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Reproductive function in young Swedish men

Time trend, prenatal and adult exposure to smoking and phthalates

Jonatan Axelsson

Department of Translational Medicine
Molecular Reproductive Medicine

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Abstract

A decrease in male reproductive function, including sperm counts, during the second half of the 20th century, has been postulated. During this period, testicular cancer has increased several-fold. Environmental and lifestyle-related factors have been proposed as explanations for a possible negative trend in male reproductive function. One important lifestyle factor is smoking. However, smoking during pregnancy appears to play a greater role for a man's reproductive function than smoking by the man himself. As regards environmental chemicals, one type of compound to which humans are exposed is phthalates. These are often used as plasticizers in different consumer products. Several phthalates have been reported as decreasing male reproductive function in laboratory animals, especially when given during the foetal period.

Between 2008 and 2010, 314 men from the general Swedish population were recruited. Their semen quality was compared with a group of men recruited in a similar manner between 2000 and 2001.

The participants also delivered serum and urinary samples and answered questionnaires concerning maternal and paternal smoking during pregnancy. Data on maternal smoking was additionally assessed through the Swedish Medical Birth Register. Through a Swedish screening program for rubella, maternal serum samples were retrieved from the men's prenatal period. We analysed metabolites of phthalates as exposure markers both in the maternal sera and in urine and serum of the men. Associations between parental smoking during pregnancy as well as phthalate metabolite levels, and parameters of male reproductive function were studied.

In summary, we found no change in semen quality between 2000-2001 and 2008-2010. However, both maternal and paternal smoking during pregnancy were associated with reduced sperm counts in men whose other parent did not smoke. In addition, prenatal exposure to diethylhexyl phthalate (DEHP) and diisononyl phthalate (DiNP) appeared to be associated with decreased semen volume, and exposure to DiNP as well with smaller testicular size. Finally, adult exposure to DEHP and dibutyl phthalate (DBP) were associated with decreased progressive sperm motility, and DEHP exposure was also linked to a higher proportion of immature sperm.

Thus, although no change in semen quality appeared to have occurred in Swedish men during the last decade, parental smoking and prenatal and adult exposure to certain phthalates may play a role in the male reproductive function.

Key words: Semen analysis, maternal exposure, paternal exposure, smoking, phthalate

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3) A smoking Humphrey Bogart from Casablanca trailer screenshot [Public domain] via Wikimedia Commons
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Contents

Populärvetenskaplig sammanfattning 7

List of papers 9

Abbreviations 11

Background 13
- Trends in sperm counts 13
- Infertility 14
- Trends in testicular cancer 14
- Trends in the incidence of male genital malformation 15
- Testicular dysgenesis syndrome 16

The male reproductive system 16
- The development of the male reproductive organs 16
- The testicle 17
- Spermatogenesis 18
- Male reproductive hormones and the hypothalamic-pituitary-gonadal axis 18

Measurement of semen quality 19

Environment, lifestyle and the male reproductive function 20
- Smoking and male reproductive function 21
- Parental smoking and reproductive function in sons 21
- Phthalates 22

Aims 27

Subjects and methods 29

Subjects 29
- Cohort A 29
- Cohort B 29

Physical examination 30

Questionnaire 30

Samples from the men 30

Semen analysis 31
Analyses of reproductive hormones 31
Register data on maternal smoking 32
Maternal sampling in pregnancy 32
Analyses of phthalate exposure 32
Analyses of maternal serum 32
Analyses of men's urine 33
Analyses of men's serum 34
Statistical methods 34
Paper I 34
Paper II 35
Paper III 36
Paper IV 36
Results 37
Time trend in semen quality over the previous decade 37
Own and parental smoking in relation to semen quality 37
Prenatal exposure to phthalates and male reproductive function 38
Adult exposure to phthalates in relation to semen quality 39
Discussion 43
Time trend in semen quality 43
Smoking exposure and semen quality 43
Phthalate exposure and male reproductive function 45
Misclassification of exposures 47
Potential confounding 48
Potential effects of childhood exposures 48
Clinical relevance 48
Summary 49
Conclusions 51
Future perspectives 53
Acknowledgements 55
References 57
Original papers 75

Populärvetenskaplig sammanfattning

Det har diskuterats huruvida mäns spermiekoncentration minskat och missbildningar i pojkars könsorgan ökat under den senare delen av 1900-talet. Med säkerhet har dock testikelcancer ökat flerfaldigt under samma tidsperiod.

Målet med denna avhandling var dels att undersöka huruvida skånska mäns spermakvalitet hade förändrats under det förra årtiondet, dels att studera samband mellan olika miljöfaktorer och manlig reproduktionsfunktion. Dessa miljöfaktorer var exponering för egen rökning, föräldrarnas rökning under graviditeten, samt för ftalater (en sorts plastmjukgörare) både under fostertiden och i vuxenlivet.

Vi rekryterade 314 unga män mellan år 2008 och 2010, främst från Pliktverket i Kristianstad i samband med mönstringen, och i viss utsträckning också män från gymnasieskolor i Malmö-Lund-området. Männen fick besvara frågor om föräldrarnas rökning under graviditeten. Uppgifter om moderns rökning inhämtades även från Socialstyrelsens födelseregister.

Vi hade tillgång till blodprov från 112 av männens mödrar, tagna under graviditeten. Dessa prov hade tagits vid en screening för röda hund, varefter proven sparats i en biobank. I dessa prov, liksom i urin- och blodprov från männen själva, mättes nedbrytningsprodukter (metaboliter) av ftalater som markörer för exponering.

De rekryterade männens spermakvalitet jämfördes med den hos män rekryterade på ett liknande sätt år 2000 till 2001. Därefter studerades samband mellan exponering för både rökning och ftalater såväl under fostertiden som i vuxenlivet, och männens reproduktionsfunktion.

Vi fann inte någon förändring i spermakvaliteten under det senaste årtiondet.

Däremot hade de män vars fäder eller mödrar rökt under graviditeten ett påtagligt lägre spermieantal. Vidare tycktes exponering under fostertiden för de två ftalaterna di-isononylftalat (DiNP) och dietylhexylftalat (DEHP) ha samband med en sänkt testikelvolym respektive spermavolym. Slutligen hade exponering för DEHP och dibutylftalat (DBP) i vuxenlivet samband med en lägre andel framåtrörliga spermier, vilket kan tänkas påverka fertiliteten.

Således verkade både exponering föräldrarnas rökning under graviditeten och för ftalater under fostertiden samt i vuxenlivet, ha samband med en sänkt manlig reproduktionsfunktion.

List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. Axelsson J, Rylander L, Rignell-Hydbom A, Giwercman A. No secular trend over the last decade in sperm counts among Swedish men from the general population. *Human Reproduction*. 2011; 26: 1012-1016

II. Axelsson J, Rylander L, Rignell-Hydbom A, Silfver K Å, Stenqvist A, Giwercman A. The Impact of Paternal and Maternal Smoking on Semen Quality of Adolescent Men. *PLoS One*. 2013; 8: e66766

III. Axelsson J, Rylander L, Rignell-Hydbom A, Lindh CH, Jönsson BA, Giwercman A. Prenatal phthalate exposure and reproductive function in young men. *Environmental Research*. 2015; 138C: 264-270

IV. Axelsson J, Rylander L, Rignell-Hydbom A, Lindh CH, Jönsson BA, Giwercman A. Phthalate exposure and semen quality in young men from the general Swedish population. *Submitted*

Abbreviations

AGD	anogenital distance
CI	confidence interval
DBP	dibutyl phthalate
DEHP	diethylhexyl phthalate
DEP	diethyl phthalate
DFI	DNA Fragmentation Index
DiNP	diisononyl phthalate
FSH	follicle-stimulating hormone
HDS	high DNA stainability
LH	luteinizing hormone
LOD	level of detection
MBP	monobutyl phthalate (a metabolite of DBP)
MBzP	monobenzyl phthalate (a metabolite of BBzP)
MCiOP	mono-(carboxy-iso-octyl) phthalate (a metabolite of DiNP)
MEHHP	mono-(2-ethyl-5-hydroxylhexyl) phthalate (a metabolite of DEHP)
MEHP	mono-(2-ethylhexyl) phthalate (a metabolite of DEHP)
MECPP	mono-(2-ethyl-5-carboxypentyl) phthalate (a metabolite of DEHP)
MEOHP	mono-(2-ethyl-5-oxohexyl) phthalate (a metabolite of DEHP)
MEP	monoethyl phthalate (a metabolite of DEP)
MHiNP	mono-(hydroxyl-iso-nonyl) phthalate (a metabolite of DiNP)
MOiNP	mono-(oxo-iso-nonyl) phthalate (a metabolite of DiNP)
MPW	masculinization programming window
SHBG	sex-hormone binding globulin
TC	testicular cancer

Background

Environmental effects on the human reproductive function is not a new issue, but has even been suggested as having caused the fall of the Rome Empire (Gilfillan 1965).

Trends in sperm counts

More recently, a 50% decline in sperm count in 50 years was suggested (Carlsen et al. 1992). This was the result of a meta-analysis including 61 papers on men without infertility from several different countries during the period. The decline in sperm count was suggested to indicate serious implications for human reproductive health (Carlsen et al. 1992). A reanalysis of the papers included and additionally 47 studies performed between 1934 and 1996, with a partial adjustment for geographic region and abstinence period, found virtually the same decline (Swan et al. 2000). Some other studies have also reported a decline, whereas others found no change or even an increase in sperm count (Fisch and Braun 2013). Few of these studies, however, included the early part of the period covered by the first papers (Carlsen et al. 1992, Swan et al. 2000).

Whether a decline in sperm count or semen quality really has occurred has been heavily debated (Brinkworth and Handelsman 2010, Pacey 2013) and no consensus has been reached. One problem of the studies reporting a decline in sperm count (Carlsen et al. 1992, Swan et al. 2000) could be that the method used for sperm counting may have differed in the earlier studies as compared to the later ones (Pacey 2013). This may have led to gradually lower estimated sperm counts, and thus a false appearance of decrease. Another problem may be that geographical differences in sperm count, even within nations, were not taken into account (Fisch and Braun 2013).

Consequently, in order to confidently assess potential time trends in semen quality, prospective studies using standardized methods to assess semen quality over time are necessary. At the beginning of this PhD project, such studies were few or non-existent.

Infertility

Infertility, defined as not achieving a pregnancy within one year of regular unprotected intercourse, is a common problem. One in six couples of reproductive age are considered to be infertile, and the male factor contributes in about 50% of these cases (Nieschlag 2010).

Trends in fertility are difficult to study due to the influence of social factors. Nevertheless, some studies have reported a time-related increase in fertility problems (Lassen et al. 2012, Priskorn et al. 2012), whereas others have not (Akre et al. 1999, Joffe et al. 2013).

Trends in testicular cancer

During the same period as it was suggested that sperm counts decreased, an increase in testicular cancer (TC), by a factor of three to four, has been reported in the Western World (Brinkworth and Handelsman 2010) also in the Nordic countries (Figure 1). TC is the most common form of cancer in 15 to 40 year-old men in most developed countries (Znaor et al. 2014).

Still, the incidence of TC varies widely between certain different countries, being much higher in Norway and Denmark than in Finland (Figure 1) (Trabert et al. 2015). At the same time, high TC incidence appears to coincide with low sperm counts in Norway and Denmark (Jorgensen et al. 2002). Also at the individual level, men with TC often have reduced sperm counts or fertility (Juul et al. 2014). In parallel, subfertile men may have an increased risk of TC (Garner et al. 2005).

Men living in a country with a low incidence of TC moving to a country with a high incidence seem to maintain their low TC risk, but if they father sons the sons will run a higher risk, more similar to that of the new country (Hemminki and Li 2002, Myrup et al. 2008). In addition, the risk of TC appears to follow birth cohorts since, for example, men born during the Second World War have a decreased risk (Olesen et al. 2007). These patterns are hard to explain by genetic factors. Consequently environmental or lifestyle-related factors (Hemminki and Li 2002, Olesen et al. 2007) possibly acting in early life or *in utero* (Myrup et al. 2008), have been proposed as causes. Indeed, some studies have reported higher levels of certain persistent pollutants in men, or mothers of men, with TC (Hardell et al. 2006, McGlynn et al. 2008).

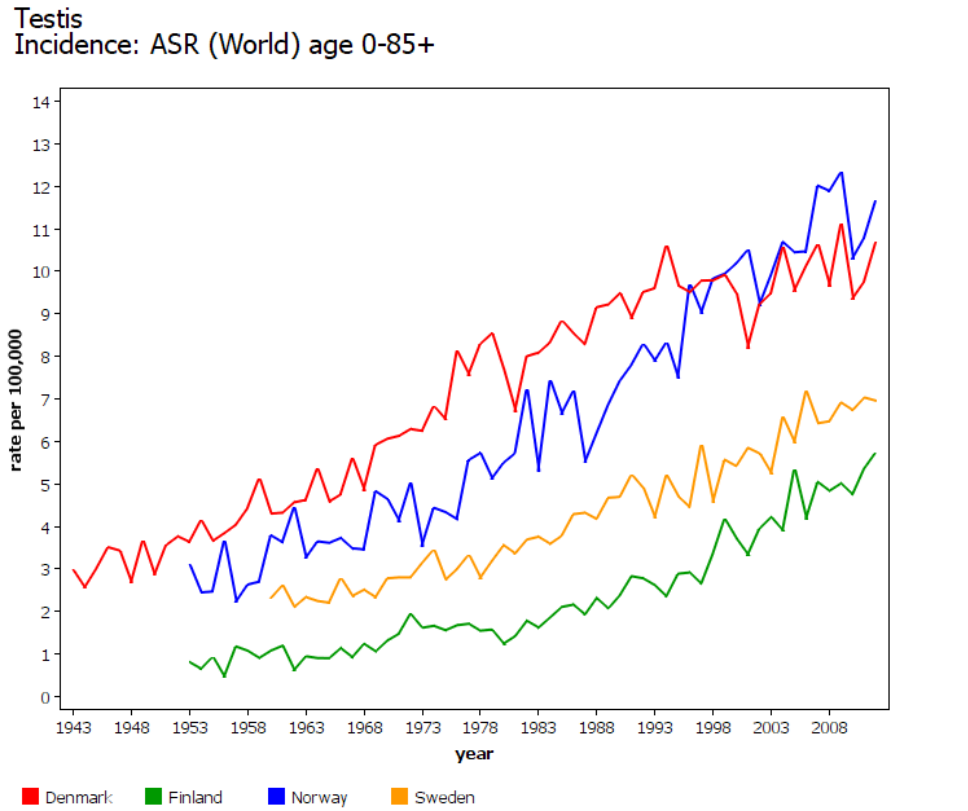

Figure 1
Incidence in testicular cancer in the Nordic countries between 1943 and 2010, according to an online analysis through the NORDCAN database and program (Engholm et al. 2010, Engholm et al. 2014)

Trends in the incidence of male genital malformation

In addition to the previously mentioned confirmed or suggested changes in male reproductive disorders, it has been proposed that an increase in male genital malformation has occurred during the same period (Nordkap et al. 2012, Toppari et al. 2001, Toppari et al. 2010).

The two most common such malformations are cryptorchidism, or undescended testis, and hypospadias (Toppari et al. 2001), the former being the overall most common birth defect in newborn boys (Toppari et al. 2010). Hypospadias is a condition when the opening of the urethra is not located at the tip of the penis, but somewhere underneath.

However, changes in the incidence of these malformations have been hard to establish due to a lack of consensus regarding diagnostic criteria and, as a result, time-related differences in registration routines (Toppari et al. 2001). In one case a modification of a national registration system led to a sudden change in registered incidence (Toppari et al. 2001). Certain countries have even stopped registering cryptorchidism due to poor reporting. Nevertheless, through a study using common methods and definitions, the incidences of cryptorchidism (Boisen et al. 2004) and hypospadias (Boisen et al. 2005, Virtanen et al. 2001) were reported to be considerably higher in Denmark than in Finland. This difference was in line with the differences in TC and sperm count between the two countries, and also in line with a later report of smaller testicles in Danish than in Finnish newborn boys (Main et al. 2006).

Although the etiology of these malformations is largely unknown, higher levels of chlorinated pesticides (Damgaard et al. 2006) and certain brominated flame retardants (Main et al. 2007) have been reported in mothers of newborn boys with cryptorchidism. These studies may indicate the general importance of environmental factors to the human male reproductive development.

Testicular dysgenesis syndrome

It has been suggested that hypospadias, cryptorchidism, TC and low sperm count have a common origin *in utero* (Skakkebaek et al. 2001). Thus, these different inter-related disorders are suggested as different symptoms of a common entity, the testicular dysgenesis syndrome (TDS). One proposed mechanism leading to TDS is a disturbed embryonal programming and gonadal development, mediated through adverse environmental factors.

A similar syndrome to TDS can be induced in rats by exposure to certain compounds of a group of chemicals called phthalate esters or phthalates, during the foetal period (Foster 2006). Thus, one pathway to TDS might be that certain chemicals alter hormonal action through an 'endocrine disruption' (Sharpe and Skakkebaek 2008).

The male reproductive system

The development of the male reproductive organs

Interference with the male sexual differentiation is an area of particular interest regarding endocrine disruption (Sharpe 2006). The basic developmental path of the human embryo is a female development, unless masculinization is actively induced

(Larsen 2001). Completion of the testicular formation needs hormonal intervention through anti-Müllerian hormone, testosterone and insulin-like factor 3 (Sharpe 2006). Hence, unlike the female development, the male development is totally hormone-dependent and therefore more susceptible to interference from endocrine-disrupting compounds. A specific window has been identified in rats, during which the masculinization of the reproductive tract is programmed. This window was suggested to occur in humans between the 8th and 14th gestational weeks (Welsh et al. 2008). In rats, exposures leading to decreased testosterone production during this window cause reduced size of the male rat's reproductive organs (Macleod et al. 2010).

The testicle

The testes produce the male gametes (sperm) and androgens such as testosterone (Weinbauer et al. 2010).

The testis has two different functional compartments: the seminiferous tubules where the sperm are produced, and the interstitial compartment containing the testosterone-producing Leydig cells (Figure 2).

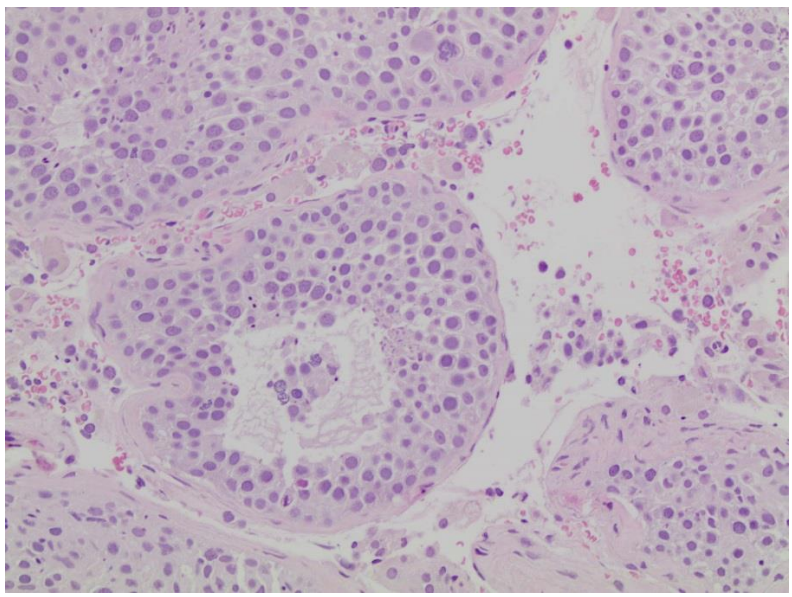

Figure 2
Biopsy of a human testis. Large rounded structures represent the seminferous tubules where the spermatogenesis takes place. The space between the tubules represents the interstitial compartment. By courtesy of Roy Ehrnström, MD, PhD, Skåne University Hospital, Malmö, Sweden.

The function of the testis and its compartments is governed by the pituitary gland and the hypothalamus in the brain.

Spermatogenesis

Spermatogenesis is dependent on the supporting Sertoli cells. These are located in the periphery of the tubules (Figure 2) and secrete a number of factors important for spermatogenesis. The total number of Sertoli cells, which appears to increase until 15 years of age, decides the final testicular volume and adult sperm production (Weinbauer et al. 2010).

Spermatogenesis begins with a division of stem cells and ends with a mature sperm, which in all takes around 74 days (Amann 2008, Heller and Clermont 1964). For unknown reasons, the release of sperm into the lumen of the seminiferous tubules (spermiation) may be affected by external factors such as hormonal modifications and toxicants (Alukal et al. 2009).

The maturation of the sperm takes place in the epididymis (Turner 2009). This involves a dramatic increase in motility and potential of fertilization, and takes only a few days (Johnson and Varner 1988). During the maturation, the sperm chromatin condensates through disulfide bonds in DNA-binding proteins called protamines (Cooper and Yeung 2010).

The durations of spermatogenesis and sperm maturation are important to keep in mind when potential effects of toxicants on semen parameters are interpreted (Evans 2007).

Male reproductive hormones and the hypothalamic-pituitary-gonadal axis

Two important hormones regulate the testicular functions (Sokol 2009). These are the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH), together called the gonadotropins, which are secreted by the pituitary gland (Weinbauer et al. 2010). The gonadotropins are, in turn, regulated by the gonadotropin-releasing hormone (GnRH), secreted by the hypothalamus in a pulsatile fashion. This leads to the secretion of gonadotropins in discrete peaks, which is most evident for LH because of its shorter half-life. Further, the secretion of gonadotropins is also regulated through a negative feed-back mechanism by gonadal hormones such as testosterone and estradiol from the Leydig cells, and inhibin B from the Sertoli cells (Figure 3).

LH stimulates testosterone production whereas FSH together with testosterone stimulates spermatogenesis (Weinbauer et al. 2010). LH is often elevated in men with a deficient production of androgens due to testicular failure (Simoni and Nieschlag 2010). Similarly, some men with deficient spermatogenesis show an elevated level of FSH. These patterns are due to a lack of the negative feedback by testosterone/estradiol

and inhibin B, respectively (Figure 3). A simultaneous elevation of FSH and LH can be observed in men with more severe testicular damage (Sokol 2009).

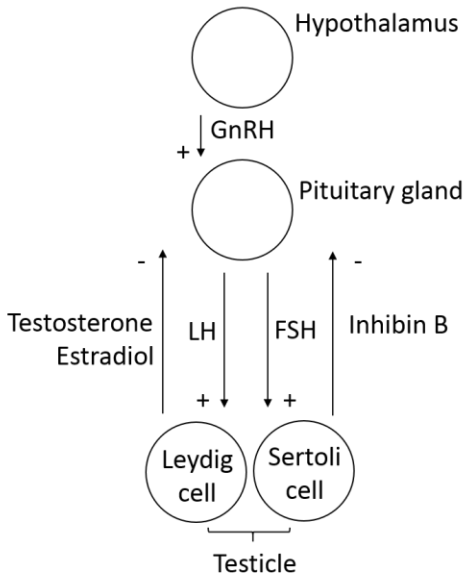

Figure 3
Hypothalamic-pituitary-gonadal axis

Testosterone in plasma is mainly bound to proteins such as sex hormone binding globulin (SHBG), so only 2% of the testosterone in plasma circulates freely and biologically active (Simoni and Nieschlag 2010). Testosterone, and its more potent product dihydrotestosterone, exert their effects through binding to the androgen receptor (Weinbauer et al. 2010). The effects of the androgens on spermatogenesis are believed to be mediated via the Sertoli cells, since the germ cells appear to lack androgen receptors (O'Donnell et al. 2006). Besides testosterone and FSH, oestrogen also seems to play a role in spermatogenesis (O'Donnell et al. 2006).

Measurement of semen quality

Male reproductive function can be evaluated through a semen analysis (Cooper 2010). The result of a semen analysis varies with the analytical method and how and where the semen was collected. Consequently, the analysis must be standardized. In order to be able to compare different results, a period of abstinence of between 48 hours and 7 days

should be observed. However, even within this time span the duration of the abstinence period affects the semen parameters (Levitas et al. 2005). Consequently the actual length of the abstinence period should be recorded (Sanchez-Pozo et al. 2013).

The ejaculate should always be examined according to the guidelines specified by WHO (World Health Organization. 1999, 2010b). For the relevant parts of this thesis the previous version (World Health Organization. 1999) was used, since the most recent guidelines were not published during the initial recruitment and semen analysis of the participants.

The most obvious changes between the 1999 and 2010 versions may be the shift in what is considered to be normal values of different semen parameters, such as of sperm concentration from 20 x 10^6/mL to 15 x 10^6/mL and of the proportion of progressively motile sperm from 50% to 32%.

Environment, lifestyle and the male reproductive function

It appears reasonable that the apparent changes, and geographical differences, in male reproductive health are caused by environmental factors (Bonde and Toppari 2010).

Possibly, even a major part of male reproductive problems in the Western World is caused by such factors, acting either prenatally or postnatally on testicular development and function (Nordkap et al. 2012).

Environmental contaminants, including certain pesticides and organohalogens such as PCB, have been associated with different reproductive disorders in wild-life species including mammals (Hamlin and Guillette 2010, Toppari et al. 1996). Further, experimental exposure of pregnant rats to chemicals with an endocrine-disrupting potential clearly leads to symptoms reminiscent of TDS in the male offspring (Fisher et al. 2003, Hass et al. 2007). This has contributed to an increase of concern for similar effects in humans.

As concerns potential effects of lifestyle and environmental factors in humans, several pesticides, PCB and air pollution appear to negatively affect different aspects of semen quality (Jurewicz et al. 2009). Further, there is some support that mobile phones may adversely affect sperm motility and viability (Adams et al. 2014). However, concerning obesity, no consistent association with semen parameters appears to be present (Barazani et al. 2014). Alcohol intake may affect semen parameters such as sperm counts and morphology (Jensen et al. 2014), but potential effects seem greatest in men with a high intake. Finally, several occupational exposures such as heat, ionizing radiation, welding and several chemicals seem to show strong evidence for adverse effects on the male reproductive function (Jensen et al. 2006).

Nevertheless, evident connections between specific toxicants in the general environment and male reproductive diseases have not been completely established (Nordkap et al. 2012).

Therefore, further research appears necessary to identify potential underlying factors for male reproductive dysfunction. Two exposures of possible importance are smoking and phthalates.

Smoking and male reproductive function

Smoking is causally related to diseases in nearly all the organs of the body as well as to harm to the foetus (United States. Public Health Service. Office of the Surgeon General. and National Center for Chronic Disease Prevention and Health Promotion (U.S.). Office on Smoking and Health. 2014). In accordance with this, smoking seems to be associated both with decreased semen quality (Barazani et al. 2014, Li et al. 2011, Practice Committee of the American Society for Reproductive 2012, Vine 1996) and with an increase in genetic abnormalities both in the sperm (Soares and Melo 2008) and in the cord blood of the offspring (Laubenthal et al. 2012). Nevertheless, whether a man's mother smoked during pregnancy appears to play a larger role than his own smoking, as considers his sperm counts (Paasch et al. 2008, Ravnborg et al. 2011).

Parental smoking and reproductive function in sons

Prenatal exposure to tobacco smoke has been suggested as an explanation for possible time-related decline in semen quality (Storgaard et al. 2003). Maternal smoking has rather consistently been associated with reduced semen quality in sons, whereas associations with the other entities of TDS (TC, cryptorchidism, hypospadias) seem to be less clear (Hakonsen et al. 2014).

Concerning paternal smoking, no statistically significant associations have been reported with sons' reproductive function (Virtanen et al. 2012). Nevertheless, the potential of reduced fertility in sons of smoking men through germ line mutations has been discussed (Shiverick 2011), and daughters of smoking men have been reported to have a shortened reproductive life span (Fukuda et al. 2011).

However, the often high correlation between maternal and paternal smoking may lead to difficulties in separating one from the other as concerns potential associations with sons' reproductive function. In addition, not all studies had access to register-based data on smoking during pregnancy, which may be more accurate than data from questionnaires filled in many years later.

Phthalates

Phthalates, or phthalic acid esters (Figure 4), may belong to the most abundant synthetic chemicals in the environment (Grady and Sathyanarayana 2012). They are used in many diverse products. Some phthalates are used as plasticizers, mostly in soft polyvinyl chloride (PVC). Phthalates differ in length and, consequently also in their chemical properties.

Around one million tons of phthalates is produced per year in Western Europe (Wittassek et al. 2011). The long-chain phthalates are most often used in PVC, were they may constitute up to 40% of the product. They are often found in building materials, cables, floorings and materials in contact with food. The short-chain phthalates are used in non-PVC applications such as in certain personal care products, paints, adhesives and coatings of pharmaceutical tablets (Wittassek et al. 2011).

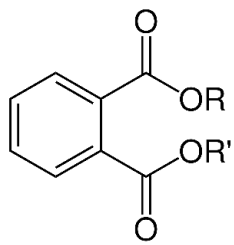

Figure 4
General structure of phthalates. R and R' are general placeholders which vary in legth depending on the phthalate
Source: "Phthalates" by User: Bryan Derksen - Own work. Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Phthalates.svg#mediaviewer/File:Phthalates.svg

The most common phthalate was for many years the long-chain phthalate di(2-ethylhexyl) phthalate (DEHP) (Figure 5). DEHP has, however, gradually been replaced by di-isononyl phthalate (DiNP) and other long-chain phthalates (Wittassek et al. 2011).

Since phthalates are not chemically bound to the product, they may gradually leak out. As a result the general population is continuously exposed to several phthalates, as shown by the presence of their metabolites in nearly all of samples from different individuals (Wittassek et al. 2011).

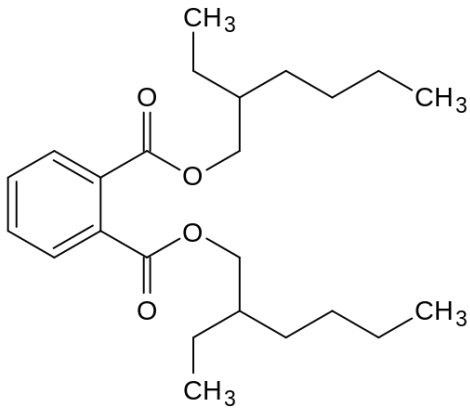

Figure 5
Chemical structure of di(2-ethylhexyl) phthalate (DEHP)
Source: "Bis(2-ethylhexyl) phthalate" by Dschanz - Own work using: BKchem. Licensed under Public Domain via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Bis(2-ethylhexyl)_phthalate.svg#mediaviewer/File:Bis(2-ethylhexyl)_phthalate.svg

Toxicity in animals

Robust reporting in animal literature supports the fact that both DEHP and dibutyl phthalate (DBP) adversely affect the development of the male reproductive tract (Kay et al. 2014). These effects are suggested to be due to a negatively affected function of the Leydig cells. Effects of foetal exposure in male rats include hypospadias, cryptorchidism, malformations of the accessory sex glands such as the seminal vesicles (Albert and Jegou 2014), and reduced weight of the male reproductive organs (Kay et al. 2014).

As mentioned, a specific window of sensitivity has been identified for these disorders of masculinization to occur (Welsh et al. 2008). This period has been called the masculinization programming window (MPW), and is proposed as the period during which the foetal testosterone levels program the formation (Welsh et al. 2008) and the future size (Macleod et al. 2010) of the male reproductive organs.

Nevertheless, administration of certain phthalates also to adult rats can induce testicular effects, including reduction of spermatogenesis (Kay et al. 2014) and sperm motility (Aly et al. 2015, Madkour 2014).

Studies in human tissue

In contrast to animal studies, experimental studies on human testicular tissue have suggested that phthalate exposure does not lead to antiandrogenic effects in the human foetal testis (Kay et al. 2014, Spade et al. 2014). Nevertheless, phthalate exposure has been reported to decrease testosterone production in the adult human testis (Desdoits-

Lethimonier et al. 2012) and to reduce the number of germ cells in the foetal testis of both humans and rats (van den Driesche et al. 2015).

Epidemiologic studies on adult exposure and semen quality

According to a previously mentioned review (Kay et al. 2014), studies examining associations between human phthalate exposure and semen quality have given inconsistent results, although exposure to DBP and DEHP was often associated with some decrease in semen quality. Two later studies reported that urinary or serum phthalate metabolite levels were negatively associated with semen parameters (Kranvogl et al. 2014, Specht et al. 2014). In spite of this, a recent study found no adverse effects of phthalate exposure on couple fecundity (Specht et al. 2015).

Nonetheless, it is generally believed that the foetus is more sensitive to phthalates than the adult individual (Kay et al. 2014).

Epidemiologic studies on prenatal exposure and male reproductive function

Previous studies have considered proxy markers of male reproductive function at early ages, such as cryptorchidism, hypospadias and the distance between the anus and the genitals. This distance is called the anogenital distance (AGD) and has been reported as a marker for androgen action during the foetal life (Dean and Sharpe 2013). The AGD may also be a marker for the adult male reproductive function, including semen quality (Eisenberg et al. 2011, Mendiola et al. 2011), fertility (Eisenberg et al. 2011) and testosterone levels (Eisenberg et al. 2012).

Associations between phthalate exposure and cryptorchidism, hypospadias and AGD were recently reviewed (Albert and Jegou 2014, Kay et al. 2014). One of the reviews concluded that the epidemiological data overall was insufficient (Albert and Jegou 2014), whereas the other concluded that limited evidence was present for an association between prenatal phthalate exposure and a shorter AGD, but that data was insufficient as concerns malformations (Kay et al. 2014). However, in several studies that were included, prenatal exposure was not measured during the critical window, the MPW (Kay et al. 2014).

Since these reviews appeared, two additional studies have been published focusing on prenatal phthalate exposure during the MPW in relation to AGD. The smaller of these studies found that metabolites of DiNP were negatively associated with AGD (Bornehag et al. 2015) whereas the other found a similar association for metabolites of DEHP (Swan et al. 2015), but not for the sole measured metabolite of DiNP.

Another recent study failed to find a negative association between phthalate metabolite levels and levels of testosterone in amniotic fluid, and found no association with cryptorchidism (Jensen et al. 2015). This appears to be in line with the data from experimental studies on the human foetal testis. Nevertheless, the study (Jensen et al. 2015) found that metabolites of DEHP were negatively associated with insulin-like factor 3, which may be a better marker than testosterone as concerns negative impact

on the foetal testis (Anand-Ivell and Ivell 2014). Thus, androgen output may be heavily confounded by acute regulation from the hypothalamic-pituitary-gonadal axis, and further, exhibits a high level of variance both within and between individuals (Anand-Ivell and Ivell 2014). These circumstances may, therefore, limit the use of testosterone as a parameter of Leydig cell function.

General considerations regarding previous epidemiologic studies

Of the previous studies on phthalate exposure and male reproductive outcomes, only a few studies included measurements of the secondary metabolites of the long-chain phthalates, which are suggested as being the most reliable exposure markers (Wittassek et al. 2011)

Regarding the studies concerning phthalate exposure in adulthood, these have mostly been performed on subfertile men (Kay et al. 2014). Those men may differ from men in the general population as concerns potential associations between exposure and reproductive function. Further, sampling of the exposure and semen parameters was not always performed the same day, despite the short half-lives of the phthalate metabolites (Wittassek et al. 2011). Finally, account was not always taken of the abstinence period despite its association with most variables of semen quality (Levitas et al. 2005), nor always for the urinary dilution which seems to play a role in the concentration of phthalate metabolites in urine (Fromme et al. 2007, Hoppin et al. 2002, Peck et al. 2010).

Regarding previous studies concerning prenatal phthalate exposure, only a few studies focused on exposure during the most sensitive period of the pregnancy, the MPW. In addition, these studies have as yet only used childhood proxy markers for adult male reproductive function, such as AGD or genital malformation. The relevance of these outcomes as concerns semen quality is not fully clear, at least as concerns AGD (Parra et al. 2015). No study to date has examined an association between prenatal phthalate exposure and adult reproductive function, including semen quality.

Taken together, more studies appear to be necessary to clarify associations between both pre- and postnatal exposure to phthalates, and parameters of male reproductive function.

Aims

The overall aims of this thesis were to study whether a recent time-related trend in the male reproductive function of young Swedish men appeared to be present, and to examine associations with environmental factors in order to possibly increase the chances of preventing male reproductive disorders.

The specific aims were:

1. to study whether semen quality in young Swedish men had changed over the previous decade;

2. to examine associations between prenatal and adult exposure to smoking and semen quality;

3. to study associations between prenatal exposure to phthalates and male reproductive function, including both semen quality and hormones;

4. to examine associations between adult exposure to phthalates and semen quality.

Subjects and methods

Subjects

For this thesis, two different cohorts of men were used (Cohort A and B).

In both cohorts, the men received SEK 500 (EUR 55) for their participation and signed their informed consent.

Cohort A

This group of men was gathered in 2000 to 2001. During this period about 95% of all Swedish 18-year-old men underwent a medical health examination at the National Service Administration in Sweden (Pliktverket) concerning their possible military service (Richthoff et al. 2002). All 2 255 men who lived within 60 km of the city of Malmö were invited to participate, and 305 men accepted (14%). Of these, the 224 men who were born and raised in Sweden, with mothers born and raised in Sweden, were included in this thesis.

This cohort was used in Paper I only, to study if semen quality had changed between 2000-2001 and 2008-2010.

Cohort B

Between 2008 and 2010, 314 Swedish men (17-20 years old) were recruited, mostly through the medical examination at the National Service Administration. Due to savings in the military budget, only about 25% of all Swedish men around 18 years old underwent this examination during the period. All 1 681 men who were examined from 1 December 2008 to 27 May 2010, fulfilling the criteria above, were invited to participate. Of these, 241 men accepted (14%). In order to achieve a number of approximately 300 men as in Cohort A, another 73 were recruited men through advertisements in schools and as friends of participants.

This cohort was used in all papers (I-IV) included in this thesis.

Physical examination

All men were examined by a physician for varicocele and testicular size, estimated by use of Prader's orchidometer (Figure 6). Total testicular size was calculated by adding the size of the right and the left testicles.

Figure 6
Prader's orchidometer

Questionnaire

All participants filled in information about length and weight, own smoking habits, maternal and paternal smoking habits during pregnancy, indoor parental smoking during childhood and previous testicular trauma leading to discoloration or swelling.

Samples from the men

The day of the examination, the men delivered samples of semen, urine and serum. The semen samples were collected after masturbation at the hospital. The men were asked to keep an abstinence period of 48 to 72 hours (which 42% did), but in each case the actual length was recorded.

Semen analysis

The semen samples were analysed according to the WHO guidelines (World Health Organization. 1999). The laboratory used serves as a reference unit for the external quality control of the European Society of Human Reproduction and Embryology, and the Nordic Association for Andrology.

In addition to standard parameters of semen quality, the proportion of sperm with DNA breaks and of sperm with a low DNA compaction were analysed by use of the Sperm Chromatin Structure Assay (SCSA) (Evenson et al. 1999). These two parameters of sperm DNA structure are called the DNA fragmentation index (DFI) and High DNA stainability (HDS). The latter term refers to a higher binding of a staining chemical in sperm DNA with a low compaction, believed to represent immature sperm (Evenson et al. 2002).

The following parameters of semen quality were used in the studies included in this thesis:

1) Semen volume (Papers I-IV)
2) Sperm concentration (Papers I-IV)
3) Total sperm count (Papers I-IV)
4) Proportion of progressively motile sperm (Papers I-IV)
5) Proportion of normal sperm (Papers II-IV)
6) DFI (Papers II-IV)
7) HDS (Papers III-IV)

Analyses of reproductive hormones

For Paper III, serum samples obtained from the men were analysed for reproductive hormones at the Laboratory of Clinical Chemistry, Skåne University Hospital. The following hormones were analysed: testosterone, FSH, LH, SHBG and estradiol. The concentration of free testosterone was calculated according to a previously-published method (Vermeulen et al. 1999)

Register data on maternal smoking

For Paper II, information on maternal smoking in gestational weeks 8 to 12 was derived from the Medical Birth Register (Socialstyrelsen 2009b). This data is recorded by a midwife on the first visit in the maternity ward. This data is missing in only about 5% of the cases and has a low rate of error (Cnattingius et al. 1990).

Maternal sampling in pregnancy

In early pregnancy in Sweden, screening for antibodies to rubella (German measles) is routinely carried out. Unless the woman declines, part of the sample is stored in a biobank.

In order to study associations between prenatal exposure to phthalates and reproductive outcomes (Paper III), we were able to retrieve the maternal samples of 112 men. These samples were taken from the 6th to the 35th week of pregnancy (mean 12 weeks), whereof 69% were taken within the suggested MPW, between 8 and 14 completed gestational weeks.

Analyses of phthalate exposure

Levels of phthalate metabolites in maternal serum (Paper III) and in the participating men's urine and serum (Paper IV) were analysed by use of liquid chromatography-tandem mass spectrometry (LC/MS/MS). Levels below the level of detection (LOD) were given the value of LOD divided by 2.

Analyses of maternal serum

In maternal serum (Paper III), we analysed the secondary metabolites of DEHP and DiNP, and a metabolite of nicotine called cotinine.

Following phthalate metabolites were analysed:

For DEHP:

1) Mono-(2-ethyl-5-hydroxylhexyl) phthalate (MEHHP);
2) Mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) and;

3) Mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP).

For DiNP:

4) Mono-(hydroxy-iso-nonyl) phthalate (MHiNP);
5) Mono-(oxo-iso-nonyl) phthalate (MOiNP) and;
6) Mono-(carboxy-iso-octyl) phthalate (MCiOP).

Description of analytical method

For analysis, 100 µL of serum was added to isotopically labelled internal standards for all evaluated compounds. The samples were digested with glucuronidase, and proteins precipitated using acetonitrile. The samples were analysed using a triple quadrupole linear ion trap mass spectrometer coupled to a liquid chromatography system. The analysis was performed in a negative ion mode.

Analyses of men's urine

For the men's urine, ten different metabolites of five different phthalates were analyzed according to a previously-described method (Toft et al. 2012):

Primary metabolite of DEHP:

1) mono-(2-ethylhexyl) phthalate (MEHP)

Secondary metabolites of DEHP:

1) MEHHP
2) MEOHP
3) MECPP

Secondary metabolites of DiNP:

1) MHiNP
2) MOiNP
3) MCiOP

Metabolite of DBP:

1) monobutyl phthalate (MBP)

Metabolite of butylbenzyl phthalate:

1) monobenzyl phthalate (MBzP)

Metabolite of diethyl phthalate:

1) monoethyl phthalate (MEP)

Analyses of men's serum

In the men's serum, we analysed the already mentioned secondary metabolites of DEHP and DiNP according to another, previously described method (Specht et al. 2014).

Statistical methods

For statistical analyses, the SPSS computer program (Statistical Package for the Social Sciences) version 17 to 22 was used. In all the papers included in this thesis, linear regression models, or more exactly general linear models, were used which allow inclusion of categorized variables. Statistical analyses with semen parameters as outcome variables were adjusted for abstinence period, categorized as < 48, 49-72, 73-96, 97-120 and > 120 hours. Due to skewed distribution of residuals, different variables were transformed in the different papers for a more normal distribution, since normally distributed residuals is an assumption of the statistical method (Tabachnick and Fidell 2013b). We checked improvements in distribution through values of skewness and kurtosis, and through plots to assess normality, scedasticity and linearity.

Urinary levels were adjusted for urinary dilution by dividing the molar concentration of the metabolites with that of creatinine.

Phthalate metabolite levels were transformed by the natural logarithm to increase statistical prediction (Tabachnick and Fidell 2013b).

Differences in logarithmically transformed reproductive variables were back-transformed into ratios.

Paper I

In this paper, we wished to study whether semen quality had changed during the last decade in young Swedish men from the general population (between 2000-2001 [Cohort A] and 2008-2010 [Cohort B]).

We excluded 8 men from Cohort A and 19 men from Cohort B due to a lack of information regarding abstinence period, sperm concentration and due to classification difficulties regarding smoking.

As potential confounders, we included the following variables: abstinence period, cigarette smoking (yes/no), and Body Mass Index (BMI).

Further, we dichotomized sperm concentration (with cut-off limit: 20 x 10^6 and 40 x 10^6, respectively) and progressive sperm motility (cut-off limit: 50%), to calculate whether men in any of the two cohorts were more likely to have a value above the cut-off limit. This was carried out by use of logistic regression.

Paper II

In this paper, we studied associations between parental smoking during pregnancy and semen quality in sons.

The same 19 men were excluded as in Paper I. We chose to use the data from the Medical Birth Register as the source of information regarding maternal smoking due to the risk of a recall bias in the questionnaires answered approximately 18 years later. Data on paternal smoking, however, was only available through the questionnaires.

The values of sperm concentration and total sperm count were transformed by the natural logarithm. Abstinence period was included as a potential confounder.

At first we included subjects' own smoking and maternal and paternal smoking during pregnancy one by one in the analyses, without a simultaneous adjustment for the other two types of exposure.

Secondly, we included all the three exposures in the model at the same time, to adjust for each other.

In order to test the robustness of the results, we adjusted for the extent of maternal smoking (none, 1-9 cigarettes per day, or 10 or more) instead of using maternal smoking as a dichotomized variable. Subsequently, we separately additionally adjusted for indoor parental smoking during childhood, BMI, age, varicocele at examination and previous testicular trauma.

Finally, we studied if associations between one type of exposure (own, maternal or paternal smoking) and an outcome was dependent on any of the other two types. Consequently, the following interaction terms were included one at a time in the model: paternal smoking*maternal smoking, paternal smoking*own smoking, and maternal smoking*own smoking.

Paper III

In this paper, we studied associations between phthalate metabolite levels in maternal serum, and semen parameters and reproductive hormone levels in sons.

We transformed DFI, HDS, testosterone, free testosterone, LH, FSH, SHBG and foetal age by the natural logarithm, and sperm concentration and total sperm count by the cubic root (Sanchez-Pozo et al. 2013).

Associations between exposures and reproductive parameters were studied with adjustment for the following potential confounders: men's BMI, current smoking (yes/no) and paternal smoking during pregnancy (yes/no), as well as foetal age (days), maternal age (years) and level of cotinine at maternal sampling. Semen parameters were additionally adjusted for the abstinence period and hormone levels additionally for the time of day at men's sampling.

Exposure levels were modelled both as continuous variables and as categorized in tertiles. For categorized exposure, we studied differences in the reproductive parameters between the lowest and the highest exposure tertile of each metabolite.

In order to minimize the risk that phthalate exposure in adulthood would be responsible for associations between prenatal levels of a metabolite and a reproductive outcome, we separately additionally adjusted the statistically significant findings for the levels of the same particular metabolite in sons' urine.

Paper IV

In this paper we studied associations between the levels of phthalate metabolites in men's serum and urine, and their semen quality.

Phthalate metabolite levels and semen parameters were transformed as in Paper III, but semen volume was additionally transformed by the natural logarithm.

The following 5 variables were considered as potential confounders: abstinence period, BMI, own smoking and maternal and paternal smoking during pregnancy. After adjusting for these variables, we studied associations between the phthalate metabolite levels (both as continuous variables and as categorized in quartiles) and the semen variables.

Any statistically significant association between a metabolite and an outcome was recalculated with an additional adjustment for levels of the same metabolite in maternal serum during the pregnancy.

Results

Time trend in semen quality over the previous decade

No change in sperm count or motility was found during the period (between 2000-2001 and 2008-2010). Likewise, no change was found in the chances of showing a sperm concentration above 20 or 40 x 10^6/mL, or a progressive sperm motility above 50% (**Paper I**).

Own and parental smoking in relation to semen quality

We found no association between the men's own smoking and parameters of semen quality (**Paper II**).

However, in the analyses adjusted for abstinence time only, paternal smoking during pregnancy was associated with a 33% lower total sperm count (95% confidence interval [95% CI]: 9.5-50%), and with a 4.8 (95% CI: 0.35-9.2) percentage points lower proportion of progressively motile sperm.

In the model that included both abstinence period, own smoking and maternal as well as paternal smoking during the pregnancy, the men whose fathers had been smoking had 31% (95% CI: 4.9-50%) lower total sperm count and 0.51 (95% CI: 0.07-0.94) mL lower semen volume. This was robust to the alternative adjustment for the extent of maternal smoking, and for the additional adjustment for indoor parental smoking during childhood, BMI, age, varicocele and testicular trauma.

We found that maternal and paternal smoking interacted as considers sperm concentration and total sperm count. Thus, both variables were decreased in paternally exposed men (by 35% [95% CI: 8.1-55%] and 46% [95% CI: 21-64%], respectively) only if the mother was a non-smoker (unadjusted total sperm count shown in Figure 7). Likewise, maternal smoking was associated with a reduced sperm concentration (by 36% [95% CI: 3.9-57%]) only in men whose fathers were non-smokers.

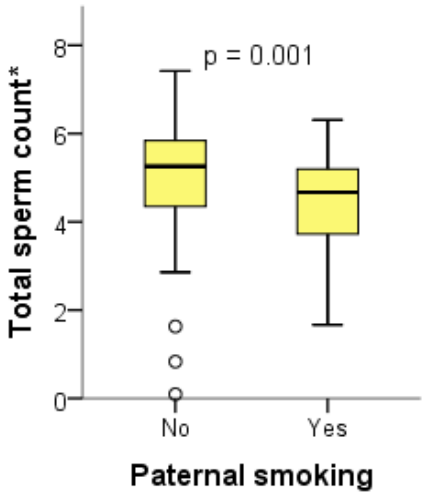

Figure 7
Unadjusted total sperm count in relation to paternal smoking in men of non-smoking mothers
*original scale (x 10^6) here transformed by the natural logarithm

Prenatal exposure to phthalates and male reproductive function

Men in the highest maternal exposure tertile of the DiNP metabolite MCiOP had 4.3 (95% CI: 0.89-7.6) mL lower total testicular size and 0.87 (95% CI: 0.28-1.5) mL lower semen volume than men in the lowest exposure tertile (**Paper III**).

Further, men in the highest tertile of the DEHP metabolite MEHHP had 0.70 (95% CI: 0.09-1.3) mL lower semen volume than men in the lowest exposure tertile. As continuous variables, maternal levels of both MCiOP and MEHHP were negatively associated with semen volume (p = 0.048 and 0.005, respectively [for MEHHP shown unadjusted in Figure 8]).

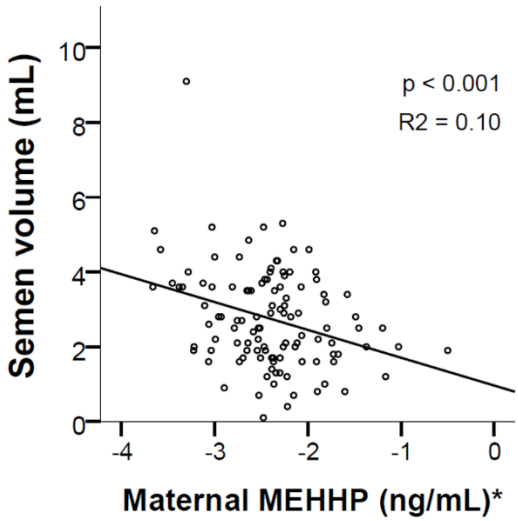

Figure 8
Unadjusted levels of the DEHP metabolite MEHHP in maternal serum during pregnancy, and semen volume in sons
*transformed by the natural logarithm

Men in the highest tertile of MCiOP had 30% (95% CI: 3.6-63%) higher levels of FSH than men in the lowest tertile.

A similar association for the DiNP metabolites MCiOP and MOiNP as continuous variables was found with FSH (p = 0.02 and 0.01, respectively) and for MHiNP and MOiNP with LH (p = 0.01 for both).

These results are summarized in Table 1, and were robust to additional adjustment for the men's own urinary levels of the same metabolites.

Adult exposure to phthalates in relation to semen quality

Exposure markers as continuous variables

Levels of all DEHP and DBP metabolites (MEHP, MECPP, MEOHP, MEHHP and MBP) in urine and MECPP in serum, were negatively associated with progressive sperm motility (p < 0.001-0.03) (**Paper IV**). Urinary MECPP was associated also with HDS (p = 0.04).

Finally, MCiOP was associated with semen volume (p = 0.04).

Categorized exposure markers

When comparing men in the highest urinary exposure quartile with those in the lowest, progressive sperm motility was 11 (95% CI: 5.0-17) percentage points lower in the highest exposure quartile of MECPP (unadjusted difference in Figure 9), 8.7 (95% CI. 2.8-15) percentage points lower in the highest quartile of MEHHP and 6.9 (95% CI: 1.1-13) percentage points lower in the highest quartile of MEOHP.

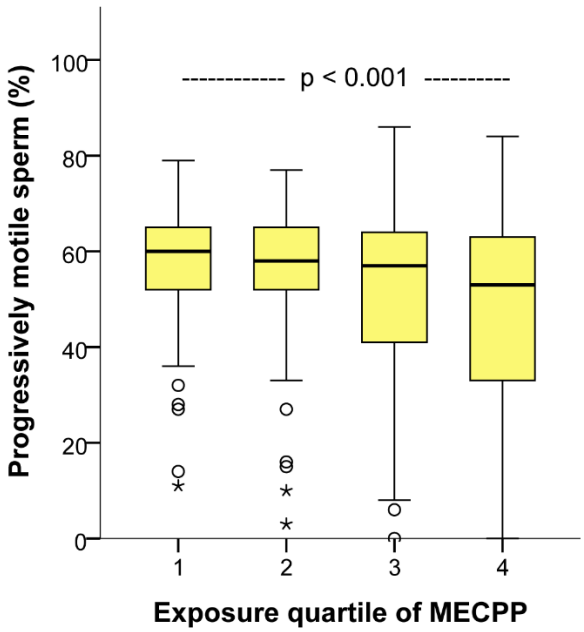

Figure 9
Unadjusted progressive motility according to levels of the DEHP metabolite MECPP in men's urine
Note: boxes include values from the 25th to the 75th percentile; bold horizontal lines depict median values; T-bars extend 1.5 times the height of the box or if less, to minimum and maximum; circles represent outliers, and stars extreme outliers.

Further, men in the highest exposure quartile of MEHP had 26% (95% CI: 5.2-52%) higher HDS than men in the lowest exposure quartile.

All these results are summarized in Table 1, and were robust to additional adjustment for maternal levels of the same metabolite during pregnancy.

Table 1
Summary of statistically significant findings ($p < 0.05$) between prenatal and adult phthalate metabolite levels and parameters of male reproductive function.

PARENT PHTHALATE	METABOLITE	TESTIS SIZE (ML)		SEMEN VOLUME (ML)		FSH (IU/L)		LH (IU/L)		PROGRESSIVE MOTILITY (%)		HDS (%)	
Time of exposure		Prenatal	Adult	Prenatal	Adult	Prenatal	Adult	Prenatal	Adult	Prenatal	Adult	Prenatal	Adult
DiNP	MCiOP	↓	=	↓	↑[a]	↑	=	=	=	=	=	=	=
	MOiNP	=	=	=	=	↑	=	↑	=	=	=	=	=
	MHiNP	=	=	=	=	=	=	↑	=	=	=	=	=
DEHP	MEHP	=	=	=	=	=	=	=	=	=	↓	=	↑
	MECPP	=	=	=	=	=	=	=	=	=	↓	=	↑
	MEOHP	=	=	=	=	=	=	=	=	=	↓	=	=
	MEHHP	=	=	↓	=	=	=	=	=	=	↓	=	=
DBP	MBP	=	=	=	=	=	=	=	=	=	↓	=	=

Abbreviations: FSH, follicle-stimulating hormone; HDS, high DNA stainability; IU/L, international units per litre; LH, luteinizing hormone

[a] Disappeared after additional adjustments for prenatal levels.

Discussion

Time trend in semen quality

The absence of a negative trend in sperm count in our men (**Paper I**) seems consistent with the results of another prospective study (Jorgensen et al. 2012) performed in Denmark and published during the course of this PhD project. This study found no decline in semen quality but a slight increase in sperm counts between 1996 and 2010.

However, these results do not exclude a decrease in sperm count having taken place earlier in time. In addition, environmental or lifestyle-related factors during the foetal or adult period may still explain why some men have a higher degree of reproductive dysfunction than others.

We believe that the men included in this thesis provide a fair representation of the population. In support of this, a Danish study with a similar participation rate as ours reported that Danish men, recruited prior to their military service, showed equal levels of hormones that reflect spermatogenesis to men refusing semen sampling (Andersen et al. 2000). This indicates that the participants were not biased in terms of reproductive potential. In addition, we found similar semen parameters in the men recruited prior to possible military service and those recruited through schools or as friends of participants (Paper I).

Smoking exposure and semen quality

In contrast to much other data (Li et al. 2011, Vine 1996), we found no association between own smoking and semen quality (**Paper II**). However, maternal smoking during pregnancy has been more strongly associated with reduced testicular function than has a man's own smoking (Paasch et al. 2008, Ravnborg et al. 2011). This agrees with our findings of an association between maternal smoking and sperm concentration, albeit only in men whose fathers did not smoke during the pregnancy. The association between maternal smoking and reduced sperm counts in sons has been rather consistently reported (Hakonsen et al. 2014, Virtanen et al. 2012). Indeed, the higher prevalence of smoking during pregnancy in Danish than in Finnish women

(Jensen et al. 2004) has been suggested as contributing to the difference in male reproductive health between the two countries (Main et al. 2010).

We found a negative association with the sons' sperm counts also for paternal smoking during the pregnancy.

In the study by Carlsen and colleagues (Carlsen et al. 1992) reporting a downward trend in sperm counts from 1940 to 1990, the men for whom information on age was available were around 30 years old. Thus, the pregnancies of these men should have taken place around 1910 to 1960. Since the decrease in sperm counts seemed to be most apparent from 1940 to 1970 (Carlsen et al. 1992), this would reflect pregnancies taking place from around 1910 to 1940. During this period, smoking in men was far more prevalent than in women, and was continuously increasing (Löfdahl and Ström 2007, Ronneberg et al. 1994, Waldron 1991), whereas smoking in women seems to have begun to increase more firmly around 1930.

If the increase in smoking during the 20th century explains a decrease in sperm counts between 1940 and 1990 through prenatal exposure, the decrease in smoking in both genders in Sweden since around 1975 (Socialstyrelsen 2009a) might explain why we found no decrease in semen quality after 2000 (Paper I).

Some previous studies have analysed whether paternal smoking was associated with sons' semen quality (Jensen et al. 2005, Paasch et al. 2008, Ramlau-Hansen et al. 2007, Storgaard et al. 2003), but none of those studies found an association. However, only two of the studies analysed associations with the total sperm count (Paasch et al. 2008, Ramlau-Hansen et al. 2007), which was the variable most firmly associated with paternal smoking in our study.

Further, in view of a generally high correlation between maternal and paternal smoking, it may be difficult to separate their potential associations with the semen variables of their sons. In our study, however, the correlation between maternal and paternal smoking was rather low, with a correlation coefficient of 0.31 (Spearman's rho), which would safely allow inclusion of both variables in the model (Tabachnick and Fidell 2013a). In addition, we had the opportunity to adjust for maternal smoking based on reliable register data from the Swedish Medical Birth Register.

If, in previous studies, maternal and paternal smoking were more strongly correlated with each other than in our study, this might explain the inconsistent results. A parallel possibility is a higher frequency of mothers smoking more than 10 cigarettes per day in other studies. Since the associations of maternal smoking with the sperm counts of their sons is stronger at such high consumption (Storgaard et al. 2003), this might additionally increase the difficulty to distinguish a potential paternal effect.

Tobacco smoke contains established mutagenic and carcinogenic agents (Savitz 2003). In addition, spermatozoa have limited DNA repair ability (Wright et al. 2014) and lack cytoplasmic enzymes for protection against oxidative damage (Agarwal et al. 2014). Thus, we cannot exclude that the association found between paternal smoking and the

lower sperm counts in the sons could be due to epigenetic or genetic changes (Damodaran 2011) transmitted via the fathers' sperm at conception (Shiverick 2011). Accordingly, an elevated degree of mutations in a repetitive DNA sequence (minisatellite) has been reported in children of smoking fathers (Linschooten et al. 2013). This may be regarded in view of an overall much higher number of mutations arising via the father than via the mother (Kong et al. 2012).

Further, paternal smoking has been associated with reduced reproductive life span in daughters (Fukuda et al. 2011) and seems, compared with maternal smoking, to be more strongly associated with childhood cancer in the offspring (Boffetta et al. 2000, Liu et al. 2011). Given experimental evidence of a male-mediated developmental toxicity (Cordier 2008), one explanation for those patterns might be effects mediated via the spermatozoa (Brinkworth 2000, Davis et al. 1992).

Nevertheless, the fact that no other previous study has found paternal smoking during pregnancy to be associated with sperm count in sons indicates the possibility that this finding occurred by chance. This discrepancy in results displays the necessity of further studies before drawing any firmer conclusions.

Phthalate exposure and male reproductive function

In **Paper III**, we found that both DEHP and DiNP, through levels of certain metabolites in maternal sera, appeared to be associated with decreased semen volume. Maternal exposure to DiNP also appeared to be associated with lower testicular size and higher levels of FSH and LH. These hormones are often elevated in men with testicular dysfunction (Simoni and Nieschlag 2010).

Since maternal phthalate metabolite levels are reported to be associated with metabolite levels in cord blood of the newborn child (Lin et al. 2011), our findings indicate that prenatal exposure to DEHP and DiNP is negatively associated with the adult male reproductive function.

The largest part of the semen volume is produced by the seminal vesicles (Mortimer 1994). Consequently, a reduced growth of these vesicles during development would result in a lower semen volume in adulthood (Sharpe and Skakkebaek 2008). Rat studies have indicated that prenatal phthalate exposure negatively affects the formation of both the seminal vesicles and the testicles (Andrade et al. 2006, Gray et al. 2000, Macleod et al. 2010, Stroheker et al. 2005). However, exposure must include the window of susceptibility, the MPW, for effects to occur (Macleod et al. 2010, Welsh et al. 2008). Thus, a strength of our study was that most of the maternal samples (69%) were collected within the proposed human window for foetal programming of the future size of the male reproductive organs (Macleod et al. 2010, Welsh et al. 2008).

Our results appear to corroborate those of a recent Swedish study (Bornehag et al. 2015) which measured maternal urinary levels of phthalates during the MPW and found that metabolites of DiNP were associated with a shorter AGD, whereas a similar association for metabolites of DEHP lacked statistical significance. Since DEHP is considered to be of higher potency than DiNP (Gray et al. 2000), a potential explanation to the stronger associations for metabolites of DiNP may be their longer half-lives (Cantonwine et al. 2014, Fromme et al. 2007). This would make them more stable as exposure markers. Nevertheless, the largest study to date only found metabolites of DEHP, but not the sole included metabolite of DiNP (MCiOP), to be associated with a shortened AGD (Swan et al. 2015). This might indicate that the associations between phthalate metabolite levels and male reproductive outcomes were due to other factors, differently associated with phthalate metabolite levels in different countries. Such factors may be dietary sources of phthalates, in some way negatively associated with one or more of the reproductive parameters.

The findings in this study seem to contrast with experimental data suggesting that the human foetal testis is refractory to phthalate-induced antiandrogenic effects (Albert and Jegou 2014, Kay et al. 2014, Spade et al. 2014). Still, the possibility that the experimental studies performed have been based on too few individuals to find an effect if only a fraction of a population is sensitive, cannot be completely excluded. In addition, persistent testicular effects might be caused through other pathways than decreased production of testosterone (Spade et al. 2014). As concerns the negative effect on germ cell numbers in human foetal testis recently reported (van den Driesche et al. 2015), one potential reason for finding no associations with sperm numbers in our study may be the much lower exposure occurring in real life than in experimental studies. Another reason might be the great variability in phthalate metabolite levels occurring in humans through environmental exposure (Fisher et al. 2014) which would lead to difficulties in identifying associations. A high variability in sperm counts compared with other semen parameters (Jarow et al. 2013) may add to those difficulties.

As considers the adult exposure to phthalates (**Paper IV**), levels of metabolites of DEHP and DBP were negatively associated with progressive sperm motility. Further, levels of some DEHP metabolites were associated with HDS, which might indicate a negative effect on sperm maturation (Evenson et al. 2002). We also found a positive association between one metabolite of DiNP and semen volume, which was lost after additional adjustments.

Taken together, these findings seem to be in line with DEHP and DBP to belong to the most toxic phthalates to the male reproductive system (Martino-Andrade and Chahoud 2010), and with DEHP and DBP to be the phthalates most often associated with some decrease in semen quality in epidemiologic studies (Kay et al. 2014, Kranvogl et al. 2014). Nevertheless, previous studies in men from the general population did not find an association between phthalate metabolite levels and sperm motility (Han et al. 2014, Joensen et al. 2012, Jönsson et al. 2005, Specht et al. 2014).

Still, only one (Joensen et al. 2012) of these studies included measurements of the most appropriate (Wittassek et al. 2011) secondary DEHP metabolites in samples taken the same day as the semen was sampled. This may be important given the metabolites' short half-lives of less than 24 hours (Wittassek et al. 2011). In the same epidemiologic study (Joensen et al. 2012) however, no adjustment was made for abstinence period which seems to play a role in sperm motility (Levitas et al. 2005). In addition, no adjustment was made for urinary dilution which seems to affect the concentrations of phthalate metabolites in urine (Fromme et al. 2007, Hoppin et al. 2002, Peck et al. 2010). These circumstances may have led to decreased ability to detect associations between exposure and outcomes.

Nonetheless, several other epidemiologic studies have reported negative associations between DEHP or DBP exposure and sperm motility, including two occupational studies (Huang et al. 2014, Huang et al. 2011) and some studies on exposure from the general environment (Duty et al. 2003, Hauser et al. 2006, Jurewicz et al. 2013, Kranvogl et al. 2014, Pant et al. 2014, Pant et al. 2011, Pant et al. 2008).

In addition, negative effects of DEHP or DBP on motility have been reported both in human sperm *in vitro* (Fredricsson et al. 1993, Pant et al. 2011) and in rodent spermatozoa (Agarwal et al. 1986, Aly et al. 2015, Erkekoglu et al. 2011, Kwack et al. 2009, Lamb et al. 1987, Madkour 2014, Zhou et al. 2010).

One potential mechanism explaining the association found between DEHP metabolites and sperm immaturity, might be a preterm detachment of the germ cells from the Sertoli cells, as reported in animal tissue (Breslin et al. 2013, Erkekoglu et al. 2011, Gray 1986, Gray and Beamand 1984, Guibert et al. 2013). This might also explain the association found with reduced sperm motility, since immature sperm may have reduced motility (Giwercman et al. 2003). Another mechanism could be through induction of oxidative stress, which phthalate exposure seems associated with in humans (Ferguson et al. 2011, 2012) and which, through phthalate exposure, is reported to negatively affect both the sperm motility of rats (Zhou et al. 2010) and the epididymis (Zhou et al. 2011) where sperm motility is acquired.

Since the associations in **Paper III** and **Paper IV** were robust to the additional adjustments for prenatal and adult exposure to smoking and for the levels of the same metabolite in the opposite window of exposure (prenatal vs adult), this indicates that the exposures were associated with outcomes with some level of independence.

Misclassification of exposures

It seems reasonable that our data on paternal and maternal smoking (**Paper II**) were not completely accurate. Nevertheless, such a misclassification of exposure would have reduced the strength of the observed associations, leading to underestimated findings.

In parallel, the short half-lives of the phthalate metabolites (Wittassek et al. 2011) included in this thesis, may have increased the risk of misclassifying the exposure to the different phthalates. Therefore, also the associations between phthalate exposure and reproductive outcomes may be underestimated and if so, probably more than the associations found for parental smoking during pregnancy.

Potential confounding

However, given the observational nature of our studies, we cannot exclude that unidentified factors were associated both with exposures and reproductive outcomes. Consequently, it is hard to draw conclusions regarding causality. However, other experimental and epidemiologic data may indicate that an association is causal, which might seem most conceivable for the association between adult DEHP and DBP exposure and reduced sperm motility (**Paper IV**). At the same time it appears difficult to exclude that the other associations in these papers were causal.

Potential effects of childhood exposures

As concerns parental smoking during pregnancy, the associations we found did not seem to be due to indoor parental smoking during childhood (**Paper II**). This is in line with another study taking this potential confounder into account (Ravnborg et al. 2011).

Concerning the prenatal exposure to phthalates, although foetuses may be more sensitive than newborns (Kay et al. 2014), we cannot exclude that exposure in early childhood, for example through bottle-feeding or PVC-flooring (Carlstedt et al. 2013), could have aggravated (Macleod et al. 2010) potential effects. However, it seems less probable that childhood exposure would have affected sperm motility, which evolves in a few days during adulthood.

Clinical relevance

The seemingly unchanged semen quality during the last decade may indicate an unchanged reproductive potential in young Swedish men during this recent period. In addition, if sperm counts are associated with the risk of TC, our finding may also indicate a levelling off in the increase in TC incidence in 10 to 15 years, since the mean age for this malignancy is between 30 and 35.

Concerning the associations we found between the different exposures and sperm count and sperm motility, those are hard to translate in terms of fertility, especially due to uncertainties regarding causality.

The only exposures associated with sperm counts were paternal and maternal smoking during pregnancy. Nevertheless, both sperm counts and sperm motility are reported to be related to pregnancy rates (van der Steeg et al. 2011, World Health Organization. 2010a). These correlations appear to be continuous in the range of values found in our men (van der Steeg et al. 2011).

Given that the association between sperm motility and pregnancy rate in some studies lacked a threshold (Larsen et al. 2000, van der Steeg et al. 2011), the linear associations between both DEHP and DBP metabolite levels and progressive sperm motility might indicate lower fertility in men with high exposure. Accordingly, elevated phthalate (Wang et al. 2015) and phthalate metabolite levels (Buck Louis et al. 2014, Tranfo et al. 2012) have been reported in men with decreased fertility, albeit inconsistently (Specht et al. 2015). However, it appears more difficult to find associations for these metabolites with half-lives of less than 24 hours with fertility variables, such as time to pregnancy which is counted in months, than with sperm motility which evolves in a few days (Johnson and Varner 1988).

Taken together, if the associations with sperm counts and motility were causal, both prenatal exposure to parental smoking and adult exposure to DEHP and DBP might decrease human fertility at population level. The relative contributions of these factors would, however, be difficult to determine.

Finally, despite the results of the experimental studies contradicting antiandrogenic effects of phthalates in the human foetus, the associations we found between prenatal phthalate exposure and the different reproductive outcomes seem to indicate that the exposure levels present around 20 years ago during the prenatal period of the participants, were negatively associated with their masculinization.

Summary

In all, although no decline in sperm counts appears to have occurred during the last decade in this region, this thesis may have added some additional clues as to how smoking and exposure to phthalates might be associated with altered reproductive function in certain men.

Conclusions

I) No obvious change in semen quality was found during the previous decade in young Swedish men from the general population.

II) Parental smoking during pregnancy was associated with lower sperm counts in sons, which seemed most pronounced for paternal smoking.

III) Levels of certain metabolites of the phthalates DiNP and DEHP in maternal serum from the pregnancy were negatively associated with semen volume. For metabolites of DiNP, associations were also found with a lower testicular size and with altered reproductive hormones.

IV) Adult exposure to the phthalates DEHP and DBP, through levels of their metabolites, were negatively associated with sperm motility.

Future perspectives

Future studies on smoking and reproductive outcomes may benefit from having maternal cotinine levels during pregnancy as markers for maternal smoking, instead of data based on registers or questionnaires. The cotinine levels measured for Paper III were not available during the publication of Paper II, but may be used in a subsequent study. For other future studies it would be valuable if more robust data on paternal smoking was available than that from questionnaires filled in 18 years after the pregnancy. In addition, to be able to separate potential effects through the father's sperm from other pathways, more specified data on paternal smoking (before, during or after conception) would be necessary.

Further, given the association found between paternal smoking and lower sperm counts and the association reported between paternal smoking and mutations in the offspring (Linschooten et al. 2013), it would be interesting to study whether smoking men show a higher degree of such mutations in their sperm.

Concerning phthalate exposure and reproductive function, a repeated measurement of metabolites would probably increase the statistical power, given the high diurnal variation in metabolite levels. For adult exposure, performing prospective studies in for example semen donors may be considered, in order to see whether a variation in exposure within individuals is associated with a change in semen parameters such as sperm motility.

Finally, more studies appear necessary to identify additional potential confounders to the associations found in this thesis.

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Original papers