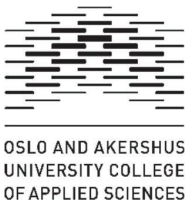
Semen quality in relation to body mass index, sperm fatty acid composition, and anti-Müllerian hormone

Doctoral thesis by

Jorunn M. Andersen



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Oslo, June 2016

Jorunn M. Andersen

List of papers

Paper 1:

Body mass index is associated with impaired semen characteristics and reduced levels of anti-Müllerian hormone across a wide weight range

J.M. Andersen, H. Herning, E.L. Aschim, J. Hjelmesæth, T. Mala, H.I. Hanevik, M. Bungum, T.B. Haugen, O. Witczak.

PLoS ONE, 2015, e0130210.

Paper II:

Fatty acid composition of spermatozoa is associated with BMI and with semen quality

J.M. Andersen, P.O. Rønning, H. Herning, S.D. Bekken, T.B. Haugen, O. Witczak.

Accepted for publication in Andrology, 2016.

Paper III:

Anti-Müllerian hormone in seminal plasma and serum: Association with sperm count and sperm motility

J.M. Andersen, H. Herning, O. Witczak, T.B. Haugen.

Human Reproduction, 2016 May 24 (Epub ahead of print).

List of abbreviations

AMH	Anti-Müllerian hormone
BMI	Body mass index
DFI	DNA fragmentation index
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FAI	Free androgen index
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
HPG	Hypothalamus-pituitary-gonadal
LH	Luteinizing hormone
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
SCSA	Sperm chromatin structure assay
SFA	Saturated fatty acid
SHBG	Sex hormone binding globulin
WHO	World Health Organization
TUNEL	Terminal uridine nick-end labelling

1. Introduction

1.1. Background

Concern about declining sperm counts emerged in the 1970s (Nelson & Bunge 1974; Bostofte *et al.* 1983), and the interest and debate escalated with a publication in 1992 (Carlsen *et al.* 1992). Carlsen and co-workers presented a meta-analysis of 61 studies published between 1938 and 1990, including data from 14 947 men, and it was suggested that semen quality had declined by 40% in 50 years. Several other groups investigated the possibility of a dramatic impairment of semen quality in the years following this publication. Some supported a decline in semen quality (Auger *et al.* 1995; Irvine *et al.* 1996; Swan *et al.* 2000), while others disputed this finding (Handelsman 1997), and the question of whether sperm counts are declining or not is still debated (Fisch & Braun 2013). Other possible threats to male reproductive health have been suggested, including influence of environmental pollutants (Toppari 1996; Perry 2008; Wilcox & Bonde 2013; Lassen *et al.* 2014) and lifestyle (Jensen *et al.* 2004b; Ravnborg *et al.* 2011). Interestingly, regional differences in semen quality in the general population have been found between east and west in the Nordic-Baltic area, and Norwegian and Danish men were found to have poorer semen quality than men from Finland and Estonia (Jorgensen *et al.* 2002).

The prevalence of obesity has more than doubled since 1980 and is becoming a global health problem (Ng *et al.* 2014; World Health Organization 2015). The World Health Organization (WHO) has defined overweight and obesity as "abnormal or excessive fat accumulation that may impair health", and in 2014 it was estimated that of adults worldwide, 39% were overweight, and 13% were obese. Norway is no exception, and overweight and obesity are an increasing problem in the Norwegian population (Norwegian Institute of Public Health 2011; Ng *et al.* 2014). It is interesting that a possible decrease in semen quality seems to appear to be in parallel with the epidemic increase in overweight and obesity. As weight gain is also seen in younger men (Norwegian Institute of Public Health 2011), it is important to gain knowledge about possible effects of overweight and obesity on male reproductive health.

Overweight and obesity are reckoned as important factors for female infertility, strongly associated with menstrual disorders, polycystic ovary syndrome, infertility and recurrent miscarriages (Pasquali *et al.* 2003; Gesink Law *et al.* 2007). During the past decade, some studies have indicated a negative association between overweight and male reproductive

health. Increased risk of couple infertility with high male body mass index (BMI) has been indicated in epidemiological studies (Sallmen *et al.* 2006; Nguyen *et al.* 2007; Ramlau-Hansen *et al.* 2007), and obesity is consistently associated with low levels of testosterone (Hofstra *et al.* 2008). Negative association between BMI and semen quality has also been shown (Jensen *et al.* 2004a; Belloc *et al.* 2014), but results are conflicting (Teerds *et al.* 2011; Sermondade *et al.* 2013), and mechanisms linking obesity to impaired semen quality are unclear.

1.2. Male reproduction

The male reproductive system consists of the penis, the testes, and a complex duct system with accessory sex glands. The testes are essential for the production of sperm and for synthesis of reproductive hormones, and are composed of more than 200 lobules with 2-3 coiled seminiferous tubules located within each lobule (Figure 1). The interstitial space between the tubules contains blood vessels and Leydig cells that produce testosterone.

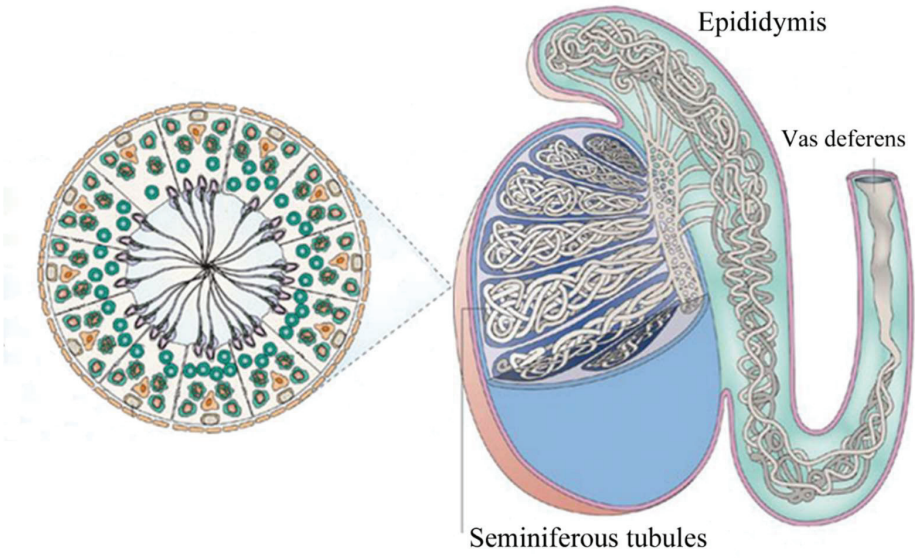

Figure 1: Schematic illustration of the testis. *Cross-section of the testis and the epididymis, and a single seminiferous tubule. Adapted from figure by Arnheim and Calabrese (Arnheim & Calabrese 2009) and figure by Krawetz (Krawetz 2005). Figures are used with permissions from Nature Publishing Group.*

The germinal epithelium within the seminiferous tubules consists of Sertoli cells and germ cells at different stages of differentiation. The Sertoli cells have multiple specialized functions (Russel & Griswold 1993). They have a supportive role, keeping the germinal cells in place, they provide nutrition, e.g. lipids and amino acids, to the developing germ cells, and secrete a wide variety of proteins, like hormones. Between adjacent Sertoli cells, specialized junctions create the blood-testis barrier that restricts entry of molecules from the intestinal space and create a microenvironment suited for germ cell development.

1.2.1. Spermatogenesis

The production of spermatozoa, the spermatogenesis, is a highly complex process (Figure 2). It takes about 75 days from the process is initiated until a spermatozoa is released into the lumen of the seminiferous tubules.

The most immature germ cells, type-A spermatogonia are present within the seminiferous tubules close to the basement membrane. When the type-A spermatogonium divides, one daughter cell will become a new type-A spermatogonium, maintaining the pool of immature germ cells. The other daughter cell progresses to a type-B spermatogonium, a cell committed to proliferation that enters meiosis and divides into primary spermatocytes. The primary spermatocytes undergo the first meiotic division into secondary spermatocytes.

The second meiotic division produces four haploid round spermatids that through an extensive cell remodelling will become elongated spermatids and eventually mature spermatozoa. The process of elongation and differentiation from round spermatids to spermatozoa is referred to as spermiogenesis. This process includes nuclear condensation, acrosome formation, development of the sperm tail, and cytoplasmic reduction. Spermatozoa released from the Sertoli cells are transported through the rete testis to the epididymis, where they mature and gain their motility. Spermatozoa reside in the epididymis for a mean of 10 – 14 days and can be stored for approximately one month. After that, the cells are expelled or reabsorbed. The spermiogenesis and the maturation in the epididymis are temperature sensitive.

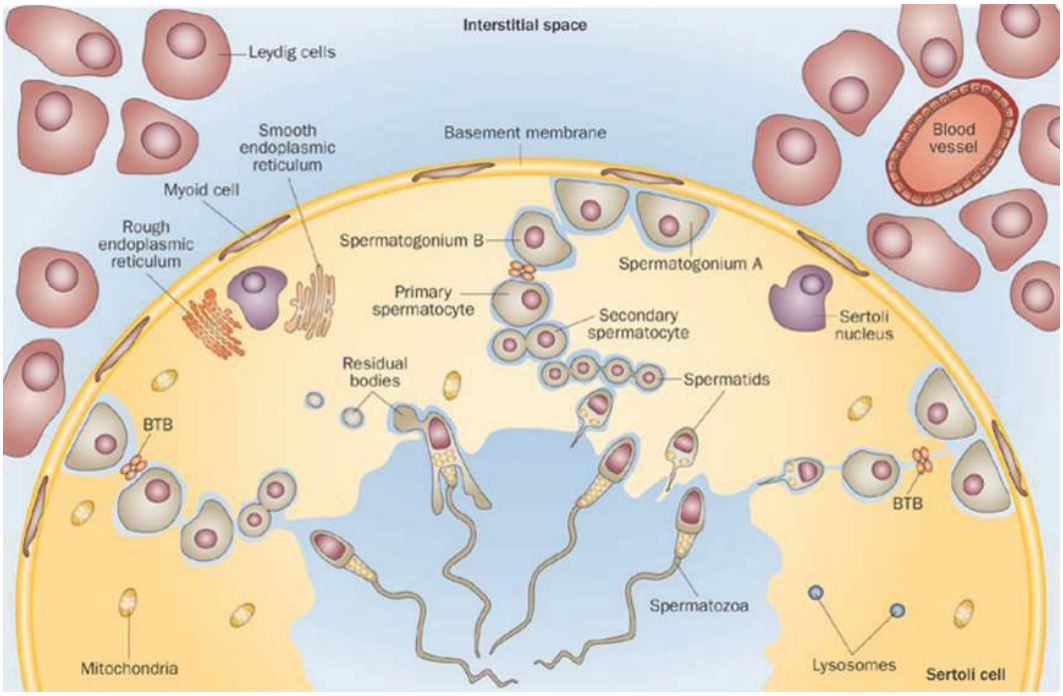

Figure 2: Schematic illustration of spermatogenesis. *The seminiferous epithelium is composed of Sertoli cells and developing germ cells at different stages. Leydig cells and blood vessels are located in the interstitium. Through several rounds of cell divisions, spermatogonia develop into spermatids that migrate toward the lumen of the seminiferous tubules. Finally, fully formed spermatozoa are released. Reproduced from Rato and co-workers (Rato et al. 2012) with permission from Nature Publishing Group.*

The mature spermatozoon consists of a head, a mid-piece, and a tail, covered by a sperm plasma membrane (Figure 3). The head contains the nucleus with tightly packed DNA and the acrosome consisting of granules with enzymes. The enzymes are released upon contact with the oocyte, making it possible to penetrate the oocyte membrane. The mid-piece contains mitochondria that produce energy needed for cell movement, driven by the motile force of the sperm tail.

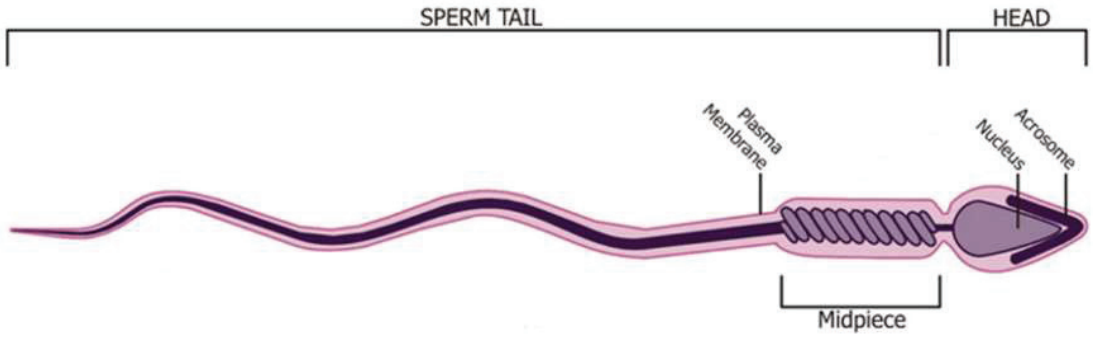

***Figure 3**: **Mature spermatozoon.** Illustration of a spermatozoon with head, midpiece, and tail, covered by the plasma membrane. The nucleus and the acrosome are located in the head. The midpiece consists of mitochondria that provide the energy needed for movement. Adapted from Borg and co-workers (Borg et al. 2010) with permission from Oxford University Press.*

Spermatozoa are ejaculated in a mixture of secrets form the testis, the epididymis, the prostate, seminal vesicles, and the bulbourethral glands (World Health Organization 1999). About 90% originate from the prostate and seminal vesicles (Weiske 1994). Semen is released in boluses. The first fraction of the ejaculate is rich in spermatozoa and contains secretions from the prostate and the epididymis (Mortimer 1994). The second fraction has lower numbers of spermatozoa and contains mostly secretion from the seminal vesicles.

1.2.2. Reproductive hormones

Spermatogenesis is regulated by reproductive hormones through the hypothalamus-pituitary-gonadal (HPG) axis (Figure 4). Production of the two gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), is controlled by gonadotropin releasing hormone (GnRH) produced in the hypothalamus. LH stimulates testosterone production in the Leydig cells. FSH and testosterone stimulate the Sertoli cells, and are important for the regulation of spermatogenesis, but the exact mechanism of regulation is not fully elucidated. Testosterone can be converted into oestrogen by aromatization, and both hormones provide negative feedback to the hypothalamus and the pituitary to decrease the levels of GnRH and gonadotropins.

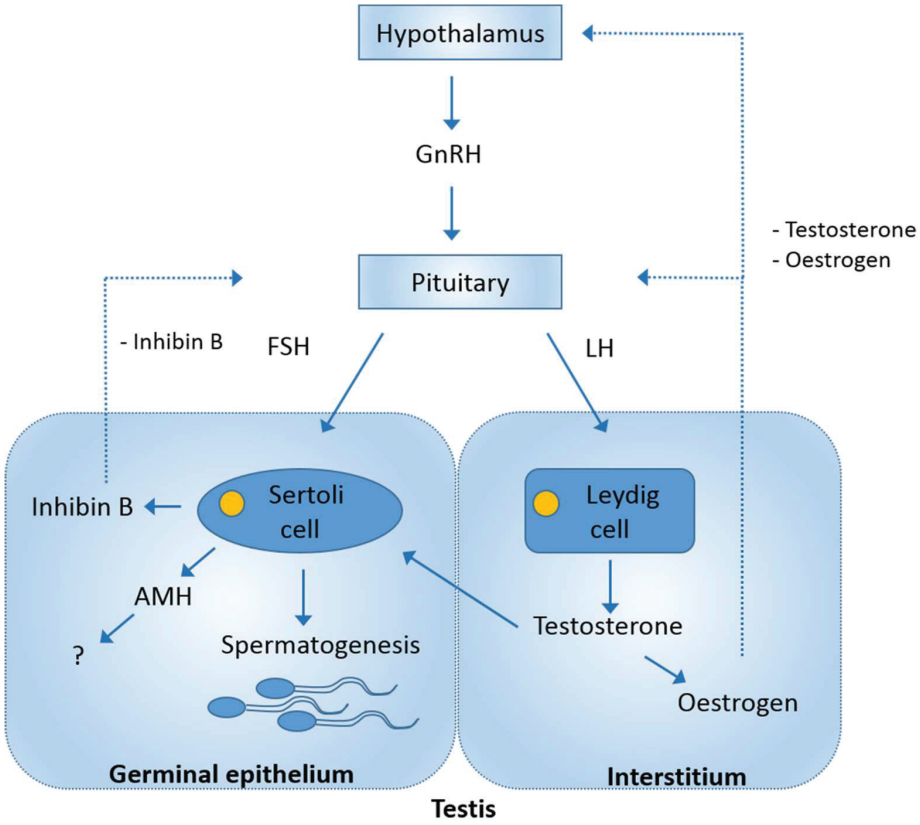

Figure 4: The Hypothalamus-pituitary-gonadal axis in the male. *GnRH is secreted from the hypothalamus and stimulates synthesis of LH and FSH by anterior pituitary cells. LH promotes secretion of testosterone from Leydig cells. FSH and testosterone can bind to Sertoli cells and regulate spermatogenesis. Solid arrows represent positive feedback, and dotted arrows indicate negative feedback. Illustration is made by the author.*

Most of the circulating testosterone and oestrogen are bound to sex hormone binding globulin (SHBG), a lesser fraction to albumin, while 1-2% are unbound and biologically active. The role for oestrogen in spermatogenesis is not clear, but it is believed to influence the numbers of spermatogonial stem cells and sperm maturation (Carreau *et al.* 2002).

The Sertoli cells produce inhibin B that signals negative feedback to the pituitary, decreasing the FSH production. Inhibin B is closely related to Sertoli cell function and spermatogenesis (Pierik *et al.* 1998). It is regarded as a marker of spermatogenesis (Pierik *et al.* 1998; Al-Qahtani *et al.* 2005; Goulis *et al.* 2008; Matuszczak *et al.* 2013), and levels of inhibin B have

been found to correlate well with sperm count (Andersson *et al.* 2004; Jørgensen *et al.* 2010). Inhibin B has a predictive value in the evaluation of male infertility, if used with an appropriate reference population of fertile men (Andersson *et al.* 2004).

AMH is also secreted by the Sertoli cells (Rey *et al.* 2003) and is responsible for the regression of the Müllerian ducts during male sex determination in the fetus (Lee & Donahoe 1993). In adult males, AMH has been detected in serum (Aksglaede *et al.* 2010) and seminal plasma (Fallat *et al.* 1996), but its action in the adult male is poorly understood. AMH is positively regulated by FSH and strongly downregulated by testosterone (Josso *et al.* 1998; Rey *et al.* 2003; Sinisi *et al.* 2008). It has been suggested as a marker of spermatogenesis, like inhibin B (Muttukrishna *et al.* 2007). However, studies of the association between serum levels of AMH and semen quality have shown conflicting results (Al-Qahtani *et al.* 2005; Tuttelmann *et al.* 2009). AMH is not known to have a feedback mechanism within the HPG axis.

1.2.3. Male infertility

Infertility has been defined by the WHO as "the inability of a sexually active, non-contracepting couple to achieve spontaneous pregnancy in one year". In infertile couples, male infertility is the cause in 20-50% of the cases (Thonneau *et al.* 1991). A summary of factors that can influence male fertility and semen quality is listed in Figure 5.

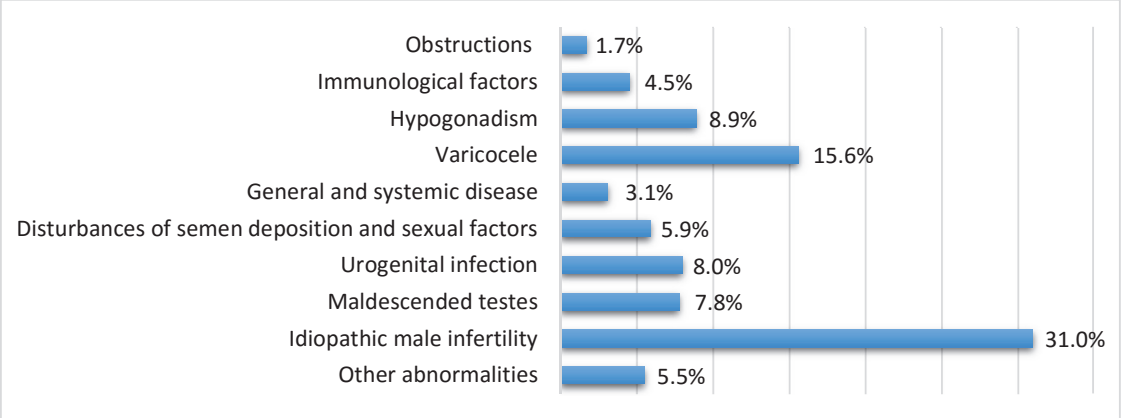

***Figure 5: Factors influencing male infertility**. Data retrieved from Hauser and co-workers (Hauser et al. 1995). Histogram is made by the author.*

Male hypogonadism is a collective term for conditions causing low testosterone levels. It is a common endocrine disorder that also may affect spermatogenesis (P. Kumar *et al.* 2010; Fraietta *et al.* 2013). In primary hypogonadism, the low testosterone levels are caused by testicular failure. It is also associated with an increase in FSH and LH. It is often seen in cases with abnormal testicular development, like in Klinefelter syndrome, after injuries to the testes from e.g. cancer treatment, or with aging. Secondary hypogonadism, or hypogonadotropic hypogonadism, is caused by congenital or acquired dysfunction of the HPG axis, negatively affecting the release of GnRH and levels of LH and FSH. It can be caused by disorders in the pituitary or the hypothalamus, as a side effect of medication, or in association with factors like inflammatory disease and obesity. Interestingly, between 30-40% of men with poor semen quality have normal findings in physical and endocrine examination. This is referred to as idiopathic male infertility.

1.2.4. Semen analysis

Analysis of ejaculated semen is used in the evaluation of male infertility. The number of sperm in the ejaculate reflects the function of the testes and its ducts, while the volume of the seminal fluid reflects the secretory activity of the accessory sex glands (World Health Organization 2010). The WHO has provided standardized methods for the analysis of semen (World Health Organization 1999). First published in 1980, the WHO manual has been edited several times. The latest edition was published in 2010, complying with new knowledge and recommendations from technicians and scientists. It provides more detailed descriptions of the different methods compared to previous versions, new guidelines for assessment of sperm motility, and evidence-based reference values for semen characteristics (Table 1). The reference group was men whose partners had a time to pregnancy of 12 months or less (Cooper *et al.* 2010; World Health Organization 2010). Standard semen analysis includes assessment of semen volume, sperm concentration, total sperm count, sperm motility, normal sperm morphology, and sperm vitality. Frequently used nomenclature describing semen quality is presented in Table 2. Combinations of these terms are used for men or groups of men having two or more of these parameters below the lower reference limit.

Table 1: Reference values for human semen characteristics (World Health Organization 2010).

Semen parameters	5th centile (95% CI)
Semen volume (ml)	1.5 (1.4–1.7)
Sperm concentration (10^6/ml)	15 (12–16)
Total number (10^6/ejaculate)	39 (33–46)
Progressive motility (%)	32 (31–34)
Normal forms (%)	4 (3.0–4.0)
Vitality (%)	58 (55–63)

Table 2: Nomenclature frequently used in semen quality.

Aspermia	No semen
Azoospermia	No spermatozoa in the ejaculate
Oligozoospermia	Total sperm count or sperm concentration below the lower reference limit.
Asthenozoospermia	Percentage of progressively motile spermatozoa below the lower reference limit.
Teratozoospermia	Percentage of morphologically normal spermatozoa below the lower reference limit.
Normozoospermia	All parameters equal to or above the reference limits

The standard semen analysis is widely used in the evaluation of male fertility. Studies have indicated that the likelihood of pregnancy increases with sperm concentration up 40-55 x 10^6/ml (Bonde *et al.* 1998; Guzick *et al.* 2001; Slama *et al.* 2002). Sperm motility has been associated with time to pregnancy (Larsen *et al.* 2000; Haugen *et al.* 2006; Nallella *et al.* 2006), however, low predictive value has been indicated with regard to probability of conception (Bonde *et al.* 1998; Slama *et al.* 2002). Although sperm morphology has been associated with fertility (Bonde *et al.* 1998; Slama *et al.* 2002), changes in reference range and scoring criteria have complicated the interpretation of this parameter (Tomlinson *et al.* 2013). Although semen characteristics may provide useful information about the male fertility

potential, it is important to note that results from semen analysis are not sufficient for the diagnosis of male infertility (Guzick *et al.* 2001). Furthermore, it has been discussed how applicable the reference values are in clinical practice (Skakkebaek 2010). Men with sperm concentration below a threshold of 40-55 x 10^6/ml (Bonde *et al.* 1998; Guzick *et al.* 2001; Slama *et al.* 2002) may be considered sub-fertile, although the semen quality is regarded as normal according to the WHO reference values.

Spermatozoa are susceptible to DNA damage (Aitken *et al.* 2003), due to oxidative stress induced by e.g. elevated scrotal temperature (Esfandiari *et al.* 2002; Sergerie *et al.* 2007; Garolla *et al.* 2015), ageing (Moskovtsev *et al.* 2006), and smoking (Fraga *et al.* 1996; Sipinen *et al.* 2010). There are several methods available for assessment of DNA fragmentation in spermatozoa, like comet assay, terminal uridine nick-end labelling (TUNEL) assay, and sperm chromatin structure assay (SCSA) (Evenson *et al.* 1999). DNA integrity is measured as DNA fragmentation index (DFI). SCSA is frequently used in a clinical setting, and with this method, DFI ≥ 30% has been associated with prolonged time to pregnancy and increased risk of infertility (Spano *et al.* 2000; Evenson & Wixon 2006). Infertility treatment by intracytoplasmic sperm injection may be beneficial for men with DFI ≥ 30% (Bungum *et al.* 2007).

1.3. Fatty acids

Lipids are a diverse group of organic molecules that are generally insoluble in water, like fats and oils (Alberts *et al.* 2002). Lipids have several functions. They serve as a source of energy, are constituents of fat-soluble vitamins and hormones, and are important for cell function and structure. Phospholipids are the major class of membrane lipids (Alberts *et al.* 2002). They consist of a hydrophilic phosphate containing head group (polar) and a hydrophobic tail (non polar) of two fatty acids that form the lipid bilayer of cell membranes. The general structure of a phospholipid is shown in Figure 6.

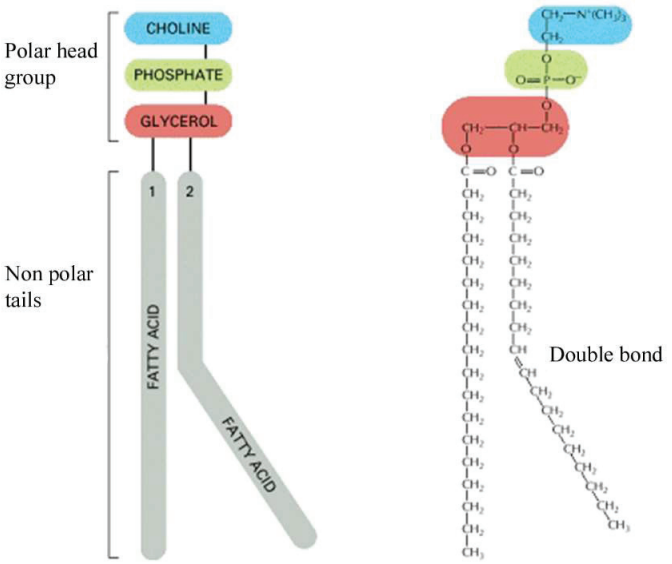

Figure 6: Structure of a phospholipid. *The structure is illustrated schematically and as a formula. Figure shows the polar head group and two hydrophobic tails. Double bonds create a kink in the fatty acid tail. One fatty acid in the tail is usually unsaturated while the other is saturated. Figure adapted from Alberts and co-workers (Alberts et al. 2002).*

Fatty acids are the simplest forms of lipids and important building blocks of the more complex lipid structures like phospholipids and triacylglycerides. Fatty acids are carboxylic acids consisting of a hydrophilic carboxylate group attached to a hydrocarbon chain.

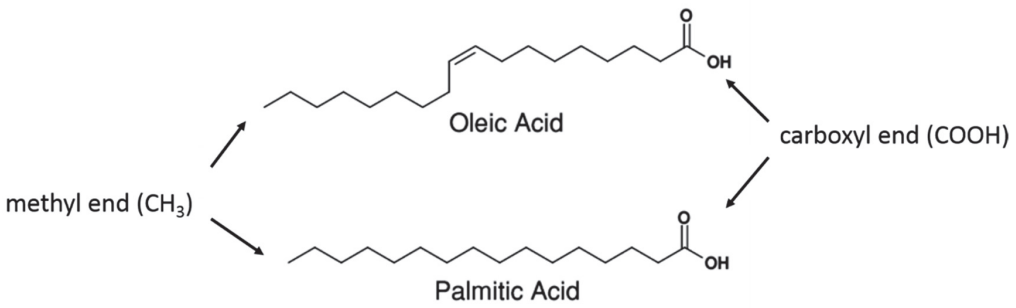

Figure 7: Structure of fatty acids. *Saturated fatty acid is exemplified by palmitic acid. Monounsaturated fatty acid is exemplified by linoleic acid. Illustration is made by the author.*

Fatty acids are commonly referred to by trivial names, like linoleic acid, while systemic names are based on the chemical structure of the fatty acid molecule. Additionally, short name nomenclature is frequently used. The n minus (n-) system, also referred to as the omega

system, indicates the number of carbon atoms, in addition to the number and position of double bonds, counting from the methyl or omega end of the molecule. An overview of common, systemic, and short names of relevant fatty acids is presented in Table 3.

Fatty acids are grouped into different classes based on their molecular structure. Saturated fatty acids (SFAs) are straight chained with no double bonds (Figure 7), and the most common SFAs contain between 12-22 carbon atoms. Unsaturated fatty acids contain one or more double bonds in the structure, introducing a kink in the molecular shape (Figure 7). Monounsaturated fatty acids (MUFAs) have one double bond in the carbon chain. The double bond is often found at the n-9 position, and in mammals, *cis*-conformation is most common. Fatty acids with two or more double bonds are referred to as polyunsaturated fatty acids (PUFAs).

Table 3: Overview of fatty acid nomenclature.

Common name	Systemic name	Short names
Saturated fatty acids		
myristic acid	*n*-tetradecanoic	C14:0
palmitic acid	*n*-hexadecanoic	C16:0
stearic acid	*n*-octadecanoic	C18:0
Monounsaturated fatty acids		
palmitoleic acid	*cis*-9-hexadecenoic	C16:1 n-7
oleic acid	*cis*-9-octadecenoic	C18:1 n-9
Polyunsaturated fatty acids		
n-3 (omega-3)		
α-linolenic acid (ALA)	all-*cis*-9,12,15-octadecatrienoic	C18:3 n-3
eicosapentaenoic acid (EPA)	all-*cis*-5,8,11,14,17-eicosapentaenoic	C20:5 n-3
docosahexaenoic acid (DHA)	all-cis-4,7,10,13,16,19-docosahexaenoic	C22:6 n-3
n-6 (omega-6)		
linoleic acid (LA)	*cis*-*cis*-9,12-octadecadienoic	C18:2 n-6
arachidonic acid (AA)	all-*cis*-5,8,11,14-eicosatetraenoic	C20:4 n-6

1.3.1. Metabolism of essential fatty acids

Lipids in the human diet originate from lipid-containing structures and storages in plants and animals. Most SFAs and MUFAs can also be synthetized from carbohydrates. Mammalian cells lack the enzyme needed to introduce the double bond at the n-9 position. The PUFAs linoleic acid and α-linolenic acid need to be obtained through diet and are therefore referred to as essential fatty acids.

Essential fatty acids can be converted into long chain PUFAs in human cells. Desaturases insert new double bonds between the existing double bond and the carboxyl group, and elongases add carbon atoms to the chain (Figure 8). Linoleic acid is the precursor of the n-6 family of essential fatty acids. It is an important component in plant lipids and the precursor for aracidonic acid, a major component in mammalian phospholipids. α-linolenic acid is the precursor for the n-3 family. It is found in plants and can be converted into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in humans by desaturation and elongation. The main dietary sources for EPA and DHA are fish oil. The fatty acid composition of ingested lipids is reflected in serum phospholipids, and especially EPA and DHA have been found to correlate well with dietary intake (Hedrick *et al.* 2012; Serra-Majem *et al.* 2012).

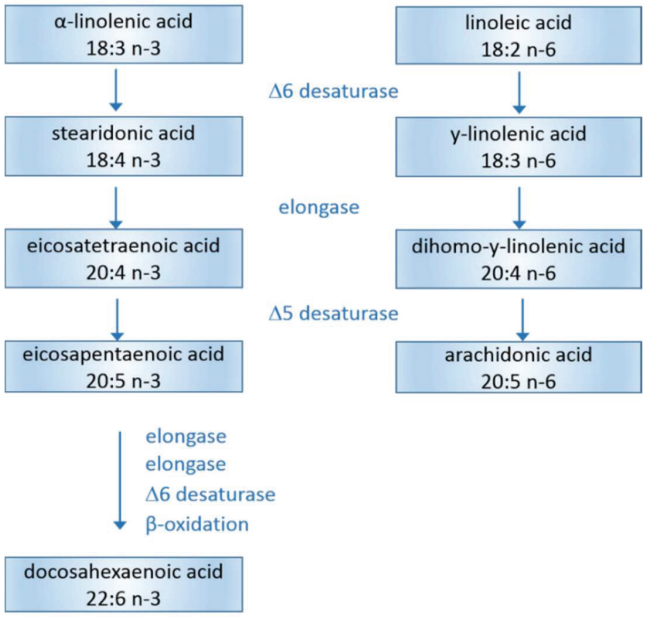

Figure 8: Conversion of essential fatty acids to long chain polyunsaturated fatty acids. *Illustration is made by the author.*

1.3.2. Fatty acid metabolism in testis

Testis has a high content of PUFAs (Nissen *et al.* 1978). Studies of rat testis have shown that in Sertoli cells the main fraction of PUFAs is in the form of triacylglycerides, while the main fraction in germ cells is phospholipids (Beckman & Coniglio 1979). It has been shown that Sertoli cells have high expression of desaturases, while low expression of these enzymes was observed in germ cells (Saether *et al.* 2003). This indicates that Sertoli cells have a high capacity for conversion of essential fatty acids into long chain PUFAs, and it is believed that PUFAs are transported from Sertoli cells to germ cells during spermatogenesis. As spermatozoa are produced, the Sertoli cells are constantly drained of fatty acids (Saether *et al.* 2003), and it is believed that Sertoli cells facilitate the supply of fatty acids to the germ cells (Retterstol *et al.* 2001).

1.3.3. Fatty acid composition of spermatozoa

In spermatozoa, as much as 30% of the fatty acids are DHA (Aksoy *et al.* 2006; Tavilani *et al.* 2006; Martinez-Soto *et al.* 2013). DHA content has been shown to be positively associated with sperm count, sperm morphology, and sperm motility (Zalata *et al.* 1998; Conquer *et al.* 1999; Aksoy *et al.* 2006; Tavilani *et al.* 2006; Martinez-Soto *et al.* 2013). The composition of fatty acids in spermatozoa has also been associated with sperm vitality and has implications for the fusion of sperm and oocyte during fertilization (Lenzi *et al.* 1996; Lewis & Maccarrone 2009). Unsaturated fatty acids increase fluidity of cell membranes, and fluidity of spermatozoa cell membranes is believed to be important for sperm motility (Nissen & Kreysel 1983; Connor *et al.* 1998). MUFAs seem to be negatively correlated with both sperm motility and sperm concentration (Aksoy *et al.* 2006; Martinez-Soto *et al.* 2013), while the relation between SFAs and semen characteristics is inconsistent (Zalata *et al.* 1998; Aksoy *et al.* 2006; Tavilani *et al.* 2006).

1.4. Reproductive health in overweight and obese men

1.4.1. Reproductive hormones

BMI is calculated by dividing weight in kilograms by the square of height in meters (kg/m^2), and is commonly used to classify grades of overweight and obesity as it correlates with total

body fat (National Heart Lung and Blood Institute 1998). Classification of overweight and obesity based on BMI is shown in Table 4.

The physical changes associated with high BMI may negatively impact male reproductive health. Deposits of adipose tissue in the scrotum and the surrounding areas may increase the scrotal temperature and affect the temperature sensitive stages of the spermatogenesis (Garolla *et al.* 2015), especially if joined with a sedentary life style (Hammoud *et al.* 2011). Levels of testosterone are negatively affected by sleep apnea (Grunstein *et al.* 1989), a common comorbidity in obesity.

Table 4. The classification of adult overweight and obesity according to BMI.

WHO category	BMI (kg/m^2)	Categories in the thesis
Normal weight	18.50-24.99	**Normal weight**
Overweight	≥25.00	**Overweight**
Preobese	*25.0-29.9*	
Obese	≥30.00	**Obese**
Obese class I	*30.00-34.99*	
Obese class II	*35.00-39.99*	**Severely obese**
Obese class III	*≥40.00*	

Increased adiposity leads to increased conversion of testosterone into oestrogen due to large amounts of aromatase in the adipose tissue. There is a strong negative association between high BMI and serum levels of testosterone (Jensen *et al.* 2004a; Ramlau-Hansen *et al.* 2010; Camacho *et al.* 2013; Luconi *et al.* 2013), and male obesity-associated secondary hypogonadism has been reported (Isidori *et al.* 2005; Calderón *et al.* 2016). Elevated levels of oestrogen have been found in association with BMI (Jensen *et al.* 2004a; Pauli *et al.* 2008; Foresta *et al.* 2009; Chavarro *et al.* 2010), but not consistently (Aggerholm *et al.* 2008; Macdonald *et al.* 2013). Oestrogen mediates negative feedback to the hypothalamus, and increased levels may contribute to downregulating levels of GnRH, FSH, and LH.

BMI has been negatively associated with LH levels (Giagulli *et al.* 1994; Hart *et al.* 2015) and positively associated with FSH levels (Strain *et al.* 1982; Qin *et al.* 2007; Pauli *et al.* 2008) in obese populations. However, many studies report unchanged levels of LH and FSH in men with high BMI (Jensen *et al.* 2004a; Aggerholm *et al.* 2008; Chavarro *et al.* 2010; Macdonald *et al.* 2013). This indicates that dysregulation of the HPG axis is not the only possible mechanism linking high BMI with reduced semen quality.

As with testosterone, it is well known that SHBG is negatively associated with BMI (Giagulli *et al.* 1994; Vermeulen *et al.* 1996; Jensen *et al.* 2004a; Fejes *et al.* 2005; Aggerholm *et al.* 2008; Chavarro *et al.* 2010). The decrease in SHBG can be attributed to the increased insulin levels often seen in overweight and obese men (Gascon *et al.* 2000; Hautanen 2000), causing reduced synthesis of SHBG in the liver. The decrease in SHBG contributes to lower circulating testosterone levels (Giagulli *et al.* 1994), as the half-life of unbound testosterone is reduced.

Inflammatory cytokines and adipokines are produced in adipose tissue. An increase in white adipose tissue results in increased secretions of adipose derived hormones that may influence the HPG axis (Du Plessis *et al.* 2010) (Figure 9). One important adipokine is leptin. Its main role is the regulation of food intake, but it is also believed to have a role in reproduction (Jope *et al.* 2003; Hofny *et al.* 2010).

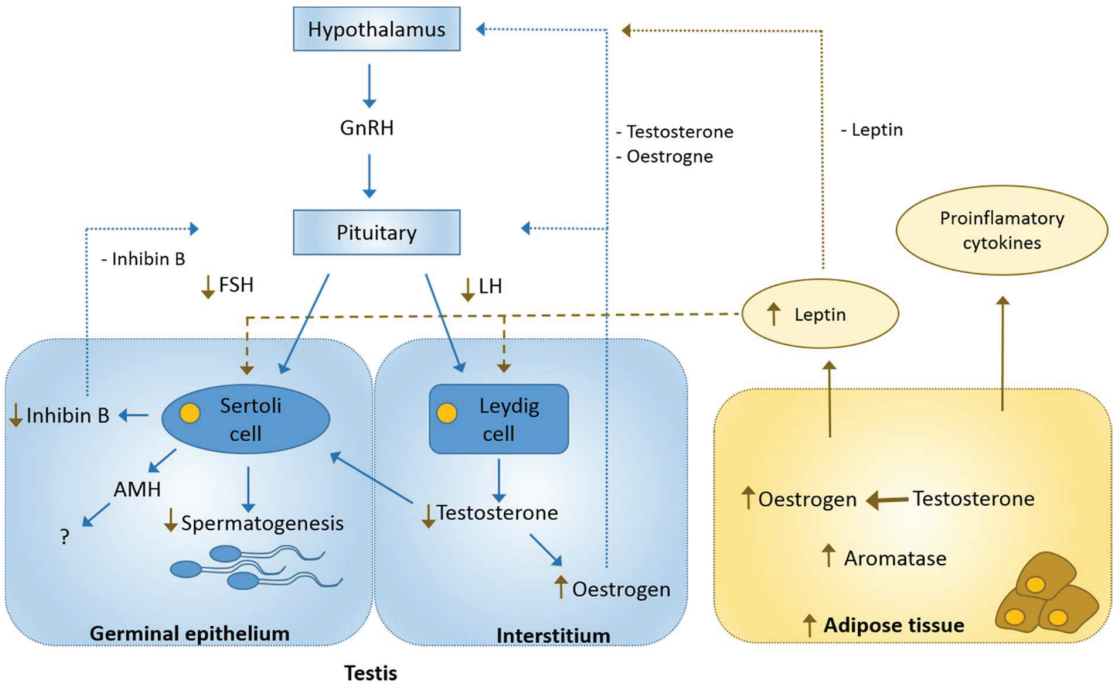

Figure 9: Possible effects of obesity*. Increased amount of adipose tissue contributes to higher levels of aromatase, and more testosterone is converted into oestrogen. Higher levels of proinflammatory cytokines and adipokines, like leptin, are produced in the adipose tissue. Leptin signals negative feedback to the hypothalamus and the pituitary and may decrease levels of FSH and LH. Leptin may influence Sertoli cell and Leydig cell function (indicated by dashed arrows). Eventually, spermatogenesis may be impaired. All dotted arrows indicate negative feedback. Solid blue arrows represent positive feedback. Solid brown arrows indicate possible effects of obesity. Illustration is made by the author.*

Circulating levels of leptin are positively correlated with amount of adipose tissue (Considine *et al.* 1996), and high levels can be found in obese individuals (Maffei *et al.* 1995; Tatti *et al.* 2001; Hofny *et al.* 2010). This adipokine can influence testosterone production through the HPG axis by downregulating GnRH (Nagatani *et al.* 1998; Cunningham *et al.* 1999) and possibly through direct effect on Leydig cells (Isidori *et al.* 1999).

Several studies have found a negative association between BMI and inhibin B levels (Jensen *et al.* 2004a; Winters *et al.* 2006; Paasch *et al.* 2008). Inhibin B is closely related to the number of Sertoli cells, and it has been suggested that obesity in adolescence may have a negative impact on the number of Sertoli cells in adulthood (Winters *et al.* 2006). Like inhibin B, AMH is a testis specific hormone in men. The association between BMI and serum AMH

levels has been addressed (Hakonsen *et al.* 2011; Robeva *et al.* 2011; Pietilainen *et al.* 2012), but is unclear. Any association between BMI and seminal plasma AMH levels has previously not been addressed.

1.4.2. Semen quality

Increased risk of couple infertility with high male BMI has been demonstrated in epidemiological studies (Sallmen *et al.* 2006; Nguyen *et al.* 2007; Ramlau-Hansen *et al.* 2007). In contrast, associations between high BMI and standard semen characteristics show varying results. In studies of semen quality, including men from the general population and from fertility clinics, it was found that high BMI was negatively associated with sperm concentration (Jensen *et al.* 2004a; Hammiche *et al.* 2012; Belloc *et al.* 2014), total sperm count (Chavarro *et al.* 2010; Belloc *et al.* 2014), sperm motility (Hammiche *et al.* 2012; Belloc *et al.* 2014), and sperm morphology (Macdonald *et al.* 2013). On the other hand, there are also studies reporting no association between BMI and semen parameters (Qin *et al.* 2007; Aggerholm *et al.* 2008; Pauli *et al.* 2008). The relationship between BMI and semen quality has also been investigated in two meta-analyses. No associations were found in the analysis of MacDonald and co-workers (MacDonald *et al.* 2010). Sermondade and co-workers identified a significantly increased risk of azoospermia and oligozoospermia in overweight and obese men (Sermondade *et al.* 2013). However, they did not find any association between BMI and sperm concentration or total sperm count when comparing means across BMI groups.

The association between BMI and sperm DNA damage has been addressed, but no clear conclusion has been reached so far. While some studies report a positive association between BMI and DNA damage I spermatozoa (Kort *et al.* 2006; Chavarro *et al.* 2010; Dupont *et al.* 2013), other report no association (Smit *et al.* 2010; Hakonsen *et al.* 2011; Rybar *et al.* 2011; Thomsen *et al.* 2014).

Although overweight and obesity are likely to influence male reproductive health, it is unclear whether weight loss can improve semen quality. Weight loss by life style change, has been associated with normalization of testosterone levels (Kaukua *et al.* 2003), and similar results are seen after bariatric surgery (Aarts *et al.* 2014; Sarwer *et al.* 2015). To date, only one study has examined the effect of weight loss on semen quality (Hakonsen *et al.* 2011). In this study,

serum and semen samples from 26 severely obese men were evaluated before and after a 14 week weight loss programme based on exercise and a healthy diet. Improvement of total sperm count and semen volume was seen for the group of men with the largest weight reduction. They also observed increased levels of testosterone, SHBG, AMH, and free androgen index (FAI).

1.4.3. Fatty acids in spermatozoa

Overweight and obesity are associated with a diet high in saturated fats. The diet in western countries is very rich in n-6 fatty acids, while it contains low levels of n-3 fatty acids (Simopoulos 2001). Fatty acids in the n-6 family have been associated with negative effects like increased amount of adipose tissue (Amri *et al.* 1994) and systemic inflammation (James *et al.* 2000), while n-3 fatty acids are likely to have opposing effects (Simopoulos 2001). Fatty acids in the diet are to some extent reflected in serum phospholipids (Hjartaker *et al.* 1997; Hagfors *et al.* 2005), and especially long chain PUFAs, like DHA, are found to be good biomarkers of dietary intake (Hedrick *et al.* 2012; Serra-Majem *et al.* 2012). BMI has been negatively associated with levels of n-3 fatty acids in serum phospholipids compared to normal weight populations (Fernandez-Real *et al.* 2003; Klein-Platat *et al.* 2005; Micallef *et al.* 2009; Pickens *et al.* 2015).

Studies investigating associations between diet and semen quality indicate that dietary fat can influence semen characteristics (Attaman *et al.* 2012; Jensen *et al.* 2013; Chavarro *et al.* 2014; Eslamian *et al.* 2015). Intake of SFAs has been negatively associated with sperm concentration (Attaman *et al.* 2012; Jensen *et al.* 2013), while a diet rich in n-3 PUFAs has been positively associated with sperm morphology (Attaman *et al.* 2012; Eslamian *et al.* 2015). No studies have investigated the relationship between BMI and fatty acid composition of spermatozoa.

2. Aim of the study

The aim of this project was to study semen characteristics, sperm fatty acid composition, and reproductive hormones in relation to BMI, in a population with a wide BMI range. We also wanted to examine the association between anti-Müllerian hormone levels in serum and seminal plasma, and semen characteristics. The specific objectives addressed were:

- To investigate if BMI is associated with semen quality and reproductive hormones in serum (paper I).

- To investigate whether BMI is associated with fatty acid composition of spermatozoa, and if semen characteristics are associated composition of fatty acids in spermatozoa and serum (paper II).

- To investigate if the levels of AMH in seminal plasma and serum are associated with semen characteristics, and to provide values for normal levels of AMH in seminal plasma and serum of adult males (paper III).

3. Summary of papers

Paper I:

Body mass index is associated with impaired semen characteristics and reduced levels of anti-Müllerian hormone across a wide weight range

J. M. Andersen, H. Herning, E. L. Aschim, J. Hjelmesæth, T. Mala, H. I. Hanevik, M. Bungum, T. B. Haugen, O. Witczak.

In paper I we investigated how BMI was associated with semen characteristics and reproductive hormones. This cross-sectional study comprised 166 men, including 38 severely obese men. We found that BMI was negatively associated with sperm concentration, total sperm count, progressive sperm, normal sperm morphology, and percentage of vital spermatozoa, adjusted for age and time of abstinence. A negative relationship was observed between BMI and total testosterone, inhibin B, and AMH in serum. Our main findings were that BMI was negatively associated with semen characteristics and serum levels of AMH.

Paper II:

Fatty acid composition of spermatozoa is associated with BMI and with semen quality.

J. M. Andersen, P. O. Rønning, H. Herning, S. D. Bekken, T. B. Haugen, O. Witczak.

The main aim in paper II was to investigate if fatty acid composition of spermatozoa was associated with BMI and with semen characteristics. This cross-sectional study included fatty acid analysis of serum and spermatozoa from 144 men with BMI between 18.8 - 62.7 kg/m^2. We found that BMI was negatively associated with levels of DHA, the main PUFA in spermatozoa, and palmitic acid, the main SFA. We did not find any associations between BMI and sperm levels of n-6 PUFAs. DHA was positively correlated with total sperm count, sperm concentration, sperm vitality, progressive sperm motility, and normal sperm morphology. A negative association was seen between DHA levels and DNA fragmentation index. Levels of palmitic acid of spermatozoa correlated positively with total sperm count, while linoleic acid correlated negatively. There was no correlation between DHA in serum and spermatozoa. Our main findings indicate that BMI may affect fatty acid composition of

spermatozoa. Although the mechanism is not known, it is possible that obesity may affect the regulation of fatty acid metabolism in testis.

Paper III:

Anti-Müllerian hormone in seminal plasma and serum: association with sperm count and sperm motility

J. M. Andersen, H. Herning, O. Witczak, T.B. Haugen.

In paper III, the aim was to investigate if AMH levels in seminal plasma and serum were associated with sperm count and sperm motility. The study population included 126 participants with BMI from 19-39 kg/m^2. We found that AMH in seminal plasma was positively associated with sperm concentration, total sperm count, and sperm motility, after adjusting for age, BMI and time of abstinence. We did not find any association between serum AMH and semen characteristics. Serum levels of inhibin B were strongly correlated with AMH in seminal plasma and AMH in serum. AMH levels showed large inter-individual variation in seminal plasma. The central 95% intervals for AMH levels in seminal plasma (n=120) and in serum (n=126) may reflect normal variation for AMH in adult men. Our results indicate that AMH in seminal plasma may be of importance for spermatogenesis. AMH in seminal plasma may serve as a marker of sperm production, however, in the lower range the predictive value is low. Further studies are needed to clarify the mechanisms of AMH regulation in spermatogenesis.

4. Discussion

4.1. Methodological considerations

Description of the methods is presented and discussed in the papers included. Some important aspects of study design, recruitment process, and analysis of semen, AMH and fatty acids are discussed in this section.

4.1.1. Participants and study design

General inclusion criteria for men participating in the study were age of 18 years and older and BMI ≥ 18,5 kg/m^2. Normal weight men from a fertility clinic were invited to participate if the couple was diagnosed with female factor infertility and age was ≥ 35 years. Exclusion criteria were history of cryptorchidism and cancer.

In the recruitment process, information about the project was distributed by advertisement in newspapers and public notices in the Oslo area. Men recruited from the fertility clinic got information about the project by a nurse or medical doctor. In advance, those who wanted to participate in the study received an e-mail with detailed information about the project, instructions for collection of blood and semen samples, a questionnaire regarding general health, and a consent form.

It was important to include a large group of men with BMI above 35 kg/m^2, as there was a lack of studies of BMI and semen quality including men with severe obesity. This was achieved by recruiting men from obesity clinics. Eligibility for bariatric surgery in Norway is regulated by national guidelines. Patients must have BMI ≥ 40 kg/m^2 with obesity-related reduced quality of life, or BMI between 35 – 40 kg/m^2 with co-morbidities like sleep apnea, cardiovascular disease, and/or diabetes. Patients cannot have psychological disorders and/or abuse alcohol. In the three papers presented in this thesis, we have included hormone levels and semen characteristics from both normal weight and severely obese men. By recruiting from hospitals, there might be a bias towards recruiting obese men with serious health problems. This may especially apply to the men with BMI between 35 – 40 kg/m^2 where co-morbidities are required to receive treatment by bariatric surgery. However, it is reasonable to believe that obesity-related health issues are high in any population with severe obesity.

Whether the group of men recruited from obesity clinics differs from men with BMI above 35 kg/m^2 recruited from the general population is unexplored.

The majority of normal weigh responders were young, often students, while overweight and obese men were older. This could be a weakness of the study as BMI, as well as reproductive status may be related to age. To get a more even age distribution for the normal weight group, men with BMI between 18.5 and 25 kg/m^2 at the age of 35 years and older were recruited from the fertility clinic. We hypothesized that men from couples with female factor were likely to have semen quality comparable to men recruited from the general population. In the normal weight group, approximately 50% of the men were recruited from the general population and 50% in the fertility clinic. As described in paper I, we addressed possible differences in semen quality and levels of reproductive hormones between the two subgroups of normal weight men and found them comparable. Furthermore, linear regression analysis for associations between BMI and semen characteristics was repeated without men from the fertility clinic. This did not alter the statistical significance for any of the associations seen between BMI and semen characteristics.

This study has a cross-sectional design, and as an observational study, it is suitable to investigate associations between variables (Kirkwood & Sterne 2003). However, as all data are collected at one point in time, the associations cannot be used to conclude about the sequence of events. Cross-sectional studies can therefore not provide evidence for causal relationship between associated variables. Nevertheless, indicating the existence of an association can be of value in understanding the aetiology of disease and important to generate hypotheses.

Cross-sectional studies are sensitive to bias due to non-response as the characteristics of the non-responders may differ from the characteristics of the responders. However, a study addressing recruitment bias in semen quality studies reported small differences between responders and non-responder (Stewart *et al.* 2009). Another concern is related to using volunteers, as persons with a particular characteristics may be more likely to take part in a study (Handelsman 1997). Such bias can limit the applicability of conclusions from a study to the general population. In our study population, there may be a bias towards recruiting men

with fertility problems. As we did not register history of infertility or fatherhood, we cannot rule out this confounder. As response rates in studies of semen quality are often low, it is difficult to recruit men that are representative of the general population (Cohn *et al.* 2002). However, recruitment of men with possible fertility problems is expected to be the same across BMI categories, and results may still be of value in attempting to understand the relation between adiposity and male reproductive health (Cohn *et al.* 2002).

4.1.2. Ethical aspects

The Regional Committee for Medical and Health Research Ethics, South East, Norway, approved the study and the project biobank**.** All participants provided a written consent after receiving both written and oral information about the project and their rights as participants.

4.1.3. Measurements of adiposity

BMI is simple index used to assess degree of obesity in a population, as it is easy to calculate based on height and weight. BMI is therefore commonly used to categorize adults as normal weight, overweight and obese. The classification of BMI according to the WHO is shown in Table 4. However, BMI does not reflect body composition as it does not discriminate between fat and lean mass. It can therefore be difficult to categorize accurately in the intermediate BMI groups, as lean persons with low BMI can have a fat distribution that is unfavourable (Romero-Corral *et al.* 2008). Nevertheless, BMI is the most common way to classify overweight and obesity.

In the presented papers, the WHO categories for obesity class II and III, were combined into one category referred to as severe obesity (Table 4). This modification was made in order to acquire BMI groups of similar size. In order to evaluate if BMI was an appropriate index for categorization of the study population, waist circumference and fat percentage were also measured.

Waist circumference correlates well with amount of abdominal fat, and a waist circumference ≥ 102 cm is associated with obesity-associated risk factors in men (National Heart Lung and Blood Institute 1998). However, this measure is not as useful to differentiate persons with BMI above 35 kg/m^2, as all will have waist circumference above ≥ 102 cm. To perform a

correct measurement, a tape should be placed between the upper border of the hip bone and the lower border of the rib cage. Measurement of waist circumference is prone to measurement errors (Verweij *et al.* 2013), especially in very obese individuals where these bones can be difficult to identify (Ulijaszek & Kerr 1999; Nordhamn *et al.* 2000). It is important that the persons making the measurements do it correctly, and that it is measured in the same way for all study participants.

Bioelectrical impedance analysis is an easy, relatively inexpensive, and non-invasive way to discriminate between fat and lean mass (Dehghan & Merchant 2008a) and to determine fat percentage. Analysis of fat percentage was performed using a simple hand held instrument as described in paper I. Relative body fat based on underwater weighing (Wagner & Heyward 1999) is recognised as the gold standard, and a high correlation with hand to hand measurements has been shown (Demura *et al.* 2002). However, factors like food intake, medication or body composition can influence the accuracy of the measurements (Dehghan & Merchant 2008b). Participants in our study were fasting over night before attendance where fat percentage was measured, and influence of food intake should therefore be minimalized. However, other factors that could affect the accuracy was not explored. A problem with the hand to hand instrument, was that weight ≥ 200 kg was not possible to enter. We were therefore not able to measure fat percentage in all participants with this method. However, fat percentage was strongly correlated with BMI in participants with weight < 200 kg.

Both waist circumference and fat percentage correlated strongly with BMI, indicating that BMI was an appropriate measure of adiposity for this study population.

4.1.4. Semen analysis

This study was started in 2008 and semen analysis was therefore performed according to the 1999-version of the WHO manual (World Health Organization 1999). However, sperm motility was recorded, stored as video files, and analysed when recruitment was completed in 2012. All video files were analysed for sperm motility in an external laboratory where the latest edition of the WHO manual had been implemented by this time. Results for sperm motility were therefore presented according to the latest version of the WHO manual (World

Health Organization 2010). The difference between the two editions regarding sperm motility is that the two original categories, rapid and slow progressive motility, were combined and categorized as progressive motility.

As semen analysis is a manual method based on microscopic assessment of semen characteristics, the analysis is prone to subjectivity. To achieve reproducible results from semen analysis, it is essential that technicians performing the analysis are properly trained, and that appropriate quality control is performed. Project staff was trained in basic semen analysis, and both internal and external quality control programmes were implemented in the laboratory. Training course in basic semen analysis and quality assurance programme were provided through The European Society of Human Reproduction and Embryology.

Each participant in the study provided one semen sample. Although there may be individual variation in semen composition over time, one sample is found to be representative in studies with a high number of participants (Rylander *et al.* 2009; Hart *et al.* 2015). Participants were allowed to collect the sample at home if they felt that it was more convenient, and were provided with a written instruction and a pre-weighed sample container. The instruction explained the importance of colleting a complete sample, to record the time of sample production, and to carry the sample close to their body during transport to the laboratory. They were asked to deliver the sample to the laboratory as soon as possible and within 2 hours after collection. Although the WHO recommends analysis within 1 hr, the 2 hrs limit was practiced as some participants wanted to collect the sample at home, and lived too far away to reach the laboratory within 1 hr. The time from the sample was collected until it reached the laboratory was registered. Sperm motility was only analysed for samples delivered before 2 hrs. In total, 77% of the samples were delivered and analysed within one hour after ejaculation. The delay of analysis for samples brought from home is a possible confounder. This issue is further addressed in the discussion in the part concerning statistical considerations (section 4.1.7.).

4.1.5. Fatty acid analysis

Analysis of sperm fatty acid composition was performed on centrifuged samples, and fatty acids were extracted from cells in the pellet. We cannot exclude contribution of fatty acids

from other cell types, like white blood cells. The presence of round cells was commented, but not quantified by the technicians performing semen analysis. However, round cells were observed in few samples (approximately 5%), and samples containing round cells were evenly distributed between the BMI groups. As the levels of DHA in white blood cells are low (Witte *et al.* 2010), the presence of this cell type is not expected to influence the significance of the association between DHA content in spermatozoa and semen characteristics presented in paper II.

The analysis of phospholipids in serum was performed in two different laboratories using comparable methods of gas chromatography. For half of the samples, fatty acids were extracted and analysed at HiOA. For the other half of the samples, fatty acids extraction and analysis were performed at Vitas AS. Results from HiOA and Vitas correlated strongly (*r*-values between 0.944 and 0.999), and values obtained at HiOA were adjusted using regression equation. All analyses of fatty acids in spermatozoa were performed at Vitas AS.

4.1.6. AMH analysis

The Gen II AMH assay is found to give stable results for levels of AMH in serum, and the results are not affected by proteolysis (A. Kumar *et al.* 2010). In 2013, the manufacturer of the Gen II AMH assay reported that interference from complement in the sample could give wrong results (Beckman Coulter 2013). Low reproducibility of AMH values was a problem that had been reported earlier. AMH concentrations were found to increase significantly if samples were stored at room temperature (Rustamov *et al.* 2012; Fleming *et al.* 2013) or if samples were pre-diluted (Han *et al.* 2014). Revised instructions for use of the assay were introduced in June 2013 to eliminate the complement interference problem. In the revised assay protocol, dilution of all samples and controls in assay buffer before they were added to the microplate was found to produce stable results. They also reported that the change gave higher AMH values than those obtained with the old protocol. Studies independent of the kit manufacturer confirmed that AMH concentrations were higher compared to the original assay (Bonifacio *et al.* 2015; Craciunas *et al.* 2015), in line with the study by Han and co-workers (Han *et al.* 2014). The dilution step was found to diminish the problem of room temperature storage, providing more reliable results than the original assay. It was also concluded that results obtained with the original Gen II assay should be treated with caution. AMH analysis

of serum and seminal plasma in our study were performed according to the new assay procedure introduced in June 2013 (Beckman Coulter 2013). As a consequence of the changes in the protocol, and the fact that higher AMH concentrations are obtained with the new method, our results cannot be directly compared with results from previous studies using the assay manufactured before 2013.

The Gen II AMH assay is optimized for serum, and as we wanted to use the same assay for to analyse AMH levels in seminal plasma, method testing was required. Testing of dilution curves and parallelism was performed by the external laboratory that also performed the serum analysis. The results from these tests were found satisfactory, verifying that the assay could be used to analyse AMH levels in seminal plasma. As it is shown that the assay is robust with regard to proteolysis in serum, it should also be valid for seminal plasma. Additionally, time to freezing was similar to that of serum samples.

AMH is produced in the testis, and AMH concentrations in seminal plasma are dependent on both the AMH production and the semen volume. Therefore, values for total AMH in ejaculates were used in the statistical analysis in paper III.

4.1.7. Statistics

Bias due to confounding factors are important to be aware of in association studies. To adjust for possible confounding factors, multiple linear regression (papers I-III) and partial correlation (paper II) were used for statistical analyses. Confounders were selected among the available variables based on biological plausibility. Age is a well-known confounder that has a strong influence on testosterone (Harman *et al.* 2001; Wu *et al.* 2008), and is also found to affect semen quality (Molina *et al.* 2010). All associations were adjusted for age as this variable ranged from 22 to 61 in the total cohort. There was a considerable difference in time of abstinence among the participants (1-21 days), and for this reason associations with semen characteristics were adjusted for this variable. Time of abstinence is likely to influence semen characteristics (Agarwal *et al.* 2016), and possibly also DNA fragmentation and AMH levels in seminal plasma. Adjustment for smoking was considered, as it may be a confounder for semen quality (Chia *et al.* 1994). However, the effect of smoking is not consistently

associated with semen characteristics (Davar *et al.* 2012; Hart *et al.* 2015). Additionally, as most participants in this study were non-smokers (83%), this variable was not adjusted for.

In paper II and III, time to analysis was added as a covariate, as this is a factor that may influence sperm motility (World Health Organization 1999). Samples collected at home were often delivered later than 1 hr after ejaculation. Compared to normal weight men, a larger percentage of severely obese men collected the sample at home. In paper II, partial correlations and multiple regression analyses of sperm motility in relation to other variables were therefore adjusted for a dichotomized time to analysis variable (≤ 1 hr and > 1 hr). Multiple regression analysis of sperm motility and other variables in paper I were not adjusted for time to analysis. However, adding this variable did not alter the finding of an association between sperm motility and BMI at a 5% significance level. In paper III, associations between sperm motility and other variables were adjusted for site of sample collection (at home or on site). In paper III, multiple regression models were also adjusted for BMI, as we have previously shown that BMI is associated with levels of several hormones, including, AMH, and semen characteristics.

4.2. General discussion

4.2.1. BMI and reproductive characteristics

Our observation of a negative association between BMI semen characteristics is in accordance with some previous studies (Jensen *et al.* 2004a; Koloszar *et al.* 2005; Qin *et al.* 2007; Belloc *et al.* 2014; Eisenberg *et al.* 2015). However, opposing results have been reported (Aggerholm *et al.* 2008; Chavarro *et al.* 2010; Martini *et al.* 2010; Ramlau-Hansen *et al.* 2010; Eskandar *et al.* 2012), and two meta-analyses (MacDonald *et al.* 2010; Sermondade *et al.* 2013) failed to identify clear associations between BMI and semen parameters. A problem when comparing results from studies of semen quality is that the methods of semen analysis varies greatly (Sanchez-Pozo *et al.* 2013). It is e.g. difficult to directly compare results obtained with computer assisted sperm analysis to results obtained using the manual WHO method. Additionally, many studies are based on cohorts of patients from fertility clinics (Chavarro *et al.* 2010; Macdonald *et al.* 2013; Belloc *et al.* 2014), and populations with a low

percentage of obese men (Martini *et al.* 2010; Ramlau-Hansen *et al.* 2010; Eskandar *et al.* 2012). The strength of our study is the large group of men with BMI ≥ 35 kg/m^2 recruited outside fertility clinics, and the even distribution or participants across the BMI categories.

The mechanism for how obesity may influence spermatogenesis is not clear, but it is likely that multiple factors play a role. Impairment of the HPG axis is one hypothesis often used to explain the association between obesity and impaired male reproductive health. Several studies have shown that obesity is associated with low testosterone levels (Jensen *et al.* 2004a; Camacho *et al.* 2013; Luconi *et al.* 2013). This was also apparent in our study. We found that over 30 % of the severely obese men had testosterone values below the lower reference value (10 nmol/l), while the normal weight men where all within the normal range. Testosterone is the major hormone in regulation of the spermatogenesis. Although testosterone levels in serum are low, our results in indicated that FAI in serum was unchanged in obese males. This can be explained by the parallel reduction in SHBG. Whether obesity is associated with changes in the intra-testicular concentration of testosterone remains unknown. However, it could be speculated that also testicular levels could be altered in disfavour of spermatogenesis.

We observed a positive association between BMI and oestrogen levels in serum. This finding is in line with results from other studies (Strain *et al.* 1982; Jensen *et al.* 2004a; Aggerholm *et al.* 2008; Ramlau-Hansen *et al.* 2010), but not a consistent finding (Fejes *et al.* 2005; Eskandar *et al.* 2012; Macdonald *et al.* 2013). Oestrogen mediates negative feedback to the hypothalamus and the pituitary, and increased levels may contribute to downregulating levels of GnRH, FSH, and LH (Hammoud *et al.* 2006). In addition, decreased levels of SHBG may increase the fraction of unbound oestrogen. Interestingly, we did not find associations between BMI and gonadotrophins in our study. It is possible that the increase in oestrogen is too small to exert a negative effect upon the secretion of GnRH and gonadotrophins. A recent review concluded that as the role for oestrogen in spermatogenesis is so complex, its exact role is difficult to determine (Schulster *et al.* 2016). Higher seminal plasma levels of oestrogen have been demonstrated in azoospermic men when compared to normozoospermic men (Zhang *et al.* 2010). However, if BMI is positively associated with seminal plasma levels of oestrogen is unexplored.

Sertoli cells are crucial for the development of germ cells (Mruk & Cheng 2004). Inhibin B is strongly correlated with Sertoli cell function (Anawalt *et al.* 1996) and sperm concentration (Pierik *et al.* 1998; Jørgensen *et al.* 2010), and it has been suggested that obesity may have a direct negative effect on the Sertoli cells (Winters *et al.* 2006). We observed a strong negative association between serum inhibin B levels and BMI, in line with other studies (Jensen *et al.* 2004a; Aggerholm *et al.* 2008; Pauli *et al.* 2008; Chavarro *et al.* 2010), suggesting that obesity may impair Sertoli cell function.

In paper I we also found a negative association between BMI and AMH in serum. The relationship between BMI and AMH levels in seminal plasma was examined in paper III, but no significant correlation was found. It could be speculated that the bidirectional secretion of AMH from the Sertoli cells is affected by adiposity, and that secretion towards the lumen is less affected. This association therefore needs to be further investigated before conclusions about the relationship between BMI and AMH level in seminal plasma can be drawn. We did not measure inhibin B in seminal plasma, which would have been an interesting comparison.

4.2.2. Fatty acids in spermatozoa

Sertoli cells are important for the metabolism of PUFAs that are incorporated into the cell membrane of spermatozoa (Rato *et al.* 2012). PUFAs are essential constituents in the membrane of spermatozoa, and found to be important for sperm function (Lenzi *et al.* 1996; Lenzi *et al.* 2000). We found a negative correlation between sperm DHA levels and semen characteristics. This is in line with other studies (Nissen & Kreysel 1983; Aksoy *et al.* 2006; Tavilani *et al.* 2006; Martinez-Soto *et al.* 2013), indicating that the composition of fatty acids are important for semen quality.

We also observed that the composition of sperm fatty acids, especially levels of DHA, were less favourable in men with high BMI. Levels of DHA in serum did not different between normal weight and obese men, indicating that dietary intake of this fatty acid was similar in the two groups. We therefore hypothesized that obesity may affect the efficiency of the enzymes needed for fatty acid metabolism in Sertoli cells, which could possibly contribute to impaired spermatogenesis in obese men. A study of cultured rat Sertoli cells indicated that

testosterone levels may influence biosynthesis of PUFAs (Hurtado de Catalfo & de Gomez Dumm 2005). Testosterone seemed to positively modulate the activity of delta 5- and delta 6-desaturase. Whether low testosterone levels in obese men can contribute to impaired metabolism of fatty acids in Sertoli cells remains to be explored.

4.2.3. DNA fragmentation in spermatozoa

Increased scrotal temperature may be a result of increased adipose tissue surrounding the testis, and may contribute to increased oxidative stress (Hammoud *et al.* 2006). In paper I, we found a difference in sperm DFI between normal weight men and severely obese men when comparing the two groups. However, no linear relationship was found between the two variables when age and time of abstinence was adjusted for. As there was a difference in age between normal weight and severely obese men, age is likely to explain the difference. Our results are in agreement with other studies using SCSA for detection of DNA fragmentation (Smit *et al.* 2010; Thomsen *et al.* 2014; Bandel *et al.* 2015). However, the relation between obesity and DNA damage in spermatozoa remains unclear, as a positive association has been reported using SCSA (Kort *et al.* 2006), comet assay (Chavarro *et al.* 2010), and TUNEL assay (Dupont *et al.* 2013). The different methods for measuring DNA fragmentation, are likely to detect different types of DNA damage (Bungum *et al.* 2007) which could explain some of the discrepancies between the different studies.

As there are high levels of DHA in sperm cell membrane, it has been indicated that spermatozoa are susceptible to attack from reactive oxygen species, contributing to an increase in DNA damage (Jones *et al.* 1979; Lewis *et al.* 2013). We found a negative correlation between levels of DHA and DFI, indicating that DHA has a protective effect on DNA in spermatozoa. A recent study indicated that men with a healthy diet, rich in fish, fruit, and green vegetables, had lower DFI compared to men with a more unhealthy dietary pattern (Jurewicz *et al.* 2016). However, specific fatty acids were not measured in this study, and the role of DHA, for which the main dietary source is fish, was not mentioned. We did not observe correlation between DFI and any of the other fatty acids measured.

4.2.4. AMH and male reproductive characteristics

We observed that AMH levels in seminal plasma were positively associated with sperm count and sperm motility. We also revealed great individual differences in seminal levels of AMH compared to serum levels.

The relationship between serum AMH levels and semen characteristics has been addressed previously, but with inconsistent results. A positive correlation between serum AMH levels and both sperm concentration and semen volume in a group of men undergoing infertility evaluation has been shown (Appasamy *et al.* 2007). The same group also reported that AMH levels were lower in a cohort of oligozoospermic men compared to controls (Al-Qahtani *et al.* 2005). In contrast, a German study found no difference in serum AMH levels between men with normal sperm concentration and oligozoospermic men (Tuttelmann *et al.* 2009). We did not find any significant association between serum AMH levels and semen characteristics. Our results support the finding that levels of serum AMH are not a useful predictor of spermatogenesis in general.

We found an association between levels of inhibin B and AMH in both serum and seminal plasma. This is expected as both hormones are produced by the Sertoli cells. AMH levels in both serum and seminal plasma were negatively correlated with FSH. Although AMH is initially positively regulated by FSH, the down-regulating effect of testosterone is more powerful (Josso *et al.* 1998). Our finding is in line with other studies (Muttukrishna *et al.* 2007; Tuttelmann *et al.* 2009). When adjusting for age and BMI, no significant relationship was found between levels of serum or seminal plasma AMH and total testosterone. This is not surprising as serum testosterone does not reflect the intra-testicular testosterone levels.

Our observations support a role for AMH in spermatogenesis and highlight the need to investigate the underlying mechanisms of AMH secretion in the adult male. Our data for distribution of AMH levels in seminal plasma and serum, may also reflect normal values in adult men. To our knowledge, this has not been indicated for seminal plasma levels previously. With regard to serum, our results may reflect normal AMH concentrations for samples analysed with the improved AMH immunoassay.

5. Conclusions

In this study, we have shown that standard semen characteristics and reproductive hormones are associated with BMI in an unfavourable manner. We have also described a negative association between BMI and fatty acid composition in spermatozoa, especially with DHA levels. Furthermore, DHA was positively correlated with all the semen characteristics and negatively correlated with sperm DNA fragmentation.

Our results support the notion that obesity has a negative impact on spermatogenesis. They also indicate that adiposity may contribute negatively to the fatty acid composition of spermatozoa, which could partly explain the relation between BMI and semen quality.

We found a strong correlation between AMH levels in seminal plasma and semen characteristics, indicating that AMH is important for spermatogenesis. AMH levels in seminal plasma showed large inter-individual variation. Our data may reflect normal range of AMH levels in serum and seminal plasma in the adult male population.

6. Future perspectives

Obesity seem to have an unfavourable impact on semen quality, however, it is still an open question if weight loss has a positive effect on semen quality. As the prevalence of overweight and obesity is increasing among young men, it is important to know if semen characteristics can be improved by changing lifestyle and diet. For severely obese men seeking bariatric surgery, it is also of importance to know how intervention by surgery may affect their reproductive health. It would therefore be interesting to investigate how weight loss, especially by bariatric surgery, affects semen characteristics.

The association between BMI and fatty acid composition in spermatozoa should be further investigated, especially with regard to the poor correlation between DHA composition in serum and spermatozoa. It would be interesting to conduct a similar study where information about diet and fat intake is obtained by using a food frequency questionnaire, in addition to analysis of fatty acid composition of spermatozoa and serum. Another study of interest would be to perform an intervention study where obese men with poor semen quality were given dietary supplement of DHA.

7. References

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8. Errata

Page	Line	Original text	Correction/change	Corrected text
8	4	decrease	Correction	Increase
13			Change of layout: Figurelegend for figure 8 was moved up, in line with figure	

9. Papers I – III