

Deleterious effects of obesity upon the hormonal and molecular mechanisms controlling spermatogenesis and male fertility

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Deleterious effects of obesity upon the hormonal and molecular mechanisms controlling spermatogenesis and male fertility

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Short title: Obesity and spermatogenesis

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Abstract

Worldwide obesity rates have nearly doubled since 1980 and currently over 10% of the population is obese. In 2008, over 1.4 billion adults aged 20 and older were overweight; of these, over 200 million men and nearly 300 million women were obese. While obesity can have many ramifications upon adult life, one growing area of concern is that of reproductive capacity. Obesity affects male infertility by influencing the hypothalamic-pituitary-gonadal axis, thus causing detrimental effects upon spermatogenesis and subsequent fertility. In particular, evidence indicates that excess adipose tissue can alter the relative ratio of testosterone and oestrogen. Additional effects involve the homeostatic disruption of insulin, sex-hormone-binding-globulin, leptin, and inhibin B, leading to diminished testosterone production and impairment to spermatogenesis. Aberrant spermatogenesis arising from obesity is associated with downstream changes in key semen parameters, defective sperm capacitation and binding, and deleterious effects on sperm chromatin structure. More recent investigations into trans-generational epigenetic inheritance further suggest that molecular changes in sperm that arise from obesity-related impaired spermatogenesis, such as modified sperm RNA levels, DNA methylation, protamination, and histone acetylation, can impact upon the development of offspring. Here, we summarise our current understanding of how obesity exerts influence over spermatogenesis and subsequent fertility status, and make recommendations for future investigative research.

Keywords: Obesity, spermatogenesis, body mass index, infertility, hypothalamic-pituitary-gonadal axis

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24 **Introduction**

25 In 2010, 48.5 million couples globally were affected by infertility (Mascarenhas et al.,
26 2012). Specifically, infertility is estimated to affect one in seven couples in the Western
27 world, with male factor infertility contributing to approximately 40% of these cases, female
28 factor infertility to 45%, and the remaining proportion due to idiopathic causes (Fritz &
29 Speroff, 2011). Interestingly, there has been a steady decline in fertility rates over the past 50
30 years, which has occurred in parallel with an increasing rate of obesity (Hammoud et al.,
31 2012). Obesity is a medical condition where excess body fat has accumulated to an extent
32 where it poses serious health risks, and is determined by body mass index (BMI), a
33 measurement relating weight and height (DuPlessis et al., 2010). According to the National
34 Institutes of Health, individuals with a BMI between 25 and 29.9 kg/m² are classified as
35 ‘overweight’, while those with a BMI greater or equal to 30kg/m² are classified as ‘obese’
36 (National Institutes of Health, 1998). Between 1980 and 2008, the worldwide prevalence of
37 obesity nearly doubled, and more than 10% of the world’s adult population is obese
38 (Finucane et al., 2011).

39 While there has been substantial investigative research targeted to the impact of
40 obesity upon female infertility, specific investigations of the exact relationship between
41 obesity and male factor infertility are relatively rare and tend to be far less conclusive. One
42 study reports that in patients seeking treatment for male infertility, there has been a three-fold
43 increase in the prevalence of obesity (Kasturi et al., 2008). While current data remains
44 highly conflicting, mounting evidence indicates that male obesity is associated with an
45 increased time to conception, reduced pregnancy rates, an an increase in pregnancy loss in
46 couples undergoing assisted reproductive techniques (ART) (Nguyen et al., 2007; Ramlau-
47 Hansen et al., 2007; Hinz et al., 2010; Keltz et al., 2010; Bakos et al., 2011a).

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3 48 In order to produce healthy fertile sperm from testicular germ cells, the process of
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5 49 spermatogenesis must occur in a normal and natural manner. However, spermatogenesis is a
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7 50 highly complex and specialised process under strict regulatory mechanisms, which involve
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9 51 the hypothalamus, pituitary, Leydig cells, Sertoli cells, and sex steroids (Rawanpura et al.,
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11 52 2010). Large amounts of testosterone in the local vicinity, bound by androgen binding
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13 53 protein in the seminiferous tubules, are required to maintain successful spermatogenesis.
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15 54 Normal luteinizing hormone (LH) secretion drives testosterone production by the interstitial
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17 55 Leydig cells, while normal follicle stimulating hormone (FSH) secretion activates Sertoli
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19 56 cells to nourish developing sperm cells throughout the different phases of spermatogenesis.
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21 57 Fluctuations in this specialist environment, particularly with regard to temperature and
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23 58 hormones, have a strong impact upon the process of spermatogenesis.
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25 59 Any disorder affecting gonadotropin releasing hormone (GnRH) secretion from the
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27 60 hypothalamus, or FSH and LH secretion from the pituitary, can impair spermatogenesis and
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29 61 thus fertility (Anawalt, 2013). While the most common cause of male infertility is the
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31 62 idiopathic failure of spermatogenesis, endocrinologists often encounter populations of men
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33 63 with treatable causes of sub-fertility, for example endocrinopathies such as obesity and
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35 64 hyperprolactinaemia (Anawalt, 2013).

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41 65 This review explores how obesity in human males can result in hormonal imbalance
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43 66 with deleterious effects upon sperm formation, how impaired spermatogenesis can affect
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45 67 fertility status, and how paternal obesity at the specific time of conception can influence
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47 68 subsequent health of the offspring.

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70 **Obesity can lead to deleterious alterations in important hormonal profiles**

71 One of the most important roles of the hypothalamic-pituitary-gonadal (HPG) axis is
72 in the regulation of reproductive function, and it is widely believed that obesity can lead to

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dysregulation of this vital physiological cascade. A bi-directional relationship has been established between hypogonadism and obesity (Rao et al., 2013), largely due to the complex interplay between hypothalamic hormones and the adipocytokines that control the pituitary-testicular axis. Endocrine changes associated with male obesity can thus result in conditions such as hypogonadotropic hyperoestrogenic hypoandrogenemia, which may adversely affect fertility by reducing testicular function, modifying spermatogenesis, or reducing sexual drive (Reis & Dias, 2012).

Aromatase over-activity can result in a reduced ratio of testosterone and oestrogen

Disruption of the HPG axis can result in reduced levels of testosterone and increased levels of oestrogen. These indications have long been associated with sub-fertility and are thus common markers of reproductive health (Handelsman & Swerdloff, 1985). The HPG axis governs the production of testosterone and is regulated via the direct negative feedback of testosterone upon the hypothalamus. The pulsatile release of GnRH by the hypothalamus drives LH secretion, which ultimately stimulates the testes to produce and secrete testosterone. In adipose tissue, testosterone is metabolised to estradiol by the cytochrome P450 enzyme aromatase, which is responsible for a key step in the biosynthesis of oestrogens and is expressed in higher levels, and with increased activity, in white adipose tissue (Duplessis et al., 2010). The increased bioavailability of aromatase in obese individuals results in the increased conversion of androgens to oestrogens, thereby simultaneously producing increased levels of circulating oestrogen. Increasing BMI has been shown to have a significant association with a reduced testosterone:oestrogen ratio (Hajshafiha et al., 2013).

Since oestrogen is more biologically active than testosterone and abnormally high levels of oestrogen can elicit negative feedback upon the HPG axis via kisspeptin neurons (Rao et al., 2013), the high levels of oestrogen in obese males consequently result in a

reduction in testosterone production, subsequently affecting spermatogenesis (Schneider et al., 1979; Jensen et al., 2004; Hammoud et al., 2006, 2008; Roth et al., 2008; Chavarro et al., 2010; Macdonald et al., 2010). It has been hypothesized that kisspeptin secretion might be the central pathway linking obesity, testosterone deficiency and environmental factors (Figure 1). Oestrogens further act directly on the testes to regulate their function; different animal models exposed to high levels of oestrogenic chemicals showed a reduction in gonad size and a decrease in sperm count and quality in males (Akingbemi, 2005).

The only somatic cells directly in contact with developing male germ cells are Sertoli cells, which provide nutrients and support. The adhesion of Sertoli cells to the developing germ cells is dependent upon testosterone, with a reduction in testosterone leading to the retention and phagocytosis of mature spermatids (Kerr et al., 1993ab). Furthermore, epithelial function in seminiferous tubules is disrupted by reduced intra-testicular levels of testosterone, thus affecting spermatogenesis (Jensen et al., 2004).

Levels of subcutaneous and visceral fat have both been associated with reduced levels of free testosterone in men (Schneider et al., 1979; Tchernof et al., 1995; Jensen et al., 2004; Winters et al., 2006 Pasquali et al., 2007; Chavarro et al., 2010; Macdonald et al., 2010). Furthermore, adipocytokines (such as the proinflammatory cytokines TNF and IL-6) inhibit the production of testosterone by negatively feeding back to the hypothalamus. Low levels of testosterone have also been shown by CT scanning to correlate with overall obesity, increased waist circumference, and the increased accumulation of visceral fat (Svartberg et al., 2004).

Obesity-related endocrine disruption

Obesity causes other endocrine changes in the human body, such as changes in the production and regulation of insulin, sex-hormone-binding-globulin (SHBG), leptin, and

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122 inhibin B. Changes in the circulating levels of these hormones impact the
123 testosterone:oestrogen ratio, ultimately impairing spermatogenesis (Figure 2).

124 Testosterone plays a key role in insulin regulation, metabolism of lipids and body
125 composition (Jones, 2010). Hyperinsulinemia has been shown to have an inhibitory effect on
126 spermatogenesis with a significantly higher level of nuclear and mitochondrial DNA damage
127 found in sperm from affected individuals (Agbajes et al., 2007). Increasing BMI and waist
128 circumference is also associated with reduced SHBG levels in the serum (Jensen et al., 2004;
129 Fejes et al., 2005; Winters et al., 2006; Pasquali et al., 2007; Chavarro et al., 2010; Hajshafiha
130 et al., 2013). SHBG is a glycoprotein which binds sex hormones such as testosterone and
131 estradiol in order to inhibit their biological activity. Hyper-insulinemia from obesity-related
132 insulin resistance causes the hepatic production of SHBG to decline, resulting in more
133 biologically active oestrogen to negatively feedback onto the HPG-axis. This fall in SHBG
134 levels may be a homeostatic mechanism to maintain an adequate level of free testosterone,
135 due to the lowered serum levels of testosterone seen in obese males (Chavarro et al., 2010).

136 There is a strong correlation between serum leptin, a hormone secreted by adipocytes,
137 and body fat percentage. Leptin stimulates the satiety center via hypothalamic-mediated
138 effects, but also functions as a metabolic and neuroendocrine hormone in regulating sexual
139 maturation and reproduction, indicating that white adipose can act as an endocrine organ
140 (Jope et al., 2003; Wang et al., 2008; Hofny et al., 2009; Duplessis et al., 2010). Mounting
141 evidence reports a higher prevalence of obesity and high circulating levels of leptin in
142 infertile men (Wang et al., 2008; Duplessis et al., 2010; Farooq et al., 2014). Fat gain due to
143 leptin deficiency caused by the *ob/ob* gene mutation is well studied, however a majority of
144 obese patients present with elevated serum leptin levels (Considine et al., 1996). Leptin
145 normally stimulates GnRH release, however the excess of leptin associated with obesity
146 causes the HPG axis to become resistant to leptin (Isidori et al., 1999; Rao et al., 2013). Due

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3 147 to the presence of leptin receptors in testicular tissue, and on the plasma membrane of sperm
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5 148 themselves, it is likely that elevated leptin levels in the serum affect spermatogenesis in obese
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7 149 males. Leptin inhibits stimulation on Leydig cells by the gonadotropins, resulting in a further
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9 150 decline in testosterone production. It has been reported that excess leptin from adipose tissue
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11 151 has deleterious effects upon sperm production and results in increased germ cell apoptosis in
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13 152 testes (Isidori et al., 1999). It is hypothesized that due to the presence of leptin receptors on
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15 153 sperm, leptin might directly affect spermatogenesis via endocrine mechanisms independent of
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17 154 the HPG axis, however the true extent of this theory has yet to be investigated and proven
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19 155 (Jope et al., 2003; Ishikawa et al., 2007).

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23 156 The production of inhibin B by Sertoli cells is the most effective marker for normal
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25 157 spermatogenesis. Inhibin B is a growth-like factor which acts in the testes to inhibit follicle
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27 158 stimulating hormone (FSH) production and to stimulate testosterone production by Leydig
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29 159 cells (Palmer et al., 2012a). The reduced levels of inhibin B found in obese males is
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31 160 indicative of seminiferous tubule dysfunction and is hypothesized to be due to a lower
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33 161 number of Sertoli cells (Jensen et al., 2004; Winters et al., 2006; Aggerholm et al., 2008;
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35 162 Pauli et al., 2008; Macdonald et al., 2010), however a compensatory increase in FSH levels
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37 163 in response to low inhibin B has not been observed, indicating a potential for partial
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39 164 dysregulation of the HPG axis (Palmer et al., 2012b). These endocrine disruptions caused by
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41 165 obesity ultimately cause further reduction in the levels of testosterone and impair
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43 166 spermatogenesis, thus compromising fertility.
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49 168 **Impaired spermatogenesis affects fertility**

50 169 *Effects upon traditional WHO semen parameters*

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54 170 Interestingly, studies investigating how increased BMI might affect routine semen
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56 171 parameters have yielded conflicting results. While it has been reported that obese men are
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three times more likely to exhibit a reduction in sperm quality compared to men of normal weight (Sharma et al., 2013; Shulka et al., 2014), the true effect of obesity upon semen quality is a source of much debate. Numerous studies have shown that there is no relationship between increased BMI and one or more of the following semen parameters: sperm concentration, count, morphology, motility, and ejaculate volume (Jensen et al., 2004; Fejes et al., 2006; Aggerholm et al., 2008; Hammoud et al., 2008; Paul et al., 2008; Nicopoulou et al., 2009; Chavarro et al., 2010; Macdonald et al., 2010; Martini et al., 2010; Hajshafiha et al., 2013; Eisenberg et al., 2014). Conversely, other studies show that BMI is associated with one or more of the following: sperm concentration, count, motility, or ejaculate volume (Jensen et al., 2004; Fejes et al., 2006; Hammoud et al., 2006; Kort et al., 2006; Hofny et al., 2009; Chavarro et al., 2010; Sermonade et al., 2013; Eisenberg et al., 2014; Hadjkacem et al., 2014). The exact cause of poor semen parameters in obese men is difficult to assign due to a paucity of studies specifically investigating treatment of infertility in obese men, conflicting results in the studies that do exist, as well as comorbidities such as cardiovascular disease, sleep apnea, and diabetes which can also affect fertility.

The lack of conclusive evidence with regard to the specific association of obesity and semen quality suggests that spermatogenesis may require only a minimum threshold level of hormonal regulation, and can continue despite temporal fluctuation (Macdonald et al., 2010). Furthermore, there exists insufficient evidence describing the effect of weight loss on rescuing sperm production and subsequent fertility. The conflicting results evident in existing literature could also represent the fact that BMI is a poor measure of body fatness (Nguyen et al., 2007). Further conflict could have arisen from the chosen sample populations not being representative of the general population as many studies were carried out in fertility clinics showing bias towards a population of sub-fertile men, from confounding lifestyle factors of impaired sperm function (including smoking, alcohol consumption, recreational

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3 197 drug use, or co-pathologies), or under-reporting in self-reporting of lifestyle factors and BMI
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5 198 (Palmer et al., 2012).
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7 199 Another important concern to take into account when analysing the effects of male
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9 200 obesity upon semen abnormalities is the potential confounding effect of maternal obesity and
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11 201 whether this leads to semen abnormalities in male offspring. It was hypothesized in 2007 that
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13 202 maternal obesity could have a programming effect upon testicular development in foetal life
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15 203 (Ramlau-Hansen et al., 2007). The authors observed that the sons of mothers who were
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17 204 overweight or obese during pregnancy were more likely to be overweight at birth and exhibit
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19 205 a high BMI in adulthood compared to the sons of normal weighted mothers. It was also
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21 206 suggested that higher free oestrogen levels in mothers (and hence the foetus) could interfere
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23 207 with normal testicular development and future fertility (Ramlau-Hansen et al., 2007). More
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25 208 recently, the same group reported that there was no obvious relationship between high birth
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27 209 weight/prepubertal body fat and semen quality in young adult life, suggesting that the effects
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29 210 of maternal obesity during pregnancy are unlikely to have an effect upon semen quality and
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31 211 subsequent fertility (Ramlau-Hansen et al., 2010).
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36 212 The discrepancies in existing data relating obesity to traditional semen parameters, as
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38 213 well as the confounding variables evident in such studies, have led to the development of
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40 214 rodent models of paternal obesity to help better investigate the effects of obesity on male
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42 215 fertility. It is hoped that such models may, in future, provide a more specific hypothesis.
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48 217 *Deleterious effects upon molecular mechanisms in sperm*

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50 218 While traditional semen parameters are an important measure for male fertility, it is
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52 219 becoming more apparent that the content and molecular structure of sperm is critical in
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54 220 generating a healthy pregnancy. Male obesity is associated with reduced pregnancy rates and
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56 221 an increase in pregnancy loss in couples undergoing ART (Hinz et al., 2010; Kelts et al.,
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222 2010; Bakos et al., 2011a). Interestingly, one study noted that fertilisation rate was higher
223 among obese men than men of normal weight for conventional IVF cycles, and that there
224 were no significant associations between male BMI and poor quality embryos or cleavage
225 rate (Colaci et al., 2012). However, this same study showed that the odds of live birth in
226 couples with obese male partners undergoing ICSI was 84% lower than men with normal
227 BMI and concluded there is a possible deleterious effect of male obesity on the odds of
228 having a live birth rate (Colaci et al, 2012). It should be noted however, that if male BMI
229 influences whether patients are treated with ICSI, stratifying data by ICSI may lead to over
230 adjustment bias and thus confound the observed outcomes (Schliep et al., 2015).
231 Conversely, other studies report that for obese patients, rates of fertilisation (Keltz et al.,
232 2010) and live birth rates (Peterson, et al. 2013) are reduced following *in vitro* fertilisation
233 (IVF) but not following ICSI, suggesting impairment in sperm binding. In support of these
234 clinical findings, mouse models of obesity, maintained upon a high fat diet, have shown
235 impaired sperm binding and capacitation compared to healthy controls (Bakos et al., 2011b;
236 Palmer et al., 2012b). A recent study also reported that sustained high protein-tyrosine
237 phosphatase 1B (PTP1B) activity in the sperm of obese mice may represent a vital link
238 between obesity and sub-fertility (Lei et al., 2014). PTP1B is upregulated by pro-
239 inflammatory factors, such as those released during the chronic inflammatory state of obesity.
240 High levels of PTP1B impairs the reassembly of SNARE proteins causing deleterious effects
241 in the sperm acrosome reaction, and a correlation was identified between high expression and
242 activity of PTP1B and impaired acrosomal exocytosis in sperm. Furthermore, PTP1B is
243 implicated in negative regulation of leptin and insulin signalling (Lei et al., 2014).
244 It should be kept in mind that in many of these studies, the confounding effect of
245 maternal obesity is often not taken into account when associating outcomes to the incidence
246 of male obesity. A recent study reported that weight status (overweight or obese) does not

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3 247 influence fecundity in couples undergoing infertility treatment. In fact, Schliep et al. (2015)
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5 248 investigated the effects of both male and female body-mass index on pregnancy and birth
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7 249 rates following *in vitro* fertilisation, and after adjusting for partner BMI, found no significant
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9 250 differences between fertilisation rate, embryo score, pregnancy, or live birth rate compared
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11 251 with normal weight controls.
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15 16 253 *Oxidative stress as a causative mechanism of subfertility*

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18 254 Obesity causes the body to be in a chronic inflammatory state and it is hypothesized
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20 255 that due to the higher metabolic rates required to maintain normal biological processes, there
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22 256 is an increase in the formation of reactive oxygen species (ROS) which can induce damage to
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24 257 DNA and plasma membrane integrity in sperm, as well as increase stress on the testicular
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26 258 environment (Esposito et al., 2004; Dandona et al., 2005, Agarwal et al., 2006; Duplessis et
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28 259 al., 2010). Studies in humans have shown a positive correlation between levels of BMI and
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30 260 sperm oxidative stress in males (Tunc & Tremellen, 2011) and rodents (Bakos et al., 2011b),
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32 261 which has further been associated with reduced sperm motility, reduced acrosome reaction
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34 262 and lower embryo implantation rates following IVF (Zorn et al., 2003; Aitkin et al., 2004;
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36 263 Aziz et al., 2004).

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38 264 An excess of scrotal adipose tissue may alter spermatogenesis by increasing testicular
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40 265 temperature or by impacting upon intra-testicular signalling (Cabler et al., 2010).
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42 266 Physiological elevation in scrotal skin temperature has been associated with substantially
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44 267 reduced sperm motility and concentration, concomitant with increased levels of sperm DNA
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46 268 damage and oxidative stress (Paul et al., 2008ab; Shiraishi et al., 2010; Duplessis et al.,
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48 269 2010). Increased apoptosis of spermatozoa is relatively common in obese males since
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50 270 phosphatidylserine externalization is increased and mitochondrial membrane potential is
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52 271 lowered (LaVignera et al., 2012). The potential for impaired acrosome reaction ability and
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the conceivable negative effects of obesity-impaired spermatogenesis upon semen parameters via oxidative stress provides a likely explanation for the sub-fertile status of obese men.

Trans-generational inheritance of obesity

It is suggested that paternal obesity at the time of conception can affect the health of resultant offspring. Epidemiology studies have shown that obese fathers have a higher likelihood of fathering obese children (Li et al., 2009), however the individual effects that genetic, epigenetic and environmental factors contribute to this phenomena cannot be readily partitioned for discrete investigation. Therefore, animal models of paternal obesity are being increasingly relied on. There has been recent evidence from such models that paternal obesity compromises both the metabolic and reproductive health of first and second generation offspring (Fullston et al., 2012), as well as influencing the susceptibility of offspring to obesity and diabetes (Mitchell et al., 2010; Ng et al., 2010). First generation offspring have identified compromised gametes with increased oxidative stress in sperm, changes in the mitochondrial function of oocytes, as well as increased fat mass in females (Fullston et al., 2012). A hypothesis known as ‘trans-generational epigenetic inheritance’ now purports that molecular changes resulting from impaired spermatogenesis due to obesity, such as changes to DNA methylation, histone acetylation or non-coding RNA levels in sperm, are transmitted to the embryo and consequently affect subsequent development (Youngson & Whitelaw, 2011; Daxinger et al., 2012; Palmer et al., 2012). This represents an interesting area which needs to be investigated further and could help to develop and improve infertility treatments.

Epigenetic inheritance

DNA methylation is a normal requirement for spermatogenesis. For example, sperm methylation is required for inactivation of the X chromosome during meiosis and for the

establishment of paternally imprinted genes in sperm (Ooi & Henikoff, 2007). Analysis of human spermatogenesis indicates the presence of DNA methyltransferase proteins during the spermatogenic cycle, which coincide with the establishment of methylation imprinting in sperm (Jenkins & Carrell, 2012). Throughout spermatogenesis these imprints are maintained, suggesting they are a key molecular event (Marques et al., 2011). While little is known on the direct impact of obesity on the methylation status of DNA in germ cells, various metabolic disorders, which are commonly associated with obesity, including type 2 diabetes, modify DNA methylation status in somatic tissues, and are thought to have an additional effect on sperm DNA methylation (Barres & Zierath, 2011). The hypomethylation of repeat elements and imprinted genes have been associated with increased levels of sperm DNA damage and reduced pregnancy rates (Tunc et al., 2009; El Haji et al., 2011; Mino et al., 2011; Nanassy & Carrell, 2011).

Histone acetylation is vital for the replacement of histones by protamines, which play a critical role in protecting sperm DNA (Francis et al, 2014), and mouse models maintained upon a high fat diet exhibit alterations in the acetylation of late round spermatids, resulting in increased levels of DNA damage (Gaucher et al., 2010; Palmer et al., 2011). Furthermore, it has been proposed that acetylated histones may have an important effect on embryogenesis via the regulation of gene expression. However, the extent of histone replacement varies widely on a species-specific basis. In human sperm, 15% of histones remain following protamination, compared to only 1% in murine models. The retention of a proportion of histones in human sperm is believed to allow pluripotent regulatory genes (such as *Nanog*, *Oct 4*, and *Sprout*) to remain histone-bound with loci capable of somatic cell histone modifications, allowing for their immediate activation and expression post-fertilisation, thus forming the basis for paternal programming in offspring (Farthing 2008). Epigenetic modifications to acetylation of these loci, or to DNA methylation, may therefore differ in the

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sperm of obese versus non-obese males, thus affecting subsequent development of the offspring. However, investigative studies of this potential mechanism remain on-going. Furthermore, studies looking into the trans-generational effects of underweight males should also be undertaken to further investigate the trans-generational effects of weight on DNA integrity and sperm RNA levels.

Sperm RNA and obesity

Mature sperm contain a regulated reserve of mRNA and non-coding RNA thought to be important for successful fertilisation and subsequent embryonic development (Ostermeier et al., 2004; Lalancette et al., 2009; Pradowaka-Dogan et al., 2014). Sperm RNA was originally dismissed as residual from spermatogenesis, however the presence of non coding RNA in sperm of many species targeting a multitude of gene sequences unrelated to spermatogenesis suggests that the sperm RNA is more than just a residual relic and may have post-fertilisation functions including transmission of acquired characteristics (Miller & Ostermeier, 2013; Sendler et al., 2013; Gapp et al., 2014). It has been demonstrated that offspring show phenotypes of variable severity following inhibition of miRNA in male pronulei of fertilised zygotes depending on the miRNA ratio, indicating these RNAs play a role in oocyte development during fertilisation and early embryo development (Miller & Ostermeier, 2013). It remains unknown, however, whether the RNA by some means marks the genome before entry into the ooplasm, at fertilisation, of some point after fertilisation.

Studies have shown significant differences in the levels of mRNA transcripts within the testes of obese and lean mice (Ghanayem et al., 2010). However, the potential role of sperm RNA, particularly in terms of functionality and a potential feature of fertilisation and embryo development, remains a topic of some debate and represents a particular focus of many research groups.

348

349 *Sperm DNA integrity in sub-fertile obese men*

350 Numerous studies, carried out in both human and animal models, have identified a
351 relationship between obesity and a reduction in the DNA integrity of sperm, which would
352 thus have consequential implications upon fertilisation and embryonic development (Kort et
353 al., 2006; Kriegel et al., 2009; Chavarro et al., 2010; Bakos et al., 2011b, Fabriello et al.,
354 2012; LaVignera et al., 2012). In two different mouse models of obesity (high fat diet and
355 leptin deficiency), increased BMI was successfully correlated to increased levels of sperm
356 DNA fragmentation (Duale et al., 2014). An increased percentage of sperm with abnormally
357 compacted chromatin, and an increased sperm DNA fragmentation index, was also detected
358 in males with a BMI greater than 25kg/m² (Kort et al., 2006; Lavignera et al., 2012).
359 Conversely, a recent study by Eisenberg showed that increased BMI had no effect on the
360 sperm DNA fragmentation index (Eisenberg et al., 2014), further suggesting that BMI may
361 not be an appropriate index to use in such studies.

362

363 **Solutions to obesity related sub-fertility**

364 Developing treatments for infertility is challenging, as the nature of fertility is
365 multifactorial. In addition to genetic or pathological infertility, there are many lifestyle factors
366 which can affect reproductive health such as nutrition, weight, exercise, stress, environmental
367 exposures, and drug use (Sharma et al., 2013). There are numerous lifestyle choices, which
368 could lead to obesity and its associated diseases and sub-fertility. One major challenge in
369 investigating how to treat sub-fertility associated with lifestyle factors is in considering how
370 lifestyle changes leading to sub-fertility are likely to influence both male and female
371 counterparts in a given couple. Therefore, if a male is obese and presenting with sub-fertility,
372 the lifestyle choices leading to his obesity are highly likely to affect his female partner also,

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373 and therefore it is more than likely that her weight could also be a contributory factor in the
374 couple's infertile status.

375 An individual's weight is often related to their eating habits and levels of activity. If
376 one member of a couple is obese, there is a high likelihood that their partner may have a
377 similar lifestyle and is also overweight or obese. Lifestyle changes through diet modifications
378 and exercise can lead to gradual weight loss and thus help to overcome obesity and its
379 associated preventable diseases. Several studies in obese males have reported that weight
380 loss through lifestyle changes resulted in increased SHBG and serum testosterone levels, as
381 well as reduced levels of insulin and leptin (Kaukua et al., 2003; Niskanen et al., 2004).

382 Since obesity appears to exert influence upon markers for male infertility, efforts have
383 been undertaken to investigate whether measures taken to reverse the unhealthy consequences
384 of obesity can also reverse the deleterious effects upon fertility. When couples seek infertility
385 treatment and male factor infertility is diagnosed, most men present with a reduced ratio of
386 testosterone to oestrogen (Luboshitzky et al., 2002). Pharmacological interventions via
387 medication can be used to treat obesity by addressing weight loss via appetite suppressants, or
388 to treat the effects of obesity upon the male reproductive tract. Aromatase inhibitors can be
389 prescribed for males presenting with infertility problems, and who exhibit elevated estrogen
390 and reduced testosterone levels, in order to prevent the excessive conversion of testosterone
391 to estrogen (Elkhiat & Fahmy, 2011; Schlegel, 2012). Studies have shown that the
392 administration of aromatase inhibitors is not only effective at restoring normal hormone
393 levels, but can also normalize spermatogenesis and semen parameters (Raman & Schlegel,
394 2002; Zumoff et al., 2003; Roth et al., 2008). While these changes show some improvements
395 in markers for fertility, further investigation into the direct effects of such treatment and the
396 potential restoration of fertility are needed.

397

398 Conclusions

399 Increased urbanization and industrialisation in the Western world has promoted a
400 sedentary lifestyle and unfavourable diet in the general population leading to an increased
401 incidence of obesity (Meldrum et al., 2012; Stefan et al., 2013). Studies have observed a
402 parallel decrease in male fertility potential over the past decades in regions where obesity is
403 prevalent (Swan et al., 2000). The diagnosis and treatment of reduced fertility in obese men
404 requires an insight into the underlying pathology, which has hormonal, mechanical,
405 molecular, and psychosocial aspects. The aetiology is multifactorial, with emerging evidence
406 showing that obese men having a greater risk of suffering from a dysregulated HPG axis and
407 thus endocrine profile, impaired spermatogenesis, and abnormal semen parameters.
408 Emerging evidence is showing that obesity negatively affects male reproductive potential, by
409 lowering the testosterone:oestrogen ratio, reducing sperm quality, altering the structure of
410 germ cells in the testes, and altering sperm RNA.

411 While several studies demonstrate short-term improvement in markers for male
412 fertility following weight loss via lifestyle modifications or surgical intervention,
413 unfortunately however, data pertaining to the long term success of such interventions are
414 lacking. Due to the complex interplay of factors contributing to subfertility associated with
415 obesity, there is a need for a multifaceted approach for further understanding and
416 development of treatments.

417

418 Declaration of interests

419 The authors report no declarations of interest. The authors alone are responsible for the
420 content and writing of the paper.

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424

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Figure legends

Figure 1 – Effects of obesity upon kisspeptin neurons is hypothesized as the central pathway linking obesity to testosterone deficiency and subsequent male subfertility.

Excess adipose tissue results in increased circulating oestrogen, a chronic state of inflammation, and resistance to leptin and insulin. These effects upon the kisspeptin neuron result in reduced levels of kisspeptin production, consequently lowering GnRH, LH, and FSH release, ultimately reducing testosterone production and affecting spermatogenesis.

Figure 2 – The effects of excess visceral adipose tissue on the hypothalamic-pituitary-gonadal axis. Increased amounts of adipose tissue increases the quantity and activity of aromatase which converts testosterone to estradiol in adipocytes. Estradiol inhibits the HPG axis via kisspeptin neurons, leading to decreased production of testosterone. Excess adipose causes insulin resistance, resulting in increased levels of insulin which decreases sex-hormone-binding-globulin production in the liver, leading to increased levels of free oestrogen. Adipose tissue produces pro-inflammatory adipocytokines and leptin which effects the HPG through negative feedback. Leptin further inhibits the stimulation of gonadotropins on Leydig cells leading to decreased androgen production from the testes.

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For Peer Review Only

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06/03/2015

Dear Professor Leese,

Re: THUF-2014-0158. Deleterious effects of obesity upon the hormonal and molecular mechanisms controlling spermatogenesis and male fertility. (Davidson et al.)

Thank you for your letter dated 24th February 2015 informing us that the above manuscript has been recommended for publication in *Human Fertility* following minor revision. We would like to thank the reviewers and the Editor for their pertinent and highly constructive comments. We have revised our manuscript in line with comments and requests. We attach a revised version of our manuscript. We believe that the revised manuscript is much improved and hope that the manuscript is now in a form that meets publication requirements.

We look forward to hearing from you in due course.

Yours sincerely,

A handwritten signature in blue ink that reads "Kevin Coward".

Dr Kevin Coward

Director, MSc in Clinical Embryology, Nuffield Department of Obstetrics & Gynaecology, University of Oxford.
Principal Investigator, Nuffield Department of Obstetrics & Gynaecology, University of Oxford.
Fellow, Higher Education Academy.

Reviewer #1

Reviewer 1 found that the authors did an excellent job of thoroughly reviewing the male reproductive implications to obesity.

Comment 1: When mentioning the hypothesized importance of the T:E ratio, the authors should mention the data on the use of aromatase inhibitors for male infertility.

Reply 1: We thank the reviewers for their constructive comment. The following text addition has been made to mention the use of aromatase inhibitors for male infertility and the new references have been added to the reference section.

Start line 382

“Since obesity appears to exert influence upon markers for male infertility, efforts have been undertaken to investigate whether measures taken to reverse the unhealthy consequences of obesity can also reverse the deleterious effects upon fertility. When couples seek infertility treatment and male factor infertility is diagnosed, most men present with a reduced ratio of testosterone to oestrogen (Luboshitzky et al., 2002). Pharmacological interventions via medication can be used to treat obesity by addressing weight loss via appetite suppressants, or to treat the effects of obesity upon the male reproductive tract. Aromatase inhibitors can be prescribed for males presenting with infertility problems, and who exhibit elevated estrogen and reduced testosterone levels, in order to prevent the excessive conversion of testosterone to estrogen (Elkhiat & Fahmy, 2011; Schlegel, 2012). Studies have shown that the administration of aromatase inhibitors is not only effective at restoring normal hormone levels, but can also normalize spermatogenesis and semen parameters (Raman & Schlegel, 2002; Zumoff et al., 2003; Roth et al., 2008). While these changes show some improvements in markers for fertility, further investigation into the direct effects of such treatment and the potential restoration of fertility are needed.”

Comment 2: While passing mention is made in the conclusions, some discussion should be dedicated to the limited studies that explore the impact of weight loss on male fertility.

Reply 2: We thank the reviewers for their comment and suggestion to discuss the limited studies which explore the impact of weight loss on male fertility. We have added the below text to touch on this. (See also the reply to the Editor’s comment regarding lifestyle impacts on obesity).

Start line 375

“An individual’s weight is often related to their eating habits and levels of activity. If one member of a couple is obese, there is a high likelihood that their partner may have a similar lifestyle and is also overweight or obese. Lifestyle changes through diet modifications and exercise can lead to gradual weight loss and thus help to overcome obesity and its associated preventable diseases. Several studies in obese males have reported that weight loss through lifestyle changes resulted in increased SHBG and serum testosterone levels, as well as reduced levels of insulin and leptin (Kaukua et al., 2003; Niskanen et al., 2004).”

Comment 3: The Lotti reference does not appear in the references section.

Reply 3: We thank the reviewers for pointing this out. In fact, this reference has now been changed to 'Swan et al (2000)' as this paper presents a more robust analysis of 101 studies.

Start line 407

"Studies have observed a parallel decrease in male fertility potential over the past decades in regions where obesity is prevalent (Swan et al., 2000)."

Reviewer #2

Reviewer 2 found that the authors presented a strong and detailed review. The reviewer noted that the authors should check they are citing the relevant original articles. We have revised the text and references and made the corrections accordingly.

Comment 1: Line 24: "now affects around" change to "Infertility is estimated to affect one in seven...."

Reply 1: This text has been changed.

Start line 26

"Specifically, infertility is estimated to affect one in seven couples in the Western world, with male factor infertility contributing to approximately 40% of these cases, female factor infertility to 45%, and the remaining proportion due to idiopathic causes (Fritz & Speroff, 2011)."

Comment 2: Line 28: Back this up with a citation.

Reply 2: This text has now been updated with an in-text citation.

Start line 30

"Interestingly, there has been a steady decline in fertility rates over the past 50 years, which has occurred in parallel with an increasing rate of obesity (Hammoud et al., 2012)."

Comment 3: Line 31: Original article should be cited National Institutes of Health. Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults—the evidence report. *Obes Res* 1998;6(Suppl 2):51S–209S. (Also, it is the National Institutes of Health", please correct

Reply 3: We thank the reviewer for pointing out this error to us. The citation has now been changed and the original article added to the references section.

Start line 36

"According to the National Institutes of Health, individuals with a BMI between 25 and 29.9 kg/m² are classified as 'overweight', while those with a BMI greater or equal to 30kg/m² are classified as 'obese' (National Institutes of Health, 1998)."

Comment 4: Line 33–35: Authors start with rising infertility in Western world, then move to obesity epidemic in the US. But abstract addresses UK male obesity. Consistency and focus is important. If this article is meant to be generalizable to the “Western” world, I would recommend language that is consistent with this aim.

Reply 4: We thank the reviewer for pointing out this inconsistency with regards to the discussed regions of the obesity statistics in the US and UK and the infertility statistic in the Western world. In order to make the text generalizable, the text has now been changed in both the abstract and introduction to refer the worldwide prevalence of obesity and infertility rather than specific different countries. The new text is shown below, and these new references have been added to the reference section.

Start line 2

“Worldwide obesity rates have nearly doubled since 1980 and currently over 10% of the population is obese. In 2008, over 1.4 billion adults aged 20 and older were overweight; of these, over 200 million men and nearly 300 million women were obese.”

Start line 25

“In 2010, 48.5 million couples globally were affected by infertility (Mascarenhas et al., 2012).”

Start line 36

“Between 1980 and 2008, worldwide prevalence of obesity nearly doubled, and more than 10% of the world’s adult population is obese (Finucane et al., 2011).”

Comment 5: Line 42–44: Studies have actually been mixed with largest studies showing no adverse effects for male overweight in IVF couples. Should provide balanced and more up-to-date evidence (see 1) Colaci DS, Afeiche M, Gaskins AJ, Wright DL, Toth TL, Tanrikut C, et al. Men's body mass index in relation to embryo quality and clinical outcomes in couples undergoing in vitro fertilization. *Fertil Steril* 2012;98:1193–9.e1. 2) Petersen GL, Schmidt L, Pinborg A, Kamper-Jørgensen M. The influence of female and male body mass index on live births after assisted reproductive technology treatment: a nationwide register-based cohort study. *Fertil Steril* 2013;99:1654–62 and 3) Schliep KC, Mumford SL, Ahrens KA, Hotaling JM, Carrell DT, Link M, et al. Effect of male and female body mass index on pregnancy and live birth success after in vitro fertilization. *Fertil Steril*. 2015Feb;103(2):388-95.

Also, it is important when giving a balanced presentation to talk about the strengths and weaknesses of studies (e.g., how many of the studies that the authors cite take into consideration the important element of confounding by maternal BMI?).

Reply 5: We thank the reviewer for their comment pointing out that larger recent studies have shown no adverse effects for overweight males in IVF couples, for providing associated references, as well as pointing out the importance of maternal BMI as a confounding variable. The new references have been added to the reference section. The following text has been added to the introduction and body of the article to discuss the recommended articles, and is further addressed in the reply to comment 9.

Start line 43

“While current data remains highly conflicting, mounting evidence indicates that male obesity is associated with an increased time to conception, reduced pregnancy rates, and an increase in pregnancy loss in couples undergoing assisted reproductive techniques (ART) (Nguyen et al., 2007; Ramlau-Hansen et al., 2007; Hinz et al., 2010; Keltz et al., 2010; Bakos et al., 2011a).”

Start line 199

“Another important concern to take into account when analysing the effects of male obesity upon semen abnormalities is the potential confounding effect of maternal obesity and whether this leads to semen abnormalities in male offspring. It was hypothesized in 2007 that maternal obesity could have a programming effect upon testicular development in foetal life (Ramlau-Hansen et al., 2007). The authors observed that the sons of mothers who were overweight or obese during pregnancy were more likely to be overweight at birth and exhibit a high BMI in adulthood compared to the sons of normal weighted mothers. It was also suggested that higher free oestrogen levels in mothers (and hence the foetus) could interfere with normal testicular development and future fertility (Ramlau-Hansen et al., 2007). More recently, the same group reported that there was no obvious relationship between high birth weight/prepubertal body fat and semen quality in young adult life, suggesting that the effects of maternal obesity during pregnancy are unlikely to have an effect upon semen quality and subsequent fertility (Ramlau-Hansen et al., 2010).”

Start line 244

“It should be kept in mind that in many of these studies, the confounding effect of maternal obesity is often not taken into account when associating outcomes to the incidence of male obesity. A recent study reported that weight status (overweight or obese) does not influence fecundity in couples undergoing infertility treatment. In fact, Schliep et al. (2015) investigated the effects of both male and female body-mass index on pregnancy and birth rates following *in vitro* fertilisation, and after adjusting for partner BMI, found no significant differences between fertilisation rate, embryo score, pregnancy, or live birth rate compared with normal weight controls.”

Comment 6: Line 102: This should be clarified to couples seeking infertility treatment whereby male factor is diagnosed. Also, please cite original source of article, Tsai article has a different objective then assessment presented in this sentence. Cannot even find mention of “infertility treatment” in the article, is this even the right citation?

Reply 6: We thank the reviewers for their comment. The text has been clarified to indicate couples where male factor infertility is diagnosed, and the reference has been fixed.

Start line 385

“When couples seek infertility treatment and male factor infertility is diagnosed, most men present with a reduced ratio of testosterone to oestrogen (Luboshitzky et al., 2002).”

Comment 7: Line 120 delete “upon”

Reply 7: Done

Comment 8: Lines 165–168: Re: “Interestingly however, one study reported a significant association between BMI and semen quality even following an adjustment for reproductive hormones, suggesting that altered hormone profiles in obese men may not be solely responsible for infertility problems (Qin et al., 2007).” This study does not seem to support the statement, since Qin et al found that only underweight men had reduced semen quality after adjusting for hormone levels. In contrast to what the authors are advocating, Qin et al actually found that “Being underweight may be a risk factor for low sperm concentration (OR: 4.68, 95% confidence intervals [CI]: 2.01-10.91). Otherwise, being overweight may be a protected factor for low sperm concentration (OR: 0.25; 95% CI: 0.08-0.83) and low total sperm count (OR: 0.37, 95% CI: 0.15-0.87).”

Reply 8: We thank the reviewers for their comment and in pointing out that since the reduced semen quality was only found in underweight men it therefore cannot be extrapolated to make conclusions about the effects of hormone profiles in obese men and corresponding fertility status. This text has been removed.

Comment 9: Lines 212-213: Again, authors are leaving out important recent studies with among much larger studies. This is concerning in that the authors of a review article such as this should have up to date information. See comment re: lines 42-44 for studies that should be added.

Reply 9: We have taken the reviewers suggestions and added information from the recommended more recent and larger study. The new reference has been added to the reference section. The following text has been added (as well as the text in response to comment 5).

Start line 222

“Interestingly, one study noted that fertilisation rate was higher among obese men than men of normal weight for conventional IVF cycles, and that there were no significant associations between male BMI and poor quality embryos or cleavage rate (Colaci et al., 2012). However, this same study showed that the odds of live birth in couples with obese male partners undergoing ICSI was 84% lower than men with normal BMI and concluded there is a possible deleterious effect of male obesity on the odds of having a live birth rate (Colaci et al, 2012).”

Comment 10: Line 214: But if male BMI affects whether a couple is treated with ICSI or not, then adjusting for ICSI may lead to over adjustment bias (see Schliep et al 2014 for an explanation as to why stratifying on ICSI may not be appropriate).

Reply 10: We thank the reviewers for their comment and recommendation to see Schliep et al for a good explanation against stratifying results on ICSI. The text has been amended to point out the over adjustment bias that may occur when stratifying data by ICSI, and the reference has been added to the reference section.

Start line 228

“It should be noted however, that if male BMI influences whether patients are treated with ICSI, stratifying data by ICSI may lead to over adjustment bias and thus confound the observed outcomes (Schliep et al., 2015). Conversely, other studies report that for obese patients, rates of fertilisation (Keltz et al., 2010) and live birth rates (Peterson, et al. 2013) are reduced following *in vitro* fertilisation (IVF) but not following ICSI, suggesting impairment in sperm binding.”

Comment 11: Lines 250-266: But other studies the authors bring up (e.g., see Qin et al) discuss poor semen quality of underweight males. Do the authors of these cited studies look at trans-generational effects of underweight males? Do these studies control for maternal BMI?

Reply 11: We thank the reviewers for their questions. The text in this article regarding the Qin et al. study has been removed. However in response to the reviewer’s question, the authors did not look at the trans-generational effects of underweight males and did not control for maternal BMI. The following text has been added to the body of the text to indicate that in general, studies should also investigate the effects of underweight males on trans-generational effects.

Start line 325

“Furthermore, studies looking into the trans-generational effects of underweight males should also be undertaken to further investigate the trans-generational effects of weight on DNA integrity and sperm RNA levels.”

Comment 12: Lines 265-266 advocacy for potentially not treating obese males with ART seems ethically concerning given that this is novel research and full assessment has not been undertaken. And particularly when in earlier and later paragraphs you highlight that BMI might not be an appropriate marker for adiposity.

Reply 12: We thank the reviewers for their comments showing that the initial sentence could be misconstrued and misinterpreted as the authors advocating for not treating obese males with ART. The sentence has been replaced with the following text in red to avoid this potential misunderstanding:

Start line 287

“A hypothesis known as ‘trans-generational epigenetic inheritance’ now purports that molecular changes resulting from impaired spermatogenesis due to obesity, such as changes to DNA methylation, histone acetylation or non-coding RNA levels in sperm, are transmitted to the embryo and consequently affect subsequent development (Youngson & Whitelaw, 2011; Daxinger et al., 2012; Palmer et al., 2012). This represents an interesting area which needs to be investigated further and could help to develop and improve infertility treatments.”

Comments from the Editor, Professor Henry Leese

The Editor found that the authors provided a well presented review on an interesting topic.

Comment: I would like the authours however to comment more on the multifactorial nature of infertility. In particular the lifestyle choices that lead to an obese male can also effect the female partner. This will lead to the question of whose obesity is impacting lack of fertility more!

Reply: We thank the editor for his useful comment and raising the interesting point of discussion regarding subfertility in couples where both partners could be obese.

Start line 364

“Developing treatments for infertility is challenging, as the nature of fertility is multifactorial. In addition to genetic or pathological infertility, there are many lifestyle factors which can affect reproductive health such as nutrition, weight, exercise, stress, environmental exposures, and drug use (Sharma et al., 2013). There are numerous lifestyle choices, which could lead to obesity and its associated diseases and sub-fertility. One major challenge in investigating how to treat sub-fertility associated with lifestyle factors is in considering how lifestyle changes leading to sub-fertility are likely to influence both male and female counterparts in a given couple. Therefore, if a male is obese and presenting with sub-fertility, the lifestyle choices leading to his obesity are highly likely to affect his female partner also, and therefore it is more than likely that her weight could also be a contributory factor in the couple’s infertile status.”

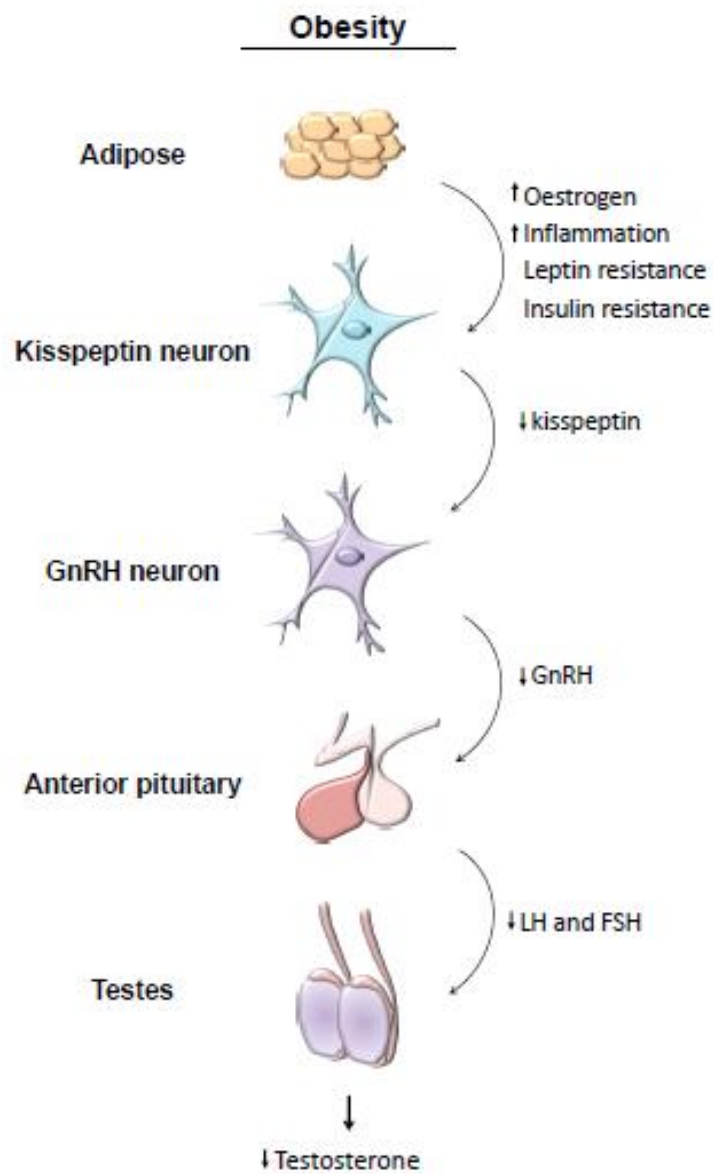


Figure 1



AUTHOR DECLARATION

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript

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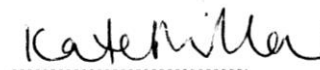
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