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Wiwat Rodprasert

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ABSTRACT

Current evidence shows increasing rates of male reproductive disorders, including cryptorchidism, hypospadias, poor semen quality, decreased testosterone level, and testicular cancer. Therefore, studies on the physiology of the male reproductive system, and the current state of male reproductive health are necessary.

In the first study, we followed the semen quality of young men for 10 years from the age of 19 years to see whether there are age-related changes in semen quality. In the second study, we compared the semen quality of young Finnish and Danish men to clarify possible differences between the countries. In the third study, we compared serum levels of reproductive hormones and insulin-like growth factor (IGF)-I) during puberty between individuals with and without a history of cryptorchidism to see whether a history of orchiopexy or delayed testicular descent is associated with alterations in the levels of these hormones.

The first study showed that sperm concentration and total sperm count do not generally increase after 19 years, indicating that the maximum sperm production capacity is often reached already in young adulthood. Sperm motility and morphology continue to improve after the age of 19 years. The second study showed that unlike in earlier studies, sperm concentrations, total sperm counts, and percentages of morphologically normal spermatozoa of Finnish and Danish men were now similar. However, Finnish men had better sperm motility. The third study showed that especially boys with a history of operated bilateral cryptorchidism had higher serum FSH and lower inhibin B levels than non-cryptorchid boys during puberty. These findings indicated an impaired Sertoli cell and/or germ cell function or number. Leydig cell function and the function of the growth hormone-IGF-I system were generally well-preserved during puberty in boys with a history of cryptorchidism.

KEYWORDS: Semen quality, sperm concentration, puberty, hormones, cryptorchidism, undescended testis

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TIIVISTELMÄ

Miehen lisääntymisterveyteen liittyvien ongelmien, kuten kivesten laskeutumattomuuden (piilokiveksisyyden), virtsaputken alahalkioisuuden, kivessyövän, heikentyneen siemennesteen laadun sekä alhaisen testosteronipitoisuuden, on raportoitu yleistyneen. Tämän vuoksi tarvitaan tutkimuksia miehen lisääntymiselimistön normaalista toiminnasta sekä miesten lisääntymisterveyden nykytilasta.

Väitöskirjan ensimmäisessä osatyössä selvitettiin tapahtuuko 19-vuotiaiden miesten siemennesteen laadussa muutoksia 10 vuoden seurannan aikana. Toisessa osatyössä verrattiin suomalaisten ja tanskalaisten nuorten miesten siemennesteen laatua, jotta nähtäisiin onko maiden välillä eroa siemennesteen laadussa. Kolmannessa osatyössä verrattiin piilokives- ja verrokkiryhmien sukupuolihormonien ja insuliinin kaltaisen kasvutekijä I:n (IGF-I) pitoisuuksia läpi murrosiän. Tässä viimeisessä osatyössä haluttiin selvittää, liittyykö hoidettuun tai spontaanisti parantuneeseen piilokiveksisyyteen muutoksia murrosiän hormoni- ja kasvutekijätasoissa.

Ensimmäisessä osatyössä todettiin, että siemennesteen siittiöpitoisuus ja siittiömäärä eivät yleisesti kasva 19. ikävuoden jälkeen. Tämä viittaa siihen, että maksimaalinen siittiötuotantokapasiteetti saavutetaan jo varhaisessa aikuisiässä. Toisessa osatyössä havaittiin, että toisin kuin aiemmissa tutkimuksissa, suomalaisten ja tanskalaisten nuorten miesten välillä ei ollut enää eroa siittiöpitoisuudessa, siittiöiden kokonaismäärässä eikä normaalien siittiöiden määrässä. Suomalaisten nuorten miesten siittiöt liikkuivat kuitenkin paremmin kuin tanskalaisten miesten siittiöt. Kolmannessa osatyössä havaittiin, että etenkin leikkaushoitoa vaatineeseen molemminpuoliseen piilokiveksisyyteen liittyi murrosiässä verrokkeja suurempi follikkelia stimuloivan hormonin pitoisuus ja verrokkeja pienempi inhibiini B:n pitoisuus. Nämä viittaavat vähentyneeseen Sertolin solujen ja/tai itusolujen toimintaan tai määrään. Murrosiän aikainen Leydigin solujen ja IGF-I-kasvuhormoni -akselin toiminta oli yleisesti ottaen normaalia piilokivesryhmässä.

AVAINSANAT: siemennesteen laatu, siittiöpitoisuus, puberteetti, hormonit, piilokives, laskeutumaton kives

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Abbreviations

AMH	Anti-Müllerian hormone
AR	Androgen receptor
ARX	Aristaless-related homeobox
ATRX	Alpha-thalassemia, mental retardation, X-linked protein
CBX2	Chromobox protein homolog 2
DAX1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DHH	Desert hedgehog gene
DHX37	DEAH-box RNA helicase
DMRT1	DM-related transcription factor 1
EMX2	Empty spiracles 2
ESR2	Estrogen receptor β
FGFR2	Fibroblast growth factor receptor 2
FSH	Follicle-stimulating hormone
GATA4	GATA binding protein 4
GW	Gestational week
hCG	Human chorionic gonadotropin
HPG	Hypothalamic-pituitary-gonadal
HSD3B2	3b-hydroxysteroid dehydrogenase type 2
HSD17B3	17b-Hydroxysteroid-dehydrogenase type 3
IGF-I	Insulin-like growth factor 1
IGFBP-3	Insulin-like growth factor binding protein 3
INSL3	Insulin-like peptide-3
LH	Luteinizing hormone
LHCGR	Luteinizing hormone/choriogonadotropin receptor
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
NR0B1	Nuclear receptor subfamily 0, group B, member 1
OpBC	Bilateral cryptorchidism with a history of operation (orchiopexy)
OpUC	Unilateral cryptorchidism with a history of operation (orchiopexy)
POR	P450 oxidoreductase deficiency
SF1	Steroidogenic factor 1

SHBG	Sex hormone binding globulin
SOX9	SRY (Sex-Determining Region Y)-Box 9
SpBC	Bilateral cryptorchidism with spontaneous testicular descent
SpUC	Unilateral cryptorchidism with spontaneous testicular descent
SRD5A2	5-alpha reductase type 2 enzyme deficiency
SRY	Sex-Determining Region Y
StAR	Steroidogenic acute regulatory protein
TDS	Testicular dysgenesis syndrome
TGCTs	Testicular germ cell tumors
TSPYL1	Testis-specific protein, Y-linked-1
WNT4	Wnt family member 4
WT1	Wilms tumor 1 (WAGR syndrome, Denys-Drash syndrome, Frasier syndrome)
ZFPM2	Zinc finger protein FOG family member 2
ZNRF3	Zinc And Ring Finger 3 genes

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Perheentupa A, Sadov S, Rönkä R, Virtanen HE, Rodprasert W, Vierula M, Jørgensen N, Skakkebæk NE, Toppari J. Semen quality improves marginally during young adulthood: a longitudinal follow-up study. Hum Reprod, 2016; 3: 502-510.

II Rodprasert W, Virtanen HE, Sadov S, Perheentupa A, Skakkebaek NE, Jørgensen N, Toppari J. An update on semen quality among young Finnish men and comparison with Danish data. Andrology, 2019; 1: 15-23.

III Rodprasert W, Koskenniemi JJ, Virtanen HE, Sadov S, Perheentupa A, Ollila H, Albrethsen J, Andersson AM, Juul A, Skakkebaek NE, Main KM, Toppari J. Reproductive Markers of Testicular Function and Size During Puberty in Boys With and Without a History of Cryptorchidism. The Journal of Clinical Endocrinology & Metabolism, 2022; 0000, 00, 1–9.

1 Introduction

The current evidence demonstrates worsening male reproductive health worldwide. Semen quality is deteriorating, and the rates of some male reproductive disorders, including, congenital cryptorchidism, hypospadias, decreased serum testosterone levels, and testicular germ cell tumors (TGCTs), have been increasing (A. M. Andersson et al., 2007; Carlsen et al., 1992; Levine et al., 2017, 2023; J. S. Park et al., 2018; Toppari et al., 2001; Travison et al., 2007; Virtanen & Toppari, 2008; Ylönen et al., 2018; Znaor et al., 2014, 2020). These findings might be because of the environmental and lifestyle factors that have changed over the years. More studies are needed to understand better these conditions' causes, trends, and physiology, which may lead to the possibility of preventing and finding better treatments.

Semen analysis is the method to assess the function and pathology of the testis, epididymis, and accessory sex glands and has an important role in evaluating male fertility (Björndahl & Holmberg, 2017; Wang & Swerdloff, 2014). In epidemiological studies, semen analysis also provides information about the fertility potential of men. The method has been standardized globally. However, some factors may influence the results of semen analysis, for example, the methods of sample collection, laboratory techniques, duration of sexual abstinence, season of sample collection, etc. These factors need to be considered when one interprets the results in clinical practice or research.

Sertoli cells are essential for the control of spermatogenesis (Meroni et al., 2019). The number of Sertoli cells is positively correlated with sperm production rate (Johnson et al., 1984). Since each Sertoli cell can support a limited number of germ cells in spermatogenesis, Sertoli cell proliferation, and final number are important for sperm production capacity. Only immature Sertoli cells proliferate. When spermatogenesis initiates, Sertoli cell proliferation stops; therefore, childhood health is essential to adult reproductive health.

Many semen quality studies are conducted in young men, 18-20 years of age; however, not much is known about the changes in semen quality after that age and whether the study of semen quality at this age reflects the full productive capacity of the testis.

Semen quality varies between geographical regions (Virtanen et al., 2017). Finnish men used to have high semen quality and low incidence of testicular cancer and cryptorchidism (K. A. Boisen et al., 2004; Jørgensen et al., 2001; Skakkebaek et al., 2016; Vierula et al., 1996). Denmark, on the other hand, was among the countries with the lowest semen quality (Jørgensen et al., 2001, 2002). Furthermore, the incidence of testicular cancer and cryptorchidism was high in Denmark (Skakkebaek et al., 2016).

Congenital cryptorchidism (undescended testis) is the most common congenital urogenital malformation in boys. The prevalence at birth among boys born with normal birth weight varies from 1.8 to 8.4% (Virtanen & Toppari, 2008). The affected individuals have an increased risk of testicular germ cell tumors (TGCTs) and infertility in adulthood (Chung & Brock, 2011; Trabert et al., 2013). Some evidence shows that cryptorchidism is associated with impaired Sertoli cell and Leydig cell function in adulthood (A. M. Andersson et al., 2004; De Gouveia Brazao et al., 2003; Lee & Coughlin, 2001). However, the testicular function during puberty in boys affected by congenital cryptorchidism is not well-known.

2 Review of the Literature

2.1 Structure, physiology, and function of the testis

Testis consists of two separate compartments: seminiferous tubule, and interstitial compartments, with differences in function and structure (A. M. Matsumoto & Bremner, 2016). The seminiferous tubule compartment is composed of germ cells and Sertoli cells and is surrounded by peritubular myoid cells (A. M. Matsumoto & Bremner, 2016). This compartment is the main component of the adult testis – contributing about 80% to 90% of the testicular volume. The interstitial compartment consists of Leydig cells, macrophages, fibroblasts, nerves, blood, and lymphatic vessels (A. M. Matsumoto & Bremner, 2016; Weinbauer et al., 2010). Testis has two important functions – hormone production and spermatogenesis.

2.1.1 Hormones in the male reproductive system

The male reproductive system is under regulation and functions through several organs and consists of numerous hormones. Hypothalamic-pituitary-gonadal (HPG) axis is the main system that controls the function of the male reproductive system through hormones. Hormones in the male reproductive system include steroids, peptides, and glycoprotein hormones. Androgens are male sex steroid hormones that induce masculine features. The main androgens are testosterone from the testis, and its active metabolite, dihydrotestosterone. Both hormones act via androgen receptors in the target cells (Handelsman, 2020). Peptide hormones include gonadotropin-releasing hormone (GnRH) from the hypothalamus and insulin-like factor 3 (INSL3) from the Leydig cells. Glycoprotein hormones include three gonadotropins - follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland, and human chorionic gonadotropin (hCG) from the placenta, thyroid-stimulating hormone (TSH) from the anterior pituitary gland, and anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), and inhibin B from Sertoli cells of the testis (Bonomi et al., 2017; Grinspon & Rey, 2010; Kanakatti Shankar et al., 2022; Rey et al., 2000; Szkudlinski, 2015). Inhibin B is the hormone in the transforming growth factor-beta (TGF-β) family (Namwanje & Brown, 2016; Robertson et al., 1995; Styne, 2020). It comprises two chains - one α

and one βB subunits - linked with a covalent bond (Robertson et al., 1995; Styne, 2020).

2.1.2 Physiology of testicular hormone production during fetal life and infancy

Fetal Leydig cells produce androgens and insulin-like peptide-3 (INSL3) starting from the gestational week (GW) 8–10 under human chorionic gonadotropin (hCG) stimulation from the placenta. Both androgens and INSL3 peak at approximately GW 16 (Achermann & Hughes, 2016; Arboleda et al., 2016; Welsh et al., 2008). The 5-α reductase enzyme converts testosterone to a more active metabolite, dihydrotestosterone, which has a crucial role in the differentiation of male external genitalia and prostate (Achermann & Hughes, 2016; Arboleda et al., 2016; Welsh et al., 2008). From the second trimester, the fetal pituitary gland is active and starts secreting follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and LH takes over hCG in the control of Leydig cell hormone production (Arboleda et al., 2016; Choi & Smitz, 2014). FSH stimulates AMH and inhibin B production from Sertoli cells. AMH induces regression of Müllerian ducts while the Wolffian ducts remain. Without AMH, Müllerian ducts develop into the uterus, the upper part of the vagina, and fallopian tubes (A. M. Matsumoto & Bremner, 2016; Rey et al., 2000). Androgens stimulate Wolffian ducts to develop into the epididymis, vas deferens, and seminal vesicle (Shaw & Renfree, 2014).

Pituitary gonadotropin levels decrease gradually towards delivery, probably due to the inhibitory effect of estrogen formed by the aromatization of testosterone (Debieve et al., 2000; Kuiri-Hänninen et al., 2014). Serum gonadotropin and testosterone levels increase again about one week after birth and reach their peaks around 1–3 months. The postnatally active HPG axis hormone production is called minipuberty (Kuiri-Hänninen et al., 2014). Subsequently, the HPG axis becomes inactive resulting in low or undetectable FSH, LH, and testosterone levels after 6 months of age(Debieve et al., 2000; Kuiri-Hänninen et al., 2014; Rey, 2014). Inhibin B level also increases during postnatal life and peaks around 3-6 months of age. In contrast to the FSH, LH, and testosterone, the inhibin level is elevated until at least the age of 15 months. Subsequently, the level decreases to the prepubertal level but is still detectable during childhood (Lanciotti et al., 2018). At puberty, the HPG axis is active again, resulting in high testosterone and normal FSH and LH levels, which continue throughout life.

At birth, AMH level is detectable and goes up significantly at three months, followed by a slight decline towards one year of age (Aksglaede et al., 2010). In childhood, the AMH level is relatively stable. When the androgens are produced

during puberty, the serum AMH level dramatically falls, and adult testis was stained negative for AMH (Lindhardt Johansen et al., 2013).

2.1.3 Testicular descent

Testis can function normally after birth when it is located in a place with a lower temperature than the core body temperature; therefore, it must descend to the scrotum at birth. The scrotal temperature is approximately 33°C, which is about 4°C lower than the core body temperature (Cobellis et al., 2014; Vikraman et al., 2016). The process of testicular descent from the abdomen to the scrotum in the fetus is divided into two phases – the transabdominal phase and the inguinoscrotal phase (Hutson et al., 2013; J. A. Mäkelä et al., 2019).

The transabdominal phase occurs approximately between GW 10 and 15 (Hutson et al., 2013). The fetal testis is anchored by the cranial suspensory ligament and the genitoinguinal ligament (gubernaculum). The former attaches the upper pole of the testis to the diaphragm, while the latter anchors the testis to the future inguinal area via the epididymis (Hutson et al., 2013; J. A. Mäkelä et al., 2019). INSL3 has an essential role in this process by binding to relaxin-family peptide receptor 2 (RXFP2) on the gubernaculum. INSL3 is a peptide hormone synthesized by Leydig cells (Harrison et al., 2019). It exerts its action by binding to a G-protein-coupled receptor (GPCR) called RXFP2 (formerly known as LGR8, GREAT, or GPR106) (Ivell & Anand-Ivell, 2011). Animal studies have revealed that INSL3 causes shortening of the proximal part of the gubernaculum and thickening of the distal end. These changes make the gubernaculum hold the testis close to the inguinal area all the time while the fetus is growing (Gorlov et al., 2002; Nef & Parada, 1999; Shono et al., 1994; Zimmermann et al., 1999). By the end of this phase, the testis is located near the internal inguinal ring.

The inguinoscrotal phase occurs approximately from GW 23-25 (Virtanen & Toppari, 2014). Androgens have an important role in this phase. The Processus vaginalis covers the testis, epididymis, and gubernaculum. These structures move together through the inguinal canal into the scrotum (Barteczko & Jacob, 2000; Virtanen & Toppari, 2014). The testis is located at the bottom of the scrotum at the end of this process.

Sertoli cells

Sertoli cells are crucial for all steps of spermatogenesis and are considered the supporters of germ cells (Meroni et al., 2019; Sharpe, 2012). They form the structural support for germ cells by surrounding spermatogonia and create a blood-testis barrier with the adjacent Sertoli cells. This barrier prevents large molecules, steroids, ions,

harmful substances, and immune cells from entering the seminiferous tubule. Only Sertoli cells and spermatogonia have direct access to the blood and the interstitial cells; therefore, Sertoli cells can send paracrine factors and nutrients to the germ cells to support and regulate spermatogenesis (A. M. Matsumoto & Bremner, 2016; Petersen & Söder, 2006). Sertoli cells are essential to all steps of spermatogenesis (O'Shaughnessy, 2014). They can support a definite number of germ cells, which is believed to be determined approximately six months before and after birth and just before puberty starts (Orth et al., 1988; Sharpe et al., 2003). Therefore, the number and function of Sertoli cells in the testis correspond to the level of sperm production (Sharpe, 2012) and determine the rate and quality of spermatogenesis (Johnson et al., 1984; Petersen & Söder, 2006; Sharpe et al., 2003). Sertoli cell proliferation in humans occurs during fetal to neonatal life and the peripubertal period (Sharpe et al., 2003). FSH increases the proliferation rate of Sertoli cells, and thyroid hormone affects the duration that proliferation can occur (Sharpe et al., 2003). Growth hormone and paracrine factors also affect Sertoli cell proliferation (Sharpe et al., 2003). Sertoli cell maturation occurs at the onset of puberty, at which Sertoli cells change their morphology, form the blood-testis barrier, and stop proliferation (Sharpe et al., 2003). Sertoli cell number also determines testicular size before puberty (Petersen & Söder, 2006). Sertoli cells express FSH and androgen receptors (A. M. Matsumoto & Bremner, 2016), both of which have a role in the regulation of spermatogenesis (O'Shaughnessy, 2014). FSH is the key regulator of Sertoli cell function (Petersen & Söder, 2006).

Leydig cells

Leydig cell population accounts for approximately 10–20% of the interstitial compartment (Fietz & Bergmann, 2017). They produce and secrete androgens, mainly testosterone (O'Shaughnessy, 2017). In fetal life, testosterone has a crucial role in the Wolffian duct differentiation into the epididymis, vas deferens, and seminal vesicles (O'Shaughnessy, 2017). Testosterone is converted by the 5-α reductase enzyme to dihydrotestosterone, which is vital for the differentiation of external genitalia to a male phenotype (Katugampola et al., 2020; Wilson et al., 1981). In blood circulation, approximately 95% of testosterone binds to two major plasma proteins – sex hormone-binding globulin (SHBG) with high affinity and albumin with low affinity (O'Shaughnessy, 2017). Free testosterone is the testosterone unbound to plasma protein; therefore, it can diffuse into the cells and exerts its action.

Also, Leydig cells produce INSL3, which has an essential role in the transabdominal phase of testicular descent in the fetus and bone metabolism in adults (Ferlin, et al., 2008; Ivell et al., 2014). INSL3 level, unlike testosterone, is not

dependent on an acute fluctuation of the HPG axis activity. INSL3 level depends on the long-term trophic effect of LH (Bay et al., 2005; Ivell et al., 2014; Ivell & Anand-Ivell, 2009); therefore it reflects the number of Leydig cells and their differentiation status (Ivell et al., 2014; Ivell & Anand-Ivell, 2009). INSL3 might act as an autocrine or paracrine in the adult testis to buffer the output from the HPG axis, which can decrease the fluctuations of the HPG axis and modulate both FSH and LH actions (Ivell & Anand-Ivell, 2009). Testosterone can convert to estradiol by aromatase enzyme (CYP19A1) in Leydig cells and peripheral tissues. Estradiol also plays an important role in the control of the HPG axis function by inhibiting hypothalamic secretion of GnRH and pituitary secretion of FSH and LH (Guercio et al., 2020; Hayes et al., 2000; Inkster et al., 1995; O'Shaughnessy, 2017; Raven et al., 2006; Serge Carreau, 2007).

2.1.4 Male puberty and regulation of testicular hormone production in adult men

The increased frequency and amplitude of the pulsatile GnRH secretion from the hypothalamus initiates puberty (Herbison, 2016; Howard & Dunkel, 2017; A. M. Matsumoto & Bremner, 2016). At the start of puberty, GnRH is secreted at night; when puberty progresses, it is secreted both day and night (Pitteloud & Dwyer, 2014). The trigger of puberty is still not clear; however, current evidence shows that GnRH secretion is regulated by kisspeptin, sex hormones, and other neuropeptides (Bonomi et al., 2017). In addition, metabolic hormones, body composition, physical growth, light, and nutritional status also play a role in controlling puberty (Castellano & Tena-Sempere, 2017; Tinggaard et al., 2012).

Kisspeptin and kisspeptin receptors have a significant role in triggering puberty (Trevisan et al., 2018). Kisspeptin, a 54-amino acid peptide, is encoded by the *KISS1* gene and secreted by the hypothalamus (Lechan, 2020; Livadas & Chrousos, 2016). Kisspeptin neurons are found in the preoptic area of the hypothalamus in human males (Bonomi et al., 2017; Herbison, 2016). Kisspeptin binds to a G-protein-coupled receptor, KISS1R (formerly GPR54), on GnRH neurons and induces GnRH release (Bonomi et al., 2017; Lechan, 2020; Styne, 2020; Trevisan et al., 2018). Pulsatile kisspeptin secretion is important for the stimulation of GnRH release (Lechan, 2020). Inactivating mutations of the *KISS1R* or *KISS1* gene cause hypogonadotropic hypogonadism with reduced GnRH secretion, resulting in decreased FSH and LH secretion from the anterior pituitary, and, finally, decreased testicular testosterone production. The patients may present with delayed puberty, incomplete puberty, congenital cryptorchidism, micropenis, and infertility (Trevisan et al., 2018). On the other hand, the activating mutations of *KISS1* or *KISS1R* lead to precocious puberty, which is a pubertal development that occurs at an earlier age

than normal (de Roux et al., 2003; Pinilla et al., 2012; Seminara et al., 2003; Tinggaard et al., 2012; Topaloglu et al., 2012; Trevisan et al., 2018). The acute injection of kisspeptin caused an increased LH and FSH secretion in healthy men (Bonomi et al., 2017). In contrast, continuous kisspeptin administration in juvenile male monkeys resulted in decreased LH secretion, which is proposed to be due to KISS1R desensitization (Styne, 2020). These kisspeptin neurons express estrogen and progesterone receptors (Lechan, 2020). Some neurotransmitters, such as neurokinin B, dynorphin, norepinephrine, and dopamine, also modulate GnRH release (Bonomi et al., 2017).

GnRH stimulates FSH and LH secretion from the anterior pituitary gland, which leads to androgen synthesis and spermatogenesis (Howard & Dunkel, 2017; A. M. Matsumoto & Bremner, 2016). The first physical sign of male puberty is generally defined as a testicular size of 3 mL or above as measured by an orchidometer (Biro et al., 1995; Palmert & Dunkel, 2012), which reflects the growth of seminiferous tubules and spermatogenesis (Tinggaard et al., 2012). This is followed sequentially by pubic hair growth, peak height velocity, spermarche (the first time that the mature spermatozoa are found in the urine), ejaculatory (first conscious ejaculation), and voice break (Tinggaard et al., 2012). Normal, healthy boys enter puberty at the Tanner stage of genitalia G2 between the age of 9.5 and 14 years (Howard & Dunkel, 2017). Puberty is associated with increased serum FSH, LH, testosterone, inhibin B, growth hormone, IGF-I, and INSL3 levels (A. M. Andersson et al., 1997; Ferlin et al., 2006; Ivell et al., 2014). The Tanner staging system of pubertal assessment is shown in Figure **1** and **Table 1** and **Table 2**.

Table 1. Assessment of stages of male pubic hair (P) by Tanner Staging System (Finlayson, Courtney Anne Styne & Jameson, 2016; Marshall & Tanner, 1970).

Tanner stages of pubic hair (P)	Descriptions
P1	Preadolescent. No pubic hair
P2	Sparse growth of long, slightly pigmented, downy hair, straight or only slightly curled. The hair appears primarily at the base of the penis.
P3	Hair is considerably darker, coarser, and mostly curled. Hair spreads sparsely over the pubic junction.
P4	Adult hair type, but the covered area is smaller than in most adults.
P5	Hair is adult in quantity and type and distributed as an inverse triangle of the classically feminine pattern. Spread has occurred to the medial surface of the thighs but not up to the linea alba or elsewhere above the base of the inverse triangle.
P6	Pubic hair spreads further beyond the triangular pattern.

Table 2. Assessment of stages of male genitalia (G) by Tanner Staging System (Marshall & Tanner, 1970).

Tanner stages of genitalia (G)	Descriptions
G1	Pre-adolescent. Testes, scrotum, and penis are of about the same size and proportion as in early childhood.
G2	The scrotum and testes have enlarged and there is a change in the texture of the scrotal skin. There is some reddening of the scrotal skin.
G3	Growth of the penis has occurred, at first mainly in length but with some increase in breadth. There has been further growth of testes and scrotum.
G4	Penis further enlarged in length and breadth with the development of the glans. Testes and scrotum further enlarged. There is also further darkening of the scrotal skin.
G5	Genitalia adult in size and shape.

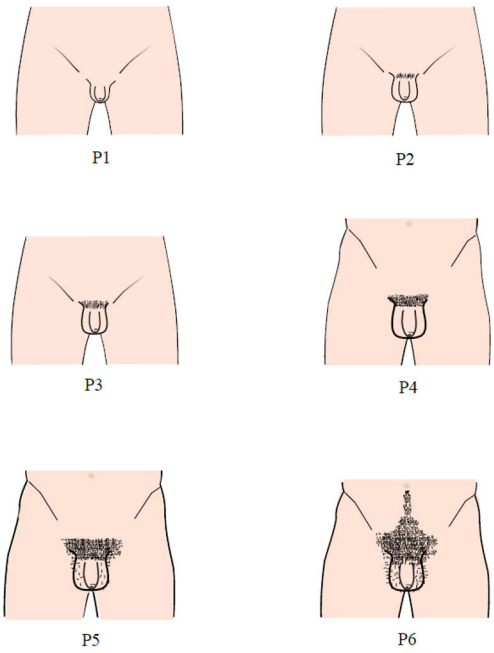

Figure 1. Tanner staging system of pubic hair in boys.

Androgens are crucial for the development and function of the male reproductive system (Luetjens, Marc C & Weinbauer, 2012). Testosterone is the main androgen secreted by Leydig cells (O'Shaughnessy, 2017). Androgens act by binding to the androgen receptor, which belongs to the steroid hormone nuclear receptor family

(Davey & Grossmann, 2016; Luetjens, Marc C & Weinbauer, 2012). The increased testosterone secretion leads to the growth of face and body hair, genitalia, spontaneous erection, increased libido, initiation of spermatogenesis, seminal fluid production, increased muscle mass, and decreased body fat (Richmond & Rogol, 2007; Wei & Crowne, 2016). The effects of androgens on bone growth, maturation and maintenance of bone mineral density are mediated via the conversion from androgens to estrogens (Cooke et al., 2017; Vanderschueren et al., 2012). In males, estrogens are produced by the conversion from androstenedione into estrone and from testosterone into estradiol by the aromatase enzyme. The aromatase enzyme is expressed in Leydig cells and Sertoli cells in the testis and peripheral tissues, including adipose tissue, brain, hair follicles, and fibroblasts. Adipose tissue is the principal source of estrogen production in males (Cooke et al., 2017; Hammes & Levin, 2019; Rochira et al., 2015). Estrogens bind to estrogen receptors expressed on the bone (Cooke et al., 2017; Hammes & Levin, 2019; Rochira et al., 2015). The number of germ cells rises markedly along with increased testicular growth and length of seminiferous tubules during puberty (Müller & Skakkebæk, 1983).

Testosterone exerts negative feedback inhibition on LH release directly by testosterone and indirectly by the conversion into estradiol (Moore & Winters, 2017). Inhibin B sends an inhibitory signal to reduce FSH release (Meachem et al., 2001; Moore & Winters, 2017). Many health conditions influence GnRH secretion, for instance, infection, malnutrition, psychological problems, and chronic diseases (Bonomi et al., 2017).

The role of growth hormone/insulin-like growth factor I system in male reproductive system

Growth hormone (GH) is a peptide hormone synthesized and secreted from the somatotrophs in the anterior pituitary gland in a pulsatile fashion (Ranke & Wit, 2018). It acts on the growth hormone receptor (GHR), a cytokine receptor found in several tissues, including the liver, muscle, bone, growth plates, and gonads (Juul & Skakkebæk, 2019). It acts on the liver and the epiphyseal growth plates in the long bones and vertebra (Ranke & Wit, 2018). At the liver, it causes an increased expression of genes encoding insulin-like growth factor (IGF)-I and IGF-II, insulin-like growth factor-binding protein 3 (IGFBP-3) and IGFBP-5 and acid-labile subunit (Ranke & Wit, 2018). IGF-I and IGF-II are the primary acting hormone for linear bone growth (Juul & Skakkebæk, 2019). Even though the major source of IGF-I is the liver, almost all other tissues can also produce and secrete IGF-I (Holly & Perks, 2012). During puberty, there is a pulsatile secretion of GH, leading to increased serum GH and IGF-I levels in association with linear growth (Juul & Skakkebæk, 2019; Styne, 2003). Serum IGFBP-3 also rises at the same time (Styne, 2003).

Some evidence suggests the role of the GH-IGF-I axis on the male reproductive system. GH and IGF-I receptors are identified in germ cells, Leydig cells, Sertoli cells, and the prostate (Juul & Skakkebæk, 2019). Growth hormone promotes growth and development of the testis and stimulates spermatogenesis and steroidogenesis in the adult testes (Hull & Harvey, 2014). Animal studies showed that IGFs control Sertoli cell number, testicular size and FSH action (Griffeth et al., 2014). They also have a role in differentiating germ cells, mediating the proliferation of fetal Leydig cells, and steroidogenic function of adult Leydig cells (Griffeth et al., 2014). Boys with isolated GH deficiency may present with micropenis or cryptorchidism (Lee, 2018; Velasquez Urzola et al., 1999). In addition, GH treatment results in penile growth in most boys (Juul & Skakkebæk, 2019). Koskenniemi et al. reported that serum IGF-I level of the boys at the age of 3 months was positively associated with the distance of the testis from the pubic bone (Koskenniemi et al., 2018). However, knowledge of the role of the GH-IGF axis in the male reproductive system during puberty is limited (Juul & Skakkebæk, 2019).

2.1.5 Human spermatogenesis

Spermatogenesis is a complex process of the production of male germ cells, which takes place in the seminiferous tubules (Kretser et al., 1998; Neto et al., 2016). It is divided into three phases – mitotic (proliferative), meiotic phase, and spermiogenesis (A. M. Matsumoto & Bremner, 2016).

In the mitotic phase, diploid spermatogonial stem cells undergo mitotic divisions and develop into spermatocytes (Goldberg & Zirkin, 2018). Ad spermatogonia have a low proliferation rate; therefore, they are considered spermatogonial stem cells (A. M. Matsumoto & Bremner, 2016). They undergo mitotic divisions to form Ap, followed by B spermatogonia. B spermatogonia undergo mitotic division to form preleptotene spermatocytes, which enter a prolonged meiotic phase of 24 days (A. M. Matsumoto & Bremner, 2016).

In the meiotic phase, diploid primary spermatocytes undergo the first meiotic division (meiosis I). Preleptotene sequentially converts to leptotene, zygotene, pachytene, and diplotene primary spermatocytes and, finally, haploid secondary spermatocytes (Kretser et al., 1998; A. M. Matsumoto & Bremner, 2016). Subsequently, secondary spermatocytes undergo a second meiotic division (meiosis II) and convert into round spermatids (Goldberg & Zirkin, 2018; A. M. Matsumoto & Bremner, 2016).

Spermatogonia and Sertoli cells stay near the basement membrane of the seminiferous tubules (Chen et al., 2017; Kretser et al., 1998). When spermatogenesis progresses, germ cells move further away from the basement membrane towards the

seminiferous tubular lumen until, finally, the mature spermatids are located near the lumen of the seminiferous tubule.

Spermiogenesis is the last phase of spermatogenesis, in which round spermatids transform to elongated spermatids and finally to mature spermatozoa (Goldberg & Zirkin, 2018; A. M. Matsumoto & Bremner, 2016). The main changes in this phase include condensation of the nucleus, movement of the nucleus to the periphery of the cells, formation of the acrosomal cap, which is a modified lysosome containing proteolytic enzymes used for penetration of the ovum, formation of the sperm tail and phagocytic removal of excess cytoplasm of spermatids by Sertoli cells (Goldberg & Zirkin, 2018; Kretser et al., 2016; A. M. Matsumoto & Bremner, 2016).

After spermiogenesis finishes, mature spermatozoa are released from Sertoli cells into the seminiferous tubule lumen, which is called spermiation (A. M. Matsumoto & Bremner, 2016; O'Donnell et al., 2011). Subsequently, they are transferred to the rete testis and then to the caput epididymis, vas deferens, and seminal vesicles, respectively (A. M. Matsumoto & Bremner, 2016). In the epididymis, spermatozoa have biochemical and morphological changes, which improve their function, including improved their movement, ability to bind with zona pellucida, to induce acrosome reaction, to fuse with vitellus and to form male pronuclei (Cooper & Yeung, 2010; A. M. Matsumoto & Bremner, 2016). As a result, spermatozoa have a better ability to fertilize oocytes (Cooper & Yeung, 2010).

The duration of spermatogenesis from Ap spermatogonia to the release of mature spermatozoa into the seminiferous tubular lumen takes approximately 74 days in humans (Neto et al., 2016). The epididymal transit time of spermatozoa is approximately 12 to 21 days. Therefore, any external factors that disturb spermatogenesis may show low sperm count after three months (Cooper & Yeung, 2010; A. Matsumoto & Bremner, 2016)

Endocrine and paracrine regulation of spermatogenesis

Spermatogenesis is mainly regulated by FSH and androgens (O'Shaughnessy, 2014). FSH has a role in the germ cells before meiosis, and androgens have a role in germ cell differentiation. LH, estradiol, inhibins, activins, follistatin and other paracrine factors also have a role in spermatogenesis (Chen et al., 2017; J.-A. Mäkelä & Toppari, 2017).

FSH

FSH controls spermatogenesis by binding to its receptor on Sertoli cells and increased intracellular cyclic adenosine monophosphate (cAMP) production (J.-A. Mäkelä & Toppari, 2017), which activates multiple intracellular signaling pathways,

including cAMP-dependent protein kinase A (cAMP-PKA), mitogen - activated protein kinase (MAPK), Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-protein kinase B (Akt), intracellular calcium and phospholipase A2 pathways (Chen et al., 2017; J.-A. Mäkelä & Toppari, 2017). FSH induces Sertoli cell proliferation in fetal life and just before puberty (Oduwole et al., 2018). After puberty, FSH controls Sertoli cell function, which supports spermatogenesis (Chen et al., 2017). The action of FSH determines the number of Sertoli cells (Oduwole et al., 2018). FSH increases spermatogonial and spermatocyte numbers and also affects spermiation, a process in which mature sperms are released from Sertoli cells into the lumen of the seminiferous tubule (O'Donnell et al., 2011; O'Shaughnessy, 2014). Men with inactivating mutations of FSHβ had azoospermia, while men with inactivating mutation of FSHR had oligozoospermia. These findings suggested that FSH is crucial for germ cell development. Evidence shows that FSH is not mandatory in spermatogenesis (Kumar et al., 1997). Men with inactivating mutations of the *FSHβ* or *FSHR* genes are fertile, probably because the FSH receptor can have some function even without FSH binding (Chen et al., 2017). Furthermore, the administration of hCG or LH in men who had FSH and LH suppression was able to restore spermatogenesis, even though the sperm counts were below half of normal (J.-A. Mäkelä & Toppari, 2017). In contrast, potent FSH receptor stimulation in men who lack androgens can cause spermatogenesis, suggesting that FSH and testosterone alone can cause spermatogenesis if its level is adequately high (Huhtaniemi, 2015).

FSH also stimulates the production of inhibin B, which belongs to the transforming growth factor-beta (TGF-β) family (Namwanje & Brown, 2016; Robertson et al., 1995; Styne, 2020). Inhibin B suppresses pituitary FSH secretion to keep the function of the pituitary-testicular axis balanced.

LH and androgens

LH regulates spermatogenesis by inducing testosterone biosynthesis from Leydig cells causing high intratesticular testosterone levels. It binds to the luteinizing hormone/choriogonadotropin receptor (LHCGR) on Leydig cells and stimulates enzymes in the testicular steroidogenic pathway and transcription factors required for sperm production (Chen et al., 2017). LHβ gene mutation caused hypogonadism with delayed puberty and infertility, low serum testosterone level, spermatogenic arrest with absent Leydig cells, and decreased spermatogenesis (Weiss et al., 1992).

Androgens

Testosterone is crucial for spermatogenesis both for the initiation and the maintenance of spermatogenesis (J.-A. Mäkelä & Toppari, 2017). All testicular cells, except germ cells, express androgen receptors (AR) (Zhou et al., 2002). Intratesticular testosterone level is markedly higher than testosterone level in serum, which is crucial for spermatogenesis (Jarow et al., 2001; Roth et al., 2010; Takahashi et al., 1982). Testosterone controls spermatogenesis by exerting its actions via Sertoli cells and peritubular myoid cells (Welsh et al., 2009). Testosterone binds to AR on Sertoli cells and induces Sertoli cell function to support spermatogenesis. Testosterone is important in the development of round to elongated spermatids and in spermiation (Huhtaniemi, 2015). It is also an anti-apoptotic factor for spermatocytes and spermatids (Huhtaniemi, 2015; O'Shaughnessy, 2014). However, a more recent study showed that high intratesticular testosterone is unnecessary for spermatogenesis since exogenous testosterone administration to the lhr-/- mice can cause spermatogenesis, even though the intratesticular testosterone level is much lower than normal (Oduwole et al., 2014).

In summary, FSH and androgens are important for spermatogenesis; however, spermatogenesis can occur when one of these hormones is adequately high (Huhtaniemi, 2015).

Other factors involved in spermatogenesis

Animal studies showed the role of estrogen, activins, inhibin, follistatin, and other paracrine factors on spermatogenesis. Estradiol is synthesized in the testis by the conversion from testosterone by aromatase enzyme (Carreau et al., 2012). In men, estrogen receptors are found on Leydig cells, young germ cells, and mature spermatozoa (Carreau et al., 2012). Animal studies suggest the role of estrogen in the proliferation, differentiation, and apoptosis of germ cells (Carreau et al., 2012). Activins are produced by pituitary gonadotrophs, Sertoli cells, and germ cells of the testis. At the pituitary gland, they have an autocrine effect of stimulating FSH secretion, which is opposite to the effect of inhibin B (A. M. Matsumoto & Bremner, 2016). At the testis, activins have a role in the maintenance of spermatogenesis and support the development and function of Sertoli cells (O'Shaughnessy, 2014).

Factors influencing spermatogenesis

Many factors are associated with decreased or impaired spermatogenesis. These factors are summarized in **Table 3**.

Table 3. Conditions associated with impaired spermatogenesis (Anawalt, 2013; Esteves, 2015; Rodprasert et al., 2019).

Classification	Diseases/conditions
Primary testicular dysfunction	Chromosomal aberrations e.g. Klinefelter syndrome
	Y-chromosome microdeletions
	Cryptorchidism
	Testicular cancer
	Pelvic irradiation
	Pelvic surgery
	Testicular trauma
	Infection e.g. mumps orchitis
	Autoimmune disease
	Drugs, chemotherapy
	Substance abuse: tobacco, marijuana
	Alcohol
	Morbid obesity
	Large varicocele
	Idiopathic
	Environmental chemicals, for example, phthalates, polychlorinated biphenyls, and dioxins
Hypothalamic-pituitary disorders	Tumors
	Infiltrative disease
	Hyperprolactinemia
	Hemochromatosis
	Congenital hypogonadotropic hypogonadism, including Kallmann syndrome, Prader-Willi syndrome
	Leptin deficiency/resistance
	Substance use: opioids, anabolic steroids
	Obesity
Thyroid disease	Hypothyroidism
	Hyperthyroidism
Adrenal disease	Cushing's syndrome
	Congenital adrenal hyperplasia
	Testicular adrenal rests

Genetic factors

Chromosomal aberrations and genetic defects can cause abnormal spermatogenesis. Klinefelter syndrome is associated with hypergonadotropic hypogonadism, very small testis, and infertility. Azoospermia is observed in 90–96% of the nonmosaic

form (47,XXY) of Klinefelter syndrome and 50–74% of mosaic forms (46,XY/47,XXY) (Samplaski et al., 2014). Y chromosome (Yq) microdeletion can be detected in approximately 7.4% of infertile men, 9.7% of azoospermic men, and 6.0% of oligozoospermic men (Massart et al., 2012).

Hormonal dysfunction

Patients with congenital hypogonadotropic hypogonadism have low serum FSH and LH, resulting in a lack of spermatogenesis and low serum testosterone level (Boehm et al., 2015; Swee & Quinton, 2019). This condition is one of the rare treatable conditions of male factor infertility (Boehm et al., 2015). In these patients, spermatogenesis can be induced by pulsatile GnRH administration by pump or gonadotropin injection by hCG injection alone or in combination with recombinant human FSH (Boehm et al., 2015). Patients with androgen synthetic defects such as mild forms of luteinizing hormone/choriogonadotropin receptor (*LHCGR*) mutations, StAR, P450scc, 3β-HSD, 17β-HSD gene mutations and patients with partial androgen insensitivity syndrome may present with oligospermia or azoospermia (Guercio et al., 2015).

Urological diseases

Some urological diseases are associated with decreased semen quality. A multicenter study in six European countries demonstrated that varicocele was associated with low semen quality, particularly the high-graded type (Damsgaard et al., 2016). Higher serum FSH and lower inhibin B levels reflecting an impaired Sertoli cell function were observed in more advanced grades of varicocele. In addition, high serum LH and normal testosterone levels were found in men with varicocele, suggesting compensated Leydig cell dysfunction (Damsgaard et al., 2016). A meta-analysis in 2016 reported that varicocele was associated with 45% lower sperm count, 27% lower sperm motility and 20% lower percentage of sperm morphology as compared with controls (Agarwal et al., 2016). Hydrocele may cause a pressure effect on the testis and is associated with the arrest of spermatogenesis (Dandapat et al., 1990). Sexually transmitted diseases from some organisms, for example, Chlamydia trachomatis, Ureaplasma spp, human papillomavirus, hepatitis B, and HIV are related to decreased sperm count and sperm motility, and cytomegalovirus infection is associated with reduced sperm motility (Gimenes et al., 2014). A history of cryptorchidism increases the risk of poor semen quality (discussed below).

Environmental chemical exposure

Some evidence suggests a relationship between postnatal exposure to some endocrine-disrupting chemicals and poor semen quality (Rodprasert et al., 2019). Polychlorinated biphenyls (PCBs) exposure is negatively associated with semen quality, particularly low sperm motility (Meeker & Hauser, 2010). Perinatal or prepubertal exposure to dioxins was associated with low semen quality (Mínguez-Alarcón et al., 2017; Mocarelli et al., 2008, 2011). Exposure to phthalates relates to poor semen quality (Cai et al., 2015; Høyer et al., 2018; Radke et al., 2018). There is also small evidence that supports an association between prenatal exposure to endocrine disrupting chemicals and low semen quality. These include perinatal exposure to 2,3,7,8 tetrachlorodibenzo-p dioxin, PCBs, and polychlorinated dibenzofurans from occupational or environmental accidents (Guo et al., 2000; Mocarelli et al., 2011). This is one of the possible explanations for the differences between geographical regions.

Obesity

Obese men have increased conversion from testosterone to estrogen by the aromatase enzyme, which is expressed in adipose tissues (Schneider et al., 1979). Obesity is associated with elevated circulating estrogen levels, which suppresses GnRH from the hypothalamus and FSH and LH from the anterior pituitary gland (Chimento et al., 2014; Neto et al., 2016), resulting in impaired spermatogenesis (du Plessis et al., 2010; Neto et al., 2016). Other mechanisms of poor semen quality in obese men are sleep apnea and elevated scrotal temperature (du Plessis et al., 2010). Numerous clinical studies showed poor semen quality in obese men, especially decreased sperm concentration and total sperm counts, and in some studies, low sperm motility (Andersen et al., 2015; Palmer et al., 2012). Furthermore, weight loss in obese men is related to increased semen volume and total sperm counts (Håkonsen et al., 2011). Since the prevalence of obesity has been markedly increasing in males worldwide (Chooi et al., 2019), obesity might also play a role in declining semen quality.

Diabetes mellitus

A meta-analysis of studies in men with type 1 and type 2 diabetes mellitus in 2016 revealed that men with diabetes mellitus had reduced semen volume and sperm motility. However, the total sperm count and percentage of sperm with normal morphology were normal (Pergialiotis et al., 2016).

Lifestyle changes

A meta-analysis of 20 studies with 5865 participants demonstrated that a man's cigarette smoking is related to decreased sperm count, sperm motility, and sperm morphology (Sharma et al., 2016). In addition, several studies showed that maternal smoking during pregnancy is associated with low semen quality in sons (Jensen et al., 2004; Virtanen et al., 2012). However, some studies found that this association is observed only in sons whose mothers smoke more than ten cigarettes per day (Storgaard et al., 2003). A meta-analysis of 15 cross-sectional studies in 16,395 men in 2016 demonstrated that alcohol consumption has adverse effects on semen volume and sperm morphology compared to no alcohol use (Ricci et al., 2017). This effect seemed limited to men who consumed alcohol daily (Ricci et al., 2017).

Abnormal spermatogenesis

Sertoli cell-only syndrome is a histopathologic condition, not a clinical diagnosis, characterized by an absolute absence of germ cells (Nieschlag et al., 2010). Seminiferous tubules contain only Sertoli cells and have a small tubular diameter, but the Leydig cells are usually normal. Therefore, affected individuals have azoospermia with normale androgenization (Nieschlag et al., 2010). Testicular volume decreases, serum FSH level increases, and inhibin B level reduces, whereas serum testosterone level is normal (A.-M. Andersson et al., 1998; Bohring & Krause, 1999; Nieschlag et al., 2010). This condition is caused by Klinefelter syndrome, Y chromosome microdeletion, cryptorchidism, irradiation, cytotoxic drugs and viral infections (A.-M. Andersson et al., 1998; Bohring & Krause, 1999; Nieschlag et al., 2010; Stouffs et al., 2016).

A spermatogenic arrest is the interruption of the maturation of germ cells (Nieschlag et al., 2010). It can be caused by trisomy, Y chromosome abnormalities, radiotherapy, chemotherapy, antibiotics, heat, liver, kidney disease, and sickle cell anemia. It is identified from the testicular biopsy in approximately 4-30% of men with fertility problems. The testicular size is normal. Testicular biopsy is the definitive diagnosis (Nieschlag et al., 2010).

Semen analysis

Semen is composed of spermatozoa and secretions from the accessory sex organs, including seminal vesicles (65-75%), prostate (25-30%), vas deferens (5-10%), bulbourethral (Cowper's) glands (1-2%) and a small amount from the epididymis (WHO, 2010; Revenig et al., 2014). The contribution from each accessory gland changes according to sexual abstinence duration and the method of the semen sample collection. Standard semen analysis consists of semen volume, pH, sperm

concentration, total sperm count, sperm motility, and sperm morphology (WHO, 2010).

The use of semen analysis as a predictor of fertility

Semen analysis is the first test for evaluating a man in a subfertile couple (WHO, 2010; Wang & Swerdloff, 2014). It also helps to predict the chance of spontaneous conception, identify causes of male infertility, and plan for assisted reproduction (Leushuis et al., 2014; WHO, 2010; Barratt et al. 2017). No single semen parameter is considered the best marker for predicting fertility over other parameters because many factors are related to infertility, such as sperm concentration variability, methods of fertilization, men's health, and female fertility (Slama, 2002).

There is an association between semen quality and fertility (Guzick et al., 2001); however, the use of semen analysis variables as predictors of fertility has low accuracy (Wang & Swerdloff, 2014). Each semen variable provides different information about spermatogenesis, sperm maturation, and the male reproductive tract and can predict fertility independently (Björndahl & Holmberg, 2017). One study found that sperm concentration of more than $48.0x10^6$ per mL, sperm motility above 63%, and morphologically normal spermatozoa of more than 12% are in the fertile range. In contrast, sperm concentration below $13.5x10^6$ per mL, sperm motility of less than 32%, and a percentage of sperm with normal morphology of less than 9% are in the subfertile range. The semen variables between the fertile and subfertile ranges designated indeterminate fertility (Guzick et al., 2001). The chance of pregnancy rises considerably when sperm concentration increases to 40 million/mL (Sharpe, 2012). However, when sperm concentration exceeds 40 million/mL, the pregnancy rate no longer increases (Bonde et al., 1998; Sharpe, 2012). Sperm concentration also has an effect on time to pregnancy up to the concentration of 55 million/mL (Slama, 2002).

The lower normal reference limit of the percentage of sperm with normal morphology is 4% according to strict criteria (WHO, 2010). The probability of conception is positively associated with the percentage of sperm with normal morphology of up to 19% by strict criteria (Slama, 2002). In addition to the prediction of spontaneous pregnancy, sperm morphology also has a role in predicting the success of in vitro fertilization. The high percentage of morphologically normal spermatozoa, notably more than 14% by strict criteria, is associated with increased fertilization and pregnancy rate from in vitro fertilization (Kruger et al., 1986, 1988). However, even men with morphologically normal spermatozoa of 0% can still have a high rate of successful spontaneous and assisted pregnancy (Danis & Samplaski, 2019). Therefore, current evidence suggests that the role of sperm morphology in

predicting the success of assisted reproductive technology is limited (Danis & Samplaski, 2019).

Studies using computer-assisted sperm analysis reported that sperm motility is associated with fertility (Slama, 2002). However, sperm motility does not have a role in in-vitro fertilization and intra-cytoplasmic sperm injection, except if motility is absent (Wang & Swerdloff, 2014).

The reference values of the semen variables stated in the World Health Organization (WHO) manual of semen analysis in 2010 are derived from data from several prospective and retrospective studies in men whose partner had a time to pregnancy of 12 months or less (Cooper et al., 2009). In 2021, WHO released updated data on semen quality by adding newly published data between 2010 and 2020 from 5 more countries, including Asian and African countries, in which the data from these continents were lacking in WHO 2010. **Table 4** shows lower reference limits (fifth percentiles and 95% confidence intervals) of semen variables from WHO 2021 (WHO, 2021). Semen analysis above the 5th percentile of each variable suggests that the primary cause of the couple's infertility is less likely male infertility.

Table 4. Lower reference limits of the semen parameters according to the WHO laboratory manual of semen analysis 2021 (WHO, 2021).

Parameter	Lower reference limit (5^{th} centiles and 95% confidence intervals
Semen volume (ml)	1.4 (1.3-1.5)
Total sperm number (10^6 per ejaculate)	39 (35-40)
Sperm concentration (10^6 per ml)	16 (15-18)
Total motility (progressive + non-progressive motility, %)	42 (40-43)
Progressive motility (%)	30 (29-31)
Vitality (live spermatozoa, %)	54 (50-56)
Sperm with normal morphology (%)	4 (3.9-4.0)

Use of semen analysis in the clinical evaluation of male reproductive health

Semen volume indicates the patency of the male duct system and the accessory gland function. The markedly low semen volume is found in men with severe androgen deficiency, obstruction of ejaculatory ducts or bilateral absence of vas deferens, or spill of the sample (Wang & Swerdloff, 2014). Sperm concentration indicates the testicular capacity to produce sperm. Sperm concentration and semen volume are

used for the calculation of sperm output (Wang & Swerdloff, 2014). Secretion from the prostate gland is acidic, whereas the secretion from seminal vesicles is alkaline (WHO, 2010). Therefore, patients with obstruction of the ejaculatory duct or congenital bilateral absence of the vas deferens have semen pH of less than 7.0, a semen volume below 1.0 mL, and a low total sperm count (Daudin et al., 2000; WHO, 2010). Low sperm motility is found in patients with infection or inflammation of the male reproductive tract or abnormal sequence of ejaculation or ejaculatory duct obstruction (Björndahl & Holmberg, 2017). Severely impaired sperm motility or immotility can be found in immotile cilia syndrome or other diseases of sperm tails. Abnormal sperm morphology is found in men with abnormal spermatogenesis. Excess residual cytoplasm is related to epididymal disorders (Björndahl & Holmberg, 2017).

Geographical differences in semen quality

Studies showed geographical differences in semen quality – both the studies in young men and fertile men (Virtanen et al., 2017). Numerous semen quality studies have been conducted around the world; however, different research protocols were used, and therefore it is challenging to compare semen quality between the countries. The multi-national studies in Europe and Japan using the same research protocol with external quality control allow us to compare semen quality between the countries.

A study in 1,082 fertile men in four cities – Copenhagen, Denmark; Edinburgh, Scotland; Paris, France, and Turku, Finland published in 2000 demonstrated that Finnish men had the highest sperm concentrations followed by Scottish, French, and Danish men (Jørgensen et al., 2001). Scottish men had the highest percentage of motile sperm, followed by Finnish, Danish, and French men. The percentages of morphologically normal spermatozoa were not statistically different (Jørgensen et al., 2001). Studies in young men from the general population in Northern Europe revealed that sperm concentrations of the men in Estonia, Finland, Lithuania, Denmark, and Norway were 57, 54, 55, 41, and 41 million/mL, respectively (Jørgensen et al., 2002; Punab et al., 2002). Using a similar research protocol as the previous study, median sperm concentrations of the young men in Japan and Spain were high, 59 and 62 million/mL, respectively (Fernandez et al., 2012; Iwamoto et al., 2013). The young German men had a sperm concentration of 42-46 million/mL, which was close to the level of the Norwegian and Danish men (Paasch et al., 2008).

Regional variation of semen quality might be explained by differences in ethnic background and environmental exposure and lifestyles. Testicular histology studies have demonstrated that there are differences in the number of testicular cells and components among the men from Chinese, Hispanic and European origin (Kretser

et al., 1998). The testicular weight was highest among Europeans, followed by Hispanics and Chinese. The amount of seminiferous epithelium was highest in the Hispanic, followed by Chinese and the Europeans (Kretser et al., 1998). These results suggested that the degree of spermatogenesis per seminiferous tubule may vary depending on ethnicity. Autopsy data revealed that Chinese men had smaller testis, lower number and function of Sertoli cells, and lower sperm production per day as compared to Hispanic or Caucasian men (Johnson et al., 1998). A US study reported that germ cell apoptosis is more common in Chinese than in Caucasian men (Sinha Hikim et al., 1998). These data demonstrated that ethnicity and geographical location have an influence on semen quality. One US study investigated semen quality of men with different ethnicity who came to a single infertility center (Khandwala et al., 2017). This study showed that white men had higher semen volume, but lower sperm concentrations and total sperm counts than those of the Asian men. A higher proportion of white men had semen quality parameters in the suboptimal range or had azoospermia. These findings remained true even after adjustment for body mass index (Khandwala et al., 2017).

Adverse secular trend of male reproductive health

Current evidence suggests that there is a decline in semen quality and an increased prevalence of some male reproductive disorders in many countries, including testicular germ cell tumors (TGCTs) and hypospadias (J. S. Park et al., 2018; Skakkebaek et al., 2016; Ylönen et al., 2018; Znaor et al., 2014, 2020). In addition, the increased prevalence of cryptorchidism in Denmark and the UK and decreased serum testosterone levels in Denmark and the US have also been reported (A. M. Andersson et al., 2007; Skakkebaek et al., 2016; Travison et al., 2007). It has been proposed that these findings are related to a testicular dysgenesis syndrome (Priskorn, Holmboe, et al., 2018).

In 1992, Carlsen et al. reported a considerable global decline of mean sperm concentration from 113 million/mL to 66 million/mL and a decrease of semen volume from 3.40 mL to 2.75 mL over a period from 1938 to 1991 (Carlsen et al., 2005). Other systematic reviews and meta-analyses have later confirmed these findings (Levine et al., 2017; Swan et al., 1997). The latest systematic review and meta-analysis in 2023 by Levine et al. found that mean sperm concentration of unselected men from all continents decreased 51.6% between 1973 and 2018. The rate of sperm concentration decline was more rapid after year 2000 – from 1.16% per year before 2000 to 2.64%/year after 2000. This meta-analysis included semen quality of men from South and central America, Asia, and Africa – the regions which lacked data in the previous publications. Therefore, the global sperm concentration decline could be confirmed (Levine et al., 2023).

A Finnish study, which included serum testosterone level data of the men aged 25-74 years who were born between 1913-1977, showed a decreasing trend in serum testosterone levels of the Finnish men (Perheentupa et al., 2013). Men of the same age who were born in earlier decades had higher serum testosterone levels than men who were born later. This finding was observed in all age ranges. A US study showed decreased serum total testosterone concentrations at a rate of 1% per year from 1987 to 2004 (Travison et al., 2007), which is in line with data from four large Danish population surveys conducted from 1982 to 2001 (A. M. Andersson et al., 2007). The decreasing testosterone levels suggested a decline in Leydig cell function. Also, the incidence of testicular cancer is increasing in many countries (J. S. Park et al., 2018). The latest study based on the national cancer registries in the Nordic countries demonstrated that the age-standardised incidence rates (ASR) of testicular cancer has increased in Finland, Norway and Sweden (Ylönen et al., 2018). In contrast, the ASR has been relatively stable in Denmark and Iceland (Ylönen et al., 2018). The incidence of testicular cancer is predicted to continue to increase in Europe, especially the countries that used to have low incidence (Znaor et al., 2020).

Denmark used to have the highest incidence of testicular cancer and lowest semen quality. However, the latest findings revealed that the semen quality of the Danish men has been relatively stable, and the incidence of testicular cancer has levelled-off (Priskorn et al., 2018; Ylönen et al., 2018). In contrast, Finland used to have a low incidence of testicular cancer and high semen quality, but recent studies showed adverse trends of these conditions (Jørgensen et al., 2011). Therefore, the comparison of the incidence of male reproductive disorders between the two countries with different background will give ideas of the nature of these conditions.

The causes of the changes in the trend of the male reproductive disorders are probably from environmental factors and lifestyle change as mentioned above rather than genetic factors (Skakkebaek et al., 2016). The above-mentioned factors affecting spermatogenesis might explain the declining semen quality around the world. However, the main culprit has not been identified. It has been believed that this trend is caused by many factors.

The hypothesis of testicular dysgenesis syndrome (TDS) states that a variety of male reproductive disorders, including cryptorchidism, hypospadias, TGCTs and low semen quality, short anogenital distance and low serum testosterone level can have the same origin during fetal development (Juul et al., 2014; Skakkebaek, 2001). Many factors, for example 45X,46XY mosaicism, Klinefelter syndrome, mutations of sex-determining region Y or androgen receptor genes, low birth weight, small for gestational age, preterm birth, maternal diabetes mellitus, hypertension in pregnancy, or endocrine disrupting chemical exposure, can disturb fetal testicular development and cause this condition (Juul et al., 2014; Skakkebaek, 2001). It is

possible that the recent adverse trends of male reproductive health is possibly due to the disturbance of the testicular development during fetal life.

Impact of low semen quality

Poor semen quality is related to low fertility. Furthermore, poor semen quality is also associated with higher morbidity and mortality. Among the male partners of the infertile couples in the US and Denmark, decreased sperm concentration, total sperm count, percentage of motility, and percentage of sperm morphology are associated with increased mortality after a mean follow-up of 7.7 years in the US study and a range of follow up of less than one year to a maximum of 38 years in the Danish study (Eisenberg et al., 2014; Jensen et al., 2009). The increased mortality among men with poor semen quality in the Danish study was due to a variety of causes. The causes of increased risk of death in the US study were not reported. A more recent US study also reported a higher risk of death among infertile men, particularly azoospermic men, as compared with men without infertility (Giudice et al., 2021). The causes of increased mortality were unknown, but cardiovascular and cancers did not explain the findings (Giudice et al., 2021). According to these findings, semen quality may not be only a marker for fertility potential but also for the general health of the men.

2.1.6 Cryptorchidism

Cryptorchidism (undescended testis, maldescended testis) is a condition in which one or both testes are not located in the normal position at the bottom of scrotum (Virtanen et al., 2007). Instead, the testis locates anywhere along the normal path of testicular descent, which can be at a high scrotal, suprascrotal, inguinal, or abdominal position (K. A. Boisen et al., 2004). However, if the testis lies outside this normal path of testicular descent, it is called ectopic testis (Ramareddy et al., 2013). Right testis is more commonly affected than the left side (Comploj & Pycha, 2012). Cryptorchidism that can be detected at birth is called congenital cryptorchidism (Wohlfahrt-Veje et al., 2009). Acquired cryptorchidism (ascending testis or ascensus testis) is a condition in which both testes are in scrotal position at birth documented by a physician, but one or both testes ascend to a cryptorchid position later (Taghizadeh & Thomas, 2008; Wohlfahrt-Veje et al., 2009). The testis that descends later than normal, i.e. a few months after birth, has an increased risk of ascent later (John Radcliffe Hospital Cryptorchidism Study Group, 1986).

Congenital cryptorchidism is one of the most common urogenital malformations in newborn boys. Prevalence at birth has been reported to vary from 1.8 to 8.4% among boys with normal birth weight (Virtanen & Toppari, 2008). Boys with low

birth weight and/or preterm boys have increased risk of cryptorchidism, with a reported prevalence of cryptorchidism of 1.1 to 45.3% (Sijstermans et al., 2008). Boisen et al. studied the prevalence of congenital cryptorchidism in 1455 Finnish and 1068 Danish boys. Prevalence at birth in Finland and Denmark were 2.4% and 9.0%, respectively (Boisen et al., 2004). Among all of the Finnish boys at birth, 1.1%, 0.1% and 0.5% had high scrotal, suprascrotal, inguinal testis, respectively, and the testis was non-palpable in 0.6% of all Finnish boys (Boisen et al., 2004). In Denmark, 7%, 0.9% and 0.8% of all boys had testis at high scrotal, suprascrotal and inguinal position, respectively. And non-palpable testis was found in 0.4% of the Danish boys (Boisen et al., 2004).

Etiologies of congenital cryptorchidism

Most of the cryptorchidism occur without other clinical manifestations, the-so-called isolated cryptorchidism (Ferlin, et al., 2008). In some cases, there are diseases or conditions that disturb the process of testicular descent that cause cryptorchidism. These include conditions associated with abnormal reproductive hormone levels or actions that are involved in testicular descent and anatomical defects of the abdominal wall. Some syndromes manifest as cryptorchidism with unknown mechanisms. **Table 5** shows diseases and conditions that can manifest as cryptorchidism.

Table 5. Diseases and conditions associated with congenital cryptorchidism.

Classifications	Diseases or syndromes
1. Sex chromosome abnormalities	47,XXY (Klinefelter syndrome and variants) 45,X/46,XY (mixed gonadal dysgenesis) 46,XX/46,XY (chimerism) Ovotesticular disorders of sex development (DSD) (46,XX/46,XY chimerism)
2. 46,XX sex reversal	Ovotesticular disorders of sex development *SRY+* Duplication of *SOX9,* mutations of *RSPO1*
3. Other chromosome abnormalities	Down syndrome
4. Disorders in 46,XY individuals	
4.1 Androgen deficiency	4.1.1 Gonadal dysgenesis Mutations in *ARX, ATRX, CBX2, DAX1 (NROB1), DHH, DHX37, DMRT1, EMX2, ESR2, FGFR2, GATA4, HHAT, MAP3K1, NR5A1, SF1, SOX9, SRY, TSPYL1, WNT4, WT1* (WAGR syndrome, Denys-Drash syndrome, Frasier syndrome), *ZFPM2*, and *ZNRF3* genes 4.1.2 Androgen biosynthetic defects Steroidogenic acute regulatory (*StAR*) protein deficiency 7-Dehydro-cholesterol desmolase deficiency (Smith-Lemli-Opitz syndrome) Cholesterol desmolase deficiency (*CYP11A1*) 3b-hydroxysteroid dehydrogenase type 2 deficiency (*HSD3B2*) 17,20-lyase deficiency or combined 17 hydroxylase/17,20-lyase deficiency (*CYP17A1*) P450 oxidoreductase deficiency (*POR*) 17b-Hydroxysteroid-dehydrogenase type 3 (*HSD17B3*) 5-alpha reductase type 2 enzyme deficiency (*SRD5A2*). 4.1.3 Hypogonadism Hypogonadotropic hypogonadism, including Kallmann syndrome Hypergonadotropic hypogonadism: Noonan syndrome Combined hypergonadotropic and hypogonadotropic hypogonadism: Bardet-Biedl syndrome, Prader-Willi syndrome
4.2 Decreased androgen action	Androgen insensitivity syndrome (Androgen receptor (*AR*) mutations) LH receptor (*LHCGR*) gene inactivating mutations
4.3 Ovotesticular disorders of sex development	46,XY ovotesticular disorders of sex development
4.4 Decreased INSL3 levels or actions	*INSL3* or *RXFP2* mutations
4.5 Decreased AMH action	Persistent Müllerian duct syndrome: *AMH, AMHR2* mutations
5. Anatomical defects of the abdominal wall	Prune-Belly syndrome Posterior urethral valve
6. Other mechanisms	See reference (Virtanen et al., 2007)

Abbreviations: *ARX*, aristaless-related homeobox; *ATRX*, alpha-thalassemia, mental retardation, X-linked protein; *CBX2*, chromobox protein homolog 2; *DAX1*, dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; *DHH*, desert hedgehog gene; *DHX37*, DEAH-box RNA helicase; *DMRT1*, DM-related transcription factor 1; *EMX2*, empty spiracles 2; *ESR2*, estrogen receptor β; *FGFR2*, fibroblast growth factor receptor 2; *GATA4*, GATA binding protein 4; *MAP3K1*, mitogen-activated protein kinase kinase kinase 1, *NR0B1*, nuclear receptor subfamily 0, group B, member 1; *NR5A1*, *SF1*, Steroidogenic factor 1; *SOX9*, SRY (Sex-Determining Region Y)-Box 9; *SRY*, Sex-Determining Region Y, *TSPYL1*, testis-specific protein, Y-linked-1 *WNT4*, Wnt family member 4; *WT1*, Wilms tumor 1 (WAGR syndrome, Denys-Drash syndrome, Frasier syndrome); *ZFPM2*, zinc finger protein, FOG family member 2; *ZNRF3*, Zinc and Ring Finger 3 genes

Testicular histology in individuals with cryptorchidism

Cryptorchid testes show several histologic changes (Mechlin & Kogan, 2014). Cryptorchid testis has reduced germ cell numbers. The number of germ cells per tubule decreases rapidly during 1–3 years of age (Dunkel et al., 1997; Hadziselimović et al., 1986; Huff et al., 1989, 1991, 2001; Kollin et al., 2012). The longer the testis stays in the cryptorchid position, the lower the number of Sertoli and germ cells remaining in the testis. Boys who underwent orchiopexy at 3 years had lower Sertoli and germ cell numbers than boys who were operated on at 9 months (Kollin et al., 2012). Also, an impaired transformation from gonocytes into adult dark (Ad) spermatogonia, which are considered to be the stem cell pool for spermatogenesis, has been reported (Hadziselimović et al., 1986; Huff et al., 1991, 2001). The spermatogonia also fail to mature into primary spermatocytes (Mechlin & Kogan, 2014). The Leydig cell population declines after the first few months of life (Hadziselimović et al., 1986; Huff et al., 1991). Leydig cell hypoplasia is also detected (Mechlin & Kogan, 2014). There is also interstitial or peritubular fibrosis, which is worsened with advancing age (K. H. Park et al., 2007; Suskind et al., 2008). Interestingly, the contralateral, descended testis of the boys with unilateral cryptorchidism also showed a lowered Ad spermatogonia number and a delayed primary spermatocyte development (Huff et al., 2001).

Long-term consequences of cryptorchidism

Individuals with cryptorchidism have an increased risk of testicular germ cell tumors (TGCTs) and infertility (Lee, 2005; Trabert et al., 2013). The risk of TGCTs increases 3.2- to 7.5-fold compared to normal individuals (Thorup et al., 2010; Trabert et al., 2013). On the other hand, approximately 10% of the men diagnosed with TGCTs had a history of cryptorchidism (Mannuel et al., 2012). The rate of successful paternity among men with a history of bilateral cryptorchidism is significantly lower as compared with men with a history of unilateral cryptorchidism and controls (65.3 vs. 89.7 vs. 93.2%, respectively) (Lee, 2005). An increased germ cell loss in cryptorchid testis, as described in the previous section, causes impaired fertility in cryptorchidism (Virtanen & Toppari, 2015).

Long-term consequences of cryptorchidism on hypothalamic-pituitary-testicular axis hormones

Studies investigating the long-term consequences of cryptorchidism on Sertoli and Leydig cell hormone levels showed mixed results, particularly Leydig cell function (Rodprasert et al., 2020). The impaired Sertoli cell function has been shown in some studies from the mini-pubertal period until adulthood, as demonstrated by low serum

inhibin B and high serum FSH levels in cryptorchid boys compared to controls (Rodprasert et al., 2020). The Leydig cell function seems to be preserved in early life; however, evidence of Leydig cell dysfunction is shown in some studies since the pubertal period (Rodprasert et al., 2020). Impaired Leydig cell function in cryptorchidism does not affect the onset of puberty (Sadov et al., 2016; Taskinen et al., 1997).

To date, only two old studies have investigated HPG axis hormones in boys with a history of cryptorchidism during puberty with conflicting results (Dickerman et al., 1979; Gendrel et al., 1977). One study found that serum FSH levels in pubertal boys with a history of cryptorchidism were higher than the normal reference range suggesting Sertoli cell dysfunction (Dickerman et al., 1979). In addition, serum LH was higher and testosterone level was lower than the normal range in boys with bilateral cryptorchidism, suggesting impaired Leydig cell function. In contrast, the other study found that boys with a history of cryptorchidism and controls had similar FSH levels; however, there was a blunted peak LH level after LHRH test and a blunted peak testosterone level after hCG stimulation test in the cryptorchid group. These results can imply that Sertoli cell function was normal in boys with a history of cryptorchidism, whereas Leydig cell function was impaired due to subnormal pituitary function (Gendrel et al., 1977). However, serum inhibin B level was not measured in these two studies, therefore the Sertoli cell product was not actually reported. More longitudinal follow-up studies, which also measure serum inhibin B levels are necessary to provide better understanding of the physiology of HPG axis hormone production of the boys with a history of cryptorchidism during puberty.

Pubertal development in boys with a history of cryptorchidism

Sadov et al. reported that the onset of puberty, defined as the age when testicular volume of one or both testes exceeds 3 ml by orchidometer, of the Finnish boys with a history of congenital cryptorchidism and controls were not different (11.7 and 11.8 years, respectively) (Sadov et al., 2016). The testicular size of the boys with a history of unilateral or bilateral cryptorchidism was smaller than that of the controls at the end of puberty (Sadov et al., 2016).

To my knowledge, there is no longitudinal study investigating the GH-IGF-I axis in boys with a history of cryptorchidism. In addition, the role of GH/IGF-I system on the testicular development and function, particularly in cryptorchidism, should be investigated.

Studies in adult men demonstrated that previously undescended testis of the men with a history of unilateral cryptorchidism and bilateral cryptorchidism are smaller than that of healthy controls (van Brakel et al., 2013). Sertoli cell function is impaired in men with a history of bilateral cryptorchidism - indicated by lower inhibin B and

higher FSH levels – as compared with controls (A. M. Andersson et al., 2004a; de Gouveia Brazao et al., 2003; Lee & Coughlin, 2001; Rohayem et al., 2017; van Brakel et al., 2013). Higher LH and comparable testosterone levels between men with and without a history of cryptorchidism reflecting compensatory Leydig cell dysfunction has been shown in one study (de Gouveia Brazao et al., 2003). However, some studies showed decompensated Leydig cell dysfunction – with decreased testosterone and elevated LH levels (Rohayem et al., 2017).

3 Aims

The aims of this doctoral thesis were as follows:

Study 1: to study whether sperm production and semen quality change during early adulthood

Study 2: to compare semen quality in contemporary Finnish and Danish men

Study 3: to study the levels of reproductive hormones and growth factors of boys with and without a history of congenital cryptorchidism during puberty. The role of HPG axis hormones and IGF-I in testicular growth was also examined.

4 Materials and Methods

Study 1: Semen quality improves marginally during young adulthood: a longitudinal follow-up study

All of the Finnish men have to attend a medical examination before military recruitment at the age of 18 years. We received a list of military conscripts who were living in Turku, Raisio or Kaarina in the Southwest of Finland from the military authorities. We invited all of them to participate in the study by sending invitation letters to their home address.

The men were asked to visit the Andrology laboratory at the Institute of Biomedicine, University of Turku for four times – at the age of 19, 21, 25 and 29 years – for medical examinations and semen sample collection. The visit was organized in the morning or afternoon. There were two cohorts in this study – cohort A, which was the main study cohort, and cohort B that was used as a validation of the findings from cohort A. The men in cohort A were born in 1979–1981 (n=336). Men in cohort B were born in 1983 (n=197). Participation rates are described in the results section.

Physical examination in each visit consisted of an assessment of pubic hair according to Tanner staging system, andrological examination, which included measurement of testicular size by orchidometer and scrotal ultrasonography. The paper questionnaire was sent to the man's home address before study visit. The questionnaire included questions about medical conditions, drug or substance use and fertility.

Semen analysis

The semen samples were analyzed at the Andrology laboratory, Institute of Biomedicine, University of Turku. Duration of sexual abstinence was recorded. The sample was collected by masturbation in the private room in the andrology laboratory or at home. If the sample was collected at home, it had to be kept in 37°C all the time and be delivered to the laboratory within one hour after collecting the sample.

Semen sample was placed in 37°C incubator during liquefaction. Semen volume was estimated by subtracting the weight of the empty container from the weight of container including sample assuming that 1 g is equal to 1 ml of semen sample. Sperm motility was assessed on a 37°C heated microscope stage with x400 magnification. The percentage of total sperm motility and percentage of progressive motility were reported. Semen sample was diluted in a solution of 0.6 mol/l $NaHCO_3$ and 0.4% (v/v) formaldehyde in distilled water for the assessment of sperm concentration. Improved Neubauer haemocytometer was used. Semen sample was smeared on a glass slide, air-dried, fixed in 95% ethanol followed by Papanicolaou staining. The assessment of sperm morphology was based on the strict criteria (Kruger et al., 1987; Menkveld et al., 1990; WHO, 2010).

One technician analyzed semen volume, sperm concentration and sperm motility. Two people assessed sperm morphology – one person for each cohort. Sperm morphology was analyzed only for the men who participated in all four rounds. Semen volume, sperm concentration, total sperm count, percentage of motile spermatozoa, percentage of morphologically normal spermatozoa and total number of motile spermatozoa were reported.

Data were analyzed by using SAS for Windows version 9.2 (Cary, NC, USA). Comparisons of the semen variables between visits were performed by using mixed model for repeated measurements. Duration of sexual abstinence was entered into the model as a covariate. Time from the last ejaculation to the start of semen analysis was also added to the model for sperm motility. Tukey–Kramer method was used for the adjustment of P-values. P value of < 0.05 was considered a statistical significance.

Study 2: An update on semen quality among young Finnish men and comparison with Danish data

This was a cross-sectional study in the young Finnish and Danish men from general population. In Finland, men who were 18–19 years of age and were living in Turku, Kaarina or Raisio in the Southwest Finland were invited to participate in the study. List of the men and their home addresses were obtained from the registration authority. In Denmark, 18- to 19-year-old men who lived and attended the medical examinations for military recruitment in the greater Copenhagen area were invited. Men in both countries were invited regardless of their health status. The invitation letters were sent to their home addresses. The study was conducted at the Institute of Biomedicine, University of Turku in Finland and the Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark. The period of examination was from 2008–2011.

A total of 289 Finnish men and 882 Danish men participated in the study. The paper-based questionnaire in Finnish or Danish were sent to the man's home address. The participant was asked to abstain from ejaculation for at least 48 hours before collecting semen sample, however, the samples collected less than 48 hours of sexual abstinence were still accepted. On the day of attendance, the man returned the questionnaire, provided one semen sample, underwent general and andrological examination by a medical doctor.

The questionnaire consisted of questions about general health, medical conditions, reproductive health, diet, lifestyle, medication and substance use and maternal pregnancy history.

Semen analysis

The semen samples of the Finnish and Danish men were analyzed at the Andrology laboratory, Institute of Biomedicine, University of Turku and the Department of Growth and reproduction, Rigshospitalet Copenhagen, Denmark, respectively.

The protocol for the collection of semen samples and semen analysis were the same as in the Study 1. Bürker-Türk haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) was used in Denmark, and improved Neubauer haemocytometer was used in Finland.

In Finland, one technician analyzed semen volume, sperm concentration and sperm motility and the other person assessed sperm morphology. In Denmark, three technicians analyzed all the semen samples. Quality control of sperm concentration between the two laboratories was performed. The technician in Copenhagen sent five fresh semen sample to Turku. The technicians in both countries assessed sperm concentrations of the same semen samples. The results from the two centers were not significantly different.

The comparison of the semen parameters between the countries, the subgroup analysis of men who were not on any medications or androgenic hormones, had no andrological diseases or fertility problems and the adjustment for the confounding factors to the semen parameters were performed by using multi-way ANOVA.

Potential confounding variables tested included age, season of collecting semen sample and duration of sexual abstinence. We also tested the effect of the time from ejaculation to the start of semen analysis on the sperm motility. The variables were transformed if needed to receive normal distribution of residuals. Sperm concentration, total sperm count, total number of morphologically normal spermatozoa and total number of progressively motile spermatozoa were cubic-root transformed. Percentage of morphologically normal spermatozoa was square-rooted transformed and sperm motility was logit-transformed. Increasing age is associated with increasing semen volume and total number of morphologically normal

spermatozoa. Longer duration of sexual abstinence is associated with increased semen volume, sperm concentration, total sperm count, and sperm motility. Percentage of morphologically normal spermatozoa was not confounded by these variables. Season had no influence on semen variables. Duration of sexual abstinence was entered into the model as three continuous variables: less than or equal to 48 hours, 49 to 96 hours and more than 96 h. Time from the ejaculation to the start of semen analysis was entered into the model as two continuous variables: below or equal to 30 minutes and above 30 minutes. The duration of more than 30 minutes was inversely associated with sperm motility. The model gave estimates of the median semen variables at a mean age, a mean abstinence period and at mean time from ejaculation to the start of semen analysis.

The analysis of data was performed by using IBM SPSS Statistics version 24.0 (IBM, Armonk, NY). Two-sided P-values of less than 0.05 were considered statistically significant. Bonferroni adjusted alpha levels of 0.006 per test were used for the comparisons of the 8 main semen variable outcomes.

Data of the Danish men had been published previously as a part of larger studies (Jørgensen et al., 2012; Priskorn, et al., 2018).

Study 3: Reproductive markers of testicular function and size during puberty in boys with and without a history of cryptorchidism

The participants were recruited from the birth cohort study on the prevalence of congenital cryptorchidism and hypospadias and the case-control study on risk factors of congenital cryptorchidism. Both studies were conducted at the Turku University Hospital in Turku, Finland. In the birth cohort study, mothers participated in the study during pregnancy, and the boys were born in 1997-99. A total of 1,494 boys were examined at birth, and 35 had congenital cryptorchidism (K. Boisen et al., 2004). In addition, 160 congenitally cryptorchid boys and 24 non-cryptorchid controls born in 1997-2002 participated in the case-control study on risk factors of congenital cryptorchidism. For each cryptorchid boy, up to two controls were chosen [matching criteria: were matched by date of birth (± 14 days), number of parity, gestational age (± 7 days), maternal history of cigarette smoking during pregnancy (smoked or non-smoked) and maternal history of diabetes mellitus (had or did not have).

The boys in the birth cohort and the case-control study were invited to participate in the longitudinal pubertal follow-up study. A total of 120 boys participated - 52 with a history of congenital cryptorchidism (35 unilateral and 17 bilateral cryptorchidism) and 68 non-cryptorchid controls (including two boys who had participated in the prospective birth cohort study and wanted to participate as extra controls). The participation rate was 31.5% for the boys with a history of

cryptorchidism and 21.2% for controls. Eight boys were excluded – five had monorchidism, one had acquired cryptorchidism, and two developed precocious puberty. Three controls did not give consent to withdraw blood. Therefore, there were 109 boys in the statistical analysis – 30 with a history of unilateral cryptorchidism, 16 with a history of bilateral cryptorchidism, and 63 controls. Fifteen boys with a history of unilateral cryptorchidism and 9 boys with a history of bilateral cryptorchidism underwent orchiopexy in childhood. The median age at the first orchidopexy was 2.0 years (range, 0.2–9.3, n=24). The maximum number of orchiopexies was four times (n=1).

There were five groups of boys classified according to the testicular position from birth to 18 months of age and the need for treatment of cryptorchidism – boys who had a history of bilateral cryptorchidism and underwent orchiopexy (OpBC), or experienced spontaneous testicular descent (SpBC), those who had a history of unilateral cryptorchidism and underwent orchiopexy (OpUC), or experienced spontaneous testicular descent (spUC), and non-cryptorchid controls.

The pubertal follow-up study was conducted between 2005 and 2019 at the Institute of Biomedicine, University of Turku, Finland. The boys were examined by researchers (n=9) every 6 months, starting from the age of 8.5 years until full pubertal maturation, defined as the testicular sizes being stable for three consecutive visits. The examinations included assessment of gynecomastia, Tanner staging of genitalia and pubic hair, measurements of body weight, height, waist and hip circumferences, testicular length and width by ruler, testicular volume by orchidometer and flaccid penile length and width. Scrotal ultrasonography (Aloka Prosound 6, linear probe, 5–13 MHz and Aloka SSD-500, linear probe, 7.5 MHz; Hitachi Aloka Medical) had been performed to measure the testicular size for the calculation of testicular volume, assessment of testicular parenchyma, and to evaluate intra-scrotal structures. Testicular volume by ultrasonography was calculated with the ellipsoid formula ($\pi/6 \times \text{length} \times \text{width}2$). Combined testicular volume of both sides were reported. Onset of puberty is indicated when testicular volume of either side by orchidometer was permanently above 3 mL (the age at the first visit was selected). Examination workshops were organized once to twice a year to standardize the measurement among the examiners.

Blood samples were collected at the age of 8.5 years, and subsequently, every visit started when the length of one or both testes was more than 20 mm. The samples were stored in -20°C until the time of the analysis. The samples were analyzed at the Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark for FSH, LH, total testosterone, inhibin B, SHBG, IGF-I, and insulin-like growth factor-binding protein 3 (IGFBP-3). Serum FSH and LH levels were measured by two-sided fluoroimmunometric analysis DELFIA (Wallac Oy, Turku, Finland) with the limit of detection (LoD) of 0.05 U/L and inter-assay coefficients of variations (CV)

were 2.7% for FSH and 1.9% for LH, respectively. Serum total testosterone and SHBG were measured by chemiluminescent enzyme immunoassay (Access 2, Beckman Coulter, Brea, CA, USA), with the LoD of 0.35 nmol/l and 0.33 nmol/l, respectively. The inter-assay CVs of both testosterone and SHBG were 5.2%. Serum inhibin B levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Inhibin B gen II, Beckman Coulter) with LoD of 3 pg/mL and inter-assay CV of 10.3%. IGF-I and IGF-BP3 were analyzed by automated chemiluminescence immunoassay ISYS (IDS) with LoD of 10 and 80 µg/L, respectively. The inter-assay CV were 7.2% for IGF-I and 13.2% for IGFBP-3. Insulin-like factor 3 (INSL3) levels were measured only at the final visit of each boy (with Tanner stage at least G4) by using liquid chromatography–tandem mass spectrometry (LC-MS/MS) in-house assay (Albrethsen et al., 2018). The technicians were blinded from the medical history of the subjects.

The previous publication of this project reported testicular growth of the boys with a history of unilateral or bilateral cryptorchidism and controls (Sadov et al., 2016). The present study presents also the hormonal data of the boys.

Statistical analysis

Data analysis and statistical modeling were performed using R version 4.1.1 (The R Project for Statistical Programming) with the package ‘nlme’ for the linear mixed effect modeling and package ‘emmeans’ for the calculation of estimated marginal means (model-based means).

Continuous variables were presented as mean (standard deviation) for normally distributed data, and median (interquartile range) for skewed data. Categorical variables were presented as percentages. Between-group differences of continuous variables were analyzed using one-way variance analysis (ANOVA) for normally-distributed data and Kruskal-Wallis test for non-normally distributed data. Chi-squared test was used to compare categorical variables between groups.

The comparison of testicular volume and hormone levels between the five study groups was performed by using the linear mixed-effect models for repeated measures. Testicular volume and hormone levels were modeled on age at examination, time of blood sampling, five groups of boys, the interactions between pubertal age and blood sampling time, and between pubertal age and groups of boys. Since the age at onset of puberty varies among individuals, pubertal age was used in the report to demonstrate the year(s) of pubertal maturation. Pubertal age 0 indicates the onset of puberty, and pubertal age -1 and 1 years indicate one year before and after the onset of puberty, respectively. Dunnett’s test was used to compare the estimated marginal means between groups. The visits with undetectable hormone

levels or with less than three participants were excluded. P value of less than 0.05 is considered statistically significant.

Ethics statement

The Joint Ethics Committee of the University of Turku and the Turku University Hospital in Finland reviewed and approved the original birth cohort study, the case-control study on congenital cryptorchidism, semen quality studies (study I and II in this thesis), and the pubertal follow-up study (study III). All of the studies were conducted according to Helsinki II Declaration. All men in the study I and II and the boys and their parents in the study III provided written informed consent before participation.

5 Results

Study 1: Semen quality improves marginally during young adulthood: a longitudinal follow-up study

In cohort A, 336 men participated at 19 years, and 111 men participated at the last visit (at 29 years of age). Sixty-one men participated in all four visits. In cohort B, 197 men attended the first visit. Of these, 90 men came at the age of 29, and 52 men came at all four visits. The participation rate at the age of 19 years was 13.4%. The examination period was from 1998-2003.

The medical characteristics of the participants who attended the first round (age 19 years) are presented in **Table 6**. Men in both cohorts had relatively similar characteristics. Varicocele was detected during examination in 22.3% of the men of cohort A and 20.0% of the men in cohort B and about 0.5-1.5% of men received varicocele treatment. The proportion of men who were treated for cryptorchidism was 0.9% in cohort A, and in cohort B it was 1.5%.

The results of the analysis of all men and men who participated in all four rounds in each cohort were similar. Semen quality of the men in cohort A is presented in **Table 7** (all men) and **8** (men who participated at all visits). In cohort A, there was significantly increased semen volume, percentage of motile spermatozoa, percentage of morphologically normal spermatozoa and total number of motile spermatozoa from the age of 19 to 29 years. Percentage of morphologically normal spermatozoa increased significantly at a later age, especially after the age of 25 years ($p<0.001$ for age 19 years vs 29 years, 21 years vs 29 years and 25 years vs 29 years among men who attended all four rounds), while sperm motility increased significantly at every visit, except age 25 years vs 29 years, as compared with the previous visit). There were no significant differences in sperm concentration or total sperm count between any age points.

Table 6. Characteristics of the participants at the first visit (at the age of 19 years).

Conditions	Cohort A (n=336)	Cohort B (n=197)
Diagnosis (%)		
Diabetes mellitus	0.0%	0.0%
Thyroid disease	0.3%	1.0%
Epididymitis	0.6%	0.0%
Chlamydia	1.2%	1.5%
Prostatitis	0.9%	0.5%
Medication use during the past 3 months (%)	14.6%	20.0%
History of treatment (%)		
Cryptorchidism	0.9%	1.5%
Testicular torsion	0.9%	1.0%
Testicular cancer	0.0%	0.0%
Varicocele	1.5%	0.5%
Inguinal hernia	6.8%	3.0%
Fertility history (%)		
Had fertility problems	0.9%	0.0%
Caused a pregnancy	2.1%	6.1%

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Semen quality of the men in cohort B is shown in **Table 9** (all men) and **10** (men who participated at all visits). Data of the cohort B also showed significantly increased percentage of morphologically normal spermatozoa and total number of motile spermatozoa from the age of 19 to 29 years. In contrast to the findings of cohort A, the change in semen volume was not statistically significant. Among men who participated all four rounds in cohort B, there was significantly higher sperm concentration at the age of 29 years as compared with that at the age of 21 and 25 years and significantly higher total sperm count at the age of 29 years as compared with that at 25 years. In addition, percentage of motile spermatozoa increased significantly only from the age of 19 to 21 years, but not in all age comparisons as in cohort A.

Table 7. Semen quality of all men in cohort A.

Age (y) **n**	**19** **336**	**21** **179**	**25** **181**	**29** **111**	**P-value*** Age of comparisons
Semen volume (mL)	3.0 (0.5-8.5)	3.4 (0.6-9.9)	3.5 (0.7-9.9)	3.5 (0.2-8.7)	0.06 19y vs 21y **<0.001 19y vs 25y** **<0.001 19y vs 29y** 0.09 21y vs 25y 0.21 21y vs 29y 1.00 25y vs 29y
Sperm concentration (million/mL)	60 (0-515)	56 (0-377)	51 (0-225)	70 (0-195)	0.37 19y vs 21y 0.61 19y vs 25y 0.81 19y vs 29y 0.98 21y vs 25y 0.20 21y vs 29y 0.27 25y vs 29y
Total sperm count (million)	193 (0-1380)	184 (0-753)	187 (0-1345)	219 (0-966)	1.00 19y vs 21y 0.46 19y vs 25y 0.06 19y vs 29y 0.52 21y vs 25y 0.08 21y vs 29 0.63 25y vs 29y
Total motility (%)	66 (1-87)	72 (15-92)	78 (19-95)	82 (36-94)	**<0.001 19y vs 21y** **<0.001 19y vs 25y** **<0.001 19y vs 29y** **<0.001 21y vs 25y** **<0.001 21y vs 29y** 0.21 25y vs 29y
Total motile sperm count (million)	124 (0-800)	128 (0-538)	138 (0-942)	184 (0-763)	0.47 19y vs 21y **0.002 19y vs 25y** **<0.001 19y vs 29y** 0.13 (21y vs 25y **<0.001 21y vs 29y** 0.23 25y vs 29y

Data presented as median (range)

*Bold text indicates that the comparison was statistically significant.

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Table 8. Semen quality of men in cohort A who attended all 4 rounds.

Age (y) **n**	**19** **61**	**21** **61**	**25** **61**	**29** **61**	**P-value*** Age of comparisons
Semen volume (mL)	3.2 (0.9-8.0)	3.7 (0.8-8.7)	3.6 (1.0-8.2)	3.9 (0.2-7.9)	0.69 19y vs 21y **0.01 19y vs 25y** **0.02 19y vs 29y** 0.19 21y vs 25y 0.26 21y vs 29y 1.00 25y vs 29y
Sperm concentration (million/mL)	54 (0-309)	47 (0-377)	56 (0-225)	66 (0-195)	0.79 19y vs 21y 0.99 19y vs 25y 0.99 19y vs 29y 0.70 21y vs 25y 0.77 21y vs 29y 1.00 25y vs 29y
Total sperm count (million)	177 (0-651)	182 (0-753)	219 (0-880)	220 (0-966)	1.00 19y vs 21y 0.41 19y vs 25y 0.43 19y vs 29y 0.3 21y vs 25y 0.31 21y vs 29y 1.00 25y vs 29y
Total motility (%)	67 (30-81)	72 (31-87)	80 (37-95)	82 (41-92)	**<0.001 19y vs 21y** **<0.001 19y vs 25y** **<0.001 19y vs 29y** **<0.001 21y vs 25y** **<0.001 21y vs 29y** 0.94 25y vs 29y
Normal morphology (%)	7.5 (4–11)	7.0 (4–11)	8.0 (4–12)	10 (6–14)	0.57 19y vs 21y 0.99 19y vs 25y **<0.001 19y vs 29y** 0.44 21y vs 25y **<0.001 21y vs 29y** **<0.001 25y vs 29y**
Total motile sperm count (million)	126 (0–417)	140 (0–538)	163 (0–689)	184 (0–763)	0.93 19y vs 21y **0.01 19y vs 25y** **0.008 19y vs 29y** **0.03 21y vs 25y** **0.02 21y vs 29y** 0.99 25y vs 29y

Data presented as median (range)

*Bold text indicates that the comparison was statistically significant.

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Table 9. Semen quality of all men in cohort B.

Age (y) **n**	**19** **197**	**21** **110**	**25** **96**	**29** **90**	**P-value** Age of comparisons
Semen volume (mL)	3.4 (0.6–9.0)	3.5 (0.4–10.9)	3.7 (1.0–8.9)	3.5 (0.8–8.9)	0.51 19y vs 21y 0.09 19y vs 25y 0.59 19y vs 29y 0.79 21y vs 25y 1.00 21y vs 29y 0.96 25y vs 29y
Sperm concentration (million/mL)	50 (0–274)	57 (0–263)	46 (0–195)	62 (0–171)	0.96 19y vs 21y 0.43 19y vs 25y 0.09 19y vs 29y 0.76 21y vs 25y **0.03 21y vs 29y** **0.007 25y vs 29y**
Total sperm count (million)	172 (0–785)	171 (0–975)	168 (0–1362)	225 (0–1077)	1.00 19y vs 21y 1.00 19y vs 25y **0.009 19y vs 29y** **0.02 21y vs 29y** 1.00 21y vs 25y **0.02 25y vs 29y**
Total motility (%)	76 (18–91)	79 (21–93)	82 (21–94)	81 (18–96)	**0.02 19y vs 21y** **0.002 19y vs 25y** **0.03 19y vs 29y** 0.85 21y vs 25y 0.90 21y vs 29y 1.00 25y vs 29y
Total motile sperm count (million)	130 0–573	132 0–711	138 (0–1034)	183 (0–717)	0.88 19y vs 21y 0.73 19y vs 25y **<0.001 19y vs 29y** 0.99 21y vs 25y **0.01 21y vs 29y** **0.02 25y vs 29y**

Data presented as median (range)

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*Bold text indicates that the comparison was statistically significant.

Table 10. Semen quality of men in cohort B who attended all 4 rounds.

Age (y) **n**	**19** **52**	**21** **52**	**25** **52**	**29** **52**	**P-value** Age of comparisons
Semen volume (mL)	3.8 (1.1–8.7)	3.8 (0.4–10.9)	4.0 (1.6–8.9)	3.4 (0.8–7.9)	0.94 19y vs 21y 0.86 19y vs 25y 1.00 19y vs 29y 1.00 21y vs 25y 0.93 21y vs 29y 0.86 25y vs 29y
Sperm concentration (million/mL)	54 (0–266)	59 (0–160)	57 (0–153)	69 (0–160)	0.99 19y vs 21y 0.56 19y vs 25y 0.12 19y vs 29y 0.53 21y vs 25y **0.03 21y vs 29y** **0.005 25y vs 29y**
Total sperm count (million)	202 (0–701)	192 (0–928)	193 (0–1362)	233 (0–1077)	1.00 19y vs 21y 0.72 19y vs 25y 0.16 19y vs 29y 0.79 21y vs 25y 0.13 21y vs 29y **0.02 25y vs 29y**
Total motility (%)	76 (47–88)	82 (21–93)	83 (44–92)	82 (18–94)	**0.04 19y vs 21y** 0.05 19y vs 25y 0.20 19y vs 29y 1.00 21y vs 25y 0.99 21y vs 29y 1.00 25y vs 29y
Normal morphology (%)	7.3 (0–19) n = 51	8.5 (0–19) n = 49	8.0 (1–21) n =49	9.0 (0–21) n =51	0.22 19y vs 21y 0.59 19y vs 25y **0.001 19y vs 29y** 0.91 21y vs 25y **0.04 21y vs 29y** **0.02 25y vs 29y**
Total motile sperm count (million)	142 (0–558)	161 (0–711)	153 (0–1034)	193 (0-979)	1.00 19y vs 21y 0.92 19y vs 25y **0.005 19y vs 29y** 0.81 21y vs 25y **0.04 21y vs 29y** **0.007 25y vs 29y**

Data presented as median (range)
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Study 2: An update on semen quality among young Finnish men and comparison with Danish data

A total of 1160 men participated in the study – 287 Finnish and 873 Danish men. The participation rate was 9% in Finland and 35% in Denmark. A total of 104 Finnish men (3.4% of non-participating Finnish men) returned the refusal letters to the researcher and provided their information about height, weight, and body mass index. These data were not different between participating and non-participating men (data not shown).

Characteristics of the men in the study are shown in **Table 11 and 12**. Men in both countries have similar features, except Finnish men were slightly younger and had a bigger testicular size. In Finland, there was less percentage of men who had sexually transmitted diseases and a higher percentage of men who had thyroid disease than the Danish men. Three Finnish men (1%) were operated for cryptorchidism in childhood, whereas 14 Danish men (1.6%) were operated, p=0.44. None in Finland received hormonal treatment for cryptorchidism, while 3 Danish men (0.3%) did. The amount and duration of cigarette smoking of the men were not different between the countries. However, a history of maternal or paternal smoking during pregnancy was less common in Finland. The amount of alcohol consumption was higher in Denmark.

Table 11. Characteristics of the men in the study – history data.

	FINLAND (N=287)	DENMARK (N=873)	P-VALUE
Age (Y)[a]	18.7 (18.5–19.1)	19.1 (18.4–23.1)	<0.001
Duration of Education (Y)[a]	11.6	12.6	<0.001
Cigarette Smokers[b] (%)	45.0%	49.5%	0.19
Smoking (Cigarette/D), All Men (Mean (Sd))	3.3 (5.7)	4.2 (6.5)	0.12
Smoking (Cigarette/D), Only Smokers (Mean (Sd))	7 (1–20)	10 (1–20)	0.52
Duration Of Smoking (Y)[a]	2.0 (0.0–6.0)	2.0 (0.0–7.0)	0.56
Alcohol Consumption During Past Week (Units)[a]	6 (0-36)	11 (0-42)	<0.001
Diagnosis (%)			
Asthma	9.8%	10.9%	0.66
Diabetes	0.7%	0.0%	0.06
Thyroid Disease	1.0%	0.0%	0.02
Varicocele	0.3%	0.7%	1.00
Hydrocele	1.4%	1.5%	1.00
Testicular Torsion	1.0%	1.5%	0.77
Hypospadias (%)	0.0%	0.6%	0.34
Epididymitis/Orchitis (%)	0.3%	0.9%	0.47
Prostatitis (%)	0.8%	0.1%	0.14
Sexually Transmitted Diseases (%)[C]	2.1%	10.7%	<0.001
Pregnancy History (%)			
Maternal Smoking During Pregnancy	11.9%	24.1%	<0.001
Paternal Smoking During Pregnancy	31.9%	42.5%	0.01
Preeclampsia (%)	2%	2.3%	0.62
Hypertension during Pregnancy (%)	4.4%	4.0%	0.95
Gestational diabetes mellitus (%)	1.6%	0.4%	0.11
History of treatment (%)			
Testicular cancer	0.0%	0.1%	1.00
Varicocele	0.3%	0.3%	1.00
Hydrocele	1.4%	1.3%	1.00
Cryptorchidism	1.0%	1.9%	0.44
Duration of sexual abstinence (h)[a]	65 (39-117)	63 (36-137)	0.02
Time from ejaculation to the start of analysis (min)[a]	45 (20-80)	35 (15-85)	0.003

h, hours; min, minutes; y, year

[a]median (5^{th}-95^{th})

[b]including both current and ex-smoker

[c]gonococcal and chlamydial infection

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Table 12. Characteristics of the men in the study – physical examination data.

	FINLAND (N=287)	DENMARK (N=873)	P-VALUE
Year of examination (%)			<0.001
2008	0.0%	15.3%	
2009	44.4%	35.4%	
2010	29.9%	35.5%	
2011	25.7%	13.7%	
Season of examination (%)			<0.001
spring	31%	34%	
summer	17%	8%	
autumn	22%	46%	
winter	30%	12%	
Body mass index (kg/m^2) [a]	22.6 (18.4–30.5)	22.4 (18.8–28.7)	0.07
Testicular size (ml) (orchidometer)[a,b]	24	21	<0.001
Tanner stage of pubic hair			0.01
iv	3.5%	0.8%	
v	11.2%	12.1%	
vi	85.3%	87.1%	
Conditions detected during examination (%)			0.64
Testicular position			
scrotal	100%	99.2%	
inguinal	0%	0.3%	
non-palpable	0%	0.5%	
varicocele	20.6%	12.8%	0.004
hydrocele	0.7%	0.7%	1.000
epididymal abnormality	2.1%	1.6%	0.60

[a] median (5th-95th)

[b]Average size of both testes.

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Table 13. Semen quality of the young men in Finland and Denmark.

Semen variables	All men (Finland n=287, Denmark n=873)			
	OBSERVED		ADJUSTED	P-value
	Mean (SD)	Median (5^{th} -95^{th})	Median (5^{th} -95^{th})	
Semen volume (mL)				
Finland	3.2 (1.6)	3.1 (0.9-6.4)	2.8 (2.7-3.0)	0.04
Denmark	3.4 (1.6)	3.2 (1.3-6.2)	3.1 (3.0-3.2)	
Sperm concentration ($x10^6$/mL)				
Finland	59 (43)	48 (9-139)	49 (44-54)	0.48
Denmark	61 (53)	48 (4-167)	47 (44-50)	
Total sperm count ($x10^6$)				
Finland	177 (147)	146 (14-450)	148 (132-165)	0.87
Denmark	197 (172)	146 (13-541)	146 (137-156)	
Normal morphology (%)				
Finland	7.5 (4.3)	6.5 (1.5-16.0)	6.9 (6.3-7.5)	0.27
Denmark	7.5 (4.8)	7.0 (0.5-16.0)	6.5 (6.2-6.8)	
Total motility (%)				
Finland	77 (16)	82 (40-92)	80 (78-81)	<0.001
Denmark	67 (14)	70 (40-87)	69 (68-70)	
Progressive motility(%)				
Finland	68 (18)	75 (21-86)	70 (68-72)	
Denmark	57 (16)	59 (28-79)	57 (56-59)	<0.001
Total number of morphologically normal spermatozoa ($x10^6$)				
Finland	14 (17)	10 (0-43)	11 (9-13)	0.38
Denmark	17 (21)	10 (0-60)	10 (9-11)	
Total number of progressively motile spermatozoa ($x10^6$)				
Finland	132 (114)	104 (3-336)	106 (94-120)	0.001
Denmark	119 (109)	89 (5-342)	84 (78-90)	

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Table 14. Semen quality of the subgroup of healthy young men in Finland and Denmark*.

Semen variables	Subgroup (Finland n=166, Denmark n=465)			
	OBSERVED		ADJUSTED	P-value
	Mean (SD)	Median (5^{th} -95^{th})	Median (5^{th} -95^{th})	
Semen volume (mL)				
Finland	3.1 (1.5)	3.1 (0.8-6.2)	2.7 (2.5-2.9)	0.05
Denmark	3.3 (1.5)	3.1 (1.2-6.2)	3.0 (2.8-3.1)	
Sperm concentration ($x10^6$/mL)				
Finland	61 (42)	51 (11-139)	53 (46-61)	0.66
Denmark	64 (53)	52 (5-166)	51 (47-56)	
Total sperm count ($x10^6$)				
Finland	175 (128)	151 (17-454)	147 (128-169)	0.78
Denmark	202 (171)	157 (15-562)	151 (139-163)	
Normal morphology (%)				
Finland	7.7 (4.3)	7.0(2.0-16.0)	6.9 (6.2-7.7)	0.23
Denmark	7.5 (4.8)	7.0 (0.5-16.0)	6.4 (6.0-6.8)	
Total motility (%)				
Finland	78 (15)	83 (48-92)	80 (78-82)	<0.001
Denmark	67 (14)	69 (39-87)	69 (67-70)	
Progressive motility (%)				
Finland	69 (18)	75 (21-86)	71 (68-73)	<0.001
Denmark	57 (16)	59 (27-79)	57 (56-59)	
Total number of morphologically normal spermatozoa ($x10^6$)				
Finland	14 (15)	9 (0-43)	10 (9-13)	0.69
Denmark	18 (21)	10 (0-64)	10 (9-11)	
Total number of progressively motile spermatozoa ($x10^6$)				
Finland	128 (99)	103 (4-337)	103 (89-119)	0.04
Denmark	121 (108)	91 (6-344)	86 (78-94)	

*Subgroup analysis of men with no medications or androgenic hormones use, no past or current andrological diseases and no fertility problem (unsuccessful attempt to achieve pregnancy with regular unprotected sexual intercourses for at least 12 months)
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Semen quality of all men in the study is shown in **Table 13**. Semen quality of the subgroup of men who did not use any medication or androgenic steroids and had no past or current andrological diseases and no fertility problem defined as unsuccessful attempt to achieve pregnancy with regular unprotected sexual intercourses for at least 12 months is shown in **Table 14**. Analysis of all men showed

that Finnish men had significantly lower semen volume but higher sperm motility than Danish men. Sperm concentrations, total sperm counts and percentage and number of morphologically normal spermatozoa of the two countries were similar. There was a higher percentage of men in Denmark than in Finland who had sperm concentration less than 15 million/mL, which is the lower reference limit according to the WHO manual 2010 (15.0% vs 9.1%, respectively, p = 0.01). Subgroup analysis showed similar findings as the analysis of all men, except that there was no significant difference in semen volume between the countries.

When three main semen variables were taken into account together and classified into low, intermediate, and high semen quality, we found that a lower proportion of Finnish men had low semen quality than Danish men (25.4% vs. 34.6%, p=0.004) **(Figure 2)**.

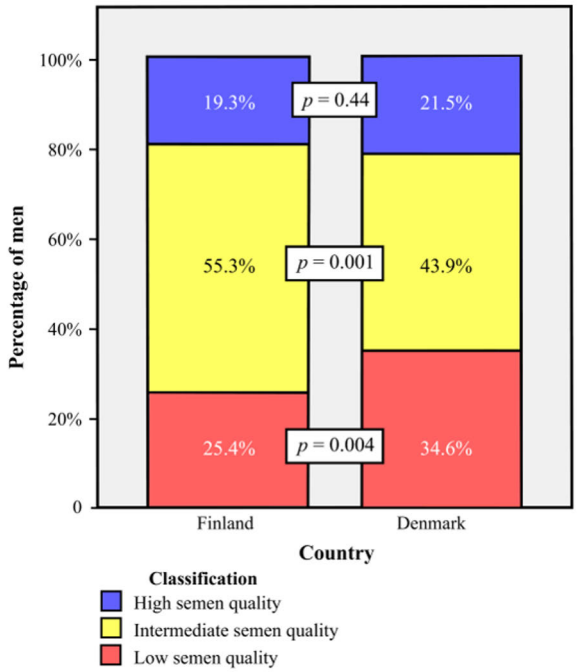

Figure 2. Classification of semen quality of the Finnish and Danish men when sperm concentration, progressive sperm motility and percentage of morphologically normal spermatozoa were taken into account together. *Low semen quality:* sperm concentration <15 million/mL or percentage of progressive (A+B) sperm motility <32% or percentage of morphologically normal spermatozoa <4% or as any combination of these. *Intermediate semen quality:* semen variables fell between low and high semen quality. *High semen quality*: sperm concentration >40 million/mL AND percentage of progressive (A+B) sperm motility >50% AND percentage of morphologically normal spermatozoa >9%. The differences between countries in each semen quality category were tested by chi-squared test. This figure is reproduced from Rodprasert W et al. Andrology. 2019;7(1):15–23 with permission.

Study 3: Reproductive Markers of Testicular Function and Size during Puberty in Boys with and without a History of Cryptorchidism

At the start of the study, 16 boys who had a history of bilateral cryptorchidism, 30 boys with a history of unilateral cryptorchidism and 63 controls came for an examination and had blood samples collected. The onset of puberty was similar between groups. Most blood samples were collected from 7.30 to 11.00 and 13.01–16.00 because most participants were available during these times **(Table 15).**

Comparisons of serum FSH and inhibin B levels between groups

FSH and inhibin B levels reflect Sertoli cell and germ cell function and/or number. **Figure 3** illustrates FSH and inhibin B levels during the study. Overall, the figure showed that boys with a history of cryptorchidism in any group had higher FSH and similar or lower inhibin B levels than controls, even though the statistical significance was not shown in all visits.

Boys in the OpBC group had significantly higher FSH levels than controls from pubertal onset until complete puberty. Inhibin B levels of the OpBC group were lower than controls from 0.5 years after pubertal onset onwards. These results indicate low Sertoli cell and/or germ cell function and/or number. FSH and inhibin B levels of boys in the SpBC group were not statistically different from controls; however, FSH went up and inhibin B fell rapidly from approximately 1.5 years after pubertal onset.

Boys in the OpUC and SpUC had similar changes in FSH and inhibin B levels. FSH levels were significantly higher than controls two years after pubertal onset onwards, whereas inhibin B levels were not statistically different from controls.

Comparisons of serum LH, testosterone and INSL3 levels between groups

Serum LH, testosterone, and INSL3 reflect Leydig cell function and/or number. Serum LH and testosterone levels increased, and SHBG decreased as puberty progressed. LH and testosterone levels were not different between groups during the study. Likewise, INSL3 levels at full puberty were not statistically different between groups (mean±SD, 1.1±0.4, 1.7±1.1, 1.2±0.6, 1.3±0.4, and 1.5±0.7 μg/L in the OpBC, SpBC, OpUC, SpUC, and controls, respectively, p=0.17). SHBG was generally similar between groups, except at 0.5 years after pubertal onset when the SHBG levels of SpBC were less than those of controls (**Figure 4**).

Table 15. Characteristics of the participants.

	OpBC	SpBC	OpUC	SpUC	Controls
N at the first visit	9	7	15	15	63
N at the final visit	7	3	11	9	48
Testicular position at birth					
Suprascrotal or higher	9	4	13	8	-
High scrotal	-	3	2	7	-
Scrotal or retractile	-	-	-	-	63
Age at the onset of puberty, yrs (mean (SD))	11.6 (1.0)	11.9 (1.8)	11.4 (0.9)	12.1 (1.4)	11.8 (1.0)
Height at the last visit, cm (mean (SD))	176.8 (5.2)	179.4 (3.6)	178.0 (5.0)	176.4 (7.0)	179.0 (6.7)
Time of blood sampling					
Total N	92	49	197	147	748
07:30-11:00	28	19	62	53	324
11:01-13:00	6	5	13	6	58
13:01-16:00	54	18	114	87	347
16:01-18:30	4	7	8	1	19

The table includes only boys who had available blood samples.
Between-group differences were not significant.
Abbreviations: OpBC, bilateral cryptorchidism with a history of operation (orchiopexy); OpUC, unilateral cryptorchidism with a history of operation (orchiopexy); SpBC, bilateral cryptorchidism with spontaneous testicular descent; SpUC, unilateral cryptorchidism with spontaneous testicular descent.
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Comparisons of serum growth factors between groups

Serum levels of IGF-I and IGFBP-3 increased as puberty progressed. The IGF-I levels did not differ between groups, except OpBC and SpUC groups had higher levels than controls one year and two years after pubertal onset, respectively. IGFBP-3 levels of the five groups were similar, except at 1.5 years after pubertal onset when boys in the SpBC group had lower IGFBP-3 levels than controls (**Figure 5**).

Comparisons of testicular volume between groups

Combined testicular volumes of the boys with a history of orchiopexy and with either unilateral or bilateral cryptorchidism (OpUC and OpBC) were smaller than those of controls one year after puberty started onwards. Boys with spontaneous testicular descent (SpBC and SpUC) had similar testicular volume to controls (**Figure 6**).

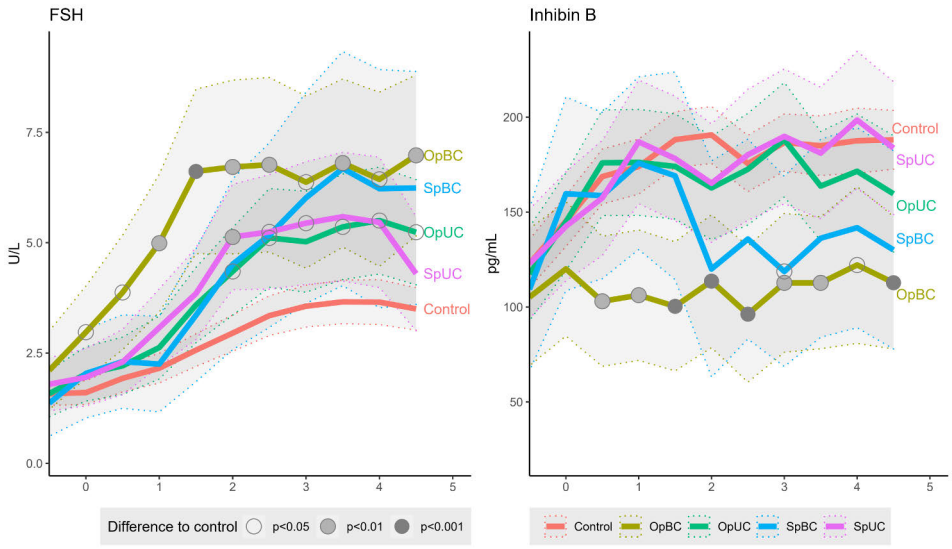

Figure 3. Serum levels of FSH (left panel) and inhibin B (right panel) of five groups of boys during puberty. The first time point is the first visit at the age of 8.5 years. The onset of puberty is denoted by 0 on the X-axis. The thick, continuous lines represent the modeled-based mean serum hormone levels. The thin, dashed lines and the shaded areas represent the 95% confidence intervals. Reproduced from Rodprasert W et al. J Clin Endocrinol Metab. 2022 Nov 25;107(12):3353-3361. with permission.

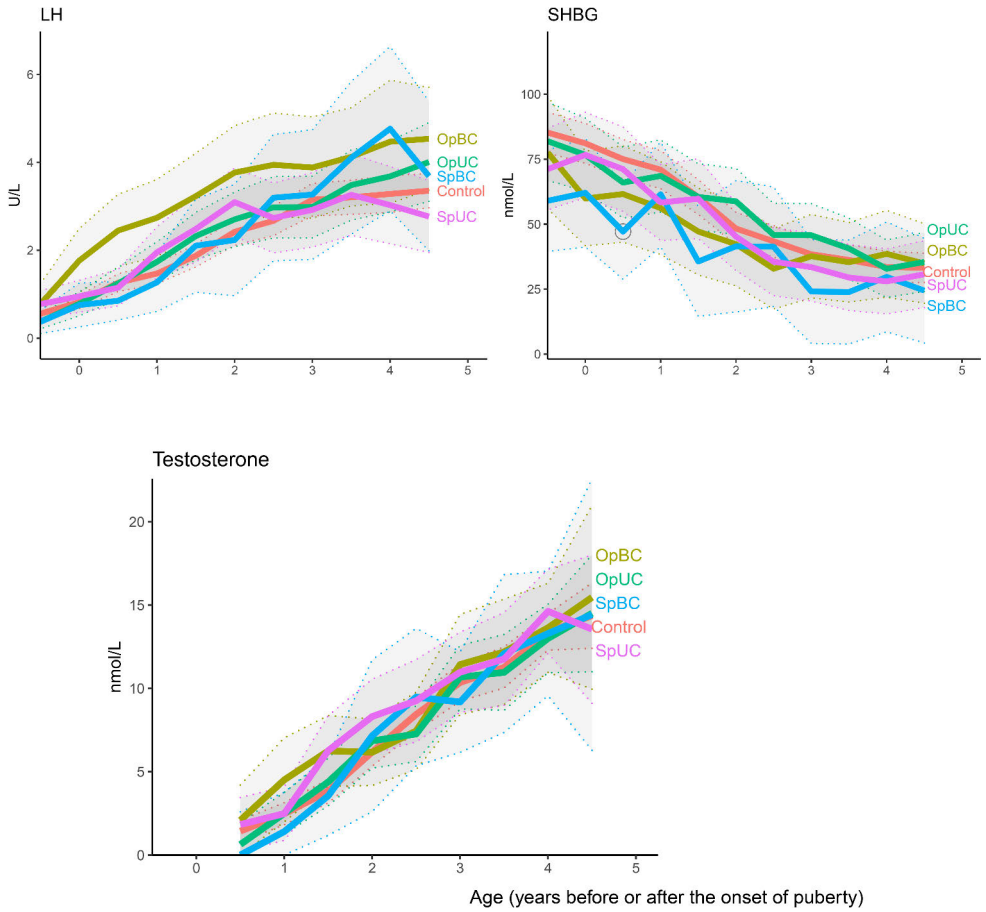

Figure 4. Serum levels of LH, testosterone and SHBG of five groups of boys during puberty. The onset of puberty is denoted by 0 on the X-axis. Testosterone levels were undetectable before 0.5 year after puberty started; therefore, the graph did not include those measurements. The thick, continuous lines represent the modeled-based mean serum hormone levels. The thin, dashed lines and the shaded areas represent the 95% confidence intervals. Reproduced from Rodprasert W et al. J Clin Endocrinol Metab. 2022 Nov 25;107(12):3353-3361. with permission.

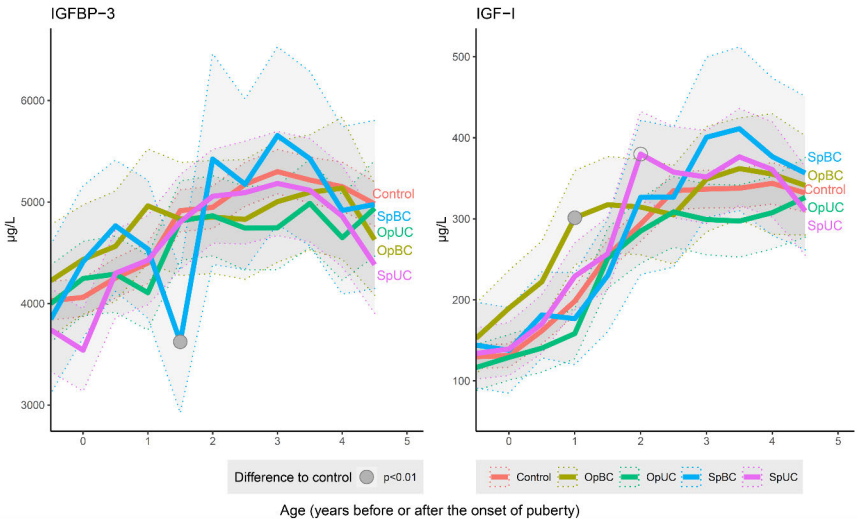

Figure 5. Serum levels of IGFBP-3 and IGF-I of five groups of boys during puberty. The first time point is the first visit at the age of 8.5 years. The onset of puberty is denoted by 0 on the X-axis. The thick, continuous lines represent the modeled-based mean serum hormone levels. The thin, dashed lines and the shaded areas represent the 95% confidence intervals. Reproduced from Rodprasert W et al. J Clin Endocrinol Metab. 2022 Nov 25;107(12):3353-3361. with permission.

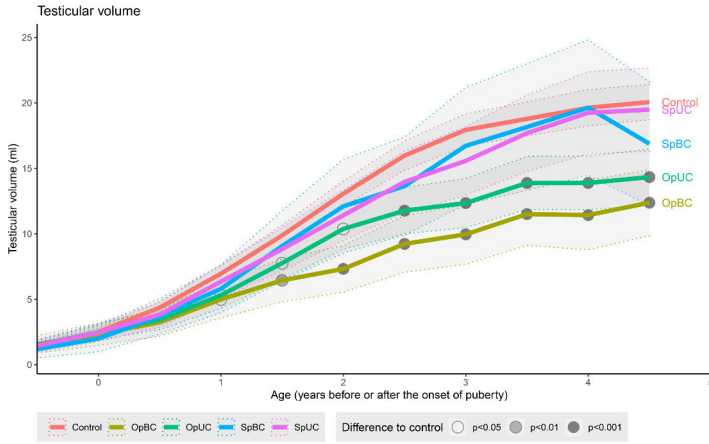

Figure 6. The combined testicular volume of both testes by ultrasonography of five groups of boys during puberty. The first time point is the first visit at the age of 8.5 years. The onset of puberty is denoted by 0 on the X-axis. The thick, continuous lines represent the modeled-based mean total testicular volume. The thin, dashed lines and the shaded areas represent the 95% confidence intervals for the means. Reproduced from Rodprasert W et al. J Clin Endocrinol Metab. 2022 Nov 25;107(12):3353-3361. with permission.

6 Discussion

Study 1: Semen quality improves marginally during young adulthood: a longitudinal follow-up study

Semen quality alters according to age. In this longitudinal follow-up study of the men from the age of 19 to 29 years, we demonstrated that at the age of 19 years, the testis almost reaches its full ability to produce sperm. Semen volume increased slightly in the exploratory cohort but not in the validation cohort. However, the maturation continued to improve further as sperm motility and morphology increased after the age of 19 years.

Carlsen et al. studied longitudinal semen quality in 158 men starting at a median age of 19.1 years for up to 4 years (Carlsen et al., 2005). The samples were collected up to 4 times per year. They found that sperm concentration, total sperm count and the percentage of morphologically normal spermatozoa did not significantly increase with advancing age. However, among men below 23 years of age, semen volume and the percentage of rapid progressive motile spermatozoa increased, while non-progressive motile spermatozoa and immotile spermatozoa decreased during the follow-up (Carlsen et al., 2005). These results were in general similar to the present study. However, we found that the percentage of morphologically normal spermatozoa increased significantly during a 10-year follow-up. This might be because this increase seemed to occur after the age of 25 years, which was beyond the period of follow-up of the Danish study. A retrospective study was conducted in 6,022 semen samples in 4,604 men aged from below 25 years to above 55 years who had sperm concentration above 20 million/mL (Levitas et al., 2007). Semen volume increased to the age of 30-35 years and lowered thereafter, which might be a result of declined function of accessory gland (Gunes et al., 2016). Total sperm count and sperm motility decreased after the age of 55 years. However, the semen samples in this study were not collected from the same men in series, therefore the variations in confounding factors might influence the results.

The higher percentages of morphologically normal spermatozoa or sperm motility in 29- as compared with 19-year-old men may result in an increase in fertility as it has been shown that higher levels of these sperm parameters are associated with increased fertility (Guzick et al., 2001; Sripada et al., 2010). On a

population level, the results of our study suggest that male fertility increases when men are approaching the age of 30 years as compared with when they are at early 20s.

The advantage of this study is a long period of follow-up. This allows the possibility to study changes of semen parameters during young adult life, especially of the same individuals. In addition, the laboratory was under a regular external quality control of sperm concentration, which showed that the results assessed by the technician were at a stable level over the study period.

The limitations of this study are the low participation rate of the men who attended all four study visits, which might limit the generalizability of the findings. A usual problem of semen quality studies is a low participation rate, which might have a concern of selection bias. This study, which was designed to follow the men for ten years, is even more difficult to have high participation, particularly for the men who attended all four visits. This study did not show the differences in the factors related to poor semen quality, for instance, a history of smoking, body mass index, or general medical illness or reproductive diseases, between the participants and non-participants as well as between the men who participated in all four visits and the ones who participated only in some visits. Furthermore, this study did not report the reasons for not participation in some visits, which might include the health-related causes. Therefore, the selection bias in this study was possible. However, the men were invited to participate in this study irrespective of their medical conditions, lifestyles, or socioeconomic status. There was low percentage of participants who reported a history of reproductive system diseases, which can be implied that having had a history of reproductive problem might not be the main reason to participate in the study.

In conclusion, our study showed that testicular sperm production capacity almost reaches its full potential at the age of 19 years, while sperm motility and morphology improve further with age. This finding indicates that sperm count at the age of 19 years can reflect sperm production capacity of the mature men.

Study 2: An update on semen quality among young Finnish men and comparison with Danish data

We demonstrated that the latest semen quality of the Finnish and Danish men was overall similar. Only sperm motility was higher in Finns as compared with Danes.

Semen quality of the young Finnish men in the present study was lower than the results from previous studies, whereas semen quality in Denmark has been relatively stable. Vierula et al showed that semen quality of the young Finnish men was stable at a high level for three decades from the examination year 1967 to 1994 (Vierula et al., 1996). The adjusted sperm concentration slightly increased from 82.4 to 85.2

million/mL, while total sperm count slightly decreased from 344.5 to 324.8 million/mL (Vierula et al., 1996). However, the adjusted median sperm concentration decreased from 67 to 48 million/mL and adjusted median total sperm count decreased from 228 to 165 million between 1998 and 2006 (Jørgensen et al., 2011). In the present study, the adjusted median sperm concentration of the young Finnish men was 49 million/mL and adjusted median total sperm count was 148 million, which was lower than that reported in the previous study. In contrast, the latest study in Denmark revealed that semen quality of the Danish men has been relatively stable during 2 decades from 1996 to 2016. The adjusted median sperm concentration in the period 1996-2000 was 43 million/mL and in 2010-2016 it was 41 million/mL (Priskorn, et al., 2018).

Epidemiological studies showed that countries with low semen quality also have high incidence of other male reproductive disorders (Serrano et al., 2013; Skakkebaek et al., 2016). These findings support the testicular dysgenesis syndrome.

The causes of decreasing semen quality of the Finnish men are still unclear. It has been proposed that a global decline in semen quality was most likely resulted from environmental rather than genetic factors, because the changes of semen quality occurred in a relatively short period of time. Numerous factors have been shown to be associated with low semen quality, for example obesity, diabetes mellitus, environmental chemical exposures, varicocele and cigarette smoking (Neto et al., 2016). Body mass index, frequency and number of cigarette smoking, amount of alcohol consumption, maternal history of cigarette smoking during pregnancy, frequency of diagnosis of sexually transmitted diseases and varicocele were similar between the men examined between 1998 and 2006 and the men in the present study, suggesting that these factors are less likely the main culprit of the deteriorating semen quality in Finland. A history of maternal and paternal cigarette smoking during pregnancy were common in both countries, particularly in Denmark, which might influence the semen quality of the sons. Studies showed that maternal or paternal smoking during pregnancy is slightly associated with cryptorchidism and low semen quality in the sons (Virtanen et al., 2012). In the future, researchers should examine the role of other lifestyle factors such as the use of computer, mobile phone, mental stress, physical activities, sauna use, diets, beverages, etc. and environmental chemical exposures on semen quality.

The participation rate in Finland as compared with Denmark was low. However, the basic information of the participants and 104 non-participants who provided their information in the refusal letters, including height, weight and body mass index, was similar. However, factors affecting semen quality, such as smoking history and medical illness, were not asked in the letters. Therefore, the risk of selection bias in our study is low, but not completely ruled out.

In conclusion, this study demonstrated that the formerly high semen quality in Finland has declined to a low level in Danish men. Our finding highlights the importance of continuing surveillance of semen quality to examine the trend further. Because the incidence of testicular cancer increases and semen quality declines, it might be interesting to see the latest prevalence of TDS-associated disorders such as cryptorchidism.

Study 3. Reproductive markers of testicular function and size during puberty in boys with and without a history of cryptorchidism

This longitudinal pubertal hormone study showed that types (unilateral or bilateral cryptorchidism) and nature (spontaneous testicular descent or requiring orchiopexy) of cryptorchidism influence FSH and inhibin B levels which are Sertoli cell/germ cell biomarkers. Leydig cell function assessed by LH, testosterone and INSL3 levels was preserved in boys with a history of cryptorchidism with or without orchiopexy. Serum growth factor levels generally did not alter in these boys.

Inhibin B is a Sertoli cell biomarker during prepuberty, because α- and βB-subunits of inhibin B are produced from Sertoli cells in this period (Andersson et al., 1998). However, after the onset of meiosis during puberty, germ cells produce βB-subunits, while Sertoli cells still produce α-subunits. Therefore, at the end of puberty, inhibin B is a marker of the cooperative function of Sertoli cells and germ cells (A. M. Andersson et al., 1998). In adult men, inhibin B and FSH levels are associated with spermatogenesis. Serum inhibin B level is positively correlated with sperm concentration and total sperm count (Jørgensen et al., 2010; Meeker et al., 2007), while serum FSH level was negatively correlated with sperm concentration, motility, and percentage of sperms with normal morphology (Meeker et al., 2007).

In this study, boys with a history of bilateral cryptorchidism generally had higher FSH and lower inhibin B levels than controls. This indicates reduced Sertoli cell and/or germ cell function or number in bilateral cryptorchidism, which was statistically significant and more apparent in individuals who underwent orchiopexy. This suggests a more severe testicular pathology in OpBC boys than in other groups. Unilateral cryptorchid boys had significantly higher FSH but similar inhibin B levels as compared with controls. This indicates reduced Sertoli cell and/or germ cell function or number with a compensated pituitary response, which is considered a less severe pathology than that found in boys with a history of bilateral cryptorchidism.

The testicular volume of boys with a history of unilateral or bilateral cryptorchidism who underwent orchiopexy was smaller than controls. In contrast, the testicular volume of those cryptorchid boys who had spontaneous testicular descent was not different from controls. Since germ cells are the main contributors

to testicular size in late puberty and adulthood, a small testis indicates a low germ cell number. This is in agreement with results from several studies, which showed that men with a history of unilateral or bilateral cryptorchidism had lower sperm concentration than healthy controls (Trsinar & Muravec, 2009; van Brakel et al., 2014), and men with a history of bilateral cryptorchidism had lower sperm concentration than men with a history of unilateral cryptorchidism (Moretti et al., 2007). Azoospermia is more frequently observed in men who had a history of bilateral cryptorchidism than unilateral cryptorchidism (Moretti et al., 2007). Operation is not considered to be a cause of further testicular damage. However, our results can imply that the testis that did not descend spontaneously had more severe pathology than controls and orchiopexy did not reverse testicular abnormalities in these boys.

Serum LH, testosterone, INSL3, and SHBG levels of all groups were similar, indicating that Leydig cell function and/or number are generally preserved in cryptorchidism. A previous study from the same boy cohort supported this finding by showing that the onset of puberty of the boys with a history of cryptorchidism was not different from controls (Sadov et al., 2016).

There is a limited number of studies evaluating reproductive hormone levels during puberty in cryptorchid boys. Two longitudinal follow-up studies published in the 1970s showed different results. Dickerman et al. found that FSH and LH levels of the cryptorchid boys were higher than normal reference range, while basal and hCG-stimulated testosterone levels were lower than normal (Dickerman et al., 1979). These results suggested Sertoli cell and Leydig cell dysfunction due to a primary testicular defect in boys with a history of cryptorchidism. However, Gendrel et al. reported that the peaked LH levels after GnRH test were lower in boys with a history of cryptorchidism from infancy to early puberty as compared with controls, while FSH levels were not different (Gendrel et al., 1977). The peaked testosterone levels were blunted in boys with a history of cryptorchidism from the age of one year to mid-puberty. This study suggested Leydig cell dysfunction due to pituitary LH secretory defect, but Sertoli cell function is preserved in cryptorchid boys (Gendrel et al., 1977). These two studies reported different results from the present study. One factor to note is that the assays used to measure serum gonadotropin and testosterone levels were different. The assays at that time had less sensitivity than the newer assays, particularly for detecting gonadotropin and testosterone at a low level during prepuberty to early puberty.

Serum IGF-I and IGFBP-3 levels of all groups were generally similar. However, the IGF-I level in the group of OpBC was higher than that of controls at one year following the onset of puberty, and the IGF-I level SpUC group was higher than that of controls at two years after pubertal onset. This transient increase of growth factor during early puberty is probably a compensatory drive of testicular growth in

cryptorchid boys who generally have small testes. Even though the role of growth factors on testicular growth is unclear, some evidence suggests the role of IGF-I on testicular growth during puberty (Juul & Skakkebæk, 2019). GH and IGF-I receptors are detected in germ cells, Leydig cells, Sertoli cells, and the prostate in rats (Lobie et al., 1990). Animal studies showed that IGF-I induces Sertoli cell proliferation and germ cell proliferation and differentiation (Cannarella et al., 2018). In addition, FSH action on Sertoli cell proliferation needs the insulin receptor and IGF-I receptor (Pitetti et al., 2013). Finnish-Danish birth cohort study demonstrated the role of IGF-I in testicular descent during mini-puberty (Koskenniemi et al., 2018). Juul and Skakkebæk have hypothesized that the very high IGF-I levels during puberty, similar to the levels found in acromegalic patients, have a role in the development and growth of the sexual organs (Juul & Skakkebæk, 2019). However, further studies are needed to gain a better understanding of the role of GH-IGF-I system on testicular growth and function.

The strength of this study is the study design that all the boys were examined at birth and the presence or absence of cryptorchidism at birth was recorded. This eliminates the risk of recall bias among the participants in the retrospective studies. In addition, the possibility of an inclusion of retractile testis or acquired cryptorchidism in the analysis was excluded. Longitudinal pubertal data were carefully collected and covered the entire period of puberty, allowing us to examine the development during puberty reliably.

The main limitation of the study is the small number of participants in some groups, particularly OpBC and SpBC groups. This limits the power to detect any statistical differences between groups. And also, because of this, we did not perform the analysis to investigate the effects of age at orchiopexy on hormone levels and testicular size. In addition, blood sampling time varied from 7.30 to 18.30, which might affect reproductive hormone levels. However, we adjusted the hormone levels by time of blood sampling in the model to reduce the influence of time on hormone levels.

In conclusion, congenital cryptorchidism, especially bilateral cryptorchidism requiring orchiopexy, was associated with a decreased testicular size and reduced Sertoli cell or germ cell function and/or number during puberty. This finding might explain infertility in adulthood. Leydig cell function was preserved during puberty. Follow-up of these participants into adulthood to see their reproductive hormone levels and fertility status and their associations with the findings during puberty will gain knowledge on congenital cryptorchidism. In addition, a separate study is needed to investigate the influence of age at orchiopexy on hormone levels and testicular volume during puberty and adulthood.

7 Summary/Conclusions

This doctoral research adds new knowledge and increases understanding about semen quality changes in young men, the updated status of semen quality of young men in Finland and Denmark, and the reproductive hormone and IGF-I levels during puberty in boys with and without a history of cryptorchidism.

The first study showed that spermatogenesis almost reaches its highest level at 19 years; however, sperm maturation continues until at least ten years later. Therefore, the epidemiological study of semen quality at 19 years represents the testicular sperm productive capacity of mature men.

The downward trend of semen quality has been demonstrated in several studies, which causes concern about the fertility rate in the future. This doctoral thesis research showed that semen quality of the young Finnish men has decreased to the low level of the Danish men. Recent studies showed that male reproductive health is worsening especially in the countries that used to have good reproductive health. These findings highlight the importance of surveillance of semen quality in the young men from general population and the rates of other male reproductive disorders that have links with TDS. Clinical, epidemiological and experimental studies that investigate the possible causes of this adverse trend are also necessary.

Cryptorchidism is the most common congenital urological malformation. It is linked with long-term consequences that need care. This doctoral thesis increases knowledge on the consequences of congenital cryptorchidism on testicular endocrine function during puberty. Boys with a history of cryptorchidism, especially bilateral cryptorchidism needed orchiopexy, have impaired Sertoli cell and germ cell function at the end of puberty, while Leydig cell function is preserved. The growth factors during puberty were generally similar to controls, with some time points that showed elevated IGF-I levels. More studies are needed to examine the long-term Sertoli and Leydig cell function of men with a history of cryptorchidism. In addition, the role of growth hormone/IGF-I system on testicular growth and function needs to be investigated further.

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