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Citation for published version (APA):

Richthoff, J. (2007). *The impact of genetic, environmental and life-style factors on male reproductive function*. [Doctoral Thesis (compilation), Reproductive medicine, Malmö]. Department of Clinical Sciences, Lund University.

Total number of authors:

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The impact of genetic, environmental and life-style factors on male reproductive function

Jonas Richthoff, M.D.

MALMÖ

2007

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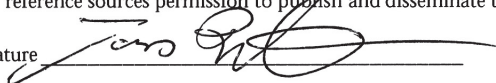
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Organization LUND UNIVERSITY	Document name DOCTORAL DISSERTATION	
	Date of issue 2007-04-27	
	Sponsoring organization	
Author(s) Jonas Richthoff		
Title and subtitle The impact of genetic, environmental and life-style factors on male reproductive function		
<p>Abstract</p> <p>Recent studies have indicated a decline in sperm number in the western world during the past 50 years, sperm concentration changing from 113 to 66 millions per mL. Furthermore, significant geographical differences in male reproductive function have been observed. In Finland, the sperm concentration was found to be substantially higher than in Denmark. In parallel, the incidence of testicular cancer (TC) and of congenital malformations of male genital organs is significantly higher in Denmark as compared to Finland. Previous studies have shown an association between decreased sperm quality and risk of TC. Recently, it was suggested that poor sperm quality, cryptorchidism, hypospadias and TC have a common cause and are symptoms of a so called Testicular Dysgenesis Syndrome (TDS). The aetiology of TDS is unknown but genetic, environmental and life-style related factors have been suggested as its causes. Genetic factors might, at least partly, explain the difference in male reproductive function between Denmark and Finland. However, Swedish and Danish populations are genetically similar and therefore any differences in reproductive parameters found between these two countries are most likely due to environmental and/or life-style related factors. The aim of this study was to compare reproductive parameters between Swedish and Danish men and to investigate the possible impact of environmental, life-style and genetic factors.</p> <p>All papers included in this thesis were based on investigations of Swedish military conscripts (n=305). Semen parameters of the Swedish conscripts were compared to previously published data on their Danish counterparts. Furthermore, reproductive parameters were evaluated in relation to exposure to persistent organohalogen pollutants (POP) and to smoking. Polymorphisms in the androgen receptor gene were also assessed. We found 30% higher sperm number as well as higher sperm concentration and semen volume in Swedish men as compared to Danish. Non-smoking men presented with 40% higher sperm number as compared to smokers. Exposure to POP, assessed by serum levels of CB-153, was associated with impairment of sperm motility but not with the number of spermatozoa. Androgen receptor CAG repeat number was only weakly associated with sperm parameters.</p> <p>Our data indicated that the reproductive function of Swedish adolescent men is superior to that of their Danish counterparts. Smoking is less prevalent in Sweden compared to Denmark and this could, at least partly, contribute to the difference in sperm number found between these countries.</p>		
Key words: Male reproductive function, sperm concentration, Cryptorchidism, testicular cancer, smoking, environment, genetic, sex hormone, accessory sex gland function, maternal smoking		
Classification system and/or index terms (if any): Jonas.Richthoff@med.lu.se		
Supplementary bibliographical information:	Language English	
	ISBN 978-91-85559-31-2	
ISSN and key title: 1652-8220		
Recipient's notes	Number of pages 138	Price
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**Lund University
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Jonas Richthoff, M.D.

MALMÖ

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ABBREVIATIONS

AIS	Androgen insensitivity syndrome
AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
BMI	Body mass index
CASA	Computer-aided sperm analysis
CB-153	2,2',4,4',5,5'-hexachlorobiphenyl
CIS	Carcinoma <i>in situ</i>
CV	Coefficient of variation
DHT	5 α -dihydrotestosterone
DDT	2,2-bis (4-chlorophenyl)-1,1,1-trichloroethane
DNA	Deoxyribonucleic acid
ED	Endocrine disrupters
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
hCG	Human chorion gonadotropin
IVF	In vitro fertilization
LH	Luteinizing hormone
PCG	Primordial germ cell
PCR	Polymerase chain reaction
POP	Persistent organohalogen pollutant
PCB	Polychlorinated biphenyls
PSA	Prostate specific antigen
AMH	Anti-Müllerian hormone
NAG	Neutral α -glucosidase
SBMB	Spinal bulbar muscle atrophy
Sg I	Semenogelin I
SgII	Semenogelin II
SHBG	Sex hormone binding globulin
StAR	Steroidogenic acute regulatory protein
SRD5A2	5 α -reductase type 2 gene
SRY	Sex determining region on the Y chromosome
T	Testosterone
TGCC	Testicular germ cell cancer
TC	Testicular cancer
TDS	Testicular dysgenesis syndrome
TEQ	Toxic equivalence
VCL	Curve linear velocity
WHO	World health organization

LIST OF PAPERS

This thesis is based on the following five papers, which will be referred according to their Roman numerals:

- I. Richthoff, J., Rylander, L., Hagmar, L. Malm, J. and Giwercman, A. (2002) Higher sperm counts in Southern Sweden compared with Denmark. *Human Reproduction*, **17**, 2468-2473.
- II. Richthoff, J., Rylander, L., Jonsson, B.A.G., Åkesson, H., Hagmar, L., Nilsson-Ehle, P., Stridsberg, M. and Giwercman, A. (2003) Serum levels of 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) in relation to markers of reproductive function in young males from the general Swedish population. *Environmental Health Perspectives*, **111**, 409-413.
- III. Giwercman, Y.L., Richthoff, J., Lilja, H., Anderberg, C., Abrahamsson, P-A and Giwercman, A. (2004) Androgen receptor CAG repeat length correlates with semen PSA levels in adolescence. *Prostate*, **59**, 227-233.
- IV. Richthoff, J., Elzanaty, S., Rylander, L., Hagmar, L. and Giwercman, A. Association between tobacco exposure and reproductive parameters in adolescent males. *International Journal of Andrology*, in press.
- V. Richthoff, J., Rylander, L., Gade-Nielsen, P, and Giwercman, A. The impact of tobacco exposure on sperm morphometric parameters in adolescent males. *Manuscript*.

INTRODUCTION

EPIDEMIOLOGICAL TRENDS

Time related decline of sperm number

Since the 1940s, the reference value for normal sperm counts has changed from $60 \times 10^6/\text{ml}$ to the present $20 \times 10^6/\text{ml}$ (WHO 1999) and in 1974 Nelson and Bunge suggested that the human semen quality actually has deteriorated (Nelson & Bunge, 1974). Carlsen and co-workers performed a meta analyze covering 61 papers from 1938 to 1990 and found a significant decline in mean sperm counts from $113 \times 10^6/\text{ml}$ to $66 \times 10^6/\text{ml}$ among men without history of infertility (Carlsen *et al.*, 1992) (Figure 1).

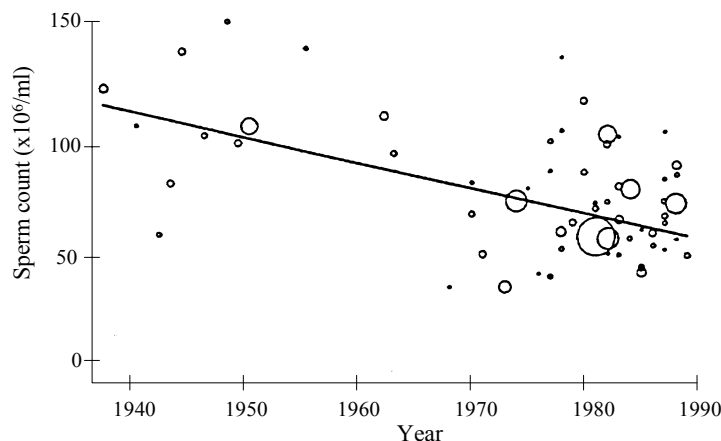


Figure 1. Decline in sperm density in the Western world during the last 50 years (modified from Carlsen *et al.*, 1992).

A later study, on healthy, fertile men from Paris, also showed a decline in sperm concentration during 1973-1992, from $89 \times 10^6/\text{ml}$ to $60 \times 10^6/\text{ml}$ (Auger *et al.*, 1995). This study also showed that motility and the proportion of normal spermatozoa were decreased. Furthermore, a study in Scotland, comparing semen

donors born before 1959 and after 1970 showed that median sperm number was lower in donors born after 1970, 301×10^6 vs. 214×10^6 per ejaculate.

The suggestion of declining sperm count has been criticised by many authors, but despite the criticism, where unchanged or even improved sperm quality during the latest decades was claimed (Brake & Krause, 1992; Olsen *et al.*, 1995; De Nully Brown *et al.*, 1989; Farrow, 1994), the support for the hypothesis of a time-related decline in male reproductive function has increased (Bonde *et al.*, 1998; Swan *et al.*, 2000; Van Waeleghem *et al.*, 1996; Auger *et al.*, 1995; Giwercman, 1995). Recently also a geographic variation in sperm counts has been suggested.

Geographic differences in reproductive function

The first study to assess geographic differences in semen quality compared 411 Danish men with 221 Finnish males (Jensen *et al.*, 2000). A considerably higher sperm concentration and sperm count in Finland ($104 \times 10^6/\text{ml}$, 304×10^6) compared to Denmark ($53 \times 10^6/\text{ml}$ and 140×10^6) was found. The authors suggested that some of this discrepancy could be explained by low participation ratio for the Danish cohort (<1%), an age difference of 3 years, or inter-laboratory variation (15%) in assessment of semen parameters.

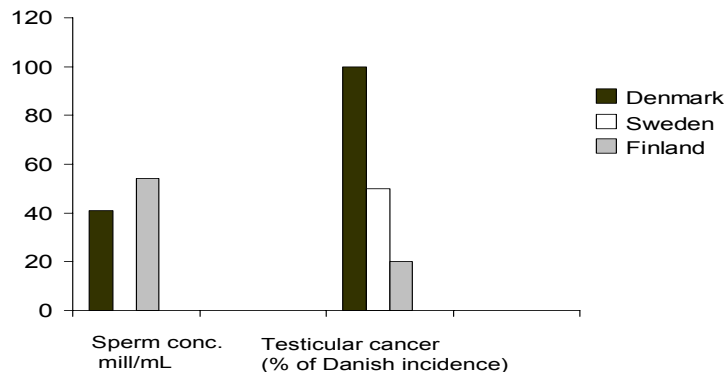


Figure 2. Differences in sperm concentration and testicular cancer incidence, in three Nordic countries.

(Denmark), Edinburgh (Scotland) and Paris (France) were compared. Lowest sperm concentration and total counts were again detected for Danish men followed by the French and Scottish men, whereas Finish men had the highest sperm counts.

A more recent study on 324 fertile Japanese men, showed the same low level of semen quality as in Denmark (Iwamoto *et al.*, 2005). The first report from the United States to compare semen quality among study centers, using standardized methods was by Swan *et al.* (Swan, 2006). In this particular study, 512 spouses to pregnant women were enrolled. Among these, reduced sperm concentration and motility in semi rural and agricultural areas compared to more urban and less agriculture exposed areas was noted.

Interestingly, the testicular cancer incidence also differs between countries, with 5 times higher incidence in Denmark compared to Finland. In this context, Sweden has an intermediate position (Adami *et al.*, Int J Cancer 1994) (Figure 2). This difference in testicular germ cell cancer (TC) incidence could also strengthen the hypothesis of a geographic variation in male reproductive health, as higher TC incidence is associated with lower reproductive capacity. This west-east difference is also present in cryptorchidism and hypospadias, where the prevalence in Finland is much lower than in Denmark (0.9 vs. 1.7%) (Toppari *et al.*, 2001; Carlsen *et al.*, 1992; Boisen *et al.*, 2004).

Testicular Dysgenesis Syndrome (TDS)

It has been proposed that reduced semen quality, cryptorchidism, hypospadias and testicular cancer are symptoms of a common underlying cause - the so called Testicular dysgenesis syndrome (TDS), which due to Sertoli and Leydig cell dysfunction is arising already in foetal life (Skakkebaek *et al.*, 2001). Features of testicular dysgenesis have been found in men with poor semen quality, hypospadias, and in the contralateral testis in patient with unilateral cryptorchidism or testicular cancer (Toppari *et al.*, 2001). The aetiology of the clinical components of TDS remains unknown. Even if some could be caused by genetic defects alone (Zhang *et al.*, 1995; Raff *et al.*, 2000; Giwercman *et al.*, 2004), most are believed to be multi-factorial (Gracia *et al.*, 2000).

Cryptorchidism

Undescended testes or cryptorchidism is the most common congenital malformation in males, occurring in 0.4-3% of all full term boys at birth in the western countries (Boisen *et al.*, 2004; Toppari & Kaleva, 1999). Physical examination reveals a non- palpable testis in the scrotum. The most common location is the inguinal canal (63%), followed by the ectopic position (11%), the external ring (9%) and the intra-abdominal position (2%) (Gracia *et al.*, 2000) (Figure 3). Within 3 months after birth, the incidence rate spontaneously decreases to less than half. Abnormal sexual differentiation is associated with cryptorchidism and this condition can be considered as the cause of infertility in 2-9% of all

infertile patients (Carizza *et al.*, 1990). Testicular cancer is 3.6-7.4 times higher in an undescended testis and 5-10% of patients with TC have a history of an undescended testis, which is the most important risk factor of testicular neoplasm.

Caucasians have been reported to have 3 times higher incidence of cryptorchidism than African-Americans (Heinonen *et al.*, 1977) although some studies did not find any difference in incidence between different ethnic groups (Berkowitz *et al.*, 1993).

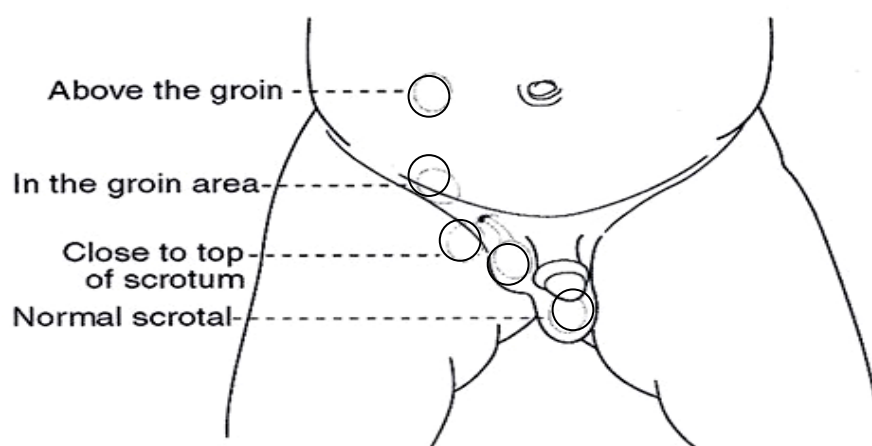


Figure 3. The most common positions of undescended testes.

The etiology of the disorder is not known, but normal hypothalmo-pitutary-gonadal axis is usually a prerequisite for normal testicular descent. Hypogonadotropic hypogonadism and androgen insensitivity are associated with cryptorchidism. Recently, a correlation between the androgen receptor (AR) GGN repeat and cryptorchidism was reported (Aschim *et al.*, 2004), showing that boys with a shift from the most common GGN variant of 23 to 24 were at higher risk for developing cryptorchidism than counterparts with GGN=23.

Hypospadias

Hypospadias (a misplaced urethral orifice along the ventral side of the penis or scrotum), is the second most frequent congenital malformation in males. In register studies, large geographic differences have been found with prevalences from 2 to 39 per 10,000 total live-borne births (Kallen *et al.*, 1986; Toppari *et al.*, 2001; Paulozzi, 1999). This might be due to discrepancies in diagnosing this condition as well as due to real regional differences. The severity of hypospadias is classified according to the anatomical position of the urethral meatus (Figure 4). It can in

mild cases be localized on the side of glans, or on the shaft of the penis, whereas more severe cases comprise scrotal and perineal anomalies. These forms are often combined with a curvature of the penile shaft.

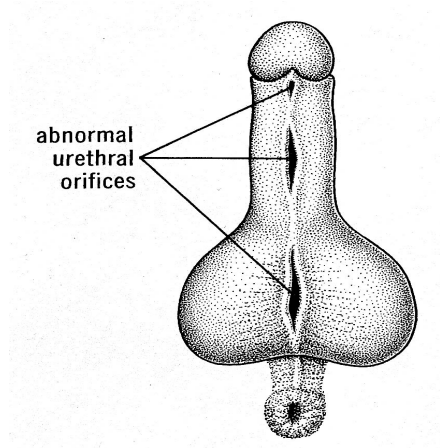


Figure 4. The localization of hypospadias.

The exact embryological processes causing hypospadias are not yet known, but it is generally considered that errors in canalization of the glans penis, incomplete fusion of the urethral folds, or incomplete fusion or merging of the labioscrotal folds are leading to glanular, penile or scrotal/perineal hypospadias. A recent study in Denmark has shown an association between fetal growth impairment and hypospadias (Boisen *et al.*, 2001). In this study also a rather high total rate of hypospadias (4.6%) was found.

Normal development of external male genitalia is dependent on the action of dihydrotestosterone (DHT) (see below). Therefore, patients with either DHT deficiency, due to mutations in the 5 α -reductase type 2 (SRD5A2) gene, or partial androgen insensitivity syndrome (AIS) due to AR mutations, often have penoscrotal hypospadias (Cuckow *et al.*, 2001). Recently, also the GGN polymorphism in the AR was linked to hypospadias, in a study showing that males with infertility and hypospadias, respectively, more often had GGN lengths of >23 than controls from the general population (Aschim *et al.*, 2004). In another study, unexpectedly low incidence of hypospadias was found among Greenlanders (Giwereman *et al.*, 2006). Compared to the Caucasian population, a more prevalent GGN length of 23 was noticed among the Inuit, indicating that GGN=23 protects against hypospadias.

Despite identifying some of genetic factors associated with hypospadias, the majority of cases remain unexplained. Similarly as for cryptorchidism, it can not be

excluded, that a combination of genetically determined predisposition and environmental or life-style related factors, represent the major underlying cause of these congenital abnormalities. However, such factors are still not identified.

Testicular cancer

Testicular cancer (TC) is the most frequent malignancy in young men. Some rare cases are discovered already before 5 years of age, although the majority is diagnosed at 20-45 years of age. In all Western countries the incidence of TC has increased, with 3-4% annually during the period when efficient cancer registration has been performed, starting in Denmark in 1943 (Richiardi *et al.*, 2004). Denmark, Norway and New Zealand have the highest incidence rates world wide, with approximately 15 cases per 100 000 persons a year.

TC is divided in non-germ cell and germ cell cancer (TGCC). Non-germ cell cancer comprises of only 5% of all cases and includes lymphomas, Sertoli cell and Leydig cell tumors, whereas TGCC constitutes more than 95% and is divided in two histological types, non-seminoma and seminoma, comprising approximately 50% each. The frequencies of these two histological types are age-dependent and before 35 years of age non-seminomas are most common, while seminomas appear later in life and are more common among patients older than 35 years.

The origin of TGCC is believed to be carcinoma in situ (CIS) cells, which are believed to arise already in early fetal life from primordial germ cells (PGC) that fail to undergo proper proliferation or differentiation (Skakkebaek *et al.*, 1987). Recently, high oestrogen levels were shown to stimulate growth of murine PGC cells *in vitro* and also to increase the frequency of PGC transformation into tumor cells (Moe-Behrens *et al.*, 2003).

Subjects with AIS, have an increased risk of CIS (Quigley *et al.*, 1995). There are also studies indicating that Müllerian inhibiting substance is involved in the regulation of germ cell differentiation or proliferation (Hirobe *et al.*, 1992), which may partially explain the strong association between cryptorchidism and TGCC (Moller & Skakkebaek, 1996). It has recently been established that patients with TC have decreased fertility prior to being diagnosed with cancer (Richiardi *et al.*, 2004; Moller & Skakkebaek, 1999).

Summary of TDS

TDS has been suggested as a syndrome which probably arises already in foetal life (Skakkebaek *et al.*, 2001). The link between the four conditions comprising TDS – hypospadias, cryptorchidism, poor sperm counts and TGCC is partly based on clinical experience. Cryptorchidism is the most important risk factor of TGCC and men with this cancer are often presenting with poor sperm counts. Furthermore, cryptorchidism and hypospadias seem to share, at least some aetiological factors. However, whether TDS is a true pathogenetic entity and which the possible causes to this syndrome are, are questions which are still unresolved. Different factors, including genetic background, life style factors and/or exposure to environmental factors in foetal life, have been suggested as possible causes of the TDS. Substantial evidence of adverse developmental effects caused by endocrine disrupters comes from both animal studies (Vos *et al.*, 2000; Guillette, 1994; Facemire *et al.*, 1995) and human studies (Toppari *et al.*, 1996; Sharpe & Skakkebaek, 1993).

However, it cannot be excluded that cryptorchidism, hypospadias, male infertility and TGCC occur as independent conditions. Although foetal life may be the most vulnerable time window for the adverse effects of environment and life style on male reproductive function, infancy and puberty might also represent time periods crucial for the development of normal male reproductive function.

Development of male genitals

Foetal period

Sex determination and differentiation comprises a cascade of events in the developing embryo. An establishment of chromosomal sex occurs, which is defined by the inheritance of an X or a Y chromosome from the father, rendering in either a 46, XX or 46, XY karyotype. Regardless of karyotype, all fetuses develop identically during the first six weeks of gestation, both being endowed with bipotential primordial gonad tissue and two internal genital ducts, Wolffian and Müllerian. In the presence of an intact Y chromosome, male gonads (testes) will develop. In the 6th week of gestation, the sex determining region Y (SRY) (Sinclair *et al.*, 1990) triggers the gonads into testis development (Figure 5). The male development is then controlled by hormones produced by the fetal testis.

The peptide hormone anti-müllerian hormone (AMH) is secreted from the Sertoli cells of the testes from early fetal life up to puberty. The Sertoli cells are responsible for the regression of Müllerian ducts in male embryos (Josso *et al.*, 1991) and for stimulating the differentiation of Leydig cells from testicular

interstitial cells. Leydig cells start to secrete the steroid hormone testosterone, first autonomously and later after stimulation by maternal human chorion gonadotropin (hCG) (Reyes *et al.*, 1989).

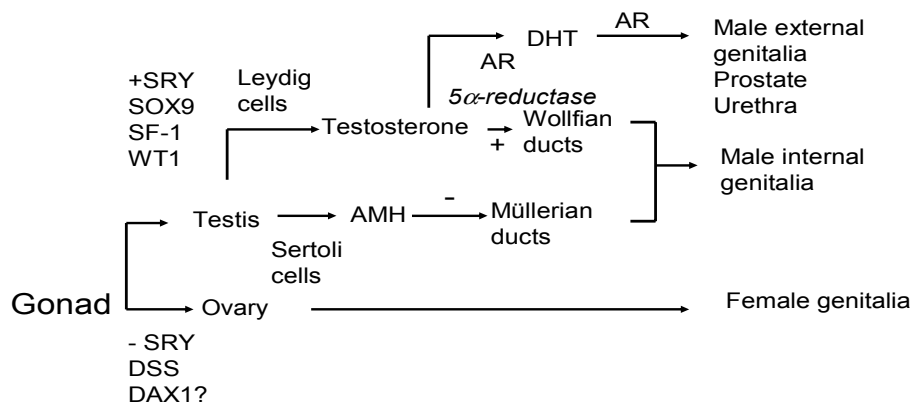


Figure 5. Development of male sex characteristics.

The male internal genital system develops from the Wolffian duct between 9 and 13 gestation weeks and consists of: epididymis, vas deferens, the seminal vesicles and the ejaculatory ducts. The Leydig cells begin to produce testosterone around week 8. DHT is a potent metabolite of testosterone, although not involved in the internal masculinization. Instead the reduction of testosterone to DHT is essential for formation of the external genital as glans penis, the shaft of the penis, scrotum, urethra and prostate (Wilson *et al.*, 1993; Imperato-McGinley *et al.*, 1992). Both testosterone and DHT are acting through the androgen receptor (AR), which is essential for both internal and external masculinization.

Neonatal period and infancy

The function of the testis is under control of the pituitary and the hypothalamus. Gonadotropin releasing hormone (GnRH) is released from the hypothalamus and controls the secretion of the gonadotropins lutenizing hormone (LH) and follicle stimulating hormone (FSH), from the pituitary. LH acts on specific receptors in the Leydig cell membrane and stimulates testicular steroidogenesis or testosterone production and FSH controls spermatogenesis via the Sertoli cells. An intratesticular, paracrine effect of testosterone is also important for spermatogenesis. Androgens exert a negative feedback action on the secretion of LH through inhibition of GnRH release and Sertoli cells produce activin and

inhibin, which regulate FSH secretion. In a newborn boy, there is a significant activation of the hypothalamus-pituitary-testicular axis. At 2-3 month age, the serum levels of gonadotropins, testosterone and inhibin B are approaching pubertal levels, but fall to non-measurable concentrations after a few months of life and remain at these low levels until the boys reach puberty.

Puberty and adulthood

At the age of 7 years, androgen production starts increasing, first due to increased secretion of dehydroepiandrosterone, by the adrenal gland. Then, at the age of 10-14 years, gonadotropin secretion starts, in a pulsatile fashion, during sleeping hours until pubertal maturation for the adult man is reached. The first sign of male puberty is growth of the testes above the infantile size of 2-4 ml, increasing gradually to normal adult size of 15-30 ml each. This testicular growth precedes Sertoli cell proliferation and subsequent initiation of spermatogenesis. The pubertal development is characterized by significant increase in serum of testosterone, inhibin B and gonadotropins. It is still unclear which factors are triggering the pubertal development of boy. Testosterone increases more than 20-fold (normal concentration for adult men is 10-30 nM) and plays a crucial role for secondary sex characteristics, such as muscle growth, bone, penis-, facial-, body- and pubic hair. All sex characteristics, with the exception of testicular growth, can be evoked by use of testosterone. However, an increase in gonadal size requires a simultaneous action of androgens and gonadotropins.

All three above mentioned time windows are vulnerable stages in male genital development and reproductive function thereafter. It is therefore of importance to clarify if the suggested time-related decline in sperm counts and increase in the risk of TGCC could be caused by the impact of environmental, genetic and/or life style related factors and in which time windows these possibly adverse effects are most pronounced.

FACTORS WITH POTENTIAL IMPACT ON DEVELOPMENT AND FUNCTION OF MALE REPRODUCTIVE ORGANS

Genetic factors

Androgen receptor

Both testosterone and DHT exert their function through the androgen receptor (AR). A functional AR is an absolute requirement for normal male sex differentiation, secondary sex characteristics at puberty and spermatogenesis thereafter. The AR consists of 8 exons and is divided into three functional domains: the NH₂-terminal transactivating domain (exon 1), the DNA-binding domain (exon 2-3) and the C-terminal hormone binding domain (exon 4-8) (Figure 6) (Lubahn *et al.*, 1988). The NH₂-terminal domain regulates the AR activity (Jenster *et al.*, 1995). There is only one AR gene, located at chromosome Xq 11-12, but there are multiple allelic variants in the general population (Sleddens *et al.*, 1992).

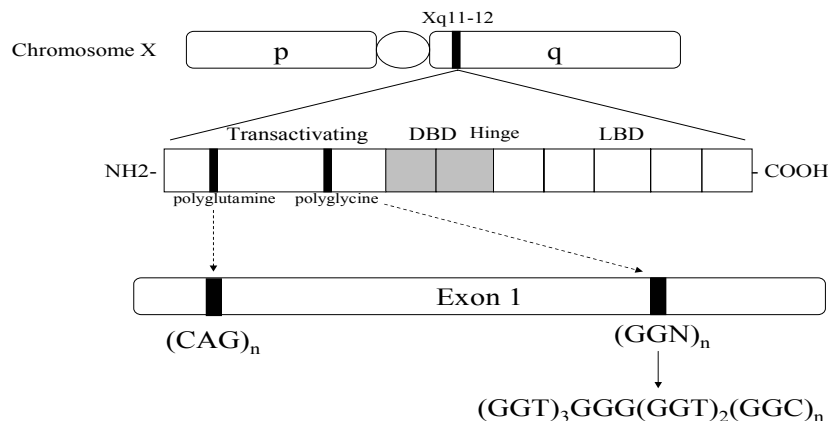


Figure 6. Human androgen receptor gene, structure and protein.

The polymorphism affects the protein coding sequence of exon 1 of the AR. This contains a glutamine repeat, CAG_nCAA and a glycine repeat, encoded by (GGT)₃GGG(GGT)₂GGC_n, generally designated the GGN repeat. There are numerous studies linking the length of the CAG repeat to androgen insensitivity *in vivo* as well as *in vitro*. In the general population the CAG repeat length varies from 10 to 30. Abnormal expansion of the CAG repeat to more than 40 repeats is

associated with spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease (La Spada *et al.*, 1991). A correlation between the expansion and the severity of the disease has been noted, which also has been supported by experimental studies, where an inverse relation between CAG length and AR transactivating capability has been shown (Tut *et al.*, 1997).

Affected males also present with signs of decreased androgen sensitivity and impaired sperm output, which has led to extensive investigations regarding other male disorders i.e. prostate cancer, testicular cancer, and diabetes mellitus, which could possibly be linked to this segment. The GGN repeat shows a completely different distribution, with two predominating alleles of 23 and 24 GGN, respectively, comprising approximately 85% of the Swedish population (Lundin *et al.*, 2003). Mutations in the first six base triplets of the GGN repeat have been suggested as a possible cause of ambiguous genitalia in 46 XY individuals (Quigley *et al.*, 1992). It was, however, recently shown that these mutations occur quite frequently in the general population, and do not seem to give rise to profound changes in the male phenotype (Lundin *et al.*, 2003), although they may be associated with subtle effects on sperm and semen parameters (Ruhayel *et al.*, 2004). Sons whose mothers smoked during pregnancy and who had short GGN lengths (GGN below 23) had higher BMI than those with other GGN lengths, no matter whether their mother had smoked or not during pregnancy. In experimental studies, a complete deletion of the GGN repeat resulted in a 30% reduction of the transcriptional activity of the AR, indicating that the GGN repeat also is of functional importance (Gao *et al.*, 1996).

Androgen synthesis

The major circulating androgen is testosterone. Almost 95% is secreted by the Leydig cells in the testis, which synthesizes about 6-7 mg testosterone per day. The remaining derives from the adrenals (Saez, 1994). The precursor of the gonadal, as well as the adrenal steroids, is cholesterol (Figure 7). The conversion of cholesterol to testosterone and DHT requires the action of five enzymes: side chain cleavage enzyme (SSC), 3 β -hydroxysteroid dehydrogenase/ $\Delta^{5,4}$ isomerase (3 β -HSD), 17 α -hydroxylase/C_{17,20} lyase, 17 β -hydroxysteroid dehydrogenase (17 β -HSD) and 5 α -reductase (SRD5A). Mutations in any of these enzymes, or associated proteins such as the steroidogenic acute regulatory (StAR) protein, affect both testosterone and cortisol synthesis. As result, either female or male androgen as well as adrenal insufficiency arises. These syndromes are known as congenital adrenal hyperplasia (Evenson *et al.*, 1993; Jensen *et al.*, 2006; Lin *et al.*, 1995). Since Leydig cells cannot store androgens, biosynthesis continually takes place and androgens are mainly secreted into the blood where testosterone is bound to albumin (54%) or sex

hormone binding globulin (SHBG) (44%). Only 3% of total testosterone circulates in a free form.

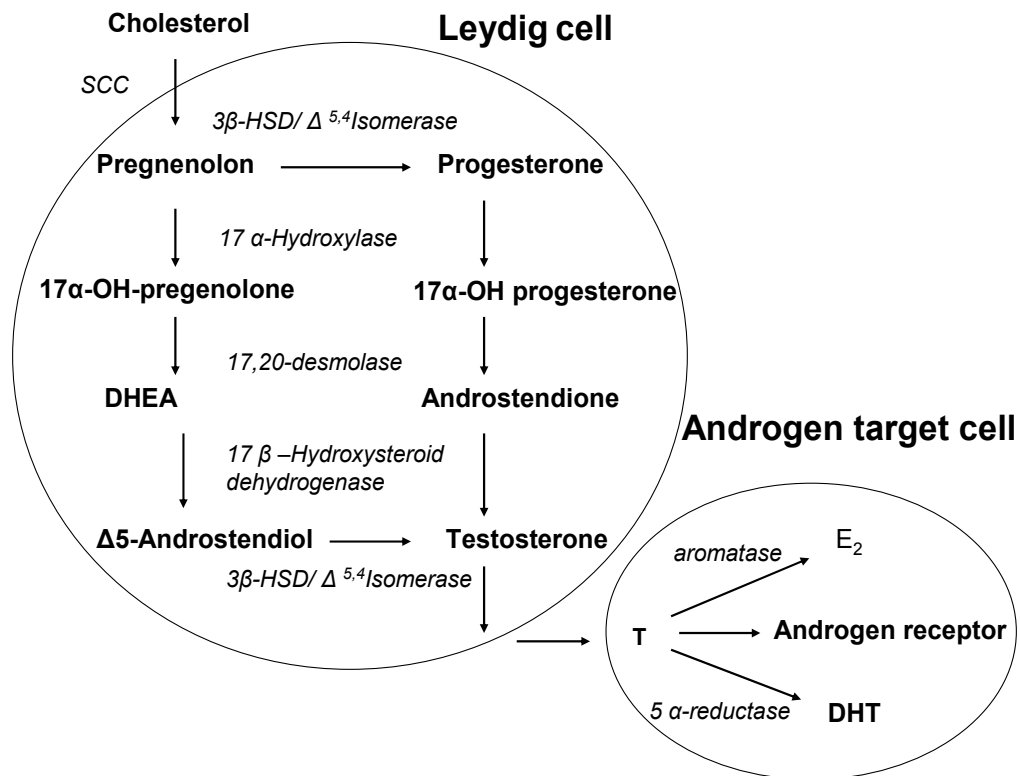


Figure 7. Steroid hormone synthesis from Leydig cells in the testis.

Testosterone is through 5 α -reduction by the enzyme SRD5A converted to DHT, and through aromatization to estradiol (Figure 7) (Simpson *et al.*, 1997). Two isoforms of SRD5A have been found. Type I is encoded by a gene located on chromosome 5 and is expressed in non-genital skin, liver and brain after birth (Tighpen 1993), whereas type II is localized on chromosome 2 and acting in androgen-dependent tissues such as the prostate, Wolffian ducts derived structures and genital skin throughout life.

Other genetic causes of hampered reproductive capability

To date, genetic defects are found in up to 10% of all infertile patients and may be associated with azoospermia, severe oligozoospermia, Sertoli cell only syndrome, spermatogenetic arrest or a positive family anamnesis for infertility e.g. an affected brother. Two common genetic aberrations are Klinefelter's syndrome and Y-chromosome microdeletions.

Klinefelter's syndrome (47,XXY) is the most frequent chromosomal abnormality affecting approximately one of 500 newborn males (Abramsky & Chapple, 1997). Two types are distinguished - numerical chromosome aberration 47, XXY and mosaic variants of this. Boys born with this chromosome defect are often not recognized before puberty, when testicular growth ceases and is followed by atrophy, resulting in micro testis (Skakkebaek *et al.*, 1994). The destruction of the seminiferous epithelium takes place and leads to azoospermia. In many cases, Klinefelter's syndrome is diagnosed when the patient is seeking medical care for infertility. The pathogenesis of Klinefelter's syndrome is so far unknown and it is remarkable that the presence of an extra X chromosome has little effect on the phenotype before puberty.

The long arm of the Y chromosome contains at least three distinct regions designated as azoospermia factors: AZFa, AZFb and AZFc, which have been shown to be essential for normal spermatogenesis (Vogt *et al.*, 1996). The loss of one of these loci is known as Y chromosomal microdeletions, which often lead to severely disturbed fertility. According to Simoni *et al.*, microdeletions are found in 5-10% of the azoospermic cases and in 2-5% of those with severe oligozoospermia (Simoni *et al.*, 1997a). In our population corresponding prevalence was 2% and 3% respectively (Ruhayel *et al.*, 2004). A deletion of the AZFc region occurs more often than AZFa or AZFb deletions (McElreavey & Krausz, 1999).

Impact of environmental factors on male reproduction

A large number of environmental pollutants e.g. dioxins, bifurans, polychlorinated biphenyls (PCB), pesticides, phenols, phthalates and others were shown to mimic the action of hormones including that of estrogens, anti-estrogens, androgens and anti-androgens (Kelce & Wilson, 1997; Sohoni & Sumpter, 1998). The term "endocrine disruptor" (ED) has been widely used to describe chemicals possessing any of these hormone-like actions.

Persistent organohalogen pollutants

Persistent organohalogen pollutants (POPs) are a group of compounds with structurally and toxicologically similar characteristics. In general, these compounds are persistent to both abiotic and biotic degradation and accumulate in the food chain. The main human exposure to POPs occurs through diet of food of animal origin, such as fish, milk, eggs, cheese and meat, being the predominate pathway. In Sweden, consumption of fatty fish from the Baltic Sea, such as herring and salmon, is the single most important source of exposure (Asplund *et al.*, 1994; Svensson *et al.*, 1995; Wetterauer & Heite, 1976). After being absorbed from the

gastrointestinal tract POPs are distributed to lipid rich tissue, which is the main storage site and where they can accumulate and remain for years.

POPs consist of accidental by-products from various chemicals and combustion processes, such as PCDD and dibenzofurans PCDFs as well as manufactured compounds, such as polychlorinated biphenyls (PCBs) used e.g. in electronically equipments and the insecticide 2,2-bis (4-chlorophenyl)-1,1,1-trichloroethane (DDT) (Brown & Lamartiniere, 1995; Lohman & Seigneur, 2001). All these compounds have, in animal as well as human studies, been associated with reproductive, developmental, and immunological disorders, and with some cancer forms (Brouwer *et al.*, 1995; Gustavsson & Hogstedt, 1997; Verkasalo *et al.*, 2004). The question is whether these compounds also affect the male reproductive system. An increasing amount of studies suggest that a deteriorated reproductive function in males might, at least partly, derive from increasing exposure to endocrine disrupters, such as POPs, during fetal life (Giwerzman, 1995; Toppari *et al.*, 1996). So far evidence of such effects is lacking.

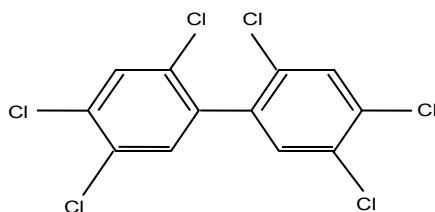


Figure 8. Molecular structure of 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153).

CB-153 a biomarker for POP

The concept of toxic equivalence (TEQ) has been introduced as an administrative tool to simplify risk assessment and regulatory control for dioxin-like compounds, i.e. biochemical and toxic response. As a biomarker for POP exposure 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) (Figure 8) is often used. CB-153 is very stable and often one of the most abundant PCB congeners. CB-153 levels have been found to correlate very well with toxic equivalents (TEQ) from total PCB in human plasma ($r=0.91$) (Grimvall *et al.*, 1997), serum (Glynn *et al.*, 2000) and in venous blood (Hagmar *et al.*, 1998) and with total PCBs ($r=0.95$) (Atuma *et al.*, 1998). CB-153 is therefore useful to elucidate the possible association between exposure to POPs and male reproductive parameters. POPs acting as endocrine disrupters are suggested to be a part of the time-related decline in sperm numbers

Impact of life style related factors on reproductive function

Several life-style related factors including hot baths, tight clothing, caffeine intake, drugs, alcohol, snuffing, cigarettes (including mother's smoking during pregnancy) could potentially have some impact on the reproductive capability (Figure 9) (Storgaard *et al.*, 2003; Vine, 1996; Fagerström & Schildt, 2003; Shafik, 1993; Saikhun *et al.*, 1998).

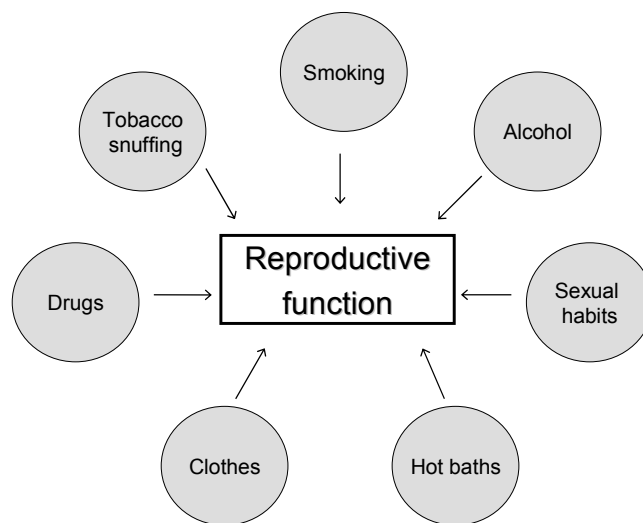


Figure 9. Life style factors that have been suggested to interfere with male reproduction.

Smoking habits

Cigarette smoking has increased worldwide during the last 100 years (Vine, 1996) and cigarette smoking habits differ a lot between different regions and countries. In 1988 5,2 trillion cigarettes were consumed by 1 billion persons (Connolly, 1992). The proportion of cigarette smoking men ranged from 15-80% according to Council on Scientific Affairs 1990 and The European report on tobacco control policy 2002. There is a significant difference in smoking habits between Swedish and Danish males (Figure 10). These numbers are in accordance with the data on smoking habits in young male conscripts, 27% of the Swedish adolescent males

being smokers (www.pliktverket.se) compared to 43% among the Danish conscripts (Jorgensen N, personal communication).

So far it has not been clarified if there is an association between cigarette smoking and reduced male reproductive capacity, but inhaled cigarette smoke contains many hazardous compounds which, at least theoretically, could be toxic to the male reproductive function. Smoking could also induce oxidative damage in the testis (Shen *et al.*, 1997), which is potentially hazardous for spermatogenesis. Some studies have demonstrated a significant association between smoking and reduced sperm output (Kunzle *et al.*, 2003; Ramlau-Hansen *et al.*, 2007), whereas other studies have not (Martini *et al.*, 2004; Sobreiro *et al.*, 2005b; Trummer *et al.*, 2002).

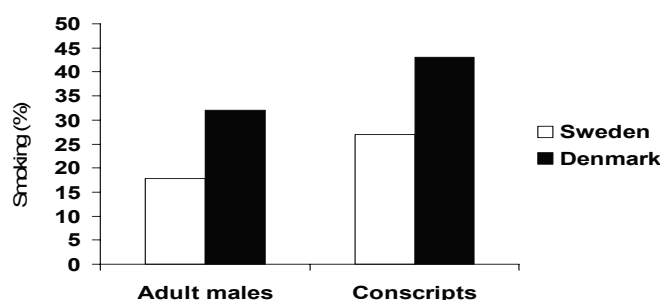


Figure 10. Differences in smoking habits between Sweden and Denmark .

Polycyclic aromatic hydrocarbon is one of the compounds in cigarette smoke that has been found to reduce fertility in both male and female mice (Mackenzie 1981). Poly aromatic hydrocarbons were also shown to impair testicular steroidogenesis and epididymal function (Inyang *et al.*, 2003). An inverse correlation between prostate size and smoking, which could indicate an anti-androgenic effect has also been reported (Kupeli *et al.*, 1997). A recent study on 154 smoking subjects showed no correlation with sperm concentration and motility, but with reduced semen volume (Pasqualotto *et al.*, 2006), possibly relating to an effect of smoking on accessory sex glands (Pakrashi & Chatterjee, 1995). Significantly lower neutral alpha glucosidase (NAG) activity was found in semen of smokers compared to non-smokers. As NAG associates to the epididymis function, this might indicate that smoking also has a negative effect on post testicular organs.

Few studies have been done on men who stopped smoking, but higher sperm concentration and increased motility was in 1969 reported in three males followed for 5-15 months after having stopped smoking (Chen *et al.*, 2004; Schirren & Gey, 1969). Another study reported that sperm motility and morphology improved in nine males, who were followed during six months after they had ceased to smoke (Sofikitis *et al.*, 1995). However, caution should be taken due to the extremely low number of subjects included in these studies.

It is difficult to conclude if cigarette smoking actually is associated with semen quality. In a meta analysis (Vine, 1996) based on 25 studies on sperm concentration in smokers vs. non-smokers, a decrease in mean sperm concentration among smokers compared to non-smokers was found in 20 studies. However, this difference was statistically significant in only eight of these studies. The average reduction was 13%. In 16 studies subjects were recruited from fertility clinics and in two of them exceptionally large differences in sperm numbers were reported why these studies were excluded. The resulting mean sperm difference between smokers and non-smoker then fell to 1%. The author concluded that future studies including subjects from general population are needed.

Maternal smoking during pregnancy

During the past few years an increasing interest has been shown for possible effects of maternal smoking during pregnancy on the reproductive capability of their sons. In an animal study, reduced fertility was reported on foetally exposed male and female mice (Mackenzie & Angevine, 1981). Some recent studies have also reported reduced sperm counts among men whose mothers were smoking during pregnancy (Storgaard *et al.*, 2003; Jensen *et al.*, 2004). The reduction was only observed among those with a mother smoking more than 10 cigarettes per day, whereas no such effect was seen in sons of mothers smoking 1-10 cigarettes per day. This leaves us with a need of further investigations to clarify the potential negative effect of maternal smoking during pregnancy on male reproductive function.

Drugs and alcohol

The effect of smoking on reproductive parameters could also be a result from psychological self selection. It is possible that other factors, which are associated with smoking, may account for the reduced semen quality. For example, smokers could be more likely than non-smokers to take drugs and medications, consume caffeine and alcohol, and to have an earlier sex-debut (Vogt *et al.*, 1986; Bracken *et al.*, 1990).

Abusers of cocaine, marijuana and opiates are hard to study; nevertheless these agents appear to have only minimal direct effect on sperm production (Bracken *et*

al., 1990). They could on the other hand, by negative feed back mechanisms, inhibit hypothalamic GnRH secretion and in that way suppress LH and testosterone levels causing reduced spermatogenesis (Cicero *et al.*, 1975).

Excessive alcohol consumption has also been suggested to reduce semen quality through testicular toxicity (Pajarinen *et al.*, 1996; Kucheria *et al.*, 1985; Saikhun *et al.*, 1998), whereas high caffeine intake (>4 cups/day) combined with heavy smoking (>20 cigarettes per day) (Marshburn *et al.*, 1989) has been associated with lowered sperm concentration. Another recent study showed improved sperm motility among those consuming more than 6 cups of coffee per day (Sobreiro *et al.*, 2005a). There are also studies suggesting increased sperm DNA damage due to coffee intake (Schmid *et al.*, 2007).

Hot baths and clothing

Tight cloths have been suggested to decrease sperm production and increase the risk of infertility. The optimal temperature for human testis is 2-3 degrees below normal body temperature of 37°C. In animal studies using dogs who were dressed in polyester trousers, a reversible reduction in sperm parameters was shown (Shafik, 1993).

Regular (weekly) sauna has been reported to decrease sperm motility and number. This parameter gradually returned to normal after cessation of sauna exposure (Brown-Woodman *et al.*, 1984; Saikhun *et al.*, 1998). Notably, in Finland, where sauna is a national tradition, the males have one of the world's highest sperm counts, which also have remained unchanged through the last decades (Vierula *et al.*, 1996). This could indicate that there are some unknown protecting genetic factors among the Finish male population, which also make them more resistant to the risk of TC.

Sexual behaviour

How long sexual abstinence time a male should have to obtain an optimal semen quality has been widely discussed. WHO (1999) now recommends 2-7 days of abstinence before leaving semen a sample for analysis. New data indicate that shorter abstinence time actually could be better for obtaining an optimal sample. A recent study from Israel indicated that only 1 day of abstinence could provide the most optimal semen sample (Levitas *et al.*, 2005). However, another study suggested that an abstinence period of 4-5 days is superior in terms of sperm motility and morphology (ELzanaty *et al.*, 2005; De Geyter *et al.*, 1998).

LABORATORY MARKERS

Spermatogenesis

The meiosis and spermatogenesis is not initiated until puberty. Sperms are produced in the seminiferous tubules of the testis encased by large Sertoli cells which provide support for the dividing germ cells. The spermatogonium is the primary cell, which after a couple of cell divisions differentiates into primary spermatocytes. After this step the first meiotic division results in secondary spermatocytes. The second meiotic division leads to round spermatids that finally mature during spermiogenesis into elongated spermatids.

Modalities and markers to determine semen quality

The following parameters are those used for determining semen quality according to WHO recommendations and references for the semen parameters according to WHO 1999 are shown in Table 1.

Table 1. Reference values of semen parameters, as defined by the WHO (1999)

Semen volume	≥ 2.0 mL
Sperm concentration	$\geq 20 \times 10^6/\text{mL}$
Total sperm count	$\geq 40 \times 10^6$ (per ejaculate)
Sperm Motility:	
Rapid progressive motility	$\geq 25\%$
Total progressive motility	$\geq 50\%$

Sperm concentration

Sperm concentration is the most commonly used laboratory marker of spermatogenesis. However, this parameter is not only dependent on the daily sperm production but also on other factors as time of abstinence (see above) and the ejaculate volume. Sperm concentration above $20 \times 10^6/\text{mL}$ is considered as normal (WHO, 1999). However, it has been shown (Bonde *et al.*, 1998) optimal chance of pregnancy is not achieved until sperm concentration reaches the level of $40\text{--}50 \times 10^6/\text{mL}$. Unfortunately, this parameter is not a very good marker of fertility, since for this, as well as for other traditional seminal parameters, there is a wide overlap between fertile and infertile subjects (Guzick *et al.*, 2001).

Sperm mophometry and morphology

According to WHO (WHO 1999), an ideal mature spermatozoon has an oval shaped head with a regular contour, being 4.0-5.0 μm long and 2.5-3.5 μm wide. The length to width ratio of the head should be 1.50 to 1.75. The sperm tail should be attached symmetrically to the broad like sperm head base. Only one tail, which should not be coiled, nicked or bent over it self, is allowed. The length of the tail should be approximately 45 μm . Immediately behind the head, is a thicker part of the tail, which is called the mid- piece. This has a maximum with of 1 μm and a length of about 7-8 μm .

Sperm motility

Sperm motility is one of the most important parameters to predict fertility. The evaluation of sperm motility is especially influenced by subjective factors. Systematic investigations have therefore been made to uniform the laboratory methods and the World Health Organization (WHO 1999) has made intensive efforts to obtain objective laboratory methods. Among the various methods for objective measurements, the tracing of video images of individual sperm cells by computer assisted sperm analysis (CASA) is the best developed. Sperm motility data obtained by CASA, have also been predictive of fertility (Larsen *et al.*, 2000; Donnelly *et al.*, 1998). Correlation with pregnancy rates *in vivo* or after *in vitro* fertilization (IVF) has been achieved using evaluation of sperm motility by CASA (Irvine *et al.*, 1994; De Geyter *et al.*, 1998). Sperm motility is dependent on a multitude of factors from the accessory sex glands (Trummer *et al.*, 2001; Schutte *et al.*, 1986) and it is not unlikely that changes in sperm motility could be a part of the suggested declining male reproductive capability.

Sperm parameters as markers of reproductive function

Sperm number, motility and morphology are traditionally used as markers of male fertility. However, use of these parameters has several limitations. As mentioned above, there is a considerable overlap between fertile and infertile men, as considers the levels of these traditional sperm characteristics (Guzick *et al.*, 2001). Considerable intra-and inter-laboratory variation (Cooper *et al.*, 1992; Neuwinger *et al.*, 1990) in assessment of semen quality represents a substantial problem when comparing results of such tests obtained at multiple occasions. Finally, all these semen characteristics are subject to intra-individual variation (Giwerzman *et al.*, 1999). One of the factors suggested to add to this variation is season, highest sperm counts at winter or early spring being reported – at least in the Northern hemisphere (Levine *et al.*, 1990). However, the issue of seasonal variation in semen parameters is still debatable (Malm *et al.*, 2004).

Accessory sex glands and epididymis

The accessory sex glands - the prostate and the seminal vesicles, represent together with the epididymis the major post-testicular organs determining the content of the seminal fluid and the function of the spermatozoa. Epididymis and the seminal vesicles have their origin from the Wolffian ducts and the prostate is derived from the urogenital sinus. The epididymis consists of three regions: caput, corpus and cauda epididymidis. Water absorption from the lumen is one of the basic functions, which is essential for normal testicular function. The epididymis secretes energy rich substrates, lipids and neutral alpha glucosidase (NAG) (Dacheux *et al.*, 2003), which are essential for the maturation of the sperms, taking place when they are transported through epididymis. It also protects spermatozoa against reactive oxygen species (Amann *et al.*, 1993).

The seminal vesicles lie inferiorly and dorsally to the bladder and are connected to the ejaculatory ducts. The most important products from the seminal vesicles are fructose, semenogelin (Sg) I, II and prostaglandins (Gerozissis *et al.*, 1982). The seminal vesicular secretion is important for semen coagulum, sperm motility and stability of sperm chromatin. Most interest has been shown in the semen coagulum, which contains SgI and II (Lundwall *et al.*, 2002). Sg I and II are probably important for providing a neutral pH and to protect the spermatozoa against the acidic pH in the vagina.

The prostate is located under the bladder and wraps around the urethra. It is divided into central and peripheral zones. The prostate gland secretion contains sodium, potassium, proteins and enzymes e.g. prostate specific antigen (PSA) (Zaneveld & Tauber, 1981). PSA is mainly important for semen liquefaction and sperm motility. PSA was shown to rapidly degrade Sg I and II into fragments, a process which has been suggested to reduce the inhibitory effect of Sg I and II on the motility of spermatozoa (Lilja *et al.*, 1989).

Seminal plasma

The seminal plasma is a mixture of various fluids from the male reproductive tract. Normally, an ejaculate contains >2 mL semen. The prostate contribution to an ejaculate is 15-30% of the total ejaculate. The spermatozoa produced in testis and the secretion of the epididymis and Cowper's gland contribute to 5%. The remaining 60-80% is produced by the seminal vesicles.

Seminal biomarkers of the accessory sex gland and epididymal function

Different substances can be measured in the ejaculate and serve as markers of epididymis, seminal vesicles and prostate function. The physiological role of NAG, secreted by the epididymis is not yet known, but it has been speculated that it might provide the spermatozoa with optimal levels of energy (Tremblay *et al.*, 1979) and

that NAG could be of importance for their maturation (Dacheux *et al.*, 2003). Another marker of epididymal function, which can be measured in seminal fluid, is L-carnitine. Fructose, which is secreted from the seminal vesicles, is also supposed to give required nutrition to the spermatozoa (Mann, 1974). So far no reliable assays for measuring levels of SgI and II are available. Zinc and PSA are predominantly secreted by the prostate and are androgen dependent. Disturbed accessory sex gland secretion could alter the seminal fluid and affect the sperm function.

Male reproductive hormones

The endocrine function of the testis, androgen production and gonad development are regulated with a negative feedback system mediated by the hypothalamus and the pituitary. The hormones involved are:

- gonadotropin releasing hormone (GnRH) released from hypothalamus;
- luteinising hormone (LH) and follicle stimulating hormone (FSH) from the pituitary;
- testosterone and oestrogen from Leydig cells in the testis, and
- Inhibin B and activin from the Sertoli cells.

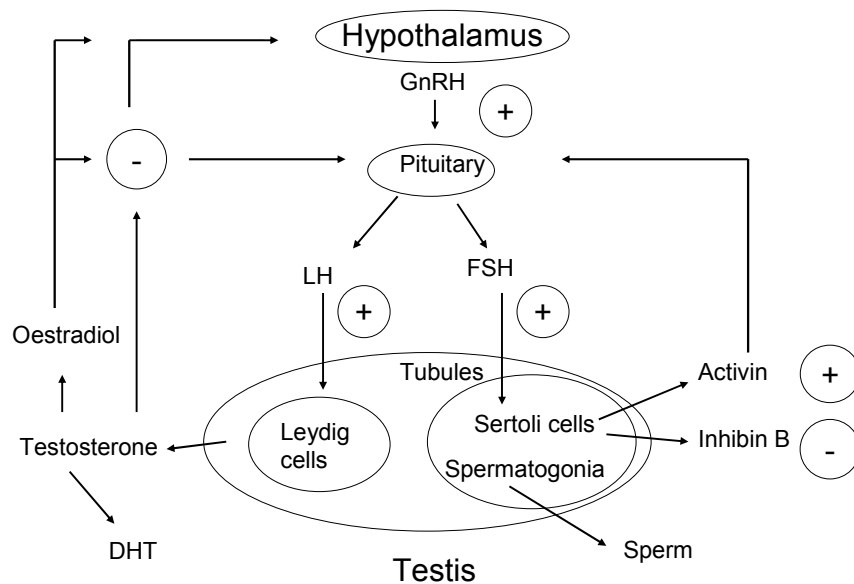


Figure 11. Sex hormone regulation of male reproductive function.

In the hypothalamic area GnRH is produced and secreted in pulses, although in absence of steroid hormones the generator becomes free-running (Lopez *et al.*, 1998). The major function of GnRH is to regulate the secretion of the

gonadotropic hormones FSH and LH from the pituitary gland (Simoni *et al.*, 1997b; Lopez *et al.*, 1998). FSH acts on Sertoli cells and is crucial for spermatogenesis, while LH acts directly on the Leydig cells and stimulates their production of testosterone, which apart from its endocrine function also acts as a paracrine factor on Sertoli cells and thereby is important for sperm production (Sharpe, 1994). Some testosterone is, both in the Leydig cells and in the peripheral tissues, reduced to oestradiol by the action of aromatase. The regulation of FSH and LH is mediated through a negative feed back system. Sertoli cells produce Inhibin B, which inhibits FSH, whereas testosterone and oestradiol inhibit LH, FSH and GnRH secretion (Figure 11).

Androgens, testosterone and DHT, acting through the intracellular androgen receptor, are essential for developing and regulating the testis, mediating masculinization of bone and muscles, maturation of secondary sexual characteristics, and stimulating spermatogenesis. Testosterone is transported in plasma bound to sex hormone binding globulin (SHBG) or albumin, only 3% circulating in a free form.

The levels of FSH, LH, testosterone, oestradiol, SHBG and Inhibin B in serum or plasma are routinely measured and serve as the markers of the function of the reproductive system. The concentrations of these hormones are subject to diurnal variation. Thus, testicular hormones, testosterone and Inhibin B (Carlsen *et al.* 1999) exhibit their highest levels in the morning decreasing during the day. FSH and LH concentration is fluctuating with several diurnal peaks and nadirs.

To summarise, a time-related deterioration as well as geographic differences in the function of male reproductive organs have been suggested. A multitude of genetic, environmental and life-style related factors are of importance for the reproductive capability and their relative impact in relation to the epidemiological trends described above, as yet unknown. Such knowledge is essential for the understanding of the normal physiology and pathophysiology of male reproduction and for developing proper treatment and prevention strategies. Several seminal and endocrine markers of male reproductive organs are available and can be used to study how genetic variation, environment and life-style affect the reproductive system of the male.

AIMS OF THE PRESENT INVESTIGATION

The overall purpose of the study was to investigate the impact of environmental factors, life-style and genetic background on the male reproductive function.

Specific aims were:

- To investigate if there are differences in sperm number and risk of congenital abnormalities between young males from two genetically similar populations, Denmark and Sweden.
- To assess if there is any correlation between androgen receptor CAG repeat length and accessory sex gland function and sperm parameters.
- To elucidate possible associations between exposure to POPs and reproductive parameters in young males from the general population.
- To investigate the impact of tobacco smoking in adolescent men on sperm number and morphology.

MATERIALS AND METHODS

Study population

Approximately 95% of all 18 years old Swedish males undergo a medical health examination prior to military service. Only men with chronic diseases are excluded.

In the current work the military health board (Pliktverket) in Kristianstad recruited the subjects to participate in the study. Invited to participate the study were 2255 men living <60 km from the city of Malmö, who underwent the health examination prior to the military service during the period May-December 2000. Among those, 305 (13.5%) accepted to be enrolled in the study, which took place at the Malmö University Hospital.

- In paper I, focusing on differences in semen parameters between Swedish and Danish conscripts, all 305 men were included;
- The total cohort of 305 men was also studied in paper II dealing with associations between serum levels of CB-153 and reproductive parameters;
- In paper III, AR CAG repeat length was evaluated in relation to seminal as well as hormonal markers of male reproductive function. Thirty one of the 305 men originally enrolled in the study were excluded because they presented with hormone levels which might indicate testicular dysfunction. Thus a total of 274 conscripts were included;
- Paper IV focused on the effects of tobacco exposure (smoking, snuffing and maternal smoking) on reproductive parameters in 301 men from whom smoking data were available;
- In paper V, morphometric parameters in spermatozoa from 150 men were evaluated in relation to their tobacco exposure.

All subjects signed an informed consent and the Ethics Committee of Lund University approved the studies.

Data collection

One physician (JR) physically examined 90% of all the participants, whereas another examiner (AG) saw the remaining 10%. The examination took place between 9 and 11 a.m. and included assessment of length and weight for calculation of body mass index (BMI), virilization as well as a careful genital investigation, including classification by Tanner stage. Presence of genital malformations, hydrocele and previous genital surgery were recorded. Testicular size was measured by use of ultrasound and orchidometer. Ultrasound scanning of

the testes was performed by use of a 7.5 MHz transducer connected to an Aloka 900 SSE scanner (Aloka, Tokyo, Japan). Each testis was investigated in two projections, and its volume calculated as length x width x depth x 0.52. Immediately thereafter, the participants delivered a semen sample which was analyzed according to WHO standards (WHO 1999). Blood samples were drawn for hormone and DNA analyses.

Semen analysis

Abstinence time

The men were asked to keep 48-72 hours of abstinence, but in each case the actual length of the period was recorded, ranging between 0.1-240 days. The period of abstinence was calculated from the date and time of previous ejaculations, which was recorded in the questionnaire.

Semen volume

Each man provided a semen sample by masturbation into a wide mouthed plastic container. The weight of the empty plastic container was subtracted from the total weight to obtain the semen volume. The measurements were done by use of Sartorius® scale and the results expressed to two decimal places.

Sperm morphometry

From fresh semen samples, smears were made for duplicate assessment. The slides were cleaned with 95% ethanol and then 6 µL of semen was placed on each slide. The aliquot was then gently pulled out into a smear with a cover slip. The slide was air dried and finally fixed with Papanicolaou stain (haematoxylin orange G6 and EA50). Morphometry parameters were assessed using the commercially available image analyser Sperm Morph (Image House Medical, Copenhagen, Denmark).

Table 2. Dilution of ejaculate for sperm count and concentration.

Spermatozoa per field of vision 40X objective	Dilution (semen+ diluent)	Semen µL	Diluent µL	Numbers of counted chambers
<15	1+4 (1:5)	100	400	25
15-40	1+9 (1:10)	50	450	10
40-200	1+19(1:20)	50	950	5
>200	1+49(1:50)	50	2450	5

Sperm concentration

The concentration was assessed by use of a modified Neubauer haemocytometer (Table 2). Positive displacement pipettes were used for proper dilution of the ejaculate. One hundred spermatozoa were counted in each of two chambers. Only three laboratory assistants performed the analyses of the ejaculates and inter-observer coefficient of variation (CV) was found to be 8.5% for concentration assessment.

Sperm motility

The semen samples were analyzed within 1 hour after collection according to the WHO recommendations (WHO, 1999) six μ l semen was placed on a glass slide, which had been kept at 37°C. The preparation was examined with a phase contrast microscope at a total magnification of x 400. For determination of sperm motility, 200 spermatozoa were scored in categories a, b, c and d; a corresponding to rapid progressive motility, b to slow progressive motility, c to non-progressive motility and d to immotility. Because there is considerable inter-observer variation in discriminating between a and b, these two scores were pooled together. Previous studies indicated that the proportion of progressively motile spermatozoa (a+b) is the most significant motility category in relation to the fertility potential of a male (Jouannet *et al.*, 1988). For that reason, and because the total sum of scores a, b, c and d will always be 100, the category c was excluded from the analysis, which thus was restricted to two types of motility, a+b and d.

CASA (Computer assisted sperm assessment)

Sperm concentration and proportion of motile, local motile and immotile spermatozoa were assessed by the use of CRISMAS (Image House, Copenhagen, Denmark) computer-aided sperm motility analyzer (CASA), as previously described (Larsen *et al.*, 2000). The classification of sperms was based on the curve linear velocity (VCL), motile spermatozoa being those with $VCL > 25 \mu\text{m/s}$, locally motile having VCL between 5-25 $\mu\text{m/s}$ and those with $VCL < 5 \mu\text{m/s}$ classified as immotile. The motility assessment was based on capture sequences of 64 images (25 MHz), and for each sample at least 100 spermatozoa were analyzed.

CASA Sperm Morph

In current study, we used the commercially available image analyser Sperm Morph (Image House Medical, Copenhagen, Denmark). Sperm Morph is a fully automated computerized analyzer using Papanicolaou stained smears for detailed classification directly comparable with the manual classification as described in the “WHO Laboratory Manual” (www.euro.who.int) and further formulated by Kruger and Menkveld as the “Strict Criteria”. Sperm Morph measures approximately 50

raw features for each sperm, using the digitalized image of the head and the mid-piece. Among features are lengths, areas, grey values, points, form and symmetry. From the raw features, 20 derived parameters are calculated and used in the Sperm Morph computer strict classification algorithm to obtain good agreement with the manual strict classification. It is therefore assumed, that the derived parameter gives a useful representation of the sperm morphology.

Initially, we selected 15 morphometric parameters based on those most commonly referred to in the literature. Among those, some were highly correlated (Pearson's $r \geq 0.85$) and we did therefore ended up with 7 different morphometric parameters, which did not so tightly associated to each other. The first parameter *Head area* (A), was calculated multiplying max sagital spin axis (a) with maximal horizontal spin axis (b) and π ($A = \pi \times a \times b$). The second parameter *Elliptic Form* was 4π area divided with perimeter^2 . The former factor is 1 for a circle and the lower the value the less round is the sperm head. *Asymmetric area* (%) was calculated by subtracting the areas right and left to the midline divided with the sum of the same areas $\times 100$. *Area difference* was calculated by subtracting the areas in front and behind the sagital midline divided with the sum of the same areas $\times 100$. *Mid line difference PMG* was calculated subtracting PMG areas in front and behind the sagital midline. *Mid-piece width* was measured on the widest part of the mid-piece and *Midline area asymmetry* was calculated by dividing the non overlapping area with the total area.

All calculations were based on scoring 200 sperms per smear. For each parameter a median value for the 200 sperms was used.

Biochemical and seminal plasma analysis

Preparing for biochemical analyses, 450 μl of the ejaculate was mixed with 50 μl 0.1 M benzamidine after 20 min of liquefaction. The mixture was centrifuged for 20 min at 4500 g, and thereafter the supernatant was poured into tubes which were kept in -80°C until analysis.

Neutral α -glucosidase

Seminal plasma activity of NAG (mU/ml) was measured using a commercially available kit (Episcreen®; Fertipro, Gent, Belgium) according to the instructions given by the manufacturer. The test is based on the measurement of the intensity of a color change evoked by the reaction between α -glycosidase and 125 μl of reagent 1 (0.09% Na-azide) which was added to 125 μl of thawed seminal plasma. The mixture was well mixed by pipetting, one diagnostic tablet (p-nitrophenyl-D-glucopyranoside) was added, and thereafter the mixture was vortexed for 60 s and then incubated for 4 h at 37°C . After obtained by reading the supernatant against

regent 2 (0.02 mol/l NaOH) a blank was set spectrophotometrically at 450 nm. This value was plotted on a standard curve and the corresponding total α -glycosidase activity was read on the abscissa. The NAG concentration was estimated by use of the corresponding table provided by the manufacturer. The CV was at 15% at a mean NAG concentration of 3 mU/ml.

Fructose

The fructose concentration (mmol/l) in seminal plasma was determined with a spectrophotometric method, described by Wetterauer and Heite 1976 (Wetterauer & Heite, 1976), run on Beckman Synchron LX20 instrument. Proteins in the sample were precipitated with perchloric acid and the absorbance of the supernatant measured. After addition of phosphoglucose isomerase, resulting in conversion of fructose to glucose, the absorbance was measured again. The absorbance difference corresponds to the concentration of fructose in the sample. The CV was 5% at a mean fructose concentration of 12.7 mmol/L.

Prostate specific antigen

The concentration of PSA (mg/l) in seminal plasma was determined with Prostatus TM kit (Wallac Oy, Finland). This is a Delfia TM method, using three monoclonal antibodies against PSA. The CV was 12% at a mean PSA concentration of 660 mg/L.

Zinc

The concentration of zinc (mmol/l) in seminal plasma was determined with a colorimetric method (Makino *et al.*, 1982). The proteins in the sample were precipitated with trichloroacetic acid, the supernatant mixed with a water soluble pyridylazo dye and the absorbance measured at 560 nm. The CV was 7% for control samples with a mean zinc concentration of 2.0 mmol/L.

Hormone analyses

Serum concentrations of FSH, LH, SHBG, testosterone and oestradiol were measured on an automated fluorescence detection system (Autodelfia, Wallac Oy, Turku, Finland). Intra- and total-assay coefficients of variation (CV) were below 4% and 7.5%, respectively. Inhibin B levels were assessed using a specific immunometric assay, as previously described (Groome *et al.*, 1996) with a detection limit of 15 ng/L and total-assay CV below 7%.

Questionnaire

All participants were asked to fill in a questionnaire at home, which was translated from Danish and had been used in a previous study (Andersen *et al.*, 2000). The men were asked for information about prenatal and postnatal factors that might

influence their reproductive function (e.g. birth place, ethnic origin and congenital malformation). Also life-style related factors such as drinking and smoking habits were noted as well as information about their mothers smoking and drinking habits during pregnancy. In addition, all subjects were asked about snuffing habits.

Genotyping

Genomic DNA was prepared from peripheral leukocytes. For determining the AR gene CAG repeat length PCR-amplification was performed, using sets of flanking primers at concentrations of 0.5 μ M. Each 50 μ l reaction was done using 50 pg DNA, 1.0 mM MgCl₂, 0.2 mM dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.4 at 70°C), and 0.1% Tween 20. Amplification was performed for 30 cycles; each cycle included denaturation for 1 min at 96°C, primer annealing at 57°C for 30 sec, and primer extension for 5 min at 72°C. One microlitre of each polymerase chain reaction (PCR) product was used for subsequent nested PCR. CAG repeat sequences were determined by Cycle Sequencing using the BIG Primer Cycle Sequencing Ready Reaction Kit and the ABI Prism 310 DNA sequencer (PE Corporation, Foster City, CA).

Determination of CB 153, biomarker of POPs

CB-153 was analyzed according to a modified method of Janak et al. (Janak *et al.*, 1999). CB-153 was extracted from serum by solid phase extraction (Isolute ENV+; IST, Hengoed, UK) using on column degradation of lipids and analysis by gas chromatography mass spectrometry. ¹³C₁₂-labeled CB-153 was used as internal standard (Cambridge Isotope Laboratories, Andover, MA, USA). Selected ion monitoring of CB-153 was performed at m/z 326 and 360, whereas m/z 338 and 372 were chosen for the labeled internal standard. The CV, calculated from samples analyzed in duplicate at different days, were 7% at 0.6 ng/mL (n=76) and 5% at 1.5 ng/mL (n=37). The detection limit was <0.05 ng/mL. The analysis of CB-153 is part of the Round Robin intercomparison program (H.Drexler, Institute and Out-patient Clinic for Occupational, Social and Environmental Medicine, University of Erlangen-Nuremberg) with analyze results within the reference limits.

Determination of lipids by enzymatic methods

Plasma concentration of phospholipids, triglycerides and cholesterol were all determined by enzymatic methods using reagents from Boeringer-Mannheim (Cholesterol and triglycerides; Mannheim, Germany) and Waco Chemicals (Phospholipids; Neuss, Germany). The total lipid concentration in plasma was calculated by summation of the amounts of triglycerides, cholesterol and phospholipids. In these calculations, the average molecular weights of triglycerides and phospholipids were assumed to be 907 and 714, respectively. For cholesterol,

we used an average molecular weight of 571, postulating that the proportion of free and etherified cholesterol in plasma was 1:2.

Statistical analysis

Paper I

Linear regression models were used for investigating the impact of ethnic origin, place of living, sampling season and abstinence period on the semen parameters. Model assumption was checked by residual analysis (corresponding analyses regarding model assumptions were performed in paper II-V). When comparisons were performed between the Swedish and the Danish conscripts (Andersen *et al.*, 2000), the confidence intervals for the mean differences were calculated based on a normal distribution (Altman, 1991). To minimize the effect of abstinence time in these country comparisons, only those who had >48 h of sexual abstinence were included. Fisher's exact test was performed when the prevalences of cryptorchidism were compared.

Paper II

The effects of CB-153 on the sperm and hormone levels were evaluated by linear regression models, adjusted for potential confounders.

Paper III

The correlations between CAG repeat lengths and abstinence time, sperm quality markers, and biochemical seminal markers, respectively, were evaluated by Spearman's correlation coefficients. For comparison of seminal PSA levels between the group with the highest and the group with the lowest serum level of free testosterone, Mann-Whitney U-test was used. Linear regression models were used for evaluating the impact of CAG number, abstinence time and hormone levels on seminal PSA and sperm characteristics, respectively.

Paper IV and V

Linear regression analyses were performed for evaluating the effect of smoking, snuffing and maternal smoking on the different reproductive and morphometry parameters, respectively. In paper V, Pearson's correlation coefficients, (r), were used to evaluate associations between the morphometry parameters and other reproductive characteristics, such as semen volume, sperm concentration, total sperm count and sperm motility.

RESULTS

Paper I

We investigated sperm count, semen volume or total sperm count among 305 Swedish conscripts. The mean values were for semen volume: 3.2 ml; sperm concentration: 72.2×10^6 /ml and for total sperm count: 208×10^6 , respectively. When we compared conscripts living in urban vs. rural areas and those with different ethnic origin, no differences between the groups were seen. The association with abstinence time was significant both with regard to sperm concentration ($p=0.001$) and total sperm count ($p< 0.001$). In the interval 24-94 h of abstinence, there was a linear association between abstinence time and sperm

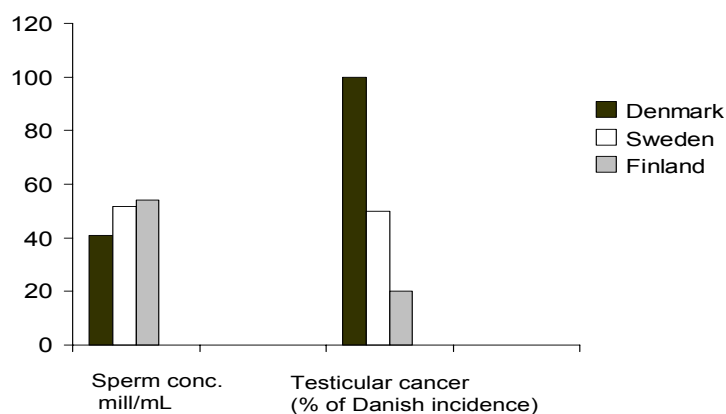


Figure 12. Differences in sperm concentration and testicular cancer incidence, in three Nordic countries .

An increase in the length of the abstinence period by 1 h corresponded to an increase in sperm number by 3.3×10^6 (CI 95% 1.9-4.6). When restricting the subjects to those borne and raised in Sweden, to make the group comparable with a former Danish study where the participants were selected according to such criteria (Andersen *et al.*, 2000), the number of subjects was reduced to 248. Comparing the results with a similar Danish population, we found 14% (95% CI=7–21%) higher (0.4 ml) median semen volume among the Swedish conscripts. We also noticed a 23% (95% CI=3–40%) higher (11.6×10^6 /ml) sperm concentration and 31% higher (95% CI=14–47%) (37.3×10^6 /ejaculate) mean sperm count in the Swedish conscripts compared to the Danish (Figure 12). The length of the abstinence time

was slightly lower in the Swedish group (89 h vs. 101 h). The Swedish conscripts reported significantly lower proportions of cryptorchidism (2.8%) compared to the Danish (12.6%). The difference was not so pronounced when looking to those actually treated for this condition, 1.2 versus 3.8% ($p=0.05$)

Paper II

A weak, but statistically significant, negative correlation was found between serum concentrations of CB-153, an index substance for POP exposure, and the proportion of CASA motile sperms and CB-153 levels (Figure 13). The same patterns of correlations, close to the level of statistical significance, were also observed for conventional motility parameters.

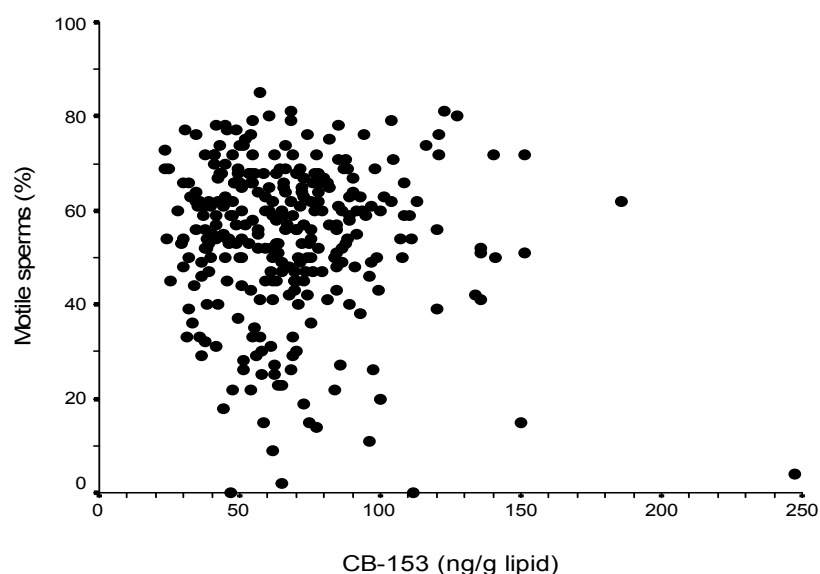


Figure 13. Correlation between CB-153 in serum and proportion of motile sperms as determined by CASA.

An increase in CB-153 levels by 10 ng/g lipid corresponded to 1.0% decrease in the percentage of CASA motility (95% CI, 0.13 to 2.0). There was no correlation between serum concentrations of CB-153 and semen volume, sperm concentration or total sperm counts.

There was also a statistically significant positive association between CB-153 and the serum levels of SHBG whereas for oestradiol and for testosterone/SHBG ratio the association with CB-153 was negative. When including BMI as a confounder the association between CB-153 and SHBG ($\beta=0.56$; 95% CI: 0.18 to 0.94) as well as testosterone/SHBG ratio ($\beta=-0.024$; 95% CI: -0.035 to -0.013) but

not oestradiol, remained statistically significant. The serum concentrations of CB-153 were not associated with the levels of LH and FSH.

Paper III

Among the 274 participants, 18 different alleles of the AR were found, CAG repeat numbers ranging from 12 to 30 (median: 22; mean: 22). There was a statistically significant negative correlation between CAG repeat length and the amount of PSA in ejaculates ($r_s = -0.13$; $p = 0.04$). No correlation was found between seminal PSA, and any of the hormone serum levels. CAG length and time of abstinence (Table 3), but neither free nor total testosterone, were statically significant predictors of PSA levels (for CAG: standardized $\beta = 0.13$; $p = 0.03$). Associations between CAG repeat lengths and levels of other biochemical markers were not significant.

A statistically significant negative correlation was found between CAG repeat number and:

- total sperm count ($r_s = -0.16$; $p = 0.01$);
- concentration of motile sperms ($r_s = -0.15$; $p = 0.02$);
- the total number of motile sperms ($r_s = -0.13$; $p = 0.004$);
- proportion of motile sperms ($r_s = -0.16$; $p = 0.01$).

In a similar analysis, the regression coefficients for the concentration of motile sperms and total sperm count were $\beta = -0.12$ ($p = 0.05$) and $\beta = -0.09$ ($p = 0.12$), respectively.

Table 3. Correlation between CAG repeat length and sperm characteristics.

	Spearman's rho	p
Abstinence time (h)	-0.06	0.30
Semen volume (mL)	-0.09	0.37
Sperm concentration ($\times 10^6/\text{mL}$)	-0.12	0.05
Total sperm count ($\times 10^6$)	-0.16	0.01
CASA motile sperms (%)	-0.18	0.01
Concentration of motile sperms ($\times 10^6/\text{mL}$)	-0.15	0.02

There was a statistically significant positive correlation between the CAG length and serum concentrations of LH ($r_s = 0.13$; $p = 0.04$), free testosterone ($r_s = 0.13$; $p = 0.03$), and androgen sensitivity index ($r_s = 0.12$; $p = 0.05$). No significant correlation was found with serum levels of total testosterone or SHBG.

Paper IV

Comparing semen parameters between smokers and non-smokers, we found 41% higher sperm counts (95% CI 4.8-88%, $p=0.02$) among non-smokers compared to smokers. Seminal volume and sperm concentration was also higher among non-smokers, but not statistically significant. The difference between the two groups was 0.3 ml for seminal volume (95% CI -0.03, 0.6 ml, $p=0.08$). Adjusted for mothers' smoking, the sperm concentration was 37% higher among non-smokers (95% CI -4, 95, $p=0.08$).

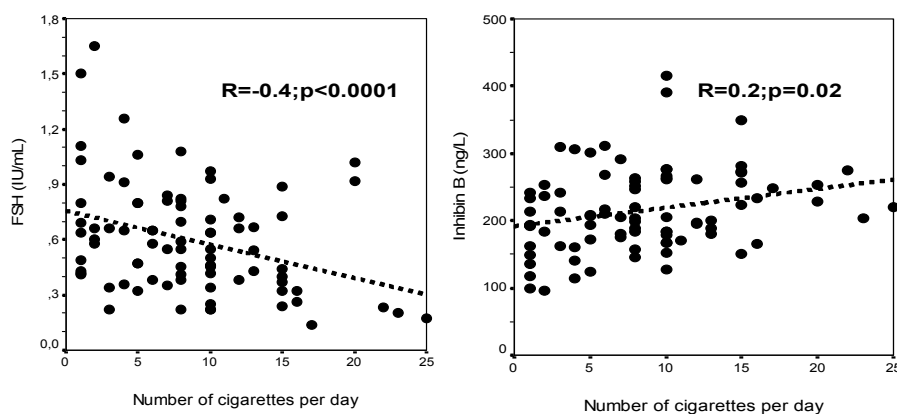


Figure 14. FSH and Inhibin B serum levels in relation to cigarette smoking

Compared to smokers, non-smokers had higher amount of the prostate markers PSA 19% (95% CI 2.9, 39, $p=0.02$) and zinc 29% (95% CI 5.9, 57, $p=0.01$) per ejaculate. However, this difference did not persist after adjusting for mothers' smoking. If the mother did not smoke during pregnancy, the NAG concentration was 25% higher (95% CI 2.8, 56) and the fructose concentration 48% higher (95% CI 18, 86, $p=0.001$) per ejaculate. No significant association between snuffing and semen parameters was obtained and no significant interactions between the different ways of exposure to tobacco (smoking; snuffing; maternal smoking) were found.

FSH concentrations were on average 17% higher (95% CI 3.0, 33, $p=0.02$) among non-smokers. When dividing the group of smokers to those smoking more and those smoking equal or less than 9 cigarettes per day the difference was even

more pronounced. Among those smoking >9 cigarettes/day, FSH concentration was 37% lower (95% CI 10, 69, P=0.005) as compared to those smoking 1-9 cigarettes/day.

Those who smoked 10 or more cigarettes per day exhibited a non-significant tendency toward higher Inhibin B concentrations compared to those smoking 1-9 cigarettes per day (mean difference: 24 ng/mL, 95% CI: -51, 1.9; p=0.07). When using the number of cigarettes per day as a continuous variable a statistically significant positive association with Inhibin B and a negative association with FSH concentration, was found (Figure 14).

Paper V

In this study, we compared morphometric parameters in 101 non-smokers and 49 smokers. We found significantly smaller sperm *head area* among smokers compared to non-smokers (mean difference: 0.25 μm^2 , 95% CI: -0.00020; 0.51, p=0.05). The value for the *elliptic form* of the sperm head was higher in smokers (mean difference: 0.010, 95%CI: 0.0010; 0.016, p=0.02) as compared to non-smokers, indicating a more round sperm head in the former group. A statistically significant difference was also observed regarding *midline area asymmetry* with lower values for smokers (mean difference 0.43, 95% CI: 0.10, 0.76, p=0.01). *Midline difference PMG* (PMG=pixel grey mean values 0-255) was close to statistical significance (p=0.08), whereas there were no differences between smokers and non-smokers regarding *asymmetric area*, *area difference* or *mid-piece width* (all p-values >0.3). None of the tested potential confounders had any impact of these results. No statistical significance was obtained when we compared morphometric parameters using mothers smoking or snuffing as independent variables (all p-values>0.5).

We found no statistically significant correlations (almost all $|r_s| < 0.01$) between the calculated sperm morphometry parameters and semen volume, sperm concentration, total sperm count or sperm motility.

DISCUSSION

Geographic differences in reproductive function

One of the major findings of this study was the observation of statistically significant higher sperm concentration, total sperm count and semen volume, among Swedish conscripts compared to a similar Danish cohort. Another marker of TDS, cryptorchidism, was also less prevalent in Sweden as compared to Denmark. Thus, for all three TDS-markers: sperm counts, cryptorchidism and TC, available data indicate a superior reproductive function among Swedish adolescent males as compared to their Danish counterparts.

This pattern seems to fit with a parallel observation of an East-West gradient in the Nordic-Baltic area regarding sperm number, TC and congenital abnormalities of male genital organs. Thus, compilation of available data indicated higher sperm counts, and lower incidence of TC and cryptorchidism/hypospadias in Finland and in the Baltic countries in relation to the numbers reported for Denmark (Andersen *et al.*, 2000; Richthoff *et al.*, 2002; Jorgensen *et al.*, 2002; Punab *et al.*, 2002; Richiardi *et al.*, 2004; Boisen *et al.*, 2004; Boisen *et al.*, 2001; Tsarev *et al.*, 2005; Toppari & Kaleva, 1999). Observations from other parts of Europe (Auger & Jouannet, 1997) and USA (Swan, 2006) have also indicated regional differences in sperm counts. However, the data from the Nordic-Baltic area are unique regarding the epidemiological link between sperm numbers and other signs of male reproductive failure.

Before discussing possible biological implications of the postulated difference between Denmark and Sweden, the validity of these findings needs to be discussed. The participation rate in the study on the Swedish conscripts was 13.5%. It could, therefore, be argued that the subjects included in this study were not representative for the general population. The participants did not differ in smoking habits or in body mass index (BMI) as compared to the whole group of Swedish conscripts (www.plikverket.se), who were considered as being representative for the general population of adolescent males. Furthermore, in most cases, it seems unlikely that these young men were aware about their reproductive capability. In a similar study from Denmark, the participation rate was 17.6% and in this study reproductive hormone levels did not differ between the participants and those who did not participate in the study (Andersen *et al.*, 2000), indicating lack of selection bias with regard to reproductive function. On the other hand, it should be kept in mind that all the Swedish conscripts were living in Southern Sweden and the Danish were recruited from Copenhagen and from Ålborg, which are areas that not necessarily are representative for the respective country.

Abstinence time is a crucial confounder in sperm analysis and this parameter was, therefore, taken into consideration by restricting the analysis to those with an abstinence time of at least 48 hours. This did not significantly influence the difference between Sweden and Denmark.

Seasonal variation has previously been suggested as a potential confounder, with highest sperm counts in the winter and autumn (Chen *et al.*, 2003; Levine *et al.*, 1990). We did not find any seasonal variation in our study.

A common source of worry when comparing the results of semen analysis performed by different laboratories is the issue of inter-laboratory variation (Neuwinger *et al.*, 1990). However, both the Copenhagen and the Malmö laboratory performed the analysis according to the most recent WHO criteria (www.euro.who.int). Furthermore, it was recently shown (Toft *et al.*, 2005) that by training the laboratory staff according to the WHO guidelines, an 8% median coefficient of variation for assessment of sperm concentration, can be achieved. Therefore, it seems rather unlikely that the more than 30% higher sperm count found among the Swedish men as compared to the Danish can be explained by selection bias, sampling schedule, abstinence time or differences in laboratory methods. On the other hand, the numbers regarding cryptorchidism incidence need to be taken cautiously, since the data were based on information obtained from the men included in the study (or their mothers) rather than on standardized investigations of newborns. However, even when only those surgically treated were included in the analysis, the difference between Denmark and Sweden remained statistically significant, although country-to-country differences in the indications for surgical corrections of this congenital malformation cannot be excluded. Interestingly, the prevalence of cryptorchidism was reported to be even lower among Latvian conscripts who also, in parallel, were presenting with higher sperm concentration as compared to Sweden (Tsarev *et al.*, 2005).

The finding of geographic differences in reproductive parameters is intriguing. However, elucidating the causes of such discrepancies may help us in understanding factors being of significance for regulation of male reproductive function.

Environmental factors

The serum level of CB-153 was previously reported to be a good marker for the exposure to the most common forms of PCBs and dioxins (Atuma *et al.*, 1998; Grimvall *et al.*, 1997). These chemicals belong to the group of ED (Kelce & Wilson, 1997; Sohoni & Sumpter, 1998), which are postulated to be possible causes of TDS (Skakkebaek *et al.*, 2001). In study II, the association between CB-153 levels and semen volume, sperm concentration or total sperm counts was

investigated. Only a weak correlation between the levels of this exposure marker and CASA sperm motility as well as SHBG concentration and the testosterone/SHBG ratio was noted. The exposure level observed in the Swedish conscripts was rather low as compared to subjects with a high consumption of PCB containing food (Sjodin *et al.*, 2002). It could, therefore, be claimed that the exposure dose in these adolescent men, was not sufficiently high to evoke the changes in sperm production. However, in a recent EU supported study (www.inuendo.dk) including Inuit men, who belong to one of the highest POP exposed populations in the world, CB-153 levels were only associated with sperm motility (Toft *et al.*, 2006), not having any impact on sperm concentration.

Thus, although both the study on Swedish conscripts and that on highly exposed men indicated no effect of POP exposure on sperm numbers, these findings are not sufficient to disprove the TDS hypothesis. It should be kept in mind that according to this hypothesis (Skakkebaek *et al.*, 2001) the major impact of the environmental exposure on male reproductive function is related to the events taking place in early foetal life, whereas measuring serum levels of CB-153 in men delivering semen samples reflects post-natal exposure to these subjects. A previous study on the semen quality of teenagers, whose mothers were heavily exposed during pregnancy to cooking oil contaminated by POPs, showed increased abnormalities in sperm morphology as well as reduced motility and ability to penetrate hamster oocytes as compared to controls not exposed during foetal life (Guo *et al.*, 2000).

The finding of an association between CB-153 concentration and sperm motility was novel. These results were not only confirmed in the already cited Inuendo study, but also a study on 29 patients recruited from an infertility clinic in USA, showed that males attending the clinic for semen evaluation had higher serum PCB levels than controls with normal sperm concentration, motility and morphology (Hauser *et al.*, 2002).

The effect of tobacco exposure

In studies IV and V the effect of tobacco exposure i.e. smoking, snuffing and maternal smoking during pregnancy on male reproductive parameters was investigated. Generally, the most pronounced effects were seen in relation to the subject's own smoking habits. The effects of maternal smoking were only related to the function of accessory sex glands and snuffing did not seem to have any impact on reproductive parameters at all.

In the context of the observed difference in sperm numbers between the Danish and the Swedish conscripts, these findings are rather intriguing. The total sperm number was more than 40% higher among non-smokers compared to smokers.

Based on the reported differences in smoking habits between the Danish and Swedish adolescent males (Manninen J, 1997),(www.euro.who.int), we estimated that smoking accounted for approximately 20% of the difference in sperm number between the conscripts in these two countries.

Apart from giving, at least partial, explanation for the observed difference in sperm numbers between the two countries, study IV also gave a hint of the mechanisms behind the effect of smoking on sperm production. The changes in hormone levels with decreased FSH concentration and increased Inhibin B in smokers, pointed to post-meiotic mechanisms as responsible for the reduction of sperm numbers in young adolescent males. Similarly to some previous studies (Pasqualotto *et al.*, 2006; Hassa *et al.*, 2006), a negative effect of smoking on the function of accessory sex glands, including a trend toward a lower semen volume, was found. Since decreased seminal volume may, at least partially, obscure the effect of smoking on sperm number, this might be one of the explanations behind the diverging results of different studies on the impact of smoking on sperm parameters if concentration is used as a marker of sperm production (Martini *et al.*, 2004; Trummer *et al.*, 2002; Vine, 1996).

The biological implications of the effect of cigarette smoking on sperm morphometric parameters are difficult to deduce, since studies on association between the morphometry and sperm function, are lacking.

We did not find any effect of maternal smoking during pregnancy on any sperm parameters. Previous Danish studies have shown reduced sperm counts in sons of heavily smoking mothers (>10 cig/day) during pregnancy (Storgaard *et al.*, 2003; Jensen *et al.*, 2004; Jensen *et al.*, 2005). In the present study we relied on retrospective information from the sons, whereas in the Danish study, access to maternal smoking data from a medical birth register was available (Storgaard *et al.*, 2003). The statistical power of our study was also lower than that in the Danish surveys. Having the differences between the two countries regarding smoking habits in mind, less maternal smoking might be a factor contributing to higher sperm numbers found among Swedish males. On the other hand, in the context of the TDS hypothesis, no strong association between maternal smoking and the risk of TC or congenital abnormalities of the reproductive organs in their sons has ever been found.

Genetic factors

Genetic factors can hardly explain the difference in reproductive function between Denmark and Sweden, since the two populations are genetically very similar (Luca Cavalli-Sforza *et al.*, 1994). In search for an explanation to higher sperm counts and lower incidence of TC and cryptorchidism in Sweden as compared to Denmark

one should, therefore, rather focus on environmental and life-style related factors. However, it can not be excluded that genetic variation is a part of the explanation as considers the differences between Denmark and Finland or the Baltic countries.

Androgens are known to play a crucial role for spermatogenesis (Tut *et al.*, 1997) and polymorphisms in the AR gene seem to have an impact on sperm production. Previous studies have indicated that long AR CAG repeats might predispose to infertility (Tut *et al.*, 1997) although these findings were challenged by others (Rajpert-De Meyts *et al.*, 2002). One study, based on men from the general population (von Eckardstein *et al.*, 2001) reported an inverse correlation between CAG number and sperm concentration. This finding was confirmed in the group of Swedish conscripts (study III). However, the association between the CAG number and sperm number was rather weak (Spearman's rho: -0.156). Thus, in a cohort of adolescent males a minor part of the variation in sperm numbers was due to variation in CAG repeat length and it is therefore not likely that the reported significant differences in sperm concentration between Denmark and Finland or the Baltic countries are due to this AR polymorphism. Moreover, the Finnish population displays the same CAG lengths as other European populations (Harkonen *et al.*, 2003).

However, one cannot exclude that other genetic factors may play an important role with respect to this matter. Polymorphisms in the 5 α -reductase II gene were recently reported to be associated with significant variations in sperm number (Elzanaty *et al.*, 2006). Another category of candidate genes are those located on the Y chromosome, which is known to play a significant role for spermatogenesis (Vogt *et al.*, 1996). A diversity in Y chromosome haplotypes with a pattern in Finland and the Baltic countries differing from that in Denmark and Sweden, has been reported (Rosser *et al.*, 2000).

GENERAL CONCLUSIONS

One of the major new findings of this thesis was the detection of higher sperm concentration, total sperm count and semen volume, in Swedish military conscripts as compared to Danish. Furthermore, our data indicated lower incidence of cryptorchidism in Sweden. We could not find any major impact of androgen receptor CAG repeat length and serum levels of CB-153, a marker of environmental POP exposure, on these sperm parameters. However, both seemed to have a weak negative effect on sperm motility. Smoking, habits are known to be one of the major lifestyle factors differing between Sweden and Denmark. We found significantly lower sperm counts among smokers compared to non-smokers. Smoking could at least partly contribute to the geographical differences in sperm numbers found between these countries.

FUTURE STUDIES

Although this study pointed to a difference between Swedish and Danish adolescent men in regard to their reproductive function, it failed to give an explanation for this phenomenon. One of the weaknesses of the study design was insufficient information regarding the foetal exposure of the enrolled subjects. Thus, future studies should focus on the impact of foetal exposure to life style and environmentally related factors.

For those subjects born in 1986 and later more detailed information regarding mothers smoking habits during pregnancy can be obtained by use of the National Birth Record Register. For military conscripts undergoing medical health examination now or in the future, such information will be available. Enrolling larger numbers of subjects would contribute to a higher statistical power and higher possibilities to disclose any effect of maternal smoking during pregnancy and through access to birth record registers we would get more accurate information about their smoking.

Foetal exposure to POPs can now be assessed through access to bio banks with rubella blood samples taken from pregnant females. Furthermore, investigations of exposure to other ED than POPs – both in the foetal life and postnatal - should be performed.

Available information about the impact of environment and tobacco exposure on male reproductive function is still insufficient. Using new methods for assessment of sperm characteristics as e.g. DNA integrity and morphometry might help us in disclosing effects not seen when using standard methods of semen analysis.

In this study we considered genetic, environmental and life style factors separately. Recent reports indicate an interaction between these parameters in regard to male reproductive function. Therefore, future studies should be performed where interaction between genetic predisposition and environmental and/or lifestyle exposure - in foetal life and in adult life – is investigated.

SVENSK SAMMANFATTNING

(Summary in Swedish)

Arvsanlagens, miljöns och livsstilens inverkan på de manliga fortplantningsorganen

Under de senaste decennierna har man misstänkt att den manliga reproduktionsfunktionen har försämrats. Detta baseras på en sammanfattning av en mängd studier, där man fann en tillbakagång av spermie antalet från $113 \times 10^6/\text{ml}$ till $66 \times 10^6/\text{ml}$ mellan 1940 och 1990. Man har också funnit stora geografiska skillnader. I Finland har man t ex noterat betydligt högre spermie kvalitet än i Danmark. Intressant nog skiljer sig testikelcancerfrekvensen också åt i många länder där den högsta frekvensen i världen är rapporterad från Danmark och Nya Zeeland till skillnad från Finland, som har bland de lägsta testikelcancernivåerna. Nyligen har också ett starkt samband mellan försämrad spermie kvalitet och risk för testikelcancer noterats.

En dansk forskargrupp under ledning av Niels Erik Skakkebeak har framkastat en ny teori angående en förmodad manlig reproduktionsförsämring. Teorin bygger på att tidiga medfödda störningar i reproduktionsorganens utveckling som hypospadi (urinrör som mynnar på felaktig plats) kryptorkism (testiklar som inte vandrat ner i pungen) och testikel cancer är symptom på ett och samma syndrom. Detta syndrom har benämnts ”testikel dysgenesi syndromet” (TDS). Man misstänker att detta syndrom anläggs redan i fosterstadiet och att ytterligare orsaker till detta syndrom kan vara genetiska, miljö och livsstils relaterade faktorer.

Manlig könsutveckling är beroende av en fungerande androgenreceptor (AR) som medierar effekterna av könshormonerna testosteron och dihydrotestosteron (DHT). AR-anlaget är beläget på den långa armen av kromosom X och består av tre olika domäner. En av dessa reglerar AR-aktiviteten. Genen innehåller hos olika individer olika långa sekvenser av CAG-baser, som kodar för aminosyran glutamin och som har betydelse för androgenkänsligheten. CAG-längden varierar normalt mellan 10-30. Man har nyligen sett att olika långa repetitioner av dessa CAG-baser är förknippade med olika sjukdomstillstånd. Ett exempel på detta är prostatacancer, där vissa studier indikerar att sjukdomen kan ha ett samband med få CAG-repetitioner, vilket också associerar med hög receptorfunktion och således en förhöjd känslighet för manligt könshormon.

Miljögifter med hormonliknande verkan har framställts som synnerligen potenta ämnen avseende interferering med normal hormonell signalering, vilket kan påverka reproduktionsfunktionen negativt. Framförallt är det polyklorerade bifenyl (PCB), vilket är ett samlingsnamn på kemiska ämnen med god

isoleringsförmåga och hög temperaturstabilitet, som blockerar hormon receptorerna genom östrogen, anti-östrogen eller anti-androgen inverkan. Dessa ämnen har ingått i mjukgörare i plaster och färger, transformatorer och kondensatorer. Då de är värme stabila och fettlösliga har de kunnat kvarstå och ackumuleras via näringskedjan till människan. CB-153 är en av de 209 PCB varianter som finns och detta ämne har en mycket god korrelation med den totala koncentrationen av PCB och kan därför användas som markör för de övriga. Hos högt exponerade män har man i tidigare studier visat en negativ påverkan på reproduktionsparametrarna. Det var därför intressant att se, om en liknande påverkan på reproduktionsfunktionen också gällde unga män, som inte har någon hög exponering.

Olika livsstilsfaktorer, som varma bad, alkoholintag, rökning, droger, kaffeintag och även sexuell aktivitet, har föreslagits interagera och påverka den manliga reproduktionsfunktionen negativt. Rökning är mycket vanligt förekommande och 1988 röktes 5,5 trillioner cigaretter av en miljard människor. Det är oroande att många unga börjar röka och att många gravida mödrar röker. Hälsoeffekterna på unga människor och foster är ännu okända. Några få studier har kunnat påvisa ett samband mellan rökning och nedsatt spermierörlighet och minskad sädesvolym, medan ett flertal andra studier inte har hittat något samband.

Eftersom man tidigare har funnit signifikanta skillnader i spermiekvaliteten mellan Finland och Danmark, och då dessa länder har olika genetisk bakgrund, var det intressant att se hur Sverige och Danmark förhöll sig till varandra, då dessa länder är mer närbesläktade genetiskt. Dessutom röker danska män i större utsträckning än svenska och har en betydligt högre testikelcancerfrekvens än svenska män.

Svaren på ovan nämnda frågeställningar var okända vid starten av detta avhandlingsarbete, som inleddes år 2000. Vi undersökte 305 män, med en medianålder på 18 år, på värnpliktskontoret i Kristianstad. Alla genomgick en andrologisk undersökning avseende manlig könsutveckling och ultraljudsskanning av testiklarna. Deltagarna fick också lämna blodprov för hormonanalyser och sädesvätskeprov. Vidare fyllde alla i ett frågoformulär angående olika livsstilsfaktorer som rökning, alkoholkonsumtion och rökvanor hos modern under graviditeten.

Vi fann (23%) högre spermie koncentration, (30%) högre spermie antal och (14%) större sädesvolym hos de svenska värnpliktssökande jämfört med de danska. Resultaten faller väl in i den hypotes, som framkastats, att spermiekvaliteten drastiskt skiljer sig i olika geografiska områden, vilket kan ha ett samband med den förmodade nedgången i spermiekvalitén, som man sett under de senaste 50 åren. Man har också konstaterat betydande skillnader i testikelcancerfrekvens bland de Nordiska och Baltiska länderna med högst frekvens i Danmark-Norge och lägst i

Finland och Baltikum. Sverige intar en mittposition i förhållande till dessa länder. Det är intressant att notera hur spermiekoncentrationerna och testikel cancerfrekvensen följs åt. Således verkar det vara så, att ju högre testikelcancerfrekvens man har inom ett visst geografiskt område, desto lägre spermiekoncentration och vise versa. Det är också intressant, att man nyligen sett ett starkt samband mellan testikel cancerfrekvens och spermiekvalité, där hög cancerincidens var förenad med sämre kvalité. I vår studie fann vi också en lägre frekvens av retinerade testiklar hos den svenska gruppen (1.2%) jämfört med den danska (3.8%), vilket också stärker fynden av de geografiska skillnaderna mellan Danmark och Sverige eftersom retinerade testiklar också är förenat med minskad spermiekvalité.

Vidare fann vi ett starkt samband mellan PSA (prostate specific antigen) i sädesvätska och AR (androgenreceptor längd). I AR-genen finns ett specifikt område av repeterade CAG-baser, som reglerar receptorns aktivitet. Testosteron omvandlas i prostata till DHT (dihydrotestosteron) och är nödvändigt för normal könsutveckling och prostatafunktion. DHT aktiverar AR, vars aktivitet/funktion reflekteras via prostatas utsöndring av PSA i sädesvätskan. Vi fann ett signifikant negativt samband mellan CAG och PSA, d v s ju färre CAG desto högre PSA-halt i sädesvätskan på grund av en känsligare och aktivare AR. Aktiv och mer känslig AR har också konstaterats vid prostata cancer. Det omvända förhållandet med höga CAG repetitionslängder innebär följaktligen minskad AR funktion och troligen också minskad fertilitet på grund av inaktivare och mindre känslig receptorfunktion. Ökad prostataaktivitet i tidig ungdom kan alltså ha positiv inverkan på fertiliteten, men i det långa loppet leda till framtida prostatasjukdomar.

I en av studierna fann vi också signifikant påverkan på spermiekvalitén hos rökarna. De som rökte uppvisade 49% lägre spermie antal än icke-rökarna. Söner till rökande mödrar uppvisade en negativ påverkan på de sekundära könskörtlarnas funktion. Vi såg också en förändring i sexualhormonproduktionen, med lägre FSH-nivåer hos rökarna. Vi fann också statistiskt signifikanta skillnader i storlek på spermierna, med mindre och rundare spermier hos rökarna jämfört med icke-rökarna. Både egen rökning och moderns rökning har således troligen en negativ inverkan på de manliga reproduktionsparametrarna.

Ytterligare fann vi ett svagt men signifikant samband mellan försämrad spermie rörlighet och serum nivåer av CB-153, vilken är en markör för miljögifter med negativ endokrin inverkan.

Sammanfattningsvis fann vi stora geografiska skillnader i spermiekvalité mellan Sverige och Danmark, som inte kan förklaras av genetiska skillnader eller miljöfaktorer. Dessa hade dock en viss negativ inverkan på spermierörligheten, vilket kan tyda på en negativ påverkan på de sekundära könskörtlarna, som också

noterades hos dem vars mödrar rökt under graviditeten. Rökning är en av de livsstilsfaktorer som markant skiljer sig mellan Sverige och Danmark. Eftersom vi fann signifikant lägre spermieantal hos rökande värnpliktssökande i Sverige jämfört med icke-rökande, kan rökning vara en av de faktorer som förklarar skillnaden i spermiekvälité mellan Sverige och Danmark. Vi kommer i framtida studier att ytterligare försöka klarlägga rökningens förhållande till spermiekvälité hos unga vuxna män och även hur exponering för miljö- och livsstilsfaktorer under fosterstadiet inverkar på den manliga reproduktionsfunktionen.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisor Professor Aleksander Giwercman, who has wholeheartedly guided me through this scientific work with enormous patience. You have always supported me when I needed it. Your work-capacity is enormous, and I am especially impressed by your good memory.

Lars Rylander, my assistant supervisor, who have guided me through the deep jungle of statistics. You also share the team of my heart, MFF!!

Lars Hagmar my assistant supervisor, who guided me into the understanding of POPs and who helped me how to write a sufficient satisfactory article.

Yvonne Giwercman, my assistant supervisor for your patience and wholehearted support.

Erna Stridsberg, Rita Hovander and Göran Svensson, working at pliktverket in Kristianstad, are thanked for their help in recruiting the military conscripts for the study; I also thank all the participants who accepted to take part in it.

Katarina Jepsson, Camilla Anderberg and Anna Bremer are thanked for their technical assistance with the blood and sperm samples.

Jan Schönebeck is thanked for his enthusiastic support in the urological and scientific world.

Claus Jorgensen and Niklas Nygaard, the heads of the surgical department at the hospital in Ljungby, are thanked for their support, always without complaints having relieved me from my clinical duties during all these years without complaints.

I thank Saad Elzanaty, Tarmo Tiido, Jacob Eberhart, Olof Stål, Patrik Romerius, Hamid Raskhani, Hanna Nenonen, Kristina Lundin and Yasir Ruhayel Anna Rignell-Hydboom who are members of our research group.

PhD students and friends at CRC (Clinical Research Centre).

I would like to thank Volvo for making so good cars that have brought me home safely when I have driven 80000 km between my home in Ljungby and the lab and hospital in Malmö.

Thanks to those who supported the study financially, Crafoordska Fund, Ove Tulefjords Fund and Foundation for Urological research.

FoU medel (Research and Development funding)

I have had a good start in life and this thanks to my parents Elsebeth and Sven-Ingvar Richthoff, who also encouraged me during all my years of studies.

I would not have been able to complete this project if it were not for my wonderful family, and I wish to express my deepest thanks to my wife Madeleine who has taken so good care of the family, my daughters Emmelie, Hanna and my sons Johan and Gustav, and not least myself, and in that way made this project possible.

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