5 ± 113.9	173.8 ± 74.8	0.95
REPRODUCTIVE HORMONE PROFILE				
LH (IU/L)	2-12IU/L	3.5 ± 1.7	3.2 ± 1.0	0.64
FSH (IU/L)	1.7-8IU/L	4.4 ± 1.9	4.2 ± 2.6	0.83
Testosterone (nmol/L)	10-30nmol/L	12.3 ± 2.5	12.7 ± 4.2	0.80
Oestradiol (pmol/L)	<190pmol/L	116.7 ± 21.2	107.5 ± 11.8	0.70
SHBG (nmol/L)	15-55nmol/L	25.2 ±8.7	21.8 ± 8.1	0.34

Data are presented at mean ± SD. P value calculated by independent samples T test for normally distributed data; Baseline refers to visit 1 (start of protocol). BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; eBMR, estimated Basal Metabolic Rate; LH, Luteinizing hormone; FSH, follicle stimulating hormone; SHBG, Sex Hormone Binding Globulin; LDL, Low Density Lipoproteins; HDL, High Density Lipoproteins; LED: Low energy diet.

Table 3-3: Baseline semen parameters of participants according to diet groups; NHS vs LED.

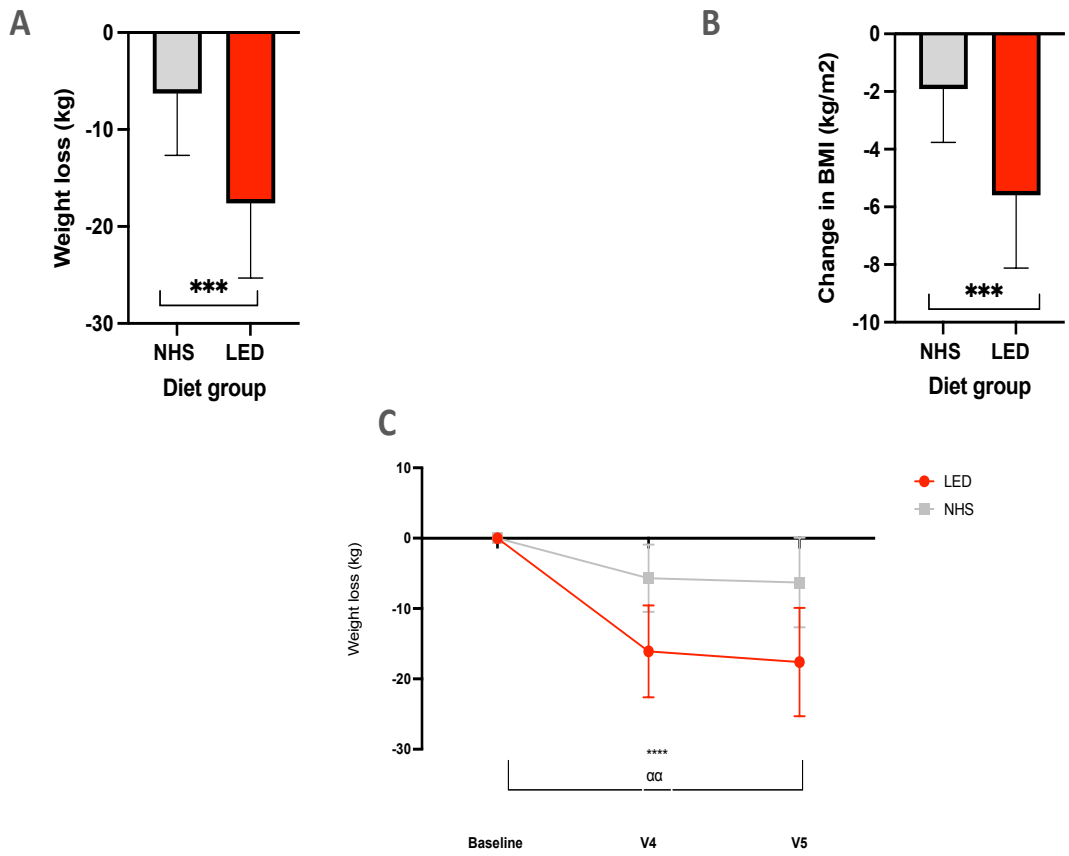
Semen parameter (units)	Reference range	Dietary intervention group		P value
		NHS arm (N = 12)	LED arm (N = 12)	
Semen Concentration (million/ml)	≥15million/ml	40.0 (8.3)	38.5 (12.8)	0.64
Sperm Volume (ml)	≥1.5ml	3.9 (1.3)	3.4 (2.6)	0.36
Progressive Motility (%)	≥32%	43.8 (9.5)	45.5 (25.8)	1.00
Total Motility (%)	≥40%	49.8 (8.6)	53.3 (27.1)	0.83
Total Motile Count (million/ejaculate) [1]	≥39million/ejaculate	91.3 (36.1)	50.4 (62.4)	0.03*
Morphology (%)	≥4%	1.8 (2.8)	1.3 (2)	0.68

Data are presented as median (IQR) as non-parametric data. P value calculated using a Mann Whitney U Test. Baseline for these parameters was calculated as the average of two semen analysis results at V1 and V2 [pre-intervention]. [1]Total Motile Count (TMC) = Sperm concentration × Sperm Volume × (Total Motility/100). * denotes where differences between NHS and LED groups were statistically significant at $p < 0.05$.

3.4.2 Weight loss observed in men undergoing dietary intervention

The 800kcal/day intervention resulted in significantly greater weight loss when compared with the NHS diet (mean change in weight in kg: - 6.3 ± 6.4, NHS; - 17.6 ± 7.7, 800kcal/day, P<0.001 vs. NHS) (Figure 3.3A) from start to end of study. Similar results were observed with change in BMI (Figure 3.3B) (mean change in BMI in kg/m^2: - 1.9 ± 1.8, NHS; -5.6 ± 2.5, 800kcal/day, P<0.001 vs. NHS) (Table 3.3B). There were no significant differences in weight loss between visit 4 and visit 5 i.e. from the end of the weight loss phase (V4) to the end of the weight maintenance phase (V5) in either of the two groups (Figure 3.3C).

Figure 3-3: Bar graphs of change in weight and BMI, and time profiles of change in weight of participants according to diet groups; NHS vs LED.



A-B: Bar graphs of the overall mean change in weight (A) and BMI (B) at the end of the dietary intervention period (visit V5) when compared with start of study (visit V1). ***, P<0.001. **C:** Time profiles of change in weight at visit 4 and visit 5 when compared with start of study (visit V1). Data points include incremental weight loss at visit 4 (end of weight loss phase, calculated as visit 4 minus visit 1) and visit 5 (end of weight maintenance phase/study, calculated as visit 5 minus visit1). Data presented as mean+/-SD. ***, P<0.0001 LED V5-V1; αα P<0.01 NHS V5-V1. LED: Low energy diet. Red=LED; Grey=NHS.

3.4.3 Effects of dietary intervention on body composition and metabolic profile

Table 3.4 summarises the changes in body composition and metabolic parameters from start to the end of the study in the two dietary arms. There were significant beneficial effects in both body composition and metabolic profile observed in the 800kcal/day arm compared with the NHS arm; a significant greater decline in diastolic blood pressure (P=0.006), waist circumference (P=0.001), fat mass (P=0.001), visceral fat (P=0.001), basal metabolic rate (P=0.001), and significant increase in lean mass (P=0.001) was observed.

Similarly, significant improvements in HbA1c (P=0.013), total cholesterol (P=0.004), LDL (P=0.005) and triglycerides (P=0.048) were observed in the 800kcal/day arm compared with the NHS advice arm (Table 3.4).

Table 3-4: Change in body composition and metabolic profiles in participants from start to end of study according to diet groups; NHS vs LED.

Parameters (units)	Dietary intervention group		P value
	NHS (N = 12)	LED (N = 12)	
BEDSIDE			
Weight (kg)	- 6.3 ± 6.4	-17.6 ± 7.7	0.001*
BMI (kg/m^2)	- 1.9 ± 1.8	-5.6 ± 2.5	0.001*
SBP (mmHg)	+1.5 ± 9.2	-6.8 ± 15.6	0.126
DBP (mmHg)	+0.8 ± 7.7	-8.8 ± 7.7	0.006*
BODY COMPOSITION			
Waist circumference (cm)	-0.8 (6.1)	- 7.8 (4.6)	0.006*
Fat Mass (%)	- 1.6 (2.6)	-7.3 (3.3)	0.001*
Fat Mass (kg)	-2.6 (6.8)	-10.3 (4.3)	0.001*
Lean Mass (%)	+1.5 (2.5)	+7.0 (3.0)	0.001*
Lean Mass (kg)	+0.8 ± 2.2	-4.6 ± 2.7	0.001*
Water weight (%)	+2.2 ± 2.0	+5.1 ± 1.9	0.001*
Visceral fat (%)	-1.7 ± 1.8	-5.0 ± 2.6	0.001*
eBMR (kcal)	-42.8 ± 87.6	-191.5 ± 106.6	0.001*
METABOLIC PROFILE			
Fasting glucose (mmol/L)	-0.0 ± 1.0	-0.3 ± 0.6	0.496
HbA1c (mmol/mol)	+0.0 (1.8)	-3.0 (4)	0.013*
Total cholesterol (mmol/L)	-0.2 (0.7)	-0.8 (0.9)	0.004*
LDL (mmol/L)	-0.0 ±0.4	-0.5 ± 0.3	0.005*
HDL (mmol/L)	+0.0 ± 0.2	+0.0 ± 0.2	0.608
Triglycerides (mmol/L)	-0.1 ± 0.7	-0.9 ± 1.2	0.048*

Data are presented as mean ± SD if normally distributed and median (IQR) if non normally distributed. Start of the study is visit 1 and end of the study is visit 5. P value calculated as independent samples T test for normally distributed data; non normally distributed data was compared using a Mann Whitney U Test. * denotes where differences between NHS and LED groups were statistically significant at $p < 0.05$. BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; eBMR, estimated Basal Metabolic Rate; LDL, Low Density Lipoproteins; HDL, High Density Lipoproteins. LED: Low energy diet.

3.4.4 Effects of dietary intervention on reproductive hormone profile

No significant changes were observed in LH, FSH, oestradiol, total and free testosterone between the dietary groups (Table 3.5) from start (visit 1) to end of the study period (visit 5). A significant improvement in SHBG was observed in the 800kcal/day group compared with NHS group (mean change in SHBG in nmol/L: 1.3 ± 4.6, NHS; 8.4 ± 7.9 ,800kcal/day, P=0.012 vs. NHS) from start to end of the study. There was a non-significant increment in total testosterone, LH, and decrease in FSH in 800kcal/day group compared with NHS group (Table 3.5).

Table 3-5: Change in reproductive hormone profiles in participants from start to end of study according to diet groups; NHS vs LED.

HORMONAL PROFILE Parameters (units)	Dietary intervention group		P value
	NHS (N = 12)	LED (N = 12)	
LH (IU/L)	-0.7 ± 1.2	+0.1 ± 1.1	0.09
FSH (IU/L)	+0.1 ± 0.8	-0.4 ± 0.8	0.12
Total testosterone (nmol/L)	+0.1 ± 1.5	+0.9 ±. 2.4	0.34
Free Testosterone[1] (nmol/L)	+0.00 (0.05)	-0.01 (0.29)	0.56
Oestradiol (pmol/L)	-12 ±. 17.2	-4.8 ±. 13.5	0.37
SHBG (nmol/L)	+1.3 ± 4.6	+8.4 ± 7.9	0.01*

Data are presented at mean ± SD if normally distributed and median (IQR) if non normally distributed. Start of the study is visit 1 and end of the study is visit 5. P value calculated by independent samples T test for normally distributed data; non normally distributed data was compared using a Mann Whitney U Test. * denotes where differences between NHS and LED groups were statistically significant at $p < 0.05$. [1]Free Testosterone was calculated using the Vermeulen equation. LH, luteinizing hormone; FSH, follicle stimulating hormone; SHBG, sex hormone binding globulin. LED: Low energy diet.

3.4.5 Effects of dietary intervention on sperm quality

Semen parameters have large intra-individual biological variability therefore we used the average of the first two visits (pre-start of diet) to represent the baseline semen values. Our primary outcome was to investigate the effect of weight loss on sperm concentration. Median sperm concentration (change in median (IQR) sperm concentration in million/ml: -2.4 (48), NHS; 12.9 (33.5),800kcal/day, P=0.48 vs. NHS) and total motile count (median (IQR) change in TMC in million/ejaculate: -2.4 (72.3), NHS; 21.8 (130.8), 800kcal/day, P=0.44 vs. NHS) increased in the LED (800kcal/day) arm from baseline to end of study compared with the NHS arm, but the increase was not statistically significant. There were no significant differences observed in any of the semen parameters from baseline to end of study between the two dietary arms. These are illustrated in Table 3.6.

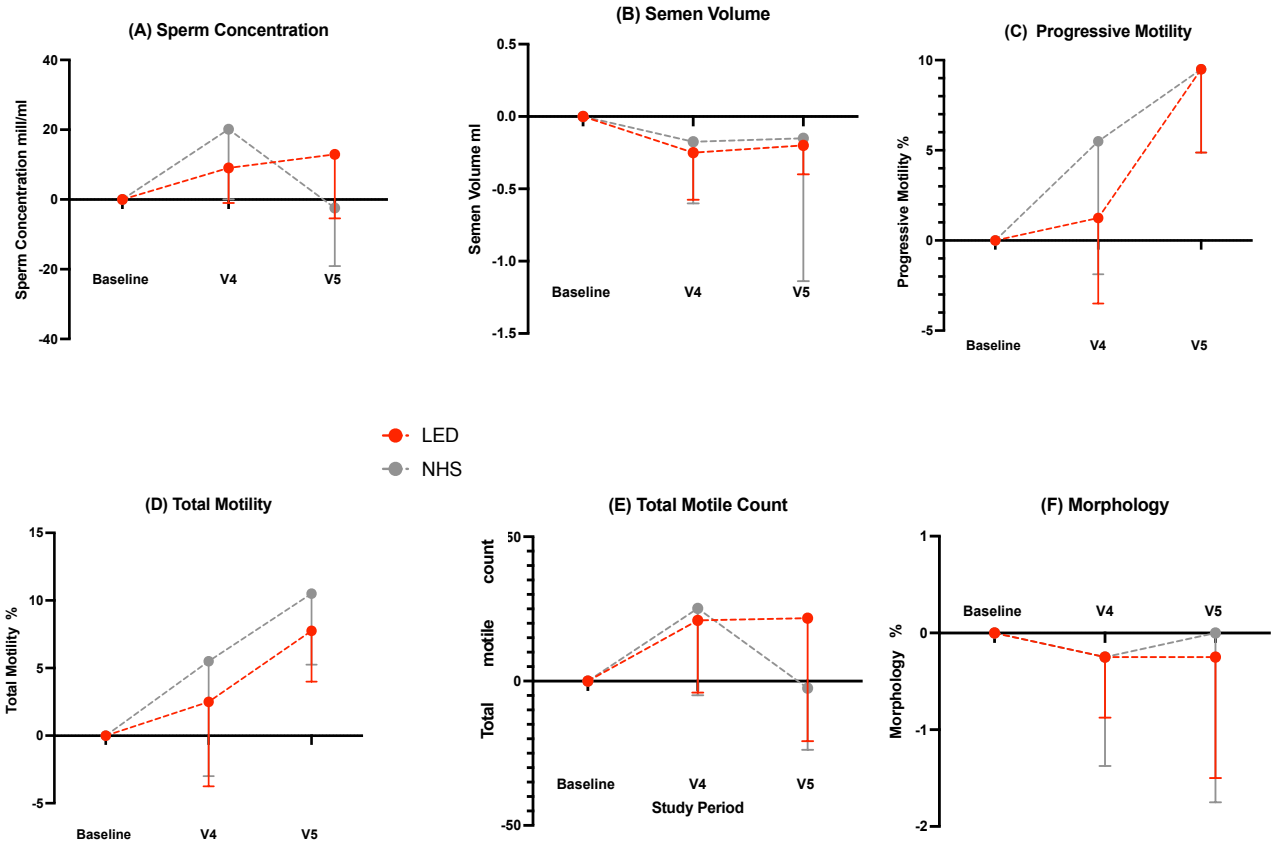
Table 3-6: Change in semen parameters in participants from baseline to end of study according to diet groups; NHS vs LED.

Parameters (units)	Dietary intervention group		P value
	NHS (N = 12)	LED (N = 12)	
Semen Concentration (million/ml)	-2.4 (48.0)	+12.9 (33.5)	0.478
Sperm Volume (ml)	-0.2 (1.4)	-0.2 (0.9)	0.71
Progressive Motility (%)	+9.5 (7.8)	+9.5 (15.1)	0.77
Total Motility (%)	+10.5 (8.7)	+7.8 (17.9)	0.99
Total Motile Count (million/ejaculate)[1]	- 2.4 (72.3)	+21.8 (130.8)	0.44
Morphology (%)	+0.0 (3.5)	- 0.3 (2)	0.54

Data are presented as median (IQR) as semen parameters were non-parametric (failed normality test). P value calculated using a Mann Whitney U Test. Baseline refers to the average of V1 and V2 (pre-diet start), therefore change in semen parameters calculated as the difference between the final visit (V5) and baseline (average of V1 and V2). [1]Total Motile Count (TMC) = Sperm concentration × sperm volume × (total motility/100). LED: Low energy diet.

We further compared time profiles of incremental change in semen parameters at multiple time points i.e. at baseline (average of visit 1 and 2, pre-start of diet), visit 4 (end of 12 weeks, weight loss phase) and visit 5 (end of study) in the two dietary arms (Figure 3.4). No significant changes were noted in the time profiles of any of the semen parameters.

Figure 3-4: Time profiles of incremental change in median semen parameters of participants according to diet groups; NHS vs LED.



Data presented as median (error bars represent IQR). Data points include each semen parameter at baseline (average of visits 1 and 2), visit 4 (end of weight loss phase, calculated as visit 4 minus baseline) and visit 5 (end of weight maintenance phase/study, calculated as visit 5 minus baseline). Total Motile Count (TMC) = Sperm concentration × Sperm Volume × (Total Motility/100); LED, Low energy diet. Red=LED; Grey=NHS.

Sperm DNA fragmentation index: We used the TUNEL assay (see section on Methods) to measure DNA fragmentation in semen samples from visit 1 (start of study) and visit 5 (end of study). Baseline DFI levels are summarised in Table 3.7. There was no statistically significant difference in TUNEL DFI values at baseline between the two diet groups. Our inhouse TUNEL assay was validated such that a cut off of 17% provided greater than 95% specificity to differentiate infertile men with DNA damage from healthy men.

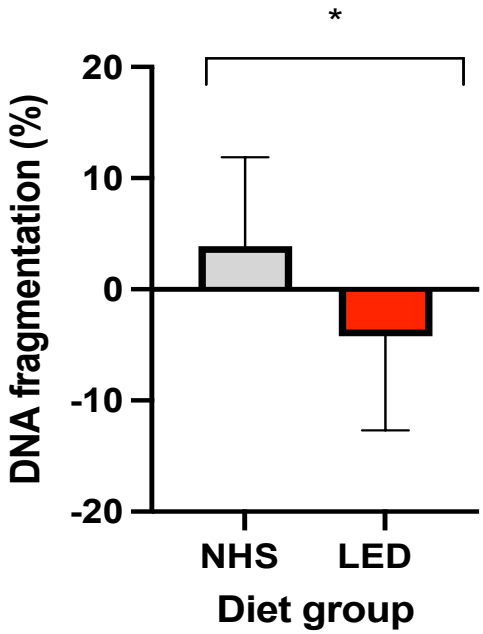
Table 3-7: Baseline DNA fragmentation as measured by TUNEL assay in male participants with obesity.

TUNEL assay	Range	Dietary intervention group		p-value
		NHS (n=12)	800kcal (n=11)	
DNA fragmentation index (%)	<18%	16.1± 6.6	11.9 ± 13.1	0.08

Frozen semen used from visit 1 (pre-start of diet). Data presented as mean+/-SEM.

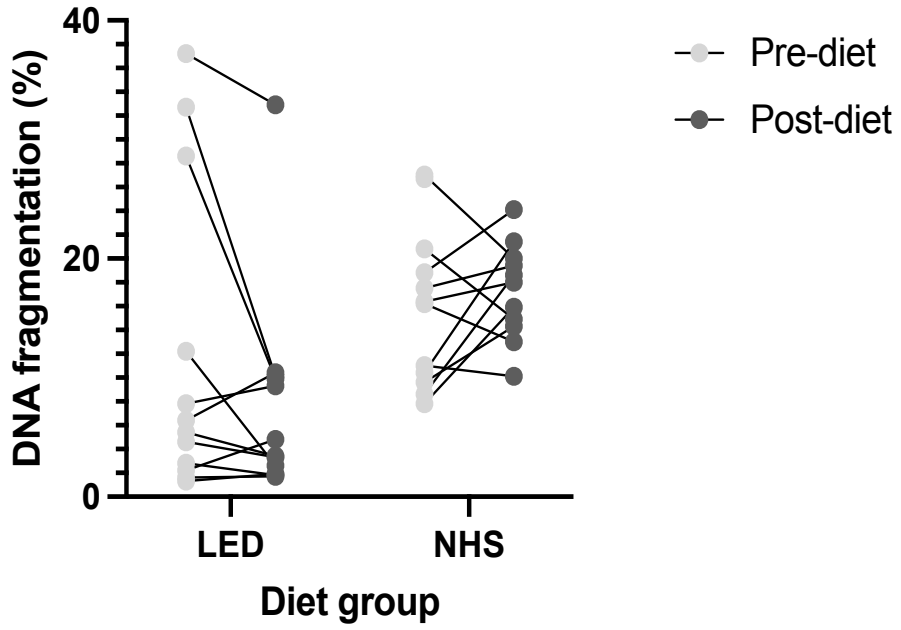
I thereafter analysed the incremental change in sperm DNA fragmentation between the start (V1) and the end of the study period (V5) (Figure 3.5). There was a significant decrease in mean sperm DFI in men in the LED group, compared with an increase in DFI in men in the NHS group ($p<0.05$). There was some inter-individual variability in DFI scores within each diet group; majority (7/12) of men had a reduction in DNA fragmentation (range -1 to -22.6%) in the LED group with 5/12 men with a slight increase in DNA fragmentation (range +0.6 to +4%). In contrast, majority (8/12) men had an increase in their DNA fragmentation (range +1.6 to +21.1%), with 4/12 men with a decrease in DFI (range -0.9 to -7%) scores (Figure 3.6).

Figure 3-5: Bar graph of change in DNA fragmentation index in men with obesity using the TUNEL assay.



Bar graphs of overall mean (+ SD) change in DFI at the end of the dietary intervention period (visit V5) when compared with a baseline visit (visit V1). *$p<0.05$

Figure 3-6: Graph illustrating change in DNA fragmentation index in each individual participant pre- (visit 1) and post diet (visit 5) .



Change in DFI from start (visit 1) to end of the dietary intervention period (visit 5) for each individual participant in LED and NHS arms.

3.4.6 Effects of dietary intervention on sexual health and quality of life scores

We evaluated changes in sexual health such as erectile function, and quality of life with validated questionnaires, SHIM and SF-36 from start to end of the study period (Table 3.8). There was no significant change in sexual/erectile function from start to end of study between the groups as evaluated by the SHIM questionnaire. However, there were only 2/12 men in each arm of the study who reported baseline erectile dysfunction, with majority of men having normal baseline SHIM scores making it difficult to assess any change. There was a significant improvement in median total SF36 scores in the LED arm compared with the NHS however these changes were not significant when subdivided into the 8 domains of the SF-36 (physical functioning, physical role limitations, bodily pain, general health perceptions, energy/vitality, social functioning, emotional role limitations and mental health). Self-rated health transition scores (not included in total SF-36 scores) were significantly higher in the LED arm compared with the NHS arm.

Table 3-8: Change in Sexual Health Inventory for Men (SHIM) and Short Form (SF-36) Survey scores of participants according to diet groups; NHS vs LED.

Questionnaire	Dietary intervention group		P value
	NHS (N = 12)	LED (N = 12)	
Sexual health Inventory for Men (SHIM)	0 (2)	0 (0.75)	0.789
Short Form (SF-36) Survey	-71.0 (144.75)	+17.5 (75.25)	0.027*
Physical health	0.0 (22.0)	0.5 (20.5)	0.063
- Physical functioning	+6.0 (20)	+14.5 (23.5)	0.057
- Physical role limitations	+0.0 (9.75)	+0.0 (0)	0.25
- Bodily pain	-10.0 (22.3)	+0.0 (34.8)	0.34
- General health perception	-3.0 (35.0)	+3.0 (25.8)	0.79
Mental health	-12.5(25.0)	0.0 (24.8)	0.004*
- Energy/vitality	-6.5 (44)	-17.5 (23.3)	0.15
- Social functioning	-25.0 (12)	-6.5 (22)	0.07
- Emotional role limitations	+0.0 (58.5)	+0.0 (0)	0.11
- Mental health	-6.0 (27)	+15.0 (36)	0.17
Self-rated health transition	+12.50 (25)	+40.0 (25)	0.03*

Values are changes in each of the scores calculated as visit 5 value minus visit 1. Data are presented as median (IQR). P value calculated using a Mann Whitney U Test. * denotes where differences between NHS and LED groups were statistically significant at $p < 0.05$.

3.5 Discussion

We carried out a randomised controlled study investigating the effects of losing >12kg weight (based on pilot results of Study 1, Chapter 2) on sperm quality using LED verses 'healthy eating' NHS advice in obese men. Prior to our study, the longest dietary intervention till date carried out to investigate effects of weight loss on sperm quality in men has been 14 weeks (Håkonsen et al., 2011), which was a longitudinal cohort study in 27 obese men, albeit lacking a control group.

LED intervention of 16 weeks was effective at inducing a greater degree of mean weight loss of -17.6kg (15.5%) compared with -6.3kg (5.3%) in the NHS (control) arm. Eleven out of the twelve participants randomised in the LED arm lost >12kg of weight loss which was pre-set as the target of LED intervention. This degree of weight loss achieved is comparative to other LED studies of similar duration, albeit with large individual variation (range of weight loss from -5kg to 36.4kg in the LED arm) as with most lifestyle interventions. The Cartilage and Osteoarthritis weight loss trial (Carot) observed that elderly obese men (mean age 62.5 years) with knee osteoarthritis lost 12.2kg (11.9%) of weight (10% of initial body weight) over a 16 week dietary programme comprising of 8 weeks of VLED or LED, followed by 8 weeks of 1200kcal/day food reintroduction (Christensen et al., 2011; Riecke et al., 2010). Similarly, another study of 668 obese men observed a mean weight loss of -11.8kg (11.8%) after 8 weeks of LED with CWP (Christensen et al., 2018). Furthermore, a metanalysis including 3017 participants from 20 studies reported a pooled mean weight change of −12.3kg during the VLED/LED (median duration of VLED/LED of 8 weeks; range 3–16 weeks) period (Johansson et al., 2014).

Men in the control (NHS) arm also lost 5% (-6.3kg) of weight, which is similar to that observed in the NHS arm in Study 1 (-3.9kg over a 8 week intervention in Study 1, compared to -6.3kg over a 16 week intervention in Study 2). Other studies have also reported weight loss with various 'healthy eating' low carbohydrate diets. The average weight loss reported in low carbohydrate diet groups ranged from 2.65 to 10.2kg and from 2.65 to 9.4kg in 'balanced diet' groups at 3–6 months post intervention (Naude et al., 2014). Similarly, a meta-analysis of different diet programs reported that a low carbohydrate diet led to a mean weight loss of -8.7kg compared with -8.0 kg at 6-month follow-up with a low-fat diet (Johnston et al., 2014). Nonetheless, as our

control (NHS) arm also lost weight, this may have underestimated the differences observed in our primary and secondary outcomes between the two groups.

Both our dietary interventions were well tolerated by our participants with no known adverse events. However, four men dropped out after being randomised due to either inability to attend study visits (1 participant) or due to dissatisfaction with dietary allocation and immediately withdrew (2 in LED arm and 1 in NHS arm). There were no significant differences in baseline demographics between the two dietary groups (LED vs NHS). Interestingly, the mean age of participants enrolled in the study, as well as the age of their respective partners (between 35-40 years) were within the prime reproductive years. Furthermore, majority of men enrolled were in long-term relationships/partnerships (21/24). This might have motivated some participants to become interested in optimising and knowing more about their fertility status. Furthermore, both study 1 and study 2 included men who had normal baseline semen parameters, with no known history of infertility, making the results more generalizable to the general obese population.

Our 800kcal/day LED intervention led to significant reductions in weight, BMI and waist circumference (WC) compared to the NHS diet group. In contrary to Study 1, we monitored body composition parameters, using bioelectrical impedance, to better define body fat composition. We observed improved body composition parameters, with significantly lower % fat mass and visceral fat and higher % lean mass in the LED group compared with the NHS arm. Similarly, significant improvements in the metabolic profile with lower diastolic blood pressure, HbA1c, total cholesterol, LDL and triglycerides were observed in the LED arm compared with the NHS arm. These cardio-metabolic benefits of weight loss are well reported in the literature (Stelmach-Mardas and Walkowiak, 2016). However, it is interesting to note that the 'healthy eating' NHS 'eatwell' advice also led to weight loss, lower waist circumference, improvements in body composition and metabolic profile. Albeit, the effect was much higher in the LED arm compared to the NHS arm, suggestive of higher weight loss dietary interventions associated with better cardio-metabolic profile. In addition, all participants kept an exercise log detailing their resistance exercise levels of 30 minutes 3 times a week during the entire study protocol, which may have helped limit any lean mass loss, as previously reported with other LED studies (Chaston et al., 2007; Christensen et al., 2011; Snel et al., 2012). In addition, these cardio-metabolic benefits mirrored significantly higher SF36 total scores in the LED vs NHS arm, with higher self-rated health transition scores.

In contrast to these clear cardio-metabolic improvements and quality of life in our LED arm, not all changes in reproductive hormone and semen quality parameters were as originally hypothesized. We observed a significant increase in SHBG with weight loss in LED compared to NHS arm. However, we only observed a non-significant increase in total testosterone in our LED arm compared to control, with no significant change in free testosterone or oestradiol in either arm. Previous studies investigating the effects of weight loss on reproductive hormones in men have been conflicting due to differences in study populations, variations in age, metabolic profiles, degree of obesity and weight loss, or differences in time points of hormone determinations (during active weight loss or during maintenance of lost weight). Leenen et al (Leenen et al., 1994) carried out a study in 37 men who had a mean weight loss of -13.5kg after 13 weeks of a calorie deficit diet and further 3 weeks of a weight maintenance diet. They did not demonstrate any significant change in total or free testosterone. However, there was a significant improvement in dehydroepiandrosterone sulphate (DHEAS) and SHBG with weight loss (Leenen et al., 1994). In contrast, Niskanen et al (2004) observed that an average weight loss of 16.3kg in 58 men, during a 9-week 800kcal/day diet, was sufficient to increase SHBG and free testosterone at statistically significant levels (Niskanen et al., 2004). Another study of 9 men who underwent 8-weeks of 300-500kcal/day diet followed by 2 weeks of 1000kcal/day diet achieved a mean weight loss of 24.4kg and noted significant increase in total, free testosterone and DHEAS (Pasquali et al., 1988). No significant increases in oestradiol, or SHBG were noted. However, contrary to our study, many of the obese participants in these previous studies had baseline pattern of increased oestradiol, reduced testosterone and SHBG concentrations. Furthermore, one may postulate that men in our study were still overweight or obese after the 16 weeks intervention, therefore, ongoing aromatisation of total testosterone to oestradiol due to the high adipose tissue, to observe any significant changes in reproductive hormonal profile. Further studies are needed to evaluate whether the return to normal weight is the prerequisite for higher degree of improvement in reproductive hormone profiles. Another potential explanation is that our oestradiol assay was unable to report oestradiol levels below 100pmol/L, which suggests that reductions below 100pmol/L were unable to be quantified, making it difficult to for us to reach any firm conclusions.

We also observed a non-significant improvement in sperm concentration and total motile count in LED arm compared to NHS; the two semen parameters considered to be the best predictors of fertility (Bonde et al., 1998; Slama et al., 2002; Zinaman et al., 2000). Our findings are in keeping with a study by Hakonsen et al

(Håkonsen et al., 2011), whereby no significant change in sperm concentration was noted in men with a median weight loss of 15% after a 14 week residential weight loss programme. However, contrary to our study, they observed a significant improvement in total sperm count in the cohort of ten men who lost the highest amount of weight (17.2 to 25.4% weight loss).

We had originally hypothesized that a longer intervention period of 16 weeks to cover an entire cycle of spermatogenesis would lead to better semen quality. However, this was not what we observed. It would be helpful to have future studies that are longer and cover more than 1 spermatogenesis cycle, as one may postulate that lifestyle interventions that may significantly improve semen parameters may take longer than 16 weeks. Furthermore, one may postulate that the higher sperm concentration and TMC with suppression in FSH observed in the LED arm, albeit statistically non-significant, may be due to negative feedback of increased testicular spermatogenesis at the hypothalamic-pituitary level with subsequent reduction in FSH. Future longer-term studies are needed to further evaluate these observations and confirm the underlying mechanisms.

Obesity has been associated with elevated levels of inflammatory adipocytokines, such as interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α), which can cause sperm cell apoptosis and DNA fragmentation (Liu and Ding, 2017b; Oliveira et al., 2017; Zhu et al., 2021). A study by Zhu et al investigated 54 subfertile men and observed that sperm DFI and sperm apoptosis were significantly increased in overweight and obese men compared with normal BMI, with increased expression of apoptosis-related factors via the activation of apoptotic signalling pathways (Zhu et al., 2021). Future randomised controlled studies are required to assess seminal plasma interleukin levels with weight loss interventions and correlate these to semen parameters and fertility outcomes.

I postulate that the higher sperm concentration with suppression in FSH in the LED arm compared to NHS arm may be due to improved survival of the spermatozoa, with reduced sperm apoptosis and DFI. Therefore we also measured DFI levels in our cohort of men pre and post intervention using the TUNEL assay (Rakesh Sharma et al., 2016)(see methodology for details) and observed a significant improvement in sperm DFI in the LED group verses an increase in sperm DFI in the NHS group. TUNEL assay is a highly sensitive and reproducible method of measuring direct DNA strand breaks. The extent of sperm DNA fragmentation, measured by the TUNEL assay, is one of the determinants of male fertility (Bungum et al., 2004; Duran et

al., 2002; Nicopoullos et al., 2019; Simon et al., 2019). Duran et al. (Duran et al., 2002) reported that pregnancy rates were lower when the TUNEL method indicated a proportion of sperm DNA damage of 12% or higher. Zhang et al. (Zhang et al., 2015) observed, in a meta-analysis of nine studies on IVF research, that a DFI lower than 27% was associated with higher clinical pregnancy rates. Osman et al. (Osman et al., 2015) reported that couples with low levels of male sperm DNA damage had higher live birth rates after IVF and/or ICSI compared to men with higher sperm DNA damage.

In summary, we observed significant improvements in cardio-metabolic profile and sperm DFI with weight loss achieved from LED compared with the NHS arm. We observed non-significant improvements in total testosterone, sperm concentration and TMC in the LED arm, which is in contrast to some findings post bariatric surgery whereby sperm counts have been noted to acutely decrease within first six months post-surgery. This is the first study to show improvements in sperm DFI in obese men with weight loss from LED, compared with a control NHS arm, highlighting the potential benefits of weight loss to male infertility in obese men.

Studies 1 and 2 have investigated the effects of weight loss on sperm parameters in men with obesity *without* baseline sperm quality associated with infertility. We next carried out Study 3 aimed to determine whether weight loss by caloric restriction improves sperm quality in men with *oligospermia* (low sperm count) as per current WHO reference range.

Chapter 4: Randomised controlled study comparing caloric restriction with standard NHS advice to improve sperm quality in obese men with oligospermia

4.1 Introduction

Study 2 (Chapter 3) suggested that weight loss >12kg with LED may significantly improve metabolic profile and sperm DFI compared with men in the NHS (healthy eating) control intervention in men with obesity and normal semen quality. Therefore, we performed the current study (Study 3) to investigate the effects of weight loss of >12kg with LED verses NHS diet in obese men with known oligospermia (low sperm count).

Oligospermia is the most common manifestation of male infertility (Kumar and Singh, 2015). Furthermore a recent systematic review reported fall in sperm counts by 50-60% since the 1970s, in North America, Europe and Australasia (Levine et al., 2017). Therefore we were interested to investigate if the physiological effects of weight loss observed in obese men with normal baseline fertility, would translate to obese men who have underlying sperm quality associated with male infertility. Oligospermia is defined as a sperm concentration <15million/ml as per WHO reference range (Cooper, 2010) which is associated with time to pregnancy and pregnancy rates (Bonde et al., 1998; Guzick et al., 2001; Slama et al., 2002; van Zyl and Menkveld, 2006) . Bonde et al investigated 430 couples over a period of 6 months, and reported that 65% of men who had a sperm concentration of >20million/ml impregnated their partners within 6 months compared to only 36.4% of men with sperm concentration <20million/ml (Bonde et al., 1998). This study was based on the previous WHO1999 cut off of sperm concentration of <20million/ml to define oligospermia (Aghazarian et al., 2020). We did not include obese men with azoospermia (i.e. no sperm seen in ejaculate) in the current study, as azoospermia represents the most severe form of male infertility and is normally due to primary testicular dysfunction resulting in failure of spermatogenesis, as opposed to obesity.

Semen oxidative stress, as measured using reactive oxygen species (ROS), is a recently identified mechanism for sperm damage and measurement of ROS is a potential tool of added value in the investigation of male fertility (Agarwal et al., 2019b; Bisht et al., 2017; Tremellen, 2008). It is suggested that use of antioxidants and lifestyle changes may reduce the risk of high ROS and sperm DFI to improve male infertility; however, there is paucity in the data with lack of good quality randomized controlled trials available. Men with idiopathic infertility and varicocele-associated infertility taking antioxidant therapy have an associated significant improvement in semen parameters and live birth rates (Cavallini et al., 2004; Imamovic Kumalic and Pinter, 2014). As such, commercial anti-oxidants are available over-the counter for men to use as

empirical therapies, and are often self-administered by infertile men to improve sperm quality and function (Balercia et al., 2005; Lenzi et al., 2004). However evidence underpinning their efficacy has been controversial (Raigani et al., 2014; Sigman et al., 2006). In addition, there are increasing concerns regarding indiscriminate anti-oxidant use causing reductive stress-mediated sperm damage (Henkel et al., 2019).

Obesity is associated with higher seminal ROS compared to men with normal BMI (Yang et al., 2016). Factors that raise semen ROS may also cause sperm DNA fragmentation although the underlying mechanisms are unclear. No previous study has investigated the effect of weight loss on seminal ROS. Therefore, to investigate the effects of weight loss on seminal ROS and sperm DFI, we measured both of these exploratory outcomes in our current study.

4.2 Hypothesis and aims

Hypothesis

Weight loss of >12kg by caloric restriction with LED would significantly improve sperm concentration compared with standard NHS advice in men with obesity who have baseline oligospermia.

Aims

To determine the following:

(i) Effects of weight loss (aiming for >12kg) by LED on WHO sperm parameters, seminal ROS and sperm DNA fragmentation compared with NHS advice in men with obesity and oligospermia.

(ii) Effects of weight loss (aiming for >12kg) by LED on reproductive hormones and metabolic profile compared with NHS advice in men with obesity and oligospermia.

Objectives

Primary objective:

1. Investigate the effects of weight loss by LED on sperm concentration in men with obesity and oligospermia.

Secondary objectives

1. Investigate the effects of weight loss on other sperm parameters (total motility, progressive motility, semen volume, morphology, TMC), seminal ROS and sperm DNA fragmentation in men with obesity.
2. Investigate the effects of weight loss on reproductive hormone parameters (serum total and calculated free testosterone, oestradiol, SHBG, LH and FSH) and metabolic parameters including waist circumference, blood pressure, HbA1c, fasting glucose and lipid profile.

4.3 Methods

4.3.1 Ethical approval and study design

Ethical Approval

Ethical approval was same as Study 1& 2.

Study Design

Study design was same as Study 2. Participants were randomised to receive either 800kcal/day or standard NHS advise ('healthy plate') during a 16-week study protocol. Participants randomised to 800kcalday received LED using LighterLife products aiming for >12kg of weight loss. We used LighterLife products for LED arm, instead of CWP due to availability and provision of LED products for research study (See Chapter 1, section 1.16). Our control arm was the NHS diet based on the standard NHS advise on a healthy balanced plate.

4.3.2 Participant recruitment

Similar recruitment methods, inclusion and exclusion criteria were used as detailed in section 2.3.2 in chapter 2 (study 1). The only additional inclusion criteria for study 3 was oligospermia (i.e. sperm concentration of <15million/ml). We excluded men who had azoospermia (i.e. no sperm seen in the ejaculate). 36 participants aged 18-60 years with BMI≥30kg/m^2 and oligospermia completed the study.

Additional recruitment methods included an online advertising platform using Clariness. Clariness is an international participant recruitment company for clinical trials. It advertised the trial through an online awareness campaign including search engine marketing, banner advertising on relevant websites and social media. Upon clicking an ad, these candidates were taken through an online prescreening process via the ClinLife® website. In this prescreening process patients were asked questions about their health based on the trial inclusion and exclusion criteria and those who pass this step were referred to us.

Written informed consent was obtained from all subjects. This study was performed in accordance with the Declaration of Helsinki.

4.3.3 Protocol

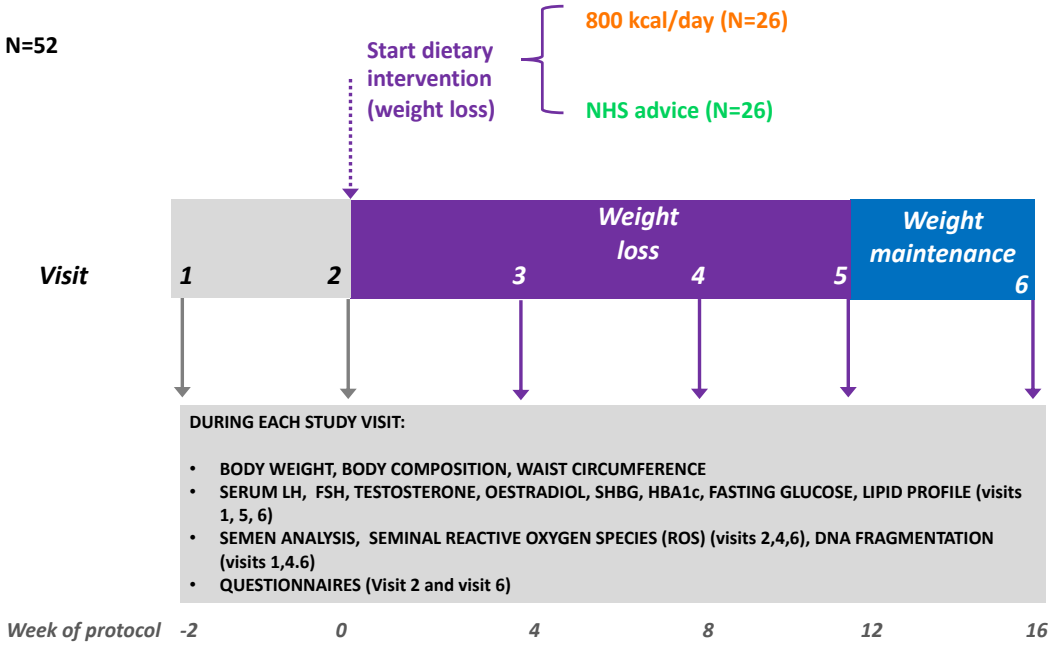
A randomized, controlled study was performed.

The protocol consisted of two phases: 1) 800kcal/day weight loss phase of 12 weeks aiming for at least >12kg weight loss, and 2) 4 weeks of weight maintenance phase with gradual food reintroduction (same as study 2, chapter 3).

Gradual reintroduction of food following a LED has been reported to assist with weight loss maintenance (Brown and Leeds, 2019; Leeds, 2014). For example, reintroducing food over a 6-week period resulted in significantly less weight regain at 52 weeks compared with a reintroduction period of 1 week (3.9 kg vs. 8.2 kg, respectively) (Gripeteg et al., 2010). Therefore, with the expertise of our nutrition team (Prof Leeds, University of Denmark and Prof Brown, University College London), we chose a 16 week study duration composed of 12 weeks of TDR phase with subsequent 4 weeks of gradual food reintroduction. (see Chapter 3, section 3.1 for more details). The control arm consisted of NHS healthy eating advice for the entire 16 weeks of the protocol.

The study protocol for study 3 is summarised in Figure 4.1.

Figure 4-1: Summary of study protocol



Thirty-six participants completed the study with 18 participants in 800kcal/day and 18 participants in NHS arm. LH, luteinizing hormone; FSH, follicle stimulating hormone; SHBG, Sex Hormone Binding Globulin

Baseline period: The initial 2-week period (weeks -2 to 0, visits 1-2) allowed the measurement of baseline values of body composition, testicular volume, reproductive hormones, metabolic profile, semen analysis and the acclimatisation of participants to study conditions.

Intervention period: During week 0 (visit 2) of the protocol, participants were initiated on one of the two randomised study groups 800kcal/day or standard NHS advise (control) for a 16-week intervention period involving further 4 visits at monthly intervals (weeks 4, 8, 12 and 16). Dr Les Huson, senior statistician, Imperial clinical research, created a randomisation list. Dr. Jayasena, PI of the study, held the list and allocated participants when requested. I had no role in randomisation.

Serial measurements of body weight, waist circumference, body composition, blood pressure and semen analysis were carried out at each of the visits. Seminal ROS was measured on fresh semen samples (see section on ROS below) at visits 2,4 and 6. Semen was stored at -20^{O}C for DNA fragmentation analysis at visits 1, 5 and 6. Fasting serum reproductive hormones and metabolic profile were measured at visits 1, 5 and

6. Validated questionnaires for erectile dysfunction (SHIM) (Rosen et al., 1999) and quality of life (SF-36) (Lins and Carvalho, 2016) were done at the start and end of study.

4.3.4 Diet groups

LighterLife products consisted of soups, shakes, porridge and bars of approximately 150kcal per product (Table 1.6, chapter 1) (https://www.lighterlife.com/). These LED products contained an array of micro and macronutrients (Table 1.6). The LED arm consisted of 4 LighterLife products plus 400mls of semi-skimmed milk per day for 12 weeks (visit 0 to visit 5). This is because four products of LighterLife would provide 611kcal/day of energy. However, in order to match the calories in 4 products of CWP (806kcal/day i.e. LED), we added 400mls of semi-skimmed milk to the 4 products of LighterLife (611kcal/day), as 400mls of semi-skimmed milk contains 206kcal, making the total calorie intake of the LED arm to 817kcal/day (see chapter 1, section 1.16 for further details). The weight maintenance phase for 4 weeks (visit 5 to visit 6) involved gradual food reintroduction by replacing one LighterLife product every 1-2 weeks with healthy meals (low carb, high protein) based on individual diet preferences (see details of *food reintroduction phase* below) in order to maintain the weight loss achieved at week 12.

NHS diet (control arm) was based on the standard NHS advise ("The Eatwell Guide," 2018) consisting of a balanced diet (1800-2200kcal/day) with general advice to reduce portion size, eating mor vegetables and fruits, less saturated fats and carbohydrates. Energy intake for weight loss for NHS arm was based on the Mifflin-St. Joer equations (Mifflin et al., 1990) and physical activity levels.

All participants were advised to drink at least 2.5litres of water per day. In addition participants on TDR (817kcal/day) were provided with additional fibre supplements in the form of ispaghula husk sachets, as when required, to prevent constipation which can be one of the side effects of LED. All men were advised to limit physical activity to resistance training of 30minutes three times a week, and to keep an exercise log throughout the study. These diets were planned with the expertise of our nutrition team (Profs Brown, UCL and Prof Leeds, University of Denmark). Compliance was checked at each visit according to both product usage and self-reporting.

Food reintroduction phase at 12 weeks for 4 weeks of weight maintenance is summarised as follows:

At 12 weeks (i.e. end of TDR), participants are on 4 LighterLife products and 400mls of semi-skimmed milk (approx. 817kcal/day)

- STEP 1 for 2 weeks (intake of approx. 1000kcal/day) from week 12 to 14 of study:
    - Take one Lighterlife product off
    - Continue 3 products and milk (650kcal/day)
    - Add 1 meal (aim approx. 350 kcal/day): healthy, low carb meal
    - Examples include:
        - Breakfast: eggs (2-3), scrambled or omelette, may add spinach/tomatoes OR high protein yoghurt with blackberries/blueberries
        - Lunch: salad with protein or steamed vegetables with protein. Examples of protein: beans/pulses/tofu (approx. 150g), or chicken breast/fish fillet/salmon (palm size, approx. 4 ounces/125g)
            - Dinner: protein with steamed vegetables/salad (similar to lunch). Add herbs/spices
- STEP 2 for next 2 weeks (intake of approx. 1200kcal/day) from weeks 14 to 16 of study:
    - Take one further Lighterlife product off
    - Stop 400mls of milk, include mil for teas and coffee as needed.
    - Continue 2 products (350kcal/day) and 2 meals (approx. 850kcal/day)
    - Meal options as above
    - Meals can also include lower carbohydrate containing fruits e.g. apples/strawberries/blueberries
- STEP 3: End of study at 16 weeks
    - Participant on 2 meals (1050kcal/day) and 1 product (150kcal/day)
    - Meals can include carbohydrates such as rice crackers/quinoa/couscous/porridge.

4.3.5 Semen sampling

Semen sampling and analysis methods were identical to methods described in Chapter 2 (section 2.3.5).

4.3.6 DNA fragmentation

DNA fragmentation methods were identical to methods described in chapter 3 (section 3.3.6)

4.3.7 Measurement of seminal ROS levels

ROS was measured using an established in-house chemiluminescence assay that was based on measurements of light emission via luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) chemiluminescence (Vessey et al. 2014). Luminol stock solution was made every 3 months and was stored in room temperature at 20-25°C in the dark. For the purposes of the current study, 50µl luminol stock solution mixed with 950µl DMSO was prepared to make up a total of 1000µl of working solution for daily use. In addition to the luminol stock solution, the following three solutions were also made up daily:

I. Negative control solution; this was made after aliquoting 400µl of phosphate-buffered saline (PBS) to an eppendorf and adding 100µl of luminol working solution. Negative control mean value had to be <120RLU/sec to allow reliable measurements.
II. Positive control solution; this was made after aliquoting 395µl of PBS to an eppendorf and adding 5µl of 30% hydrogen peroxide (H_20_2). Finally, 100µl of luminol working solution was added to complete the preparation. Positive control mean value had to be >100,000 RLU/sec to allow reliable measurements.
III. Specimen assay was made of 400µL neat (native) semen mixed with 100µL working solution containing luminol.

Each sample was vortexed to evenly disperse the samples before taking luminometer readings (GloMax; Promega Corporation; Madison, WI, USA). Negative controls were placed into the luminometer immediately after preparation, so that readings could be taken every minute for ten minutes. Once all ten readings were taken, the mean value was calculated. Chemiluminescence was expressed as mean relative light units per second (RLU/sec), as measured over 10 minutes at minute intervals. Following negative control solutions, chemiluminescence was measured for positive controls at least 20 minutes before semen sample production by the participant. Finally, chemiluminescence was measured for seminal specimens. ROS value was calculated via the following formula:

$$ROS = \frac{Mean\ seminal\ sample\ chemiluminescence - Negative\ control\ chemiluminescence}{Sperm\ concentration}$$

In-house validation was performed to ensure consistent positive and negative calibration daily. Prior to commencing the study, the assay had been run daily in the Andrology Department, Hammersmith Hospital for over a year. All analysis runs contained negative and positive control samples. The reference range for semen ROS was <3.8 RLU/sec/million sperm (Vessey et al. 2014).

Luminol chemiluminescence assay is the most commonly used technique to detect oxidized end products. Lucigenin chemiluminescence is a very similar technique using lucigenin but has the disadvantage that it can only detect extracellular free radicals, primarily superoxide. In contrast to lucigenin, luminol can detect both intracellular and extracellular deoxygenation, including hydrogen peroxide, superoxide, and hydroxyl ions (Vessey et al. 2014). We therefore used luminol chemiluminescence to detect intracellular and extracellular free radicals in the semen.

4.3.8 Measurement of reproductive hormones and metabolic profile:

Blood collection, processing and analysis of serum were identical to methods described in Chapter 2 (section 2.3.7).

4.3.9 Other measurements:

These were identical to those described in Chapter 3, section 3.3.8

4.3.10 Questionnaires

These were identical to that described in Chapter 3, section 3.3.9.

4.3.11 COVID precautions

These were as detailed in section 3.3.10, in Chapter 3, and risk assessment in appendix 3.

4.3.12 Statistical analysis

Twenty-two participants per group would give 80% power to detect a statistically significant increased sperm concentration between groups (alpha=0.05, two-sided) based on results from pilot data and study 1. Based on

previous experience, we anticipate a 20% drop-out rate, so we aimed to recruit 26 participants per group i.e. 52 participants in total.

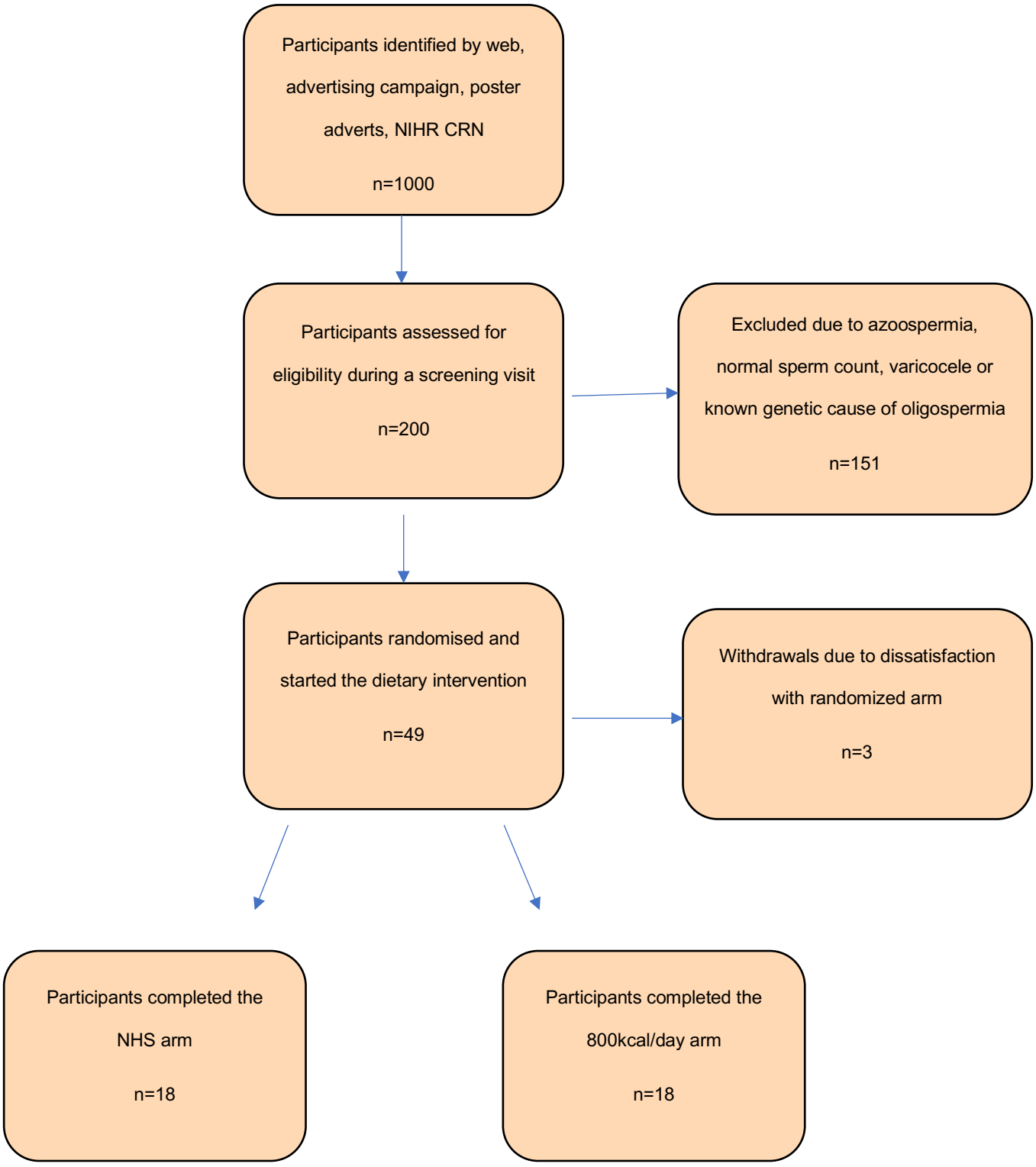
Statistical analysis was performed using GraphPad Prism v.9 (Graphpad Software Inc, La Jolla, CA, USA). Quantitative data was assessed using normal testing using the Shapiro Wilk Normality test. Data are presented as mean ± SD if normally distributed and median (IQR) if non-normally distributed. Comparison between the groups (means; if normally distributed and medians if non normally distributed) were calculated as independent samples T test for normally distributed data; non normally distributed data was compared using a Mann Whitney U Test. Categorical data was compared using a Chi Squared Fischer's Exact test. In all cases, $P<0.05$ was considered statistically significant.

4.4 Results

We screened two hundred participants at a screening visit. Of these participants, 151 men were excluded according to study inclusion and exclusion criteria (see methods section in Chapter 2, section 2.3.2, and chapter 4, section 4.3.2). Forty-nine participants were included in the study. Three participants withdrew their consent soon after allocation to a study arm (2 in LED and 1 in NHS). Forty-six participants (23 in each arm) enrolled in the study, with 36 participants having completed the study (Figure 4.2) at the time of data analysis. Three participants had COVID symptoms with confirmed COVID during the current study, and required their visits to be postponed by 2 weeks. All participants received their 1st and 2nd COVID vaccines (Pfizer or Astrazeneca) during the study period.

Figure 4-2: Patient flow diagram

NIHR: National Institute for Health and Research; CRN: Clinical Research Network



4.4.1 Baseline characteristics of participants recruited to the study

Baseline (at visit 1) demographics (Table 4.1), body composition, metabolic and reproductive hormone profiles (Table 4.2) are summarised below. No significant differences in any of these baseline parameters were observed among the two dietary groups (NHS vs LED) in men with obesity and oligospermia (Table 4.1 and Table 4.2). More than half the participants (55% in LED and 61% in NHS) in each arm had known infertility, with a median duration of infertility of 2.5 to 3 years respectively. Infertility was defined as any couple who had been trying to conceive for more than 12 months. Furthermore, 4/18 men in LED, and 3/18 men in NHS had previously undergone at least one unsuccessful IVF cycle with their current partners (Table 4.1).

For baseline semen parameters (Table 4.3), we used the means of visit 1 and 2 (pre-start of diet) to represent baseline values, and for all analysis of change. At baseline, there was a significant difference in morphology between NHS arm vs LED arm (1% vs 0% respectively), with no other significant differences observed in the other semen parameters. The baseline median sperm concentration, total, progressive motility and TMC were all below the WHO reference range. Seminal ROS was high at baseline in both the groups, with no significant difference between the groups (Table 4.3).

Table 4-1: Baseline demographics of participants according to diet groups; NHS vs LED.

Demographics	Dietary intervention group				P value
	NHS (N = 18)		LED (N = 18)		
Age (years)	37.5 ± 6.4		37.4 ± 7		0.96
Ethnicity	White	9	White	7	0.20
	Mixed	2	Mixed	0	
	Asian	5	Asian	6	
	Black	1	Black	0	
	Arab	1	Arab	5	
Occupation (Based on ISCO-08 classification)	Managers	5	Managers	1	0.25
	Professionals	3	Professionals	7	
	Technicians and Associate Professionals	4	Technicians and Associate Professionals	4	
	Clerical Support Workers	2	Clerical Support Workers	3	
	Service and Sales Workers	2	Service and Sales Workers	3	
	Unemployed	2	Unemployed	0	
Current partner	Yes	14	Yes	17	0.14
	No	4	No	1	
Partner Age	N=	14	N=	17	0.08
	Mean ± SD	36.7 ± 56.3	Mean ± SD	32.7 ± 6.2	
Children	Yes	3	Yes	2	0.63
	No	15	No	16	
History of infertility*	Yes	10	Yes	11	0.74
	No	8	No	7	
Duration of infertility (years)	N=	10	N=	11	0.96
	Median (IQR)	3 (6.5)	Median (IQR)	2.5 (6.4)	
IVF	Yes	4	Yes	3	0.67
	No	14	No	15	
Nutritional Supplements	Yes	9	Yes	10	0.74
	No	9	No	8	
Smoker	Non-smoker	10	Non-smoker	10	1.00
	Ex-smoker	8	Ex-smoker	8	
ETOH current	Yes	11	Yes	8	0.32
	No	7	No	10	
Diabetes	Yes	2	Yes	3	0.63
	No	16	No	15	
HTN	Yes	2	Yes	3	0.63
	No	16	No	15	
Hypercholesterolaemia	Yes	5	Yes	4	0.46
	No	13	No	14	
Erectile dysfunction	Yes	3	Yes	1	0.30
	No	15	No	17	

Data presented at mean ± SD if normally distributed and median (IQR) if non normally distributed. P value calculated by independent samples T test for normally distributed data; non normally distributed data was compared using a Mann Whitney U Test. All categorical data (such as ethnicity, occupation) calculated by chi-square test. HTN, hypertension; IVF, in vitro fertilisation; ETOH, alcohol; ISCO-08, International

standard of classification of occupations; LED: Low energy diet. * Infertility was defined as any couple who had been trying to conceive for more than 12 months.

Table 4-2: Baseline anthropometric, metabolic and reproductive hormone profiles of participants according to diet groups; NHS vs LED.

Parameters (units)	Reference range (if applicable)	Dietary intervention group		P value
		NHS (N = 18)	LED (N = 18)	
BEDSIDE				
Weight (kg)		115.4 ± 19.9	117.0 ± 18.2	0.8
BMI (kg/m2)		37.3 ± 6.2	37.5 ± 4.1	0.6
SBP (mmHg)		125.9 ± 13.1	125.4 ± 7.7	0.9
DBP (mmHg)		79 ± 13	78 ± 7	0.8
BODY COMPOSITION				
Waist circumference (cm)		118.9 ± 12.5	123.0 ± 10.0	0.3
Fat Mass (%)		34.5 ± 5.2	36.4 ± 6.1	0.3
Fat Mass (kg)		37.1 ± 10.5	40.8 ± 10.3	0.2
Lean Mass (%)		63.4 ± 5.3	61.9 ± 3.9	0.4
Lean Mass (kg)		73.3 ± 10.0	71.5 ± 8.3	0.6
Water weight (%)		47.8 ± 3.7	47.0 ± 2.7	0.5
Water weight (kg)		55.3 ± 7.6	54.5 ± 7.4	0.7
Visceral fat (%)		16.1 ± 4.7	17.3 ± 4.5	0.2
eBMR (kcal)		2356 ± 347.8	2304 ± 307.6	0.6
METABOLIC PROFILE				
Fasting glucose (mmol/L)	<7	5.2 ± 1.3	5.5 ± 2.3	0.6
HbA1c (mmol/mol)	<48	38.9 ± 9.0	43.8 ± 20.1	0.4
Total cholesterol (mmol/L)	<5	5.2 ± 0.9	5.1 ± 1.1	0.8
LDL (mmol/L)	<3	3.4 ± 0.8	3.3 ± 0.9	0.8
HDL (mmol/L)	>1	1.3 ± 0.5	1.2 ± 0.3	0.7
Triglycerides (mmol/L)	<1.7	1.5 ± 1.0	1.3 ± 0.5	1.0
Prolactin (mIU/L)	60-300	241.3 ± 164.6	243 ± 117.2	0.4
REPRODUCTIVE HORMONE PROFILE				
LH (IU/L)	2-12	4.3 ± 1.6	3.8 ± 1.0	0.3
FSH (IU/L)	1.7-8	5.4 ± 2.7	5.6 ± 3.0	0.9
Testosterone (nmol/L)	10-30	14.1 ± 5.0	12.7 ± 5.4	0.4
Oestradiol (pmol/L)	<190	137.2 ± 54.1	134.1 ± 42.9	0.6
SHBG (nmol/L)	15-55	23.4 ± 9.0	21.9 ± 11.1	0.7

Data are presented at mean ± SD. P value calculated by independent samples T test for normally distributed data; Baseline refers to visit 1 (start of protocol). BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; eBMR, estimated Basal Metabolic Rate; LH, Luteinizing hormone; FSH, follicle stimulating hormone; SHBG, Sex Hormone Binding Globulin; LDL, Low Density Lipoproteins; HDL, High Density Lipoproteins; LED: Low energy diet.

Table 4-3: Baseline semen parameters of participants according to diet groups; NHS vs LED.

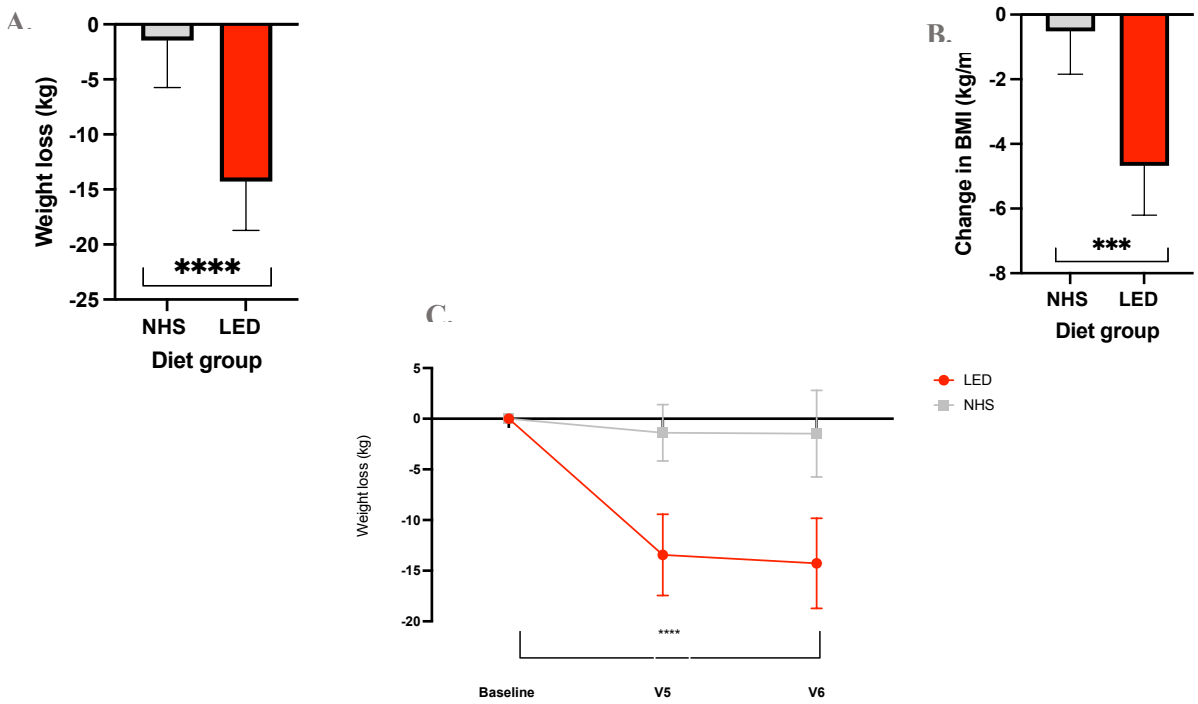
Semen parameter (units)	Reference range	Dietary intervention group		P value
		NHS arm (N = 18)	LED arm (N = 18)	
Semen Concentration (million/ml)	≥15	7.5 (8.2)	5.6 (6.4)	0.3
Sperm Volume (ml)	≥1.5	2.4 (1.8)	3.5 (3.6)	0.3
Progressive Motility (%)	≥32	28 (27.5)	27 (24.3)	0.9
Total Motility (%)	≥40	36.5 (28.9)	39.5 (28.8)	0.8
Total Motile Count (million/ejaculate) [1]	≥39	8.1 (12.4)	5.6 (7.4)	0.4
Morphology (%)	≥4	1.0 (1.5)	0.0 (1.0)	*0.048
ROS (RLU/sec/million sperm)	<3.8	60.3 (113.3)	82.1 (100.6)	0.2

Data are presented as median (IQR) as non-parametric data. P value calculated using a Mann Whitney U Test. Baseline for these parameters was calculated as the average of two semen analysis results at V1 and V2 [pre-intervention]. [1]Total Motile Count (TMC) = Sperm concentration × Sperm Volume × (Total Motility/100). * denotes where differences between NHS and LED groups were statistically significant at $p < 0.05$. LED, Low energy diet; ROS, reactive oxygen species; RLU reactive light years; IQR: interquartile range.

4.4.2 Weight loss observed in men undergoing dietary intervention

The LED intervention resulted in significantly greater weight loss when compared with the NHS diet (mean change in weight in kg: - 1.5 ± 4.3, NHS; - 14.3 ± 4.5 ,800kcal/day, P<0.0001 vs. NHS) (Figure 4.3A) from start to end of study in men with obesity and oligospermia. Similar results were observed with change in BMI (Figure 4.3B) (mean change in BMI in kg/m^2: - 1.4 ± 3.4, NHS; -4.7 ± 1.5, 800kcal/day, P=0.0016 vs. NHS) (Table 4.3B). There were no significant differences in weight loss between visit 4 and visit 5 i.e. from the end of the weight loss phase (V5) to the end of the weight maintenance phase (V6) in either of the two groups (Figure 4.3C).

Figure 4-3: Bar graphs of change in weight and BMI, and time profiles of change in weight of participants according to diet groups; NHS vs LED.



A-B: Bar graphs of the overall mean change in weight (A) and BMI (B) at the end of the dietary intervention period (visit V6) when compared with start of study (visit V1). ***, P<0.001. **C:** Time profiles of change in weight at visit 5 and visit 6 when compared with start of study (visit V1). Data points include incremental weight loss at visit 5 (end of weight loss phase, calculated as visit 5 minus visit 1) and visit 6 (end of weight maintenance phase/study, calculated as visit 6 minus visit1). Data presented as mean+/-SD. ***, P<0.001; ****, P<0.0001; LED V6-V1; **** P<0.0001 vs NHS V6-V1. LED: Low energy diet. Red=LED; Grey=NHS.

4.4.3 Effects of dietary intervention on body composition

Table 4.4 summarises the changes in body composition from start to the end of the study in the two dietary arms in men with obesity and oligospermia. There were significant beneficial effects in body composition observed in the LED arm compared with the NHS arm; a significant greater decline in waist circumference ($P<0.0001$), fat mass ($P=0.0005$), visceral fat ($P=0.0135$), basal metabolic rate ($P<0.0001$), and significant increase in lean mass ($P<0.0001$) was observed.

Table 4-4: Change in body composition in participants from start to end of study according to diet groups; NHS vs LED.

Parameters (units)	Dietary intervention group		P value
	NHS (N = 18)	LED (N = 18)	
BEDSIDE			
Weight (kg)	-1.5 ± 4.3	-14.3 ± 4.5	<0.0001*
BMI (kg/m^2)	-1.4 ± 3.4	-4.7 ± 1.5	0.0016*
SBP (mmHg)	-0.4 ± 15.5	-6.0 ± 15.4	0.3514
DBP (mmHg)	- 2.0 ± 15.3	-8 ± 22.0	0.7877
BODY COMPOSITION			
Waist circumference (cm)	-2.0 ± 6.0	-11.2 ± 6.0	<0.0001*
Fat Mass (%)	-0.8 ± 2.9	- 5.7 ± 4.8	0.0005*
Fat Mass (kg)	-0.3 ± 2.7	-9.1 ± 3.6	<0.0001*
Lean Mass (%)	+ 0.5 ± 2.5	+ 4.5 ± 3.1	<0.0001*
Lean Mass (kg)	+ 0.2 ± 3.9	-4.1 ± 2.6	0.0002*
Water weight (%)	- 0.4 ±1.9	+2.7 ± 2.8	0.0006*
Visceral fat (%)	- 1.5 ± 4.0	-4.0 ± 3.6	0.0135*
eBMR (kcal)	-0.4 ± 1.5	-3.9 ± 1.6	<0.0001*

Data are presented as mean ± SD if normally distributed and median (IQR) if non normally distributed. Start of the study is visit 1 and end of the study is visit 5. P value calculated as independent samples T test for normally distributed data; non normally distributed data was compared using a Mann Whitney U Test. * denotes where differences between NHS and LED groups were statistically significant at $p < 0.05$. BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; eBMR, estimated Basal Metabolic Rate; LED: Low energy diet.

4.4.4 Effects of dietary intervention on metabolic and reproductive hormone profile

Due to the COVID pandemic and lack of capacity to process research samples in the laboratory, we do not have the results for metabolic and hormone profile for the end of the study.

4.4.5 Effects of dietary intervention on sperm quality and seminal reactive oxygen species

Semen parameters have large intra-individual biological variability therefore we used the average of the first two visits (pre-start of diet) to represent the baseline semen values. There were no significant differences observed in any of the semen parameters from baseline to end of study between the two dietary arms in men with obesity and oligospermia. However, a significant difference was observed in seminal ROS between the two groups, with a significant higher decline in the LED arm, compared to the NHS arm. These are illustrated in Table 4.5. This median change in seminal ROS between the two groups is also illustrated in Figure 4.5A, with a significant correlation between change in seminal ROS and change in weight loss (Figure 4.5B).

Although the study was not powered to determine the effect of weight loss on pregnancy rates. Two couples in the LED arm successfully conceived (1 spontaneously, and 1 through IVF) during the study and with no reported pregnancies in the NHS arm.

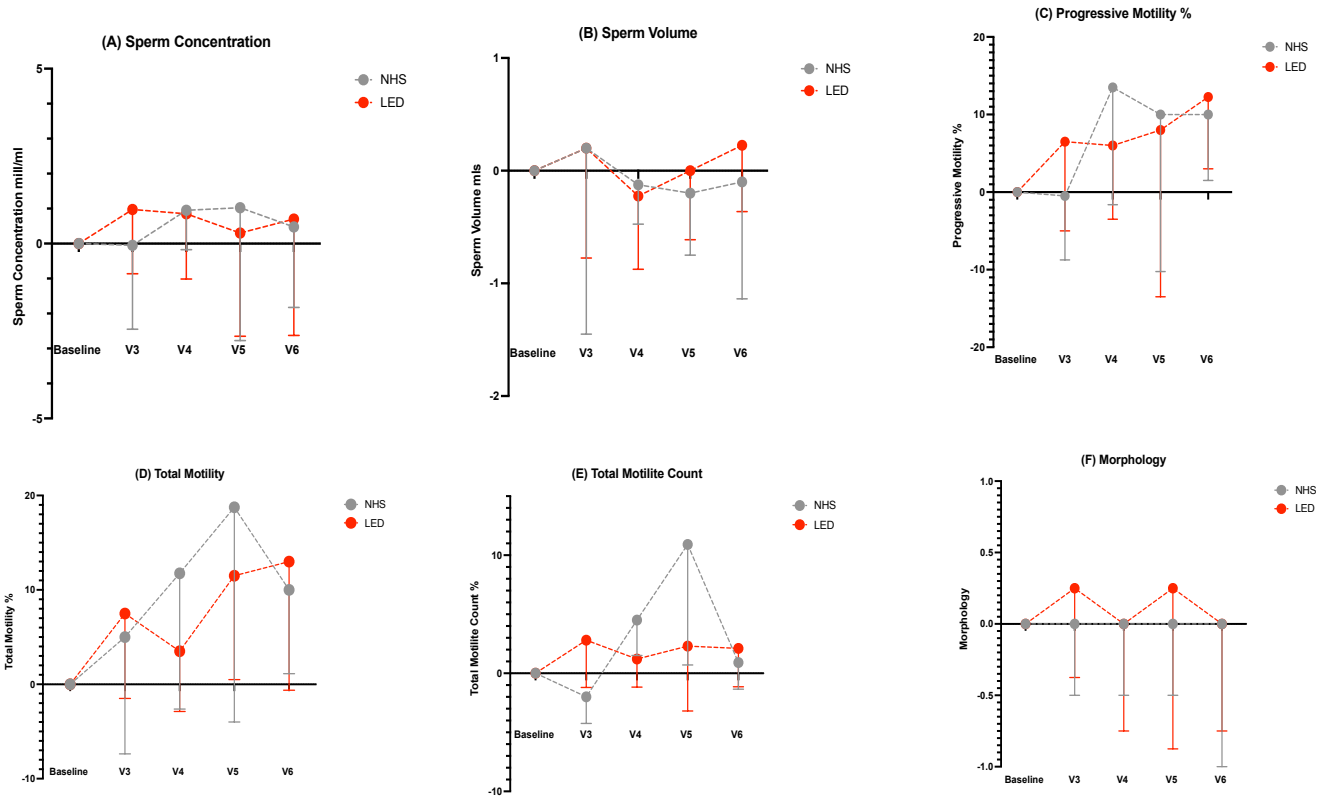
Table 4-5: Change in semen parameters and ROS in participants from baseline to end of study according to diet groups; NHS vs LED.

Parameters (units)	Dietary intervention group		P value
	NHS (N = 12)	LED (N = 12)	
Semen Concentration (million/ml)	+ 0.5 (6.5)	+ 0.7 (14.4)	0.7
Sperm Volume (ml)	-0.1 (1.5)	+ 0.2 (1.0)	0.2
Progressive Motility (%)	+ 9.0 (13)	+ 12.3 (28.4)	0.2
Total Motility (%)	+ 8.0 (14.5)	+ 13.0 (31.9)	0.1
Total Motile Count (million/ejaculate)[1]	+ 0.2 (9.2)	+ 2.1 (16.0)	0.4
Morphology (%)	-0.3 (1.2)	0.0 (1.8)	0.4
Reactive oxygen species (RLU/sec/million sperm)	-0.2 (61.9)	- 6.0 (137.5)	*0.0284

Data are presented as median (IQR) as semen parameters were non-parametric (failed normality test). P value calculated using a Mann Whitney U Test. Baseline refers to the average of V1 and V2 (pre-diet start), therefore change in semen parameters calculated as the difference between the final visit (V5) and baseline (average of V1 and V2). [1]Total Motile Count (TMC) = Sperm concentration × sperm volume × (total motility/100). LED: Low energy diet; ROS, reactive oxygen species.

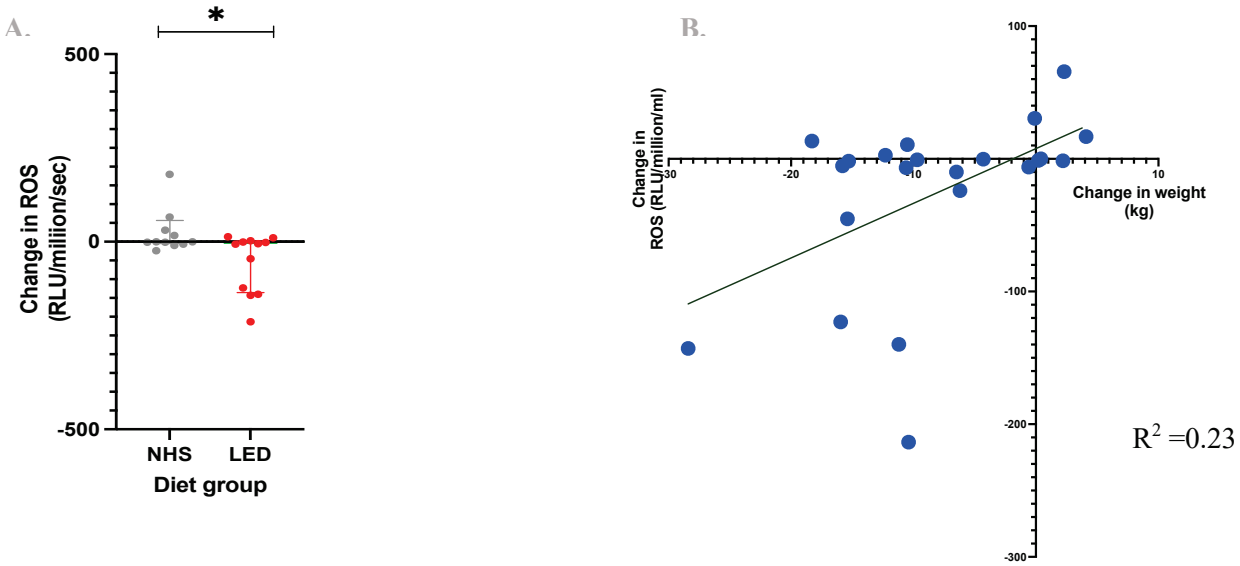
We further compared time profiles of incremental change in semen parameters at multiple time points i.e. at baseline (average of visit 1 and 2, pre-start of diet), visit 3 (4 weeks into diet), visit 4 (8 weeks into diet), visit 5 (12 weeks into diet, end of weight loss phase) and visit 6 (end of study) in the two dietary arms (Figure 4.4). No significant changes were noted in the time profiles of any of the semen parameters.

Figure 4-4: Time profiles of incremental change in median semen parameters of participants according to diet groups; NHS vs LED.



Data presented as median (error bars represent IQR). Data points include each semen parameter at baseline (average of visits 1 and 2), visit 4 (end of weight loss phase, calculated as visit 4 minus baseline) and visit 5 (end of weight maintenance phase/study, calculated as visit 5 minus baseline). Total Motile Count (TMC) = Sperm concentration × Sperm Volume × (Total Motility/100); LED, Low energy diet. IQR, interquartile range. Red=LED; Grey=NHS.

Figure 4-5: Graphs of change in median seminal ROS of participants according to diet groups; NHS vs LED, and correlation between change in seminal ROS and weight loss.



A. Bar graph of overall median change is ROS from pre-start of diet (visit 2) to end of diet (visit 6) between LED and NHS groups. Data presented as median (error bars represent IQR). **B.** Scatter graph showing correlation between change in ROS and change in weight from start to end of study period. *P<0.05; LED, Low energy diet. Red=LED; Grey=NHS; Blue – all participants.

4.4.6 Effects of dietary intervention on sexual health and quality of life scores

We evaluated changes in sexual health such as erectile function, and quality of life with validated questionnaires, SHIM and SF-36 (Ref) from start to end of the study period (Table 3.8). There was no significant change in sexual/erectile function from start to end of study between the groups as evaluated by the SHIM questionnaire. There were also no significant improvements in median total SF36 scores in the LED arm compared with the NHS in the 34 men that completed the study.

Table 4-6: Change in Sexual Health Inventory for Men (SHIM) and Short Form (SF-36) Survey scores of participants according to diet groups; NHS vs LED.

Questionnaire	Dietary intervention group		P value
	NHS (N = 17)	LED (N = 17)	
Sexual health Inventory for Men (SHIM)	0 (2)	0 (2.5)	0.46
Short Form (SF-36) Survey	23 (97.5)	27 (114)	0.76
Physical health	0.0 (10)	5.0 (13.75)	0.09
- Physical functioning	0.0 (29)	5.0 (18.75)	0.52
- Physical role limitations	0.0 (0)	0.0 (0)	0.13
- Bodily pain	0.0 (15)	5 (15.5)	0.75
- General health perception	2.50 (13.75)	10 (25.8)	0.47
Mental health	0(18)	0 (10)	0.56
- Energy/vitality	2.5 (36.25)	-7.5 (20)	0.74
- Social functioning	0 (34.75)	0 (28.5)	0.65
- Emotional role limitations	0 (33.75)	0 (0)	0.04
- Mental health	0 (11)	6 (12)	0.27
Self-rated health transition	25 (50)	37.5 (43.75)	0.10

Values are changes in each of the scores calculated as visit 6 value minus visit 1. Data are presented as median (IQR). P value calculated using a Mann Whitney U Test. * denotes where differences between NHS and LED groups were statistically significant at $p < 0.05$.

4.5 Discussion

Conventional semen analysis is the only routine diagnostic test for male infertility. It reflects the production of spermatozoa in the testes. Oligospermia is a defined as a low sperm count (<15million sperms/ml of ejaculate) (Cooper, 2010) and is the most common manifestation of male infertility associated with important fertility outcomes such as time to pregnancy and pregnancy rates (Bonde et al., 1998; Guzick et al., 2001; Slama et al., 2002; van Zyl and Menkveld, 2006). A meta-analysis based on 13,453 men demonstrated an inverse relationship between BMI and abnormal sperm count (Sermondade et al., 2013). Obese men compared with normal weight men (BMI 18.5 – 24.9 kg/m^2) have increased risk of oligospermia (sperm concentration <15million/ml) with OR 1.97 (95% CI: 1.27-3.07) (12). Furthermore, a recent cohort study (albeit lacking control groups) suggested that dietary weight loss may improve sperm counts in obese men (Håkonsen et al., 2011), however there is heterogeneity in data with other studies suggesting that dietary intervention has no effect on sperm counts (Mir et al., 2018). Therefore we carried out the current randomised controlled study to investigate the effects of losing weight with LED compared with healthy eating advice on sperm quality in obese oligospermic men.

Similar to study 2 (chapter 3), we observed significant differences in mean weight loss (-14.3kg or 12.4%) compared with the NHS arm (-1.5kg or 1.3%) after 16 weeks of intervention. Significant reductions in BMI and waist circumference were also observed in the LED arm compared with the NHS arm. As expected, these changes mirrored significant improvements in body composition parameters with lower % fat mass and visceral fat and higher % lean mass in the LED group compared with the NHS arm. The mean weight loss achieved in our study is consistent with previous studies on LED. A recent large randomised study of 306 individuals reported that total diet replacement (825-853 kcal/day formula diet for 3-5 months) followed by stepped food reintroduction (2-8 weeks) compared with best practice care led to a mean weight loss of 10·0 kg (SD 8·0) in the intervention group and 1·0 kg (3·7) in the control group (Lean et al., 2019). Similarly, a metanalysis including 3017 participants from 20 studies reported a pooled mean weight change of −12.3kg during the VLED/LED (median duration of VLED/LED of 8 weeks; range 3–16 weeks) period (Johansson et al., 2014). Compared to study 2, our NHS arm lost less weight (-6.3kg verses -1.5kg) after 16 weeks of healthy eating advice allowing better comparison of study outcomes between our LED and control groups.

Other studies have reported average weight loss range from 2.65 to 9.4kg in 'balanced diet' groups at 3–6 months post intervention (Naude et al., 2014).

Our dietary interventions were well tolerated with no adverse outcomes. However, three participants withdrew their consent (2 in LED and 1 in NHS) soon after randomisation due to dissatisfaction with the dietary allocation. There were no significant differences in baseline demographics, reproductive and metabolic profile between the two groups. Similar to study 2 (chapter 3), majority of men who took part in the current study were in long-term relationships/partnerships with couples in their prime reproductive years. Furthermore, majority of men in study 3 had a known history of idiopathic infertility with previous failed IVF cycles. This might have motivated some participants to enrol in the study to optimise their fertility status by losing weight. Furthermore, this study involved semen samples from obese men with known low sperm count, who are likely to have higher rate of pathology compared with the general population.

Despite significant weight loss and improved body composition parameters, we did not observe any significant differences in quality of life scores, or any of the sperm parameters with weight loss in LED compared with the control arm. In contrast, Hakonsen et al investigated 43 obese men (BMI 33-61kg/m2) who underwent a 14-week residential weight-loss programme (3.5-25.4kg weight loss). The subgroup with the largest weight loss had an increase in total sperm count, semen volume, testosterone and SHBG (Håkonsen et al., 2011). Another study of 105 men that underwent a 12-week weight loss programme consisting of 'healthy diet and exercise' reported significant improvements in sperm morphology and progressive motility post weight loss, but no change in sperm count (Mir et al., 2018). In all of these studies, the weight loss programmes are not described in detail and furthermore, the studies did not have control groups.

However, it is also important to mention that our study was underpowered to assess our primary outcome of change in sperm concentration. This is due to unforeseen challenges with recruitment and running of the study during the COVID pandemic. 36 participants were included in the analysis, whilst the power calculation included 52 participants (42 if excluding 20% drop outs). Therefore we are unable to firmly conclude if the lack of changes observed in sperm quality are due to the study being underpowered or if weight loss does not improve sperm quality in obese oligospermic men over a 16 week period. Recruitment efforts are currently ongoing, with remaining participants being enrolled into the study.

It is possible, that the initial weight loss leads to an acute starvation like effect with no noticeable improvement in sperm concentration, and perhaps a longer observation period post weight loss is required to truly assess the effect of weight loss and weight maintenance on sperm quality. However, one of the main strengths of this study compared to study 2 and 3 was the measurement of seminal reactive oxygen species (ROS), a sensitive and specific marker of oxidative stress in semen with limited intra-individual variation as opposed to semen analysis. Seminal ROS are released physiologically by immature or abnormal spermatozoa and leucocytes, as well as by-products of intracellular metabolic pathways and during ATP production from the sperm mitochondria. The fine balance of endogenous semen ROS and body's natural antioxidants is normally kept in close homeostasis. A number of exogenous factors, such as obesity, result in high levels of semen ROS. Spermatozoa are highly susceptible to this oxidative damage. Studies have shown that oxidative stress increases with an increase in BMI (Tunc et al., 2011). Obesity is a chronic inflammatory state whereby production of cytokines and interleukins is increased at both systemic and seminal levels (Oliveira et al., 2017). High calorie diets increase body weight, glucose and lipid levels with subsequent rise in the metabolic rate to sustain the body energy expenditure (Oliveira et al., 2017). Production of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukins (IL-1, IL-6 and IL-18) increases in parallel to the metabolic rate in obesity and induces oxidative stress suppressing the HPT axis and spermatogenesis both at the level of the hypothalamus and testis. Furthermore, cytokines such as TNF- α and IL-1 cause direct damage to the assembly of junctional proteins supporting the network of Sertoli cells with significant impairments of the seminiferous epithelium and blood-testis barrier. Therefore during spermatogenesis damage to the blood-testis barrier (BTB) integrity from obesity associated increased oxidative stress may be one of the crucial underlying factors accounting for decreased fertility (Fan et al., 2015). In addition, these pro-inflammatory cytokines may inhibit LH function at the Leydig cells leading to further low testosterone (Liu and Ding, 2017b) and Sertoli cell function/number leading to low inhibin B and poor sperm quality (Winters et al., 2006). The abdominal, suprapubic and medial thigh fat wrapping the scrotum leads to increased intrascrotal temperatures (Garolla et al., 2015) which is postulated to cause increased testicular oxidative stress and DNA fragmentation (Davidson et al., 2015). Therefore, obesity results in a low-grade systemic as well as testicular inflammatory state with high levels of ROS. Furthermore, sperm DNA fragmentation in obesity is attributed to high ROS production which surpasses seminal antioxidant capacity and impairs sperm quality leading to

infertility (Lewis et al., 2013). Tartibian and Maleki observed that sperm from recreationally active men had less oxidative stress-induced DNA damage (Tartibian and Maleki, 2012). Similarly, 6 sub-fertile obese men who underwent several months of an unreported personalised healthy lifestyle programme consisting of a 'balanced diet' and regular exercise, lost abdominal weight with significant improvement in sperm DNA fragmentation, lipid profile, testosterone: oestrogen ratio and successful pregnancy outcomes with live births (Faure et al., 2014). An Indian group studied 105 men through a 12-week weight loss programme consisting of 'healthy diet and exercise' (mean BMI decreased from 33.18kg/m^2 to 30.43kg/m^2) and reported a higher mean DNA fragmentation index before weight loss (20.2%) vs after weight loss (17.5%) p=<0.01 (Mir et al., 2018). However the exact mechanisms by which ROS causes DNA damage are not well established. One of these mechanisms postulated is through the production of lipid degradation by-products especially malonaldehyde which either causes oxidation of DNA bases (mainly guanosine) into 8′-hydroxyguanosine (which is promutagenic) or through direct interaction with the DNA strand leading to non-specific single- and double-strand breaks (Niederberger, 2012). The baseline seminal ROS levels were very high in our obese oligospermic participants reflective of underlying high levels of testicular oxidative stress due to obesity. As originally hypothesised, weight loss in LED arm, significantly decreased seminal ROS compared to the control arm. Other studies have reported higher seminal ROS levels in fertile obese men compared with fertile overweight and normal weight men (Taha et al., 2016). Furthermore, weight loss has been shown to reduce systemic oxidative stress (Melissas et al., 2006; Uzun et al., 2004), however no previous study has reported change in seminal ROS levels with weight loss.

There were multiple challenges encountered throughout the study. Firstly it was carried out during the COVID pandemic, which led to delays in all aspects of the study. It was more challenging to recruit obese oligospermic men for this study, compared to study 1 and 2 (obese men with normal fertility), perhaps suggesting that men with a known background of infertility may be less keen to enrol in such a study compared to their healthy counterparts due to the stigma attached with male factor infertility. In addition, our hospital laboratories were understandably focused on COVID and other service demands during this period. Therefore our research serum samples could not be analysed in time, for us to investigate the effect of weight loss on metabolic and reproductive hormone profile in men with obesity and oligospermia. Therefore we are unable to report on these secondary outcomes at this time. However multiple other studies have observed the

benefits of weight loss both by bariatric surgery and diet on male reproductive hormones (Corona et al., 2013b). Increased total testosterone, free testosterone, SHBG and reduced oestradiol as well as improvement in sexual function has been reported after bariatric surgery in obese men (Corona et al., 2013a; Lee et al., 2019; Wood et al., 2020). Similar benefits in male reproductive hormone profile have been reported with dietary weight loss however the findings are more heterogenous. During a 9-week very low-calorie diet (VLCD) programme, 58 abdominally obese men lost on average 16.3+/-4.5kg and maintained 14.3+/-.1 kg weight loss after a 12-month maintenance period. The study reported a significant increase in free testosterone and SHBG at 12 months compared to baseline (Niskanen et al., 2004) but no significant difference in other reproductive hormones. Similarly Stanik et al evaluated 24 moderately hyperoestrogenic, hypoandrogenic. obese men following an 8-week semistarvation (320 kcal/day) program and were able to observe a significant reduction in the oestrogen values coupled with an increase in testosterone levels without any changes in SHBG concentrations (Stanik et al., 1981). These findings were not confirmed by Hoffer et al. (8), who failed to observe any significant difference in serum total and free testosterone after a four-week 600-kcal dietary treatment with either protein or protein plus carbohydrates (Hoffer et al., 1986). It would, therefore, have been interesting to investigate if there were any effects of our weight loss intervention on the metabolic and reproductive hormone profiles of these obese oligospermic men. Furthermore, we were also unable to analyse the frozen semen samples for DNA fragmentation using the TUNEL assay due to time constraints and covid restrictions with lab work. However, I hypothesize, that, as we observed a significant decline in seminal ROS in the LED arm compared with NHS arm, that DNA fragmentation would also have significantly improved in the LED arm, as seen in study 2 (chapter 3).

Furthermore, three of our participants (all in the LED arm) contracted COVID infection during the study, and interestingly all their sperm concentrations 2 weeks post the illness were reduced to <0.1million/ml (despite delaying the study visit by 2 weeks from the illness as per our COVID risk assessment protocol, Appendix 3), suggestive of the negative impact of the acute illness on semen parameters, which may have also underestimated the differences observed in sperm parameters in this study. Although there is limited literature on the topic, the high expression of angiotensin-converting enzyme 2 (ACE2), the receptor for entry into the target cells by SARS-COV2, in somatic and germ cells of the testis, including spermatogonia, Leydig and Sertoli cells suggests that SARS-COV-2 may localise in the gonads (Wang and Xu, 2020). A study on 43

sexually active men with recent recovery from Covid-19 infection observed that 25% of these men were oligo-crypto-azoospermic, and the presence of oligo-crypto-azoospermia was significantly related to COVID-19 severity (Gacci et al., 2021). In contrast, another study of 23 men aged 20-62 years reported normal semen parameters after a median interval of 32 days from diagnosis of COVID-19 to providing semen samples (Guo et al., 2021). Future longer-term studies are required to assess normalisation of sperm parameters post infection and impact on fertility outcomes.

In summary, we report high baseline seminal ROS levels in obese oligospermic men, that significantly decrease with weight loss with LED compared with NHS diet. This is the first randomised study to report improvement in seminal ROS levels with weight loss, despite no change in semen parameters.

Chapter 5: General Discussion

Male infertility is recognized as a disease by multiple organizations including the World Health Organization and the American Medical Association (Berg, 2017; Zegers-Hochschild et al., 2009). Recent media attention on male factor infertility may represent a rise in public awareness and social acceptance. A recent systematic review reported fall in sperm counts by 50-60% since the 1970s, in North America, Europe and Australasia (Levine et al., 2017) and a variety of lifestyle factors have been implicated in this decline, with obesity being an important contributing factor. This is partly a reflection of global increase in sedentary lifestyles and changing dietary behaviours, with increasing prevalence of Westernized diets, low intakes of fruits and vegetables and high intakes of foods rich in saturated fats in men of reproductive age range (Vujkovic et al., 2009). Furthermore, male infertility is increasingly observed as a 'canary in the coal mine' for future male health conditions, with an association with cardiovascular disease, quality of life, and all-cause mortality (Choy and Eisenberg, 2018). Similarly, obesity is linked to poor sperm quality (Bendayan et al., 2018), hypogonadism and metabolic syndrome, which are all known risk factors for cardiovascular disease and increased mortality (Muraleedharan and Jones, 2014). Therefore research studies, such as the ones summarised in my thesis, investigating treatment options for both of these conditions could potentially help prevent future long-term comorbidities in men.

Weight loss by lifestyle changes are considered valuable in restoring hormone profiles and fertility especially in women. Tremendous research efforts in women have shown that weight loss results in improvements in ovulatory cycles and pregnancy outcomes, and the future health and obesity risk of the offspring. The UK Pregnancies Better Eating and Activity Trial (UPBEAT) was a multi-centre randomised controlled trial in antenatal clinics whereby obese pregnant (15-18 weeks) women were given a diet and exercise intervention and compared to women in a control group (Dalrymple et al., 2021). There was no difference in the incidence of gestational diabetes in either group. However, the intervention group was associated with lower offspring pulse rate suggestive of potentially improved cardiovascular function in the child at 3 years of age and a sustained improvement in mother's diet with lower glycaemic load and saturated fat intake at 3 years after the intervention finished. In contrast to studies in women, there is relative paucity of studies in overweight and obese men seeking fertility making it difficult to draw firm conclusions regarding the benefits of weight loss. A total of four previous studies have investigated weight loss via diet to improve male fertility (Belan et al., 2015; Faure et al., 2014; Håkonsen et al., 2011; Mir et al., 2018). These initial studies suggested

improvements in sperm concentrations, motility and morphology and sperm DNA integrity which may be linked to an improved live birth rate. However, these studies are limited due to not being randomised or have a control group. In addition, these studies are heterogenous with variable patient selection (infertility vs obesity) and length of follow-up to draw any firm conclusions. Furthermore, these studies utilized a diet and exercise combination, with none providing adequate details of the intervention.

I, therefore, performed the first three randomised controlled studies investigating the effects of dietary weight loss using LED on sperm quality in obese men with normal baseline sperm parameters and subsequently in obese men with baseline sperm parameters (oligospermia) consistent with subfertility. I measured seminal reactive oxygen species and sperm DNA fragmentation which provide additional information about male infertility potential, and examined the effects of weight loss on these markers in men with obesity. I have determined for the first time, that dietary weight loss reduces DNA fragmentation scores in men with obesity and normal sperm analysis compared to an NHS diet, I have also shown that LED reduces seminal ROS levels in men with obesity and baseline oligospermia compared to an NHS diet. Numerous studies have demonstrated that obesity is a prolonged and sustained pro-inflammatory state, which is hypothesized to cause oxidative damage to the sperm, compounded by increased intrascrotal fat deposition resulting in increased intra-testicular heat. The increased oxidative stress contributes to increased sperm DNA damage, reduced acrosomal reactions and lower successful outcomes following IVF (Marseglia et al., 2014). Results from my studies are consistent with this hypothesis, such that weight loss with LED in obese men is suggestive of reduced testicular oxidative stress as reflected by lower seminal ROS levels and improvement in sperm DNA fragmentation scores, compared to a control diet. It would therefore be very interesting in the future to conduct a multi-centre randomised controlled study to determine if dietary weight loss in men and their respective partners has additive or even synergistic effects of on live birth rates.

The studies described in this thesis used LED as the main intervention compared to a healthy eating diet. LED are specially formulated and widely available meal replacement products, in the form of liquid soups, shakes and bars offering rapid, short to medium term weight loss (up to 20 weeks) (Christensen et al., 2011; Mulholland et al., 2012), with some evidence of long-term weight loss maintenance (up to 4 years) (Lean et al., 2018). A clear advantage of formula diets is their ability to remove food from the daily decision-making process (Leeds, 2014) while providing a person with a known amount of calories (Purcell et al., 2014), making

it a convenient dietary intervention for modest weight loss. LED also allows participants to focus on introspectively looking at their relationship with food, identifying the difference between emotional and physical hunger and gaining skills to aid long-term weight maintenance. Our 1st study was a proof-of-concept study that investigated change in semen parameters with different levels of caloric restriction over an 8-week study period. This was followed by my next two studies that were longer over 16 weeks consisting of 12 weeks of weight loss with TDR and 4 weeks of food reintroduction phase compared to a control group. The decision to incorporate a longer intervention period for study 2 and 3 was to cover more than 1 cycle of spermatogenesis, as a complete cycle of spermatogenesis in men may take 64 +/- 8 (range 42 to 76) days (Heller and Clermont, 1963). One of the main disadvantages of weight loss with LED or most dietary led interventions can be weight regain due to variable adherence (Franz et al., 2007). This can be due to various reasons such as dropout from weight maintenance support, the rate of food reintroduction being too rapid, environmental factors or patients not addressing the reason for their initial weight gain. However, some studies have observed that a food reintroduction phase post TDR with extended use of LED meal replacements (e.gg 1 product a day) and high protein diets were associated with improved weight loss maintenance (Christensen et al., 2017; Johansson et al., 2014). In addition, a food reintroduction phase over 4 weeks was associated with increased dietary restraint (the tendency to eat less than desired) and reduced external eating (the tendency to eat in response to external cues such as the sight of food) compared to reintroduction lasting 1 week(Gripeteg et al., 2010). Therefore both our studies 2 and 3 had a TDR phase followed by a food reintroduction phase to aid rapid weight loss and subsequent weight maintenance. It would, however, have been interesting to assess the weight maintenance of these men 6 months to 1 year post our intervention to investigate any longer-term benefits to male reproductive parameters. During the initial stages of rapid weight loss with LED, several side effects have been reported such as hair loss, fatigue, dizziness, constipation, acute gout, cold intolerance, headaches, muscle cramps and gallstones (Saris, 2001; Wadden et al., 1983). Raised uric acid following significant weight loss may precipitate acute gallstone and gout (Christensen et al., 2011; Johansson et al., 2011). However, none of these adverse events were reported by our participants in our studies. In addition, constipation was prevented by prescribing ispaghula husk routinely to all participants randomised to LED. We also excluded all men who had a known history of gout or gallstones, which may have prevented the occurrence of these adverse outcomes.

Finally, the metabolic benefits of weight loss were confirmed in all of my 3 studies with participants in the LED arm (800kcal/day) observed to have significant reductions in their weight, waist circumference, glycaemic and lipid profiles compared to the control group. In summary, our LED intervention of 800kcal/day in our 3 studies demonstrated efficacy, tolerability and acceptability.

We failed to observe any significant changes in our primary outcome (sperm concentration) by our randomisation groups in any of the 3 studies. Post-hoc analysis in study 1 suggested that weight loss above 12kg may have significant improvement in sperm concentration. However study 2 and 3 did not confirm these findings in men with obesity with and without baseline oligospermia. Although my observations were related to semen quality, fertility in the sense of live births after the study period were not assessed in any of the studies. The standard semen analysis has its limitations and does not provide adequate information about the defects of spermatogenesis (Holstein et al., 2003). It provides descriptive parameters of the ejaculate, however, is not a direct measure of fertility (Smith et al., 1977). Furthermore, semen analysis is highly variable which may also partly explain why we did not observe any significant changes in change in sperm concentration in any of the 3 studies (Castilla et al., 2006). However novel markers of male infertility including seminal oxidative stress via reactive oxidative species (ROS) and sperm DNA fragmentation may provide more insight into underlying mechanisms of obesity associated male infertility. Recent review evidence suggest that obesity and the metabolic syndrome are associated with high level of seminal cytokines, sperm DNA fragmentation as well as systemic high sensitivity-CRP levels (Leisegang et al., 2019). Therefore, in addition to conventional semen analysis, I investigated seminal oxidative stress and DNA fragmentation in obese men with weight loss. According to a review by Ko YE et al, excess ROS production could lead to sperm DNA fragmentation and high likelihood of arrested embryo development due to fertilization of the oocyte with fragmented sperm DNA (Ko et al., 2014). Seminal ROS was significantly high in obese men with oligospermia in study 3, and was observed to reduce with weight loss with LED compared to the control arm. This observation is in agreement with Agarwal et al. who also supported that ROS values can be high in the context of obesity (Agarwal et al., 2018), however no previous study has investigated change in seminal ROS levels with weight loss. We measured semen ROS via an in-house validated chemiluminescence assay which was time-consuming. Novel technologies, such as Male Infertility Oxidative System (MiOXSYS) utilize the assessment of seminal oxidation–reduction potential (Dutta et al., 2019)

which may speed up the routine assessment of semen ROS in clinical practice. Given that obesity is associated with high semen ROS, and dietary weight loss, seems to reduce oxidative stress in semen, it would also be interesting to investigate weight loss in comparison with commercially available antioxidant therapies to target the seminal oxidative stress; some of these antioxidant therapies are associated with reduced ROS in male infertility (Balercia et al., 2005; Lenzi et al., 2004), but there are no thorough trials powered to investigate pregnancy or live birth rates yet.

Similar to seminal ROS, increase in the level of sperm DNA fragmentation has been shown to reduce chances of natural conception. Experimental studies evaluating the relationship between sperm DNA fragmentation and ART have reported worse outcomes of ART with high DNA fragmentation scores (Nicopoullos et al., 2019; Simon et al., 2019). Some experts support that sperm DNA damage scores may therefore provide additional discriminatory information for the prediction of both natural and ART live births, particularly for specific subgroups of idiopathic male factor infertility, for example male partners of couples with recurrent pregnancy loss (Agarwal et al., 2019a; Jayasena et al., 2019). Duran et al. suggested that spontaneous pregnancy is difficult if the seminal DFI shown in a TUNEL assay is higher than 12% (Duran et al., 2002). We used the alkaline comet test (study 2) and the TUNEL assay (study 3) to measure sperm DNA fragmentation scores, which directly measure sperm DNA damage. Previous studies have shown a closer correlation with pregnancy outcomes with TUNEL and comet assay than other available assays such as SCSA which measure DNA damage indirectly (Simon et al., 2017). We observed high baseline sperm DFI scores in obese men both by alkaline comet and TUNEL assays. Study 2 showed improvements in DNA fragmentation levels with weight loss with LED compared to control diet. Therefore weight loss in obese infertile men could be an important milestone to improve their metabolic health and reproductive potential by reducing systemic and testicular oxidative stress. Furthermore, semen ROS and sperm DNA fragmentation could be important additions to conventional routine semen analysis, especially on background of obesity associated male infertility.

Despite these novel findings from our 3 studies, the exact underlying molecular mechanisms remain unanswered on how weight loss may improve seminal ROS and sperm DNA fragmentation. Seminal plasma is a complex biological fluid containing a variety of organic species like low molecular weight compounds, peptides, hormones, free amino acids, proteins, and high levels of inorganic ions like Zn2+,Mg2+,Ca2+,K+,

and Na+ (Jodar et al., 2016; Sørensen et al., 1999). Previous observational studies showed that metabolomics analysis can be used to differentiate males with low sperm concentration (Courant et al., 2013) or asthenozoospermia (Gilany et al., 2014) from normozoospermic men. Seminal plasma therefore offers an accessible bodily fluid which could be analysed using metabolomics approach in obesity associated male infertility. A future potential study to assess differences in seminal plasma metabolites with weight loss by bariatric surgery verses dietary weight loss could be important as the degree and rate of weight loss with bariatric surgery verses diet may differentially affect semen parameters and the seminal plasma metabolites. This may help shed some light on the current paradoxical evidence reporting worsening semen parameters post bariatric surgery in men compared with milder dietary weight loss.

Overall, my results from the weight loss studies in obese men are encouraging however, they should be validated by larger powered studies reporting on live birth rates to objectively quantify the absolute effects of weight loss on male fertility. Equally it would be essential to conduct future randomised controlled studies looking into the effects of dietary weight loss with LED verses exercise on semen analysis, seminal oxidative stress and sperm DNA fragmentation in obese men

Finally, my studies have highlighted that weight loss by diet can be effective but difficulty in adherence to diet can result in variability in weight loss between individuals and potential weight regain. Behavioural changes and psychological support would be required to ensure successful weight loss via diet were to be implemented in complex healthcare systems. Furthermore, a successful weight loss programme would need to be tailored to be culturally sensitive taking into consideration individual beliefs and preferences.

In summary, in this thesis, I have identified that weight loss with LED may improve some but not all indices of sperm function in obese men compared to a control/NHS healthy eating diet. We observed high baseline seminal ROS and sperm DNA fragmentation scores in men with obesity. We also observed improvements in DFI scores in men post weight loss with LED compared with NHS diet, despite no significant changes observed in sperm parameters. Considering that obesity is associated with increased oxidative stress, I identified for the first time reduction in seminal oxidative stress with weight loss using LED in obese men with oligospermia in a randomised controlled setting. Due to the COVID-19 pandemic mid-way through my PhD, a lot of my latter experiments for study 3 were delayed, resulting in being unable to run DNA fragmentation samples for study 3, but it is part of my suggested future work. Overall, my work may have

implications for the development of novel diagnostic tools and management of obese men undergoing weight loss by improvement in seminal ROS and DNA fragmentation as well as their overall metabolic health.

Appendix 1: Human Inhibin B (INB) ELISA kit (Oxford Bio-Innovation, Oxford, UK) Kit information

PRINCIPLE

This INB enzyme linked immunosorbent assay applies a technique called a quantitative sandwich Immunoassay (Debieve et al., 2000; Mitchell et al., 2010). The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific for INB. Calibrators or samples are then added to the microtiter plate wells and INB if present, will bind to the antibody pre-coated wells. In order to quantitatively determine the amount of INB present in the sample, a standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody, specific for INB are added to each well to "sandwich" the INB immobilized on the plate. The microtiter plate undergoes incubation, and then the wells are thoroughly washed to remove all unbound components. Next, A and B substrate solution is added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only those wells that contain INB and enzyme conjugated antibody will exhibit a change in colour. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450 nm.

In order to measure the concentration of INB in the sample, this INB ELISA kit includes a set of calibrators. The calibrators are assayed at the same time as the samples and allow the operator to produce a calibration curve of optical density versus INB concentration. The concentration of the INB in the samples is then determined by comparing the optical density (OD) of the samples to the calibration curve. The stop solution changes the colour from blue to yellow and the intensity of the colour is measured at 450 nm using a spectrophotometer.

Table A1.1: COMPONENTS of KIT

Reagents	Quantity
Microtiter Plate	96 wells
Calibrator 1 (0 pg/mL)	1
Calibrator 2 (50 pg/mL)	1
Calibrator 3 (100 pg/mL)	1
Calibrator 4 (250 pg/mL)	1
Calibrator 5 (500 pg/mL)	1
Calibrator 6 (1,000 pg/mL)	1
Enzyme Conjugate	1 x 10ml
Substrate A	1 x 6ml
Substrate B	1 x 6ml
Stop Solution	1 x 6ml
Wash Buffer (100X concentrate)	1 x 10ml

STORAGE

All reagents provided were stored at 4°C.

SERUM SAMPLE COLLECTION AND STORAGE

We used a serum separator tube (SST) and allowed samples to clot for 30 minutes before centrifugation for 15 minutes at 3000rpm. Serum sampled aliquoted and stored at -20°C or -80°C.

REAGENT PREPARATION

Bring all kit components and samples to room temperature (18-25°C) before use.

Wash Solution

Dilute 10 mL of Wash Solution concentrate (10X) with 990 mL of de-ionized or distilled water to prepare 1,000 mL of Wash Solution (1X).

ASSAY PROCEDURE

All calibrators and samples were added in duplicate to the microtiter plate. The steps were as follows:

1. Secure the desired number of coated wells in the holder, then add 50µL of Calibrators or Samples to the appropriate well of the antibody pre-coated Microtiter Plate.

2. Add 100 µL of Conjugate to each well. Mix well. Complete mixing in this step is important. Cover and incubate for 1 hour at 37°C.

3. Wash the microtiter plate using one of the specified methods indicated below:

4. Manual Washing: Remove incubation mixture by aspiration contents of the plate into a sink or proper waste container. Using a squirt bottle, fill each well completely with wash solution, then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure four more times for a total of five washes. After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until

no moisture appears. Note: Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame.

5. Automated Washing: Aspirate all wells, then wash plate five times using wash solution. Always adjust your washer to aspirate as much liquid as possible and set fill volume at 350 µL/well/wash (range: 350-400µL). After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. It is recommended that the washer be set for a soaking time of 10 seconds or shaking time of 5 seconds between washes.

6. Add 50µL substrate A and 50µL substrate B to each well. Cover and incubate for 10 minutes at 20-25°C. (Avoid exposure to light.)

7. Add 50µL stop solution to each well. Mix well.

8. Read the optical density (OD) at 450 nm using a microtiter plate reader immediately.

CALCULATION OF RESULTS

1. This calibration curve is used to determine the amount in an unknown sample. The calibration curve is generated by plotting the average OD (450 nm) obtained for each of the six calibrator concentrations on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis.

2. First, calculate the mean OD value for each calibrator and sample. All OD values are subtracted by the mean value of the zero calibrator before result interpretation. Construct the calibration curve using graph paper or statistical software.

3. To determine the amount in each sample, first locate the OD value on the Y-axis and extend a horizontal line to the calibration curve. At the point of intersection, draw a vertical line to the X-axis and read the corresponding concentration.

4. Any variation in operator, pipetting and washing technique, incubation time or temperature, and kit age can cause variation in the result. Each user should obtain their own calibration curve.

5. The sensitivity by this assay is 1.0pg/mL.

Appendix 2: TUNEL assay

TUNEL assay procedure has been described before (Rakesh Sharma et al., 2016, 2016; Zini and Agarwal, 2011)

- Samples from the reference labs are received frozen. These are not fixed in paraformaldehyde (unless otherwise indicated).
- Thaw the sample by incubating at 37ºC for 20 min.
- Aliquot and load 6 µL of the sample on a Fixed cell chamber for manual evaluation of concentration and motility. Check the concentration of sperm in the sample. Adjust it to 2.5 x 10^6/mL. This can be done using the following calculation: $\frac{\mathbf{2.5}}{\boldsymbol{Sperm\ conc.}(\mathbf{10^6\ /\ mL})} \times \mathbf{1000} = \boldsymbol{X\ \mu L}$

  Example: Sperm concentration is 25×10^6/mL and has to be resuspended in 1.0mL of PBS:

$$\frac{2.5}{25\ (10^6\ /\ \mathrm{mL})} \times 1000 = 100\ \mu L$$

- Using a cryomarker, label (5 mL) tubes. Label each tube with the following:
  - TUNEL
  - Patient name
  - CCF No/Accession number
  - Date
- Add the required amount of seminal ejaculate into the tube. Spin the sample at 1700 rpm for 7min. and remove the seminal plasma.

Preparation of the spermatozoa positive control

- Prepare a hydrogen peroxide diluted solution (1:15 dilution) from the stock of the Andrology Laboratory (Hydrogen Peroxide 30%) by adding, for example, 100 µl of the stock to 1400 µl of PBS 1X.
- Resuspend the spermatozoa of the tube "Surname, Name, positive control" in 1 ml of the diluted H_2O_2 solution.
- Place the tube in the heater at 50°C for one hour

- Centrifuge for 7 minutes at 1700 rpm.
- Remove the supernatant and replace with 1ml of PBS.
- Centrifuge for 7 minutes at 1700 rpm.
- Remove the supernatant and replace with 1ml of PBS.
- Centrifuge for 7 minutes at 1700 rpm.
- Remove the supernatant and replace with 1ml of PBS.
- Together with the test and the negative samples, centrifuge for 7 minutes at 1600 pm. Remove the supernatant and proceed to FIXATION and PERMEABILIZATION

Fixation and Permeabilization

- Prepare a paraformaldehyde 3.7% solution by diluting the 10 ml stock formaldehyde 37% solution in 90ml PBS 1X.
- After removing the supernatant from the samples and spermatozoa controls, add 1 ml of the 3.7% paraformaldehyde solution. Incubate at room temperature for 15 minutes or store at 4 degrees centigrade for a maximum of week.
- Centrifuge for 4 minutes at 600 g (2500 rpm).
- Remove the paraformaldehyde and add 1 ml of PBS 1X.
- Centrifuge for 4 minutes at 600 g (2500 rpm).
- Perform a second wash with PBS (Repeat steps D and E)
- Remove the supernatant and replace with 1 ml of ice-cold ethanol (70%). Place the sample at 4degrees centigrade for 15-30 minutes.

Staining For TUNEL Assay

- Check the number of tubes that will be required for the TUNEL assay. It is helpful to prepare the stain for an additional 5 to 7 tubes.
- Remove the reaction buffer (green cap) from 4□C and the TdT (yellow cap) and FITC-dUTP (orange cap) from -20□C and place them at room temperature for 20 min to thaw.

  For the TdT vial, give it a quick spin (1600 rpm for 5 minutes) to bring the reagent to the bottom ofthe tube.

- Prepare the stain as shown in Table 1 for a single assay and calculate the required volumes. Always prepare an additional 4 to 5 tubes to ensure that adequate stain is available for all the tubes.

Table A2.1 Staining Solution (Single Assay)			
Staining Solution	1 Assay	6 Assays	12 Assays
Reaction buffer (green cap)	10.00 µL	60.00 µL	120.00 µL
TdT Enzyme (yellow cap)	0.75 µL	4.50 µL	9.00 µL
FITC-dUTP (orange cap)	8.00 µL	48.00 µL	96.00 µL
Distilled H2O	32.25 µL	93.5 µL	387.00 µL
Total volume	51.00 µL	306.00 µL	612.00 µL

- Add the stain in the same sequence asshown in Table 1.
- Note: The preparation of the stain andall subsequent steps must be carried out in the dark.
- For the negative controls (2) of each sample, omit the TdT enzyme from thestaining solution.
- Return the stains to appropriatestorage temperature.
- Note: The appropriate volume of Staining Solution to prepare for a
- variable number of assays is based upon multiples of the component volumes needed for 1 assay. Mix only enough Staining Solution to complete the number of assays prepared per session. The Staining Solution is active for approximately 24 hr at 4°C.
- Resuspend the pellet in each tube in 50 µL of the Staining Solution.
- Note: The same tip can be used to add the stain as long as the stain is added on the side of the tube and the tip does not come in contact with the solution.
- Incubate the sperm in the Staining Solution for 60 min at 37˚C. Cover the tubes with aluminium foil.
- Note: Record the incubation time on the aluminium foil.
- At the end of the incubation time, add 1.0 mL of Rinse Buffer (Cat# 6550AZ) (red cap) to each tubeand centrifuge at 1600 rpm for 7 min. Discard the supernatant.
- Repeat the cell rinsing with 1.0 mL of the Rinse Buffer, repeat centrifugation and discard the supernatant.
- Resuspend the cell pellet in 0.5 mL of the PI/RNase Staining Buffer (Cat# 6551AZ).
- Incubate the cells in the dark for 30 minutes at room temperature.
- Number the tubes according to the sample list. Cap the tubes and carefully cover the tubes with
- aluminium foil.

- Note: The cells must be analysed within 3 hours of staining. Cells may begin to deteriorate if left overnight before analysis.

Appendix 3: COVID-19 Risk assessment

Study visits cannot take place unless the study team member AND participant confirm beforehand they have no symptoms of COVID-19 or contacts with suspected or confirmed COVID-19 within the government guidelines for self-isolation e.g. 14 days.

1. Study team are aware they must notify line management if they or anyone in their household are exhibiting symptoms of COVID-19 and must not attend work if so.

2. Should a study team member or household member have symptoms of COVID-19, they will follow government guidelines and self-isolate as appropriate. They will not return to clinical work until they have had a negative PCR test or completed the appropriate isolation period as stipulated by government guidelines.

3. Participants will be contacted by telephone and/or email by the study team member the day before they attend, and will confirm their health status and those of their household.

The study team member will enquire specifically about the presence of COVID-19 symptoms including:

- *New continuous cough*
- *High temperature*
- *Loss of, or change in their normal sense of taste or smell (anosmia).*

The study team member will also enquire if anyone visiting, or anyone in their household has had COVID-19 symptoms within the last 14 days, that could warrant individuals in the household to self-isolate as per the current UK government guidelines.

4. Some of the study visits where feasible (e.g. visit 3 on Study 2) will be done remotely over telephone.

5. Each study visit will last less than 1 hour.

6. Only one participant at a time will be allowed in the clinic room with one study team member for the study visit.

7. Prior to participant entering the clinic area, the participant must confirm verbally to a member of the study team the considerations stipulated in point 4 above.

8. Study team member and participant must have a temperature check on entrance to the clinical area. Temperatures must not exceed ≥38 degrees Celsius. If they do not meet this criteria, or the criteria set out in point 4, they will not be admitted into clinical room, and the study visit will not take place.

9. If any participant or study team member tested positive for COVID-19, and it is likely they were infectious (i.e. they took a test within 14 days of a visit and it were positive), then there would be contact tracing as per NHS and government guidelines.

Cleaning and Hygiene

10. Participant and study team member entering the clinical area must first use the hand sanitizer or wash their hands.

11. Hands must be cleaned regularly between participants and prior to or after any procedure.

12. Clinic rooms will have contact points and surfaces disinfected after use and between study visits by study team member.

13. The weight loss intervention pack (low energy diet packaged products) will be wiped down with disinfectant before given to participant.

PPE and Distancing

14. Everyone entering the clinical department i.e. visiting participant and study team member must be wearing a mask/face covering on arrival.

15. Study team will provide the face masks for the study team and the participants. PPE will also be provided by the study team.

16. Only one participant at a time will be in the clinic room to provide enough space to maintain a 2m or greater distance from the study team, with maximum 2 persons in one clinic room at any one time.

17. A distance of 2m or greater will be maintained where possible between study team member and participant and any other individuals.

18. If it is not possible to maintain a 2m distance (e.g. taking bloods, attaching a BP cuff), PPE (gloves, aprons, masks and safety glasses/visor) will be used by study team.

19. Our study excludes patients with systemic chronic diseases. We will therefore not be recruiting patients shielding for COVID-19.

20. Gloves and aprons worn by study team member will be replaced frequently and when no longer required, gloves, aprons and masks will be disposed of in a clinical waste bin and hands sanitised immediately.

21. Participants will receive information on how to wear a mask effectively and to sanitize their hands.

22. Study team will receive instructions on 'donning and doffing' PPE.

23. Disposable masks will ideally be used for up to 4 hr periods. Masks should be replaced if soiled or difficult to breathe through.

24. Plastic safety glasses/visors must be cleaned at least once per day with soap and water.

25. Resuscitation trollies will have appropriate PPE available on them as stipulated by the Resuscitation Council guidance.

Semen and blood testing:

26. If any participant had COVID symptoms and/or tested positive for COVID, other than following the government guidelines above for isolation and testing, study protocol amendment would include delaying the next study visit by at least 2 weeks post cessation of symptoms or post positive result (if asymptomatic) to ensure study results (semen and blood results) are not affected.

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