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Reproductive health of young Danish men following prenatal exposure to persistent organic pollutants

PhD dissertation

Anne Vested

Health
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Health
Aarhus University
Department of Occupational Medicine, Aarhus University Hospital

Supervisors

Associate Professor Gunnar Toft, MSc, PhD, DMSc
Danish Ramazzini Centre, Department of Occupational Medicine
Aarhus University Hospital
Denmark

Associate Professor Cecilia Høst Ramlau-Hansen, MSc, PhD
Department of Public Health, Section of Epidemiology
University of Aarhus
Denmark

Centre leader, Adjunct Professor Sjurdur Frodi Olsen, MD, MSc, PhD, DMSc
Centre for Fetal Programming
Department of Epidemiology Research
Statens Serum Institut
Copenhagen
Denmark

Evaluation committee

Chief Physician Ulrik Schiøler Kesmodel, MD, PhD (Chairman)
School of Public Health, Section for Epidemiology, Aarhus University, Denmark and Department of Obstetrics and Gynaecology, Aarhus University Hospital, Aarhus, Denmark.

Professor Anne-Marie Nybo Andersen, MD, PhD
Section of Social Medicine, Department of Public Health, University of Copenhagen, Copenhagen, Denmark

Professor Jorma Toppari, MD, PhD
Departments of Physiology and Pediatrics, University of Turku, Turku, Finland

Preface

The work presented in this thesis was carried out between 2010 and 2013 at the Department of Occupational Medicine at Aarhus University Hospital, which provided a fantastic research environment for my PhD studies.

First of all I would like to thank my supervisors Gunnar Toft, Cecilia Høst Ramlau-Hansen and Sjurdur Frodi Olsen for excellent guidance, fruitful discussions, and highly qualified opinions and support along the way. Working with this supervisor team has been a pleasure and I have learned a lot.

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Anne Vested
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This thesis is based on the following papers:

I. A comparison of conventional and computer-assisted semen analysis (CRISMAS Software) using samples from 166 young Danish men. *Asian Journal of Andrology*. 2011; 13: 453-458.

II. Associations of *in Utero* exposure to Perfluorinated Alkyl Acids with Human Semen Quality And Reproductive Hormones in Adult Men. *Environmental Health Perspectives*. 2013; 121 (4): 543-458.

III. Human semen quality and reproductive hormone levels in adulthood following *in utero* exposure to persistent organochlorine pollutants: A follow-up study. [Submitted to *Reproduction*]

IV. Does androgen receptor gene CAG repeat length modify the effect of perfluorooctanoic acid exposure on semen quality markers? [In draft]

Contents

List of abbreviations

AGD: anogenital distance

AMH: anti-müllerian hormone

AR: androgen receptor

ART: assisted reproductive technology

CASA: computer-assisted semen analysis

CRISMAS: Copenhagen Rigshospitalet Imagehouse sperm motility analysis system

CI: confidence intervals

CPR: Civil Registration Registry

CV: coefficients of variation

DDT: dichlorodiphenyltrichloroethane

DFI: DNA Fragmentation Index

DL-PCBs: dioxin-like PCBs

FAI: free androgen index

FSH: follicle-stimulating hormone

GnRH: gonadotrophin releasing hormone

ICC: interclass correlation coefficient

LH: luteinizing hormone

MI: multiple imputation

MPW: male programming window

PCB: polychlorinated biphenyl

PFAA: perfluoroalkyl acids

PFAS: perfluoroalkyl substances

PFOA: perfluorooctanoic acid

PFOS: perfluorooctanesulfonic acid

POP: persistent organic pollutants

p,p'-DDE: dichlorodiphenyldichloroethylene

SES: socioeconomic status

SHBG: sex hormone-binding globulin

TDS: testicular dysgenesis syndrome

WHO: World Health Organization

1. English summary

Background

Persistent organic pollutants (POPs) are ubiquitous, bioaccumulative compounds suspected to act as endocrine disrupters. According to the endocrine disrupter hypothesis, exposure during critical phases of development in fetal life may interfere with hormonal signalling and cause long-term effects on male reproduction. Because POPs readily cross the placenta, *in utero* exposure to POPs is a cause for concern.

Additionally, androgen receptor (AR) gene exon 1 polymorphisms have been suggested to modify the susceptibility towards POP exposure with respect to effects on male reproductive health.

Aims

We aimed to compare the conventional and computer-assisted semen analysis (CASA) methods used for semen parameter assessment in the current study. Additionally, we aimed to investigate the hypothesis that *in utero* exposure to perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), polychlorinated biphenyls (PCBs), and dichlorodiphenyldichloroethylene (p,p'-DDE), affect semen quality and reproductive hormone levels in adulthood and to further investigate the hypothesis that CAG repeat length in *AR* exon 1 modifies the effects of *in utero* exposure to PFOA on semen quality measures and reproductive hormone levels.

Materials and methods

In 1988-1989 a pregnancy cohort was established in Aarhus, Denmark consisting of 965 women. They answered a detailed questionnaire and a blood sample was drawn in pregnancy week 30 and stored in a biobank.

In 2008-2009 we recruited 176 young male offspring from the pregnancy cohort to a physical examination, where they provided semen and blood samples and self-measured testicular volume. Semen samples were analysed by conventional semen analysis and CASA using the Copenhagen Rigshospitalet Imagehouse sperm motility analysis system (CRISMAS) software and blood samples were analyzed for reproductive hormone levels. Maternal serum samples from pregnancy week 30 were analyzed for concentrations of PFOA, PFOS, six PCBs (PCB-118, -138, -153, -156, -170, and -180), and p,p'-DDE as proxies for prenatal exposure, and the sons' number of CAG repeats in AR exon 1 were assessed by sequencing

analysis. Multivariable regression analyses adjusted for potential confounders were used in order to evaluate possible associations.

Results

The comparison of results from the conventional semen analyses with the CASA results showed large differences between the two methods for assessments of both sperm concentration and sperm motility.

Results from the follow-up studies suggested an inverse association between *in utero* exposure to PFOA and sperm concentration, total sperm count, and CASA percentage progressive motility, and a positive association between prenatal PFOA exposure and levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), whereas there were no consistent associations between *in utero* exposure to PFOS, ∑PCBs, ∑dioxin-like-PCBs, or p,p'-DDE and semen quality measures or reproductive hormone levels in adulthood.

The final study suggested that short or long *AR* CAG repeats may increase vulnerability towards PFOA exposure *in utero* with respect to markers of male reproductive health in adulthood.

Conclusions

The results are suggestive of long-term consequences of intrauterine exposure to PPOA but not PFOS, PCB or p,p'-DDE on sperm quality and reproductive hormone levels. Additionally, associations between PFOA and semen quality and reproductive hormone levels in adulthood may be modified by the number of *AR* CAG repeats.

2. Danish summary

Baggrund. Persistente organiske stoffer (POPs) er bioakkumulerende stoffer, der findes overalt i miljøet, og som er mistænkt for at være hormonforstyrrende. Ifølge hromonhypotesen kan eksponering for stoffer med hormonmodulerende virkning i kritiske faser af udviklingen i fosterlivet forstyrre den hormonelle balance og have langsigtede konsekvenser for mandlig reproduktion. Fordi POPs er i stand til at krydse placenta er der grund til bekymring i forhold til prænatal eksponering.

Formål

Et af formålene var at sammenligne den manuelle sædanalysemetode med metoden for computerassisteret sædanalyse (CASA). Begge metoder blev brugt til at estimere sædparametrene sædkoncentration og sædcellemotilitet i studierne der indgår i afhandlingen.
Derudover var formålet, at undersøge hypotesen, at prænatal eksponering for perfluorooktansyre (PFOA), perfluorooktansulfonat (PFOS), polychlorerede biphenyler (PCBer) og dichlorodiphenyldichloroethylen (p,p'-DDE) påvirker sædkvalitet og kønshormonniveauerne i voksenlivet. Desuden var formålet, at undersøge hypotesen, at længden af CAG repeatet i exon 1 af androgen receptor *(AR*) genet modificerer associationen mellem *in utero* eksponering for PFOA og sædkvalitet og kønshormonniveauerne i voksenlivet.

Materialer og metoder

I 1988-1989 blev der etableret en graviditetskohorte i Århus bestående af 965 kvinder der blandt andet besvarede et omfattende kostspørgeskema. Desuden blev der taget en blodprøve af de gravide kvinder i deres graviditetsuge 30, som blev opbevaret i en biobank.
I 2008-2009 rekrutterede vi 176 af de sønner som kvinderne ventede i 1988-89 til en til en klinisk undersøgelse, hvor de afleverede sædprøver, målte testikelvolumen og fik taget en blodprøve til kønshormonbestemmelse og opbevaring i biobank. Sædprøverne blev analyseret ved hjælp af den manuelle metode i forhold til World Health Organization (WHO) kriterier. Som supplement, blev prøverne også analyseret ved hjælp af CASA (Copenhagen Rigshospitalet Imagehouse sperm motility analysis system (CRISMAS) software). Blodprøverne fra mødrenes graviditetsuge 30 blev analyseret for koncentrationer af PFOA, PFOS, seks PCBer (PCB-118, -138, -153, -156, -170 og -180) og p,p'-DDE. Disse koncentrationer blev brugt som et udtryk for den prænatale eksponering for de undersøgte POPs. Derudover blev sønnernes antal af CAG repeats i *AR* exon 1 bestemt ved hjælp af

sekvensanalyse. Multipel regressionsanalyse, hvor der i modellen blev justeret for potentielle confoundere, blev brugt til at undersøge mulige associationer.

Resultater

Studiet, hvor vi sammenlignede sædanalyseresultater fra den manuelle metode med CASA resultater, viste, at der var stor forskel i sædkoncentrationsbestemmelse og i klassifikationen af de forskellige motilitetsgrader.

Resultaterne fra follow-up undersøgelserne viste en invers sammenhæng mellem prænatal eksponering for PFOA og sædkoncentration, totalt antal sædceller og procentdelen af progressive sædceller talt med CASA samt en positiv sammenhæng mellem prænatal PFOA eksponering og luteiniserende hormon (LH) og follikelstimulerende hormon (FSH). Der var ingen konsistente sammenhænge mellem prænatal eksponering for PFOS, summen af PCBer, summen af dioxinlignende PCBer eller p,p'-DDE og sædkvalitet eller kønshormonniveauer i voksenlivet. Det sidste studie indikerede, at korte og lange CAG repeats i *AR* øger individuel sårbarhed overfor eksponering for intrauterin eksponering for PFOA med hensyn til mandlig reproduktiv funktion i voksenlivet.

Konklusion

Resultaterne indikerer langsigtede konsekvenser af prænatal eksponering for PFOA men ikke PFOS, PCB eller p,p'-DDE på sædkvalitet og kønshormonniveauer i voksenlivet. Desuden tyder resultaterne på, at associationer mellem intrauterin PFOA eksponering og markører for mandlig reproduktivt helbred modificeres af antallet af CAG repeats i androgenreceptorgenet.

3. Introduction

A high proportion of young Danish men have a semen quality in the low/suboptimal range. According to studies identifying geographical differences in semen quality, Danish men may be among those with the poorest semen quality compared with other European countries [1-3]. Low sperm concentration is associated with a longer waiting time to pregnancy, and a sperm concentration of 40 mill/mL is regarded a threshold below which the ability to reproduce (fecundity) declines [4]. Recent studies suggest, that 40% of young Danish men have sperm concentrations in this subfertile range [1,5]. In addition, the number of annual births in Denmark after assisted reproduction technology (ART) is increasing [6,7], with an estimated 8.4% of children born in Denmark in 2012 after ART [8].

During the past two decades, increasing attention has been focused on the potential adverse effects on male reproductive health of exposure to environmental contaminants with potential hormone-modulating effects. Indications of increasing trends in the prevalence of the male genital malformations cryptorchidism (undescended testis) and hypospadias (abnormal location of the urethral meatus) as well as an increase in the incidence of testicular cancer, and indications of a secular trend of declining sperm counts in normal adult men [9] combined with a possible increase of the amount of estrogens in the environment led to the "oestrogen hypothesis" in 1993. This hypothesis suggested that altered exposure to estrogens *in utero* could be related to the increasing incidence of reproductive abnormalities via a mechanism involving inhibition of follicle stimulating hormone (FSH) secretion by excess estrogens, possibly causing a negative regulation of anti-müllerian hormone (AMH) and a reduction in Sertoli cell multiplication, having consequences for later sperm production capacity [10].

Based on a large number of animal studies and observations from wildlife settings suggesting feminization, demasculinisation, reduced fertility and offspring viability, altered sexual behaviour, and impaired hormone activity combined with the above mentioned indications of temporal trends of male reproductive disorders pointing towards environmental factors as part of the aetiology, the oestrogen hypothesis was expanded to the so called "endocrine disruptor hypothesis". The endocrine disruptor hypothesis suggests that exposure to hormone modulating compounds during fetal and childhood development may disrupt the hormonal balance and affect reproductive organ development which could be associated with long-term adverse effects on male reproductive health by mechanisms involving antiandrogenic activity,

effects on enzymes involved in reproductive hormone metabolism, or direct effects on the hormone producing organs [11,12].

In addition, it has been hypothesized that poor semen quality, testicular cancer, cryptorchidism, and hypospadias may be symptoms of one underlying pathology – the testicular dysgenesis syndrome (TDS). It is proposed that TDS has a common origin during fetal life resulting from abnormal function of Sertoli- and Leydig cells and disturbed embryonal programming and development of the reproductive organs as a result of exposure to adverse environmental factors like endocrine disrupters [13].

Persistent organic pollutants (POPs) are ubiquitous, bioaccumulative compounds with long half-lives in human serum [14-16]. Due to their comprehensive distribution and usage, they can be detected in human serum around the globe [17]. Perfluoroalkyl acids (PFAAs) like perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), the organochlorine pollutants polychlorinated biphenyls (PCBs), and the organochlorine pesticides dichlorodiphenyltrichloroethane (DDT) and its main metabolite dichlorodiphenyldichloroethylene (p,p'-DDE) are suspected of having endocrine modulating capabilities. For example, dependent on the congener type, PCBs have been shown to display estrogenic, antiestrogenic, and antiandrogenic effects [18]. Also, some PCBs are known to exhibit dioxin-like effects exerted through the aryl hydrocarbon receptor [19], and p,p'-DDE is regarded an androgen receptor antagonist [20]. PFOA and PFOS have been shown to act as estrogen receptor (ER) agonists, and to exert concentration dependent antagonistic effects on androgen receptor (AR) function *in vitro* [21].
A number of animal studies and human epidemiological studies indicate that exposure to these compounds is associated with effects on male reproductive health. They readily cross the placenta. Hence, the foetus is exposed to POPs during a vulnerable period of development, which may have long-term consequences [22-24].

3.1 Prenatal exposure to POPs and semen quality and reproductive hormones

3.1.1 POP exposure

PFOA and PFOS belong to the class of perfluoroalkyl substances (PFASs), which have been used in numerous industrial and commercial applications due to low surface tension and unique water-, dirt-, and oil-repellent properties. PFOA has been used as a processing aid in

the manufacture of components of non-stick cookware and PFOS in for example stain repellents and hydraulic fluids [25,26]. The compounds are highly persistent with estimated half-lives in human serum of 3.8 years and 5.4 years for PFOA and PFOS, respectively [14]. PFASs in the environment are likely to be a result of emission during production, disposal, use, and degradation of residual or commercial fluorochemical precursors [26]. Main routes of human exposure are thought to be through dietary intake, drinking water, and dust of indoor environments [27-31]. Accumulation of PFASs in humans occurs primarily via binding to serum albumin [26,32].

PCBs and the organochlorine pesticide DDT belong to a class of persistent and lipohlilic man-made pollutants. A wide range of consumer products like cutting oils, electrical equipment, transformers, paints, flame-retardants, sealants, and pesticide additives contain PCBs, whereas DDT has been used in disease vector control and in agriculture against insect damage to crops. The compounds accumulate in adipose tissue and bioaccumulate in the food chain, whereby they become sources for human exposure. Although the production of PCBs and DDT was banned in the late 1970s in most developed countries, compounds are still released into the environment as a result of combustion processes, volatilization, disposal, and release from materials that contain the substances [33-34].

PCBs, DDT, and PFOS are on the Stockholm convention list. The list comprises environmental contaminants that have potential adverse effects on wildlife and humans and was made and signed in 2001 by 92 countries in order to reduce release, of initially 12 environmental contaminants, into the environment. PFOS was not on the original list but was added as an amendment to the Stockholm convention list in 2009 [35]. Although many countries have signed the Stockholm convention, some of the compounds are still in use. Because malaria is a big public health problem in large parts of the world, DDT is in use today despite great concerns about effects on human health [35-36].
Generally, serum levels of the POPs studied in the present thesis are decreasing. Temporal trends suggest that serum levels of PFOA and PFOS for western countries have peaked around the year 2000 and are still declining [37-39]. Also, PCBs and p,p'-DDE serum concentrations are declining in accordance with the reduction in environmental exposure after the late 1970s [40-42].

POPs are suggested to affect hormonal balance and availability through mechanisms involving steroid receptor binding or interference with steroid biosynthesis or metabolism

[43]. Because POPs readily cross placenta, the developmental exposure of the foetus is inevitable. Together, these disturbing properties of POPs call for great concern with respect to long-term effects on the reproductive system.

3.1.2 Reproductive organ development

Reproductive organ development is directed by a complex interplay between genes and hormones involving cellular proliferation, signalling, differentiation, migration, and apoptosis. Thus, exposures affecting the hormonal balance during reproductive organ development may have irreversible long-term consequences.

Male sex determination in the early embryonic gonad is dependent on the presence of the *SRY*-gene (sex-determining region Y gene) located on the short arm of the Y chromosome. Sertoli cell differentiation is initiated by *Sry* [44], and at approximately 6-8 weeks of gestation the first sign of testis formation occurs when primordial germ cells (prospermatogonia) and early Sertoli cells form the semininferous cords, which will give rise to the semininferous tubules in the adult male [45,46]. The interstitial cells of Leydig then start secreting androgens and the Sertoli cells of the semininferous cords begin to secrete AMH. Secretion of these hormones is critical for male sexual differentiation. Due to the androgens secreted from the Leydig cells, Wolffian ducts develop into epididymis, vas deferens, and seminal vesicles and result in the formation of the penis and descent of the testes into the scrotum. AMH secretion from the Sertoli cells causes regression of the Müllerian ducts, thereby preventing development of female reproductive tract tissue [45,47]. Also, secretion of insulin-like factor 3 from the Leydig cells plays a vital role in the masculinisation process and in testes descent [48].

Sertoli cells proliferate during fetal and neonatal life and again in the peripubertal period, resulting in a fixed number of Sertoli cells in adulthood, which determines testis size and capacity of daily sperm production. FSH secreted from the pituitary is crucial for Sertoli cell proliferation and the final population size of Sertoli cells, whereas a number of genes and their downstream pathways including *SRY, SRY related HMG box 9, Fibroblast growth factor 9, and Prostaglandin D2* are suggested to be important for Sertoli cell differentiation [44, 48-50]. In the adult testis, luteinizing hormone (LH) stimulates Leydig cells to synthesise and secrete androgens that are central for spermatogenesis. LH is secreted from the pituitary as a response to gonadotrophin releasing hormone (GnRH) and is regulated by a negative feedback mechanism via testosterone secreted from the Leydig cells (Figure 1). After puberty

each differentiated Sertoli cell supports spermatogenesis of a fixed number of germ cells. Sertoli cell function is stimulated via FSH released from the pituitary [44] and they in turn secrete androgen-binding protein that binds testosterone produced from the Leydig cells and stimulates spermatogenesis. In addition, Sertoli cells secrete inhibin B, which regulates FSH release from the pituitary through a negative feedback mechanism (Figure 1) [45].

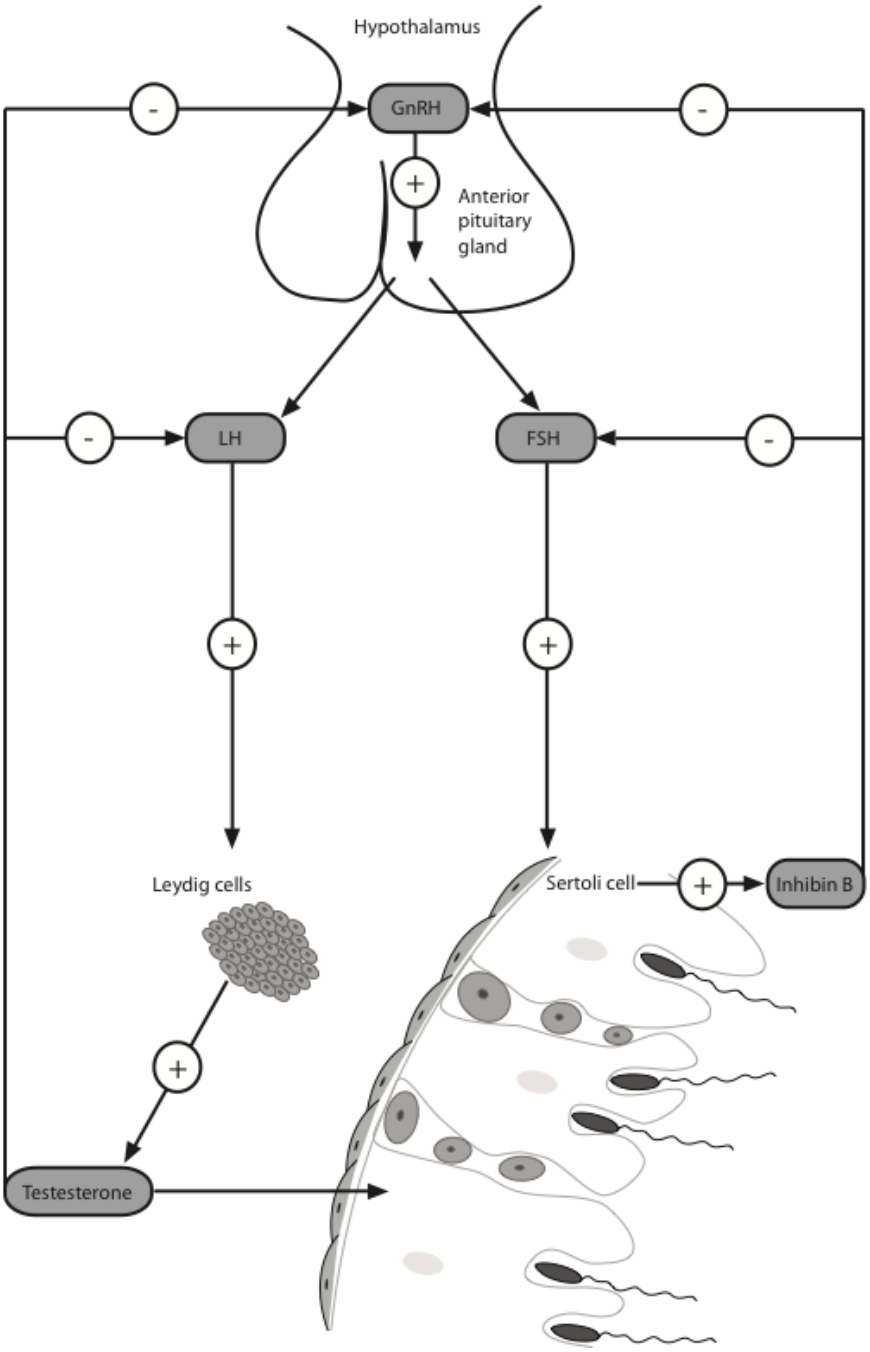

Figure 1. Hypothalamic release of gonadotophin releasing hormone (GnRH) induces pituitary secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates Sertoli cell function of supporting spermatogenesis. Sertoli cells produce inhibin B, which acts on FSH release from the pituitary gland via negative feedback. LH released from the pituitary stimulates Leydig cells to synthesize and secrete testosterone, which in turn regulates LH release from the pituitary through a negative feedback mechanism.

3.1.3 Male programming window

Studies on rats exposed to the androgen blocking agent flutamide have proposed that there is a specific time slot of male programming, corresponding to gestational weeks 8-14 in humans, during which, androgen action is critical in order to masculinise the male reproductive tract. Androgen action during the "male programming window" (MPW) is reflected throughout life by anogenital distance (AGD) in rats [51]. Additionally, it is proposed that insufficient androgen action during the MPW can induce cryptorchidism and hypospadias and that exposure to environmental contaminants compromising androgen availability during this window may have long-term consequences for male reproductive health [52,53]

3.1.4 Congenital malformations – cryptorchidism and hypospadias

Occurrence of cryptorchidism and hypospadias could reflect early signs of insufficient androgen action during MPW and fetal development. These male congenital malformations are among the most frequent among newborn boys, with a reported incidence rate of 0.1-9.0% at birth and 0.7-1.9% at 3 months of age for cryptorchidism and 0.8-3.8 cases of hypospadias per 1,000 live births [54-56]. In addition, cryptorchidism is associated with increased risk of infertility and testicular cancer in adult life [13,57].

The process of testicular descent and male genital development is, as already mentioned, dependent on normal action of key reproductive hormones (including testosterone). It has been speculated, whether exposure to endocrine modulating compounds during male reproductive organ development *in utero* is a risk factor for cryptorchidism and hypospadias [58]. A number of epidemiological case-control studies have addressed the potential effects of PCBs and p,p'-DDE on the risk of cryptorchidism and hypospadias (Table 1) but to my knowledge none have addressed the potential associations between *in utero* exposure to PFOA and PFOS and risk of these congenital malformations. In the studies reviewed in Table 1, POP levels were measured in maternal serum samples taken during pregnancy, placenta, cord blood, maternal milk samples, or maternal serum samples from the postpartum period.
There is no clear tendency in the results from these studies, ranging from no effects to small increased risks. Thus, due to the small and for the most part non-statistically significant increased risks, the results are only suggestive of weak effects of POPs on the risk of cryptorchidism and hypospadias.

Table 1. Congenital malformations – cryptorchidism and hypospadias

Reference	*Design*	*Population (n)*	*Exposure*	*Hypospadias OR*	*(95% Confidence Intervals (CI))*	*Cryptorchidism OR (95% CI)*	*(95% CI)*	*Summary of the results*
Rignell-Hydbom et al. 2012[59]	Nested case-control	Southern Sweden Maternity Cohort (SSMC) 237 cases 237 controls	PCB-153 p,p'-DDE in serum samples from early pregnancy	**PCB-153 (ng/mL)** -0.25 (ref) 1.00 <0.25-0.48 0.96 >0.48-0.76 0.75 >0.76 0.60 **p,p'-DDE (ng/mL)** -0.10 (ref) 1.00 >0.10-1.00 1.33 >1.00-2.20 1.69 >2.20 1.68	- (0.57;1.62) (0.42;1.35) (0.30;1.19) - (0.73;2.44) (0.93;3.08) (0.92;3.08)	-	-	No significant associations but a tendency towards higher p,p'-DDE being associated with higher risk of hypospadias
Virtanen et al. 2012[60]	Nested case-control	Denmark 39 cases 129 controls Finland 56 cases 56 controls	PCB-28/ 31, 60, 66, 74, 99, 105, 114, 118, 126, 138, 153, 156, 157, 167, 69, 170, 180, 183, 187, 189, and 194) in placenta	-	-	-	-	No associations between placental levels of PCBs and cryptorchidism.
McGlynn et al. 2009[61]	Nested case-control	The collaborative Perinatal project (CPP) 230 cryptorchidism cases 201 hypospadias cases 593 controls	PCB-28, 52, 74, 105, 118, 138, 153, 170, 180, 194, 203 in serum samples from third semester	**Total PCBs (µg/L)** 0-1.9 1.00 2-2.9 1.57 3.3-3.9 1.45 ≥4.0 1.69	- (1.05;2.34) (0.90;2.34) (1.06;2.68)	**Total PCBs (µg/L)** 0-1.9 1.00 2-2.9 1.27 3.3-3.9 1.32 ≥4.0 1.41	- (0.88;1.83) (0.86;2.02) (0.90;2.20)	No associations between individual PCBs or functional groupings of PCBs and hypospadias or cryptorchidism, but indications of an increased risk of hypospadias with the sum of PCBs divided into four categories.

Brucker-Davis et al. 2008[62]	Prospective case-control study	Nice, France 78 cases 86 controls	PCB-28, -52, -101, -118, -138, -153, -180, and p,p'-DDE in cord blood and maternal milk samples	-		-	**>median p,p'-DDE** At birth At 3 months **>median ∑PCB** At birth At 3 months **>median for both DDE and ∑PCB** At birth At 3 months	 2.16 1.16 2.74 2.97 3.03 1.97	 (0.94;4.98) (0.35;3.38) (1.15;6.53) (0.88;9.99) (0.99;9.21) (0.39;9.86)	Increased odds of cryptorchidism with high ∑PCB in maternal milk, and tendencies towards increased odds of cryptorchidism with higher DDE or DDE+∑PCB concentrations in maternal milk. No association between ∑PCB in cord blood and cryptorchidism.
Bhatia et al. 2005[63]	Nested case-control	The Child Health and Development Studies (CHDS) 75 cryptorchidism cases 66 hypospadias cases 283 controls	p,p'-DDE and DDT in serum samples from third trimester or postpartum period	**p,p'-DDE (ng/mL)** <27.0 27.0-43.9 44.0-60.9 ≥61 **DDT (ng/mL)** <10.0 10.0-14.9 15.0-19.9 ≥20.0	 1.00 0.81 0.68 1.18 1.00 0.81 0.45 0.79	 - (0.36;1.84) (0.28;1.64) (0.46;3.02) - (0.42;1.56) (0.16;1.28) (0.33;1.89)	**p,p'-DDE (ng/mL)** <27.0 27.0-43.9 44.0-60.9 ≥61 **DDT (ng/mL)** <10.0 10.0-14.9 15.0-19.9 ≥20.0	 1.00 1.17 0.95 1.34 1.00 0.49 2.04 1.01	 - (0.51;2.66) (0.39;2.30) (0.51;3.48) - (0.23;1.01) (1.00;4.18) (0.44;2.28)	No associations between maternal serum levels of DDE and DDT and hypospadias or cryptorchidism
Longnecker et al. 2002[64]	Nested case-control	The collaborative Perinatal project (CPP) 219 cryptorchidism cases 199 hypospadias cases 552 controls	p,p'-DDE in maternal serum from third trimester	**p,p'-DDE (ng/mL)** <21.4 21.4-42.7 42.8-64.1 64.2-85.5 ≥85.6	 1.00 1.00 1.00 0.60 1.20	 - (0.7;1.4) (0.6;1.6) (0.3;1.2) (0.6;2.4)	**p,p'-DDE (ng/mL)** <21.4 21.4-42.7 42.8-64.1 64.2-85.5 ≥85.6	 1.00 0.90 0.90 0.70 1.30	 - (0.7;1.3) (0.6;1.5) (0.4;1.3) (0.7;2.4)	No clear evidence of an effect of DDE on male reproductive development

3.1.5 Animal studies and markers of reproductive health

Experimental studies on the effects of *in utero* exposure to POPs on reproductive health measures are suggestive of effects on the male reproductive system, but results are far from unequivocal. Daily sperm production has been suggested to be reduced in male rats exposed to PCB-118 and PCB-132 [65,66], whereas no effect on sperm counts were found in mice exposed to PCB-101 and PCB-118 [67]. Increased sperm counts were reported for mice and rats exposed to the PCB mixture arochlor 1242 or PCB-77 *in utero*, respectively [68,69]. Reduced testis weights are reported in a number of rodent studies [66,67,70], but no effects on testis weights have also been found in both rat [65,71-72] and mice studies [68], and increased testis weights has been found in a study of rats perinatally exposed to PCB 77 [69]. Several studies have suggested decreased serum testosterone levels in male rat offspring perinatally exposed to different PCBs [69-70,72-73]. However, no effects on testosterone levels have also been reported for both PCB [66] and p,p'-DDE exposure [74]. Although a number of these studies point towards adverse effects on the male reproductive system, it should be noted that the exposure levels used the studies were several times higher than the exposure levels measured in general human populations.

Studies evaluating gestational and lactational effects of PFOA and PFOS on male reproductive health parameters are scarce. In a two-generational study on the reproductive toxicology of ammonium PFOA, rats were orally gavaged with doses of ammonium PFOA between 0 and 30 mg/kg/day. Testis sizes were significantly increased (testis/terminal body weight ratio) in offspring of the high-dose groups. There were no significant changes in total sperm counts, motility parameters, and sperm morphology in the exposure groups compared with controls and no signs of dose-response relationships [75], but delayed sexual maturation was observed for the first generation offspring at the 30 mg/kg/day dose-group [76]. Although AGD was assessed in the second-generation pubs the study did not report on associations between this parameter and perinatal exposure to PFOA [75-76].

3.1.6 Human studies of *in utero* exposure to POPs and male reproductive health

Knowledge on the effects of *in utero* exposure POPs on male reproductive development is sparse; however the potential effects on pubertal development have been addressed in a number of studies.

Associations between perinatal exposure to POPs and pubertal timing are diverging. One study suggests an association between delayed puberty and prenatal exposure to PCBs and p,p'-DDE [77], whereas no effects on pubertal development have also been associated with exposure to both PCBs and p,p'-DDE [78,79]. In addition, an association between earlier onset of puberty and prenatal exposure to PCBs has been suggested in a study estimating prenatal exposure from maternal serum sampled 8-9 years after birth [80]. Thus, the possible effects of early life exposures to POPs on male pubertal development are not consistent.

Although there has been much focus on the potential long-term male reproductive health effects of exposure to endocrine disrupting compounds during fetal development, only few have had the opportunity to investigate the endocrine disruptor hypothesis in humans with respect to adverse effects on semen quality in adulthood.

Important knowledge about long-term effects of high exposures to POPs during fetal development on human male reproduction has been learned from the Yu-Cheng oil-disease in Taiwan in 1979, where over 2000 men and women were accidentally contaminated with high levels of PCBs and polychlorinated dibenzofurans. Comparisons between 12 prenatally exposed men and unexposed controls showed increased morphological abnormalities of sperm, reduced sperm motility and velocity, and reduced oocyte penetration capacity in prenatally exposed men [81].

Furthermore, male reproductive health consequences of pre-and perinatal exposure to dioxins have been investigated in a cohort of sons of women exposed to dioxin after the trichlorophenol plant explosion near Seveso, Italy in 1976. Sons exposed *in utero* and postnatally through breast-feeding had significantly lower sperm concentration, total sperm count, progressive motility, and total motile sperm count compared with unexposed controls. There were no differences in any of the semen related outcomes between the sons that were only exposed *in utero* (formula fed) in comparison with the unexposed controls. In addition, sons that were both exposed to dioxin *in utero* and via breast feeding had higher FSH levels and lower inhibin B levels compared with both unexposed controls and formula fed exposed men, indicating adverse long-term consequences of perinatal exposure to dioxin on male reproductive health [82].

3.2 Androgen receptor exon 1 polymorphism as effect modifier of association between POP exposure, semen quality, and reproductive hormones.

Binding of androgens to the androgen receptor (AR) and a functional AR is crucial for male sex differentiation and reproductive development in addition to androgen signalling during pubertal development and maintenance of spermatogenesis throughout life.

The *AR* gene is located on the X-chromosome, and the AR protein belongs to the subfamily of steroid hormone receptors within the family of nuclear receptors. The receptor consists of an N-terminal region with trans-activating activity, a DNA binding domain, corresponding to the central region of the amino acid sequence, and the ligand-binding domain, comprising the C-terminal. A polymorphic glutamine repeat sequence encoded by $(CAG_n)CAA$ is located in exon 1 of the *AR* gene within the N-terminal of AR [83-84]. An inverse relationship between the number of CAG repeats in the *AR* gene and AR function was originally suggested [85]. However, recent studies indicate that rather than a linear relationship between the number of CAG repeats and AR function, an intermediate number of CAG repeats (CAG=22) is associated with optimal receptor function, and thus, a non-linear relationship between CAG number and AR function is suggested [86-87].

Suggestions of geographical and ethnical differences in semen quality and incidences of cryptorchidism, hypospadias, and testicular cancer has led to hypotheses of possible interactions between genes and environment, speculating that individuals with certain genotypes could be more susceptible to adverse exogenous exposures than others [88-89]. Indications of AR CAG repeat length as a modifier of the effect of adulthood persistent organochlorine exposure on sperm concentration, total sperm count, and DNA fragmentation index (DFI) has already been suggested [90].

3.3 Conclusions leading to the present study

Although the endocrine disruptor hypothesis was put forward almost two decades ago, knowledge is still scarce with respect to long-term male reproductive health consequences of *in utero* exposure to POPs with suspected endocrine modulating capabilities in humans.

These compounds can be traced in humans all over the world, and readily pass the placental barrier, inevitably resulting in fetal exposure during critical phases of organ development, which may have irreversible consequences for male reproductive health later in life. Only one study has assessed the association between exposure to high levels of PCBs *in utero* and early

adulthood male reproductive health consequences, whereas associations between prenatal exposure to background levels of PFAA, PCBs and p,p'-DDE and semen quality and reproductive hormone levels in adulthood have not been investigated in a longitudinal setting. If prenatal exposure to these persistent environmental contaminants is associated with reduced semen quality and altered hormone levels in adulthood more effort has to be done to limit release of these or similar compounds into the environment.

4. Aims of the thesis

The aims of the thesis were to:

- compare assessments of sperm concentration and sperm motility analysed by conventional semen analysis with those obtained by computer-assisted semen analysis (CASA) (CRISMAS 4.6 software) (Study I).

- study associations between prenatal exposure to PFOA and PFOS and semen quality measures and reproductive hormone levels in adulthood (Study II).

- study associations between *in utero* exposure to PCBs and p,p'-DDE and semen quality and reproductive hormone levels in adulthood (Study III).

- study the potential modifying effect of androgen receptor gene CAG repeat length on the association between prenatal exposure to PFOA and semen quality and reproductive hormone levels (Study IV).

5. Materials & methods

In the following, methods for the studies included in the thesis are mentioned briefly. Additional descriptions regarding the methods can be found in the manuscripts (Appendices I-IV).

5.1 Baseline and follow-up (Studies I-IV)

5.1.1 Study population at baseline

In 1988-1989 a Danish pregnancy cohort was established and included pregnant women attending a routinely scheduled pregnancy week 30 visit at a midwifery practice covering a well-defined area of the city Aarhus, Denmark. Originally, the study was designed to investigate associations between diet during pregnancy and perinatal child and pregnancy outcome measures. Due to language difficulties, women from foreign cultures were not included in the study [91]. Of the 1212 eligible women, 965 were enrolled corresponding to a participation rate of 80% [92,93].

5.1.2 Data collection at baseline

The women completed a detailed self-administered questionnaire focusing on food items that were easily quantified. In addition to the questionnaire, a 15 minute structured interview was undertaken, and a blood sample was drawn, processed, and stored in a biobank at -20°C. Additional covariates were collected from birth certificates, hospital records, and from records from the midwifery practise. Those included; information on for example maternal pre-pregnancy weight, birth weight, birth length, and gestational age. Additionally, the women provided information on alcohol consumption and smoking habits during pregnancy [92,93]. There were no differences in age, parity, gestational age, or birth weight between participants and non-participants [91].

5.1.3 Study population at follow-up

Four hundred and ninety nine male offspring from the pregnancy cohort from 1988-1989 were identified in the Danish Civil Registration Registry (CPR) (Figure 2). Thirty one were lost to follow-up due to emigration, death, illness, or declined contact by the mother. In 2008 the 468 remaining sons were invited to answer a self-administered internet-based questionnaire on general and reproductive health and lifestyle factors. A total of 115 male

offspring either did not answer the questionnaire (n=30) or filled in the questionnaire but declined further contact (n=85). Hence, a total of 353 male offspring were contacted by mail in 2008-2009 with respect to participation in a follow-up study entitled: “The importance of diet during pregnancy on children’s health”. Of the original 468 male offspring who were asked to fill in the questionnaire in 2008, a total of 176 sons agreed to participate in the physical follow-up examination, corresponding to a participation rate of 38%. When only taking the sons into account who were invited to participate in the physical examination in 2008-2009 (n=353), the participation rate was 50%.

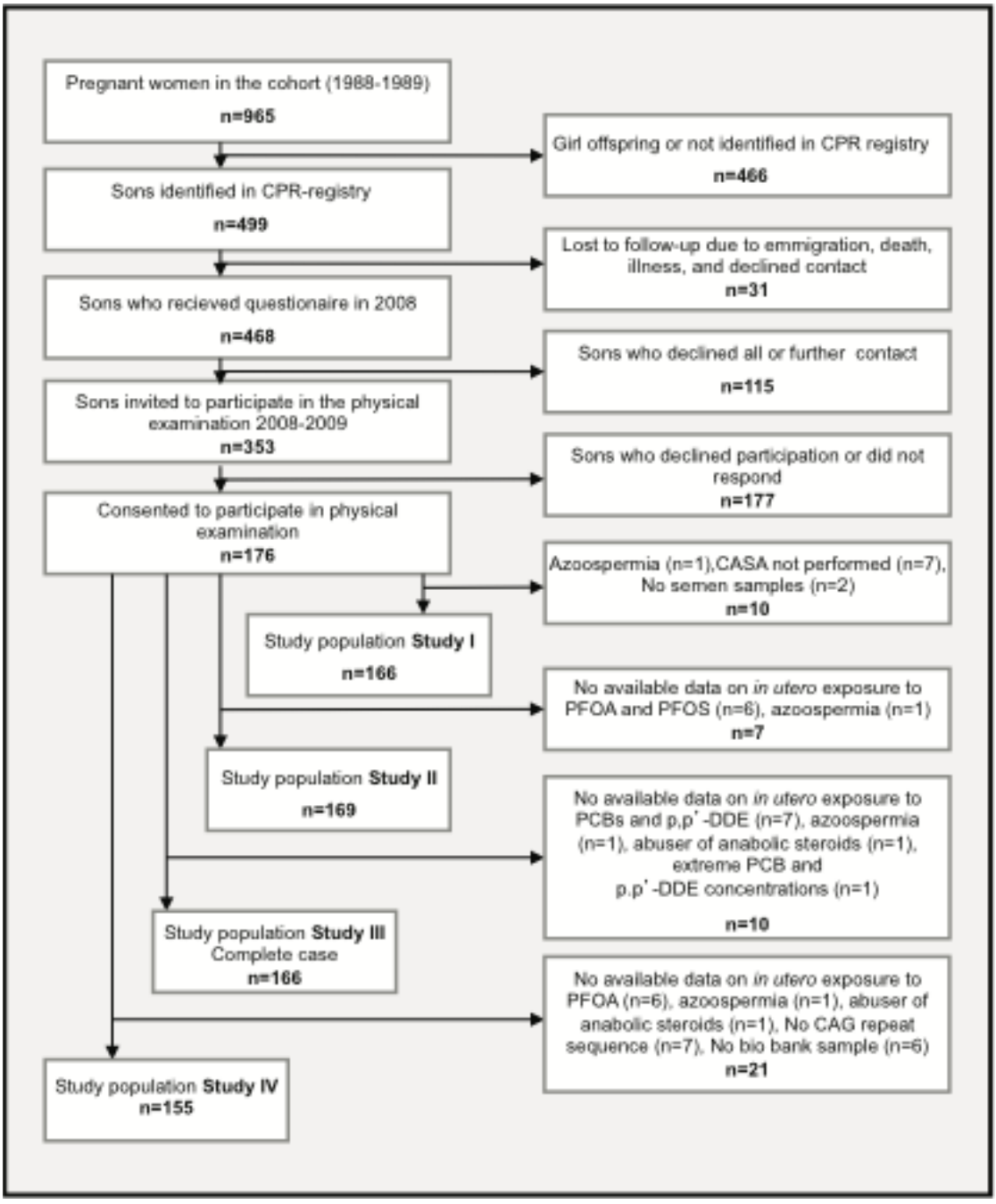

Figure 2. Flow chart for the study population

5.1.4 Data collection at follow-up

Physical examinations were conducted from February 2008 until September 2009. The participants had been informed that an abstinence period between 2 to 4 days since their last ejaculation was preferred. Participants delivered a semen sample and a blood sample was drawn for reproductive hormone analysis and for further research use (biobank). Additionally, participants measured testicular size using a Prader Orchidometer, which has been reported to be valid method for self-measurement of testicular volume [94]. Semen samples were analysed according to World Health Organization (WHO) Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus interaction [95] by conventional and computer-assisted semen analysis (CASA) methods. Two trained medical laboratory technologists performed semen analyses. One analysed semen samples from February to October 2008 and the other from October 2008 until the end of data collection in September 2009. Both participated in the European Society of Human Reproduction and Embryology external quality-control program. Blood samples were centrifuged in order to separate them into serum, plasma and buffy coat, and stored for -80°C until further analyses. Each participant provided written informed consent prior to examination and answered whether or not he wanted the result from the semen analysis and also if he gave consent to storage of blood samples in a biobank for further research use. The participants received economic compensation for their travel expenses and for participating in the physical examination (about €132). The Central Denmark Region Committees on Health Research Ethics approved this study (registration number M-20070157).

5.1.5 Conventional semen analysis

Semen sample analyses were initiated within the first hour from ejaculation for 87% of the semen samples, and all were initiated within two hours from semen sample collection.

All analyses were performed blinded to any prenatal exposures. Sperm concentration was assessed in duplicate using a Neubauer counting chamber and a phase contrast microscope. The final sperm concentration is a mean of the two counts.

Spermatozoa were classified into four motility categories: (A) rapidly progressive (>25μm/s, (B) slowly progressive (5-25 μm/s), (C) non-progressive (<5 μm/s), and (D) immotile spermatozoa. This was done for 100 spermatozoa two times and the procedure was repeated on a new slide. Thus, 400 spermatozoa were classified and the final motility numbers were means of the four category classifications. If the difference in sperm assessment exceeded the 95% confidence interval (95% CI) for difference between two measurements, the assessment was repeated [95].

Four smears for morphology analyses were made per participant and analysed according to "strickt" criteria by classifying 4×100 spermatozoa into normal or abnormal spermatozoa and using the mean for the total score. Only assessments where quality control status was in agreement with the "strict" criteria were included in the analyses on sperm morphology [96].

5.1.6 Computer assisted semen analysis

Semen samples were analysed by CASA using Copenhagen Rigshospitalet Image House Sperm Motility Analysis System (CRISMAS) Clinical software version 4.6 (Image House Medical, IHMedical A/S, Copenhagen, Denmark). Sperm concentration and total sperm count were assessed and spermatozoa classified into three motility categories: Motile (curvilinear velocity>25 μm/s), local motile (curvilinear velocity 5-25 μm/s), and immotile spermatozoa (curvilinear velocity <5 μm/s).

5.1.7 Male offspring reproductive hormone analyses

FSH, LH, estradiol, testosterone, sex hormone-binding globulin (SHBG), and inhibin B were measured by immunoassays blinded to any prenatal exposure.

5.1.8 Exposure assessment from maternal serum samples

All exposure measurements were blinded to reproductive outcome measures.

Maternal serum samples from pregnancy week 30 were analysed for levels of PFOA and PFOS in 2010-2011 at the Division of Environmental Medicine, Norwegian Institute of Public Health, Oslo, Norway) by column-switching, isotope dilution liquid chromatography/ tandem mass spectrometry methodology as previously described [97].

Concentrations of p,p'-DDE and the six PCB congeners (PCB-118, -138, -153 -156, -170, and -180) were measured in 2011-2012 by liquid-liquid extraction followed by Gas Chromatography coupled to High Resolution Mass Spectrometry at the National Institute for Health and Welfare (THL), Neulanen Research Centre, Kupio, Finland, according to a method described by Koponen et al. [98].

5.1.9 Offspring DNA purification

After gradient centrifugation, buffy coats, containing most of the white blood cells and platelets, were transferred to cryo vials and stored in the bio bank at -80°C.

Genomic DNA was purified from buffy using the Maxwell®16 instrument (Promega Corporation, Madison, USA) according to manufacturer's protocol at the Department of Clinical Biochemistry and Immunology at the Section of Neonatal Screening and Hormones, Statens Serum Institut, Denmark.

5.1.10 Offspring CAG repeat sequencing

The *AR* gene CAG repeat length was amplified by polymerase chain reaction (PCR) and PCR products were purified on columns and subjected to sequencing PCR. PCR products were ethanol precipitated, dissolved in highly deionized formamide (Applied Biosystems, Stockholm, Sweden) and sequenced on an eight-capillary AB3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The number of CAG repeats were counted by two persons, independently, and compared afterwards. The work was performed during my stay at the Department of Clinical Sciences, Molecular Reproductive Medicine at Skaane University Hospital in Malmö, Sweden.

5.1.11 Statistical analyses

All statistical analyses were performed using Stata 11.1-12.1 software (StataCorp, Collega Station, TX, USA), and a two-tailed probability level of $p<0.05$ was considered statistically significant.

5.2 Statistical analysis for study I

Data from a final study population of 166 sons were available for this study (Figure 2).
Bland-Altman plots were used to evaluate agreements between the conventional semen analysis and CRISMAS CASA results. Wilcoxon signed-rank test was used to test for differences between method medians and large one-way ANOVA was used to produce interclass correlation coefficients (ICC) between the two methods for all four semen parameters. Additionally, linear regression analysis was used to assess possible time drifts in the sperm concentration and motility results obtained by the two methods.

5.3 Statistical analysis for study II

A total of 169 sons were available for this study (Figure 2). Crude associations between untransformed outcome measures and continuous PFOA and PFOS were tested by

Spearman's rank correlation test, and adjusted trends on continuous PFOA and PFOS were tested by multivariable regression analyses. Outcome variables were natural logarithm (ln)-transformed before multivariable regression analyses for improved normal distribution of residuals. Additionally, all analyses were checked with models using the transformations leading to the most optimal normal distribution of the residuals. These transformations are listed here: cubic root transformed sperm concentration, square root transformed total sperm count, semen volume, and inhibin B, squared transformation of progressive motility, ln-transformation of FSH and free androgen index (FAI), and no transformation of morphologically normal spermatozoa, LH, estradiol, testosterone, and SHBG. Participants were divided into three groups according to tertiles of maternal pregnancy week 30 serum concentrations PFOA and PFOS. Also, for outcomes with a significant trend on continuous exposure, regression analyses using quintiles of exposure were performed.

Crude differences between exposure tertiles were tested by two-sample Wilcoxon rank-sum (Mann-Whitney) test and adjusted differences by multivariable regression analysis for each of the outcome variables using low PFOA and PFOS tertiles as reference groups. Multivariable regression analyses were adjusted for *a priori* selected potential confounders. Missings in the covariates were assigned a special category in order to be able to include participants with missing values in the covariates in the multivariable regression analyses [99]. Sons who reported spillage during semen sample collection were excluded from all analyses on total sperm count and semen volume.

5.4 Statistical analysis for study III

One of the study participants was referred to an andrological investigation because of his very low FSH and LH levels. Between study II and study III we acquired the result from the andrological investigation, revealing that this participant was an abuser of anabolic steroids. Consequently, this participant was excluded from all statistical analyses in the studies III and IV.

Another participant was excluded from all statistical analyses due to azoospermia, and one participant was excluded due to an extremely high maternal serum level of p,p'-DDE (32.18 ng/mL) and PCBs (PCB-153: 12 ng/mL). Based on new experiences indicating that results from multiple imputation (MI) analyses may possibly provide more reliable results than complete case analyses or assigning missing values their own category, we chose to use MI to impute missing values to the exposure variables, outcome variables, and covariates in study III. The final study population for the MI analyses was 173 and the study population for complete case analyses included 166 participants (Figure 2).

PCBs and p,p'-DDE were converted into molar concentrations and PCB congeners were summed in two different exposure groupings: 1. The sum of all measured PCBs (∑PCBs, pmol/mL) and 2. ∑DL-PCBs (PCB-118 and PCB-156, pmol/mL). Participants were divided into three exposure groups (low, medium, and high) based on tertiles of each of the exposure variables (∑PCBs, ∑DL-PCBs, and p,p'-DDE).

Linear trends on continuous ∑PCBs, ∑DL-PCBs, and p,p'-DDE levels and differences between tertiles were assessed by linear regression analysis with robust variance estimates. Adjusted trends and percentage differences were tested by multivariable regression analysis. All outcome variables were ln-transformed before multivariable regression analysis.

We used the same *a priori* selected confounders for the models in study III as we used in study II except that we additionally chose to adjust for serum total lipid concentration in study III [100] and abstinence time was dichotomized into ≤48 hours/ ≥49 hours.

5.4.1 Multiple imputation analysis

MI was used in order to handle missing data under the assumption that data fulfilled the missing at random criteria. The MI method uses the observed data to predict values of the missing data by creating several different plausible imputed datasets *(m>1)* and combining the results into one model. In order to account for the variability of the results between the different datasets, standard errors are calculated by using Rubin's Rules [101].

Data on the exposure variables were missing on seven participants due to missing maternal blood samples or inadequate blood sample volume. The numbers of participants with missing data in the outcome variables were as follows: sperm concentration, total sperm count, and semen volume (n=2), percentage progressive motility (n=3), percentage morphologically normal spermatozoa (n=4), testicular volume (n=1), and reproductive hormone levels (n=1). Additionally, the number of participants with missing values in the covariates were: abstinence time (n=2), spillage during semen sample collection (n=11), time from ejaculation to semen analysis (n=2), time of day of blood sampling (n=3), history of diseases in the reproductive organs (n=8), the sons' BMI (n=0), sons' smoking status (n=10), maternal smoking during pregnancy (n=7), and socioeconomic status (SES) (n=15).

For the main imputation model, 100 datasets were imputed with values for missing data. Before imputation, assumptions of normal distributions of the residuals of all continuous variables imputed on the linear scale were confirmed.

Sensitivity analyses with different imputation models were performed to check for consistency in results under different imputation models: 1) imputation based on only

outcome variables, exposure variables, and covariates; 2) imputations based on more covariates than in the main model; and 3) imputations and multiple regression analyses based on the best transformation of the outcome variables to obtain a normal distribution of the residuals. An increase in the number of imputed datasets to 150 or lowering the number of imputed datasets to 25 did not affect the results.

5.5 Statistical analysis for study IV

A final study population of 155 was available for this study (Figure 2). CAG repeats were divided into three approximately equally sized groups (<21 CAG repeats (n=49), 21-23 CAG repeats (n=57), and >23 CAG repeats (n=49)). Nenonen et al. utilized the same upper limit for CAG group division (>23), but the lower cut was in that study set at <22 [87]. Using the same groupings as Nenonen et al. (<22, 22-23, >23), would have resulted in three unevenly sized groups (<22=74, 22-23=32, and >23=49. Due to the relatively limited sample size, increasing the number of CAG categories to for example five (<20, 20-21, 22-23, 24, and >24) like in Giwercman et al. [90] was not an option. Hence, in order to obtain groups of approximately equal size, we chose to balance the group cuts around the cohort CAG repeat median (22 repeats) which has also been shown to have the highest AR activity *in vitro* when compared with CAG lengths of 16 or 28 [86]. Interactions between CAG repeat length and prenatal exposure to PFOA in relation to the different semen quality parameters and reproductive hormones were evaluated by the use of linear and multivariable regression analysis and trends on continuous PFOA for each CAG strata were tested by linear regression analysis (crude) and multivariable regression analysis (adjusted). For inclusion of participants with missing values (covariates) in the adjusted regression analyses, we assigned participants with missings in the covariates their own category in order to compare results from this study with results from study II utilizing this statistical approach.

6. Results

Main findings from the four manuscripts are listed below. Additional results can be found in the enclosed papers and supplemental materials.

Table 2. Characteristics of mothers and sons at baseline (pregnancy week 30) and follow-up according to level of participation

Characteristics	***Participating in physical examination n=176***	***Filling in questionnaire only n=157***	***Not in follow-up n=143***	***p-value***
Maternal				
PFOA	3.7 (2.8-4.7)	3.8 (3.0-5.1)	3.8 (2.9-4.8)	0.61 [a]
PFOS	21.3 (17.4-26.5)	21.9 (17.8-27.1)	22.3 (16.7-26.5)	0.76 [a]
∑PCBs (pmol/mL),	10.1 (7.3-12.9)	10.4 (7.6-13.0)	9.0 (6.6-12.2)	0.12 [a]
∑Dioxin-like-PCBs (pmol/mL) §	0.8 (0.6-1.1)	0.8 (0.6-1.1)	0.8 (0.6- 1.0)	0.11 [a]
p,p'-DDE (pmol/mL)§	8.2 (5.2-12.4)	7.3 (4.9-11.2)	8.0 (5.1-11.9)	0.48 [a]
Maternal age (years), mean (SD)	29.6 (4.4)	29.0 (4.2)	28.3 (4.5)	0.03 [b]
Prepregnancy BMI (kg/m^2)§	21.0 (19.8-22.3)	20.7 (19.5-22.7)	21.2 (19.1-23.1)	0.77 [a]
Nulliparous, n (%)	112 (64)	96 (61)	83 (58)	0.60 [c]
Smoking during pregnancy, n (%)	55 (33)	53 (37)	63 (47)	0.03 [c]
High social class, n (%)	96 (60)	87 (64)	77 (61)	0.78 [c]
Blood total lipid level (g/L)§	8.6 (7.7-9.6)	8.8 (7.9-10.0)	8.7 (7.9-9.6)	0.33 [a]
Male offspring follow-up				
BMI (kg/m^2)§ [e]	22.2 (20.5-24.1)	22.4 (20.7-24.4)		0.50 [d]
Current/occasional smoker, n (%)	83 (50)	66 (43)		0.24 [c]
History of reproductive tract disease, n (%)	26 (15)	12 (8)		0.07 [c]

[a]Kruskal-Wallis test, [b]Oneway ANOVA test, [c]Chi ^{2}-test, [d]Wilcoxon rank-sum test, [e]BMI is calculated from anthropometric measures based on self-reported values. § Median (p25-p75)

There were no statistically significant differences between any of the exposure variables when comparing PFOA, PFOS, ∑PCBs, ∑Dioxin-like-PCBs and p,p'-DDE levels according to levels of participation in the follow-up study (Participation in physical examination, filling in internet based questionnaire only, and not in follow-up) (Table 2). Also, there were no differences in maternal pre-pregnancy BMI, parity, SES, total serum lipid level, alcohol consumption during pregnancy or sons' BMI, or smoking status. Sons who did not participate in follow-up had younger mothers than sons who participated in the physical examination and mothers of the sons who participated were less likely to have smoked during pregnancy compared to those who did not participate in follow-up. In addition, sons who participated in the physical examination had a higher frequency of reproductive tract disease compared to those who only filled in questionnaires.

6.1 Semen analysis method comparison (Study I)

Median sperm concentrations, percentage rapidly progressive-, slowly progressive-, and non-progressive spermatozoa obtained by conventional semen analysis and CASA were significantly different ($p<0.001$). Median sperm concentration and percentage rapidly progressive spermatozoa assessed by conventional semen analysis were 14% and 75% lower than CASA assessments. Medians of the conventionally assessed percentage slowly progressive- and non-progressive spermatozoa were 51% and 15% higher than those obtained by CASA. The discrepancy between the results from the two methods was supported by low ICCs for rapidly progressive and slowly progressive spermatozoa (ICC=0), and non-progressive spermatozoa (ICC=0.54), whereas the ICC for sperm concentration suggested a good correlation between the methods (0.92).

Analyses investigating possible time drift in the assessments of sperm concentration and sperm motility categorisation showed that sperm concentration was counted statistically significantly different between conventional and CASA analyses in the third quarter of the data collection period, whereas there were no differences between the quarter medians for the remaining data collection period. For the assessments of rapidly progressive and slowly progressive spermatozoa, medians differed substantially in the majority of the quarters, whereas non-progressive spermatozoa medians only differed substantially in the third, fourth, and seventh quarter. Several of the quarters differed statistically significantly from reference quarter one for the conventional semen analysis method assessments of rapidly progressive- and slowly progressive spermatozoa, whereas none of the quarters differed substantially from the reference quarter in the CASA assessment.

6.2 Prenatal PFAA exposure and semen quality and reproductive hormone levels (Study II)

There was an inverse association between prenatal PFOA exposure and sperm concentration and total sperm count (*p* for trend=0.01 and *p* for trend=0.001, respectively). One ng/mL increase in PFOA was associated with 11% (95% CI: 2-19%) and 20% (95% CI: 8-31%) decrease in sperm concentration and total sperm count, respectively. This was corroborated by associations between *in utero* exposure to PFOA and CASA sperm concentration and total sperm count. Additionally, higher PFOA was associated with higher FSH and LH levels (*p* for trend=0.01 and *p* for trend=0.03, respectively) with adjusted β-coefficients (95% CI) of 0.04 (0.005; 0.07) and 0.06 (0.01; 0.10).

Additionally, CASA but not conventionally assessed results on progressive motility were suggestive of an inverse trend of lower progressive motility with higher PFOA (β-coefficient (95% CI): -0.03 (-0.05; -0.007)).

An additional analysis made after the manuscript was published suggests an additional inverse association between prenatal PFOA exposure (continuous) and testosterone/LH ratio (β-coefficient (95% CI): -0.04 (-0.06; -.0.1)). There were no associations between *in utero* exposure to PFOA and sperm morphology, semen volume, testicular volume, testosterone, inhibin B, estradiol, SHBG, or FAI and no statistically significant associations between *in utero* exposure to PFOS and any of the investigated semen quality measures, testicular volume or reproductive hormone levels.

6.3 Prenatal PCB and p,p'-DDE exposure and semen quality and reproductive hormone levels (Study III)

MI main model results were not suggestive of any dose-response relationships or threshold effects between *in utero* exposure to ∑PCBs, ∑DL-PCBs, or p,p'-DDE and semen quality measures, testicular volume, or reproductive hormone levels at the studied exposure ranges. Complete case analyses and results from the MI model using the most optimal transformations of the outcome variables indicated that higher *in utero* exposure to p,p'-DDE was associated with higher sperm concentration in adulthood (β-coefficient (95% CI): 0.03 (0.01; 0.06) and 0.02 (-0.002; 0.04), respectively).

6.4 AR CAG repeats and susceptibility towards *in utero* exposure to PFOA (Study IV)

Sperm concentration, total sperm count, FSH levels, and inhibin B levels varied considerably across CAG repeat categories with the lowest sperm concentrations and total sperm counts in the CAG category with between 21 and 23 CAG repeats and lower FSH and higher inhibin B levels in the group of men with long CAG repeats (>23) compared with the other CAG categories.

For the semen related outcomes, the initial test for interactions suggested interactions between PFOA and CAG repeat categories for progressive motility and semen volume. This was supported by a significant inverse trend on continuous PFOA and progressive motility for the <21 CAG repeat group with an adjusted β-coefficient (95% CI) of -0.05 (-0.09; -0.01) and

also for the >23 CAG repeat group with an adjusted β-coefficient (95% CI) of -0.06 (-0.11; -0.02), but not for the sons with between 21 and 23 CAG repeats. For sons in the >23 CAG repeat category, higher *in utero* exposure to PFOA was associated with higher semen volume (adjusted β-coefficient (95% CI): 0.16 (0.04; 0.28)).

Despite the lack of an overall interaction between CAG repeat and PFOA, crude and adjusted results suggested an inverse trend of lower sperm concentration with higher *in utero* exposure to PFOA in the CAG group with more than 23 repeats (adjusted β-coefficient (95% CI) of -0.27 (-0.48; -0.07)) and indications of an inverse trend of lower total sperm count with higher *in utero* exposure to PFOA for the CAG group with between 21-23 repeats (adjusted β-coefficient (95% CI) of -0.21 (-0.43; 0.009)). Also, there was an inverse association between continuous PFOA and morphological normal spermatozoa for the young men with more than 23 CAG repeats (adjusted β-coefficient (95% CI) of -0.15 (-0.29; -0.002)).

The interaction test suggested no statistically significant interactions between PFOA and CAG repeats with respect to any of the reproductive hormones, however, when stratifying on CAG repeat group there was a tendency of higher estradiol levels with higher exposure to PFOA *in utero* for sons with <21 CAG repeats and a positive association between continuous PFOA and FSH with an adjusted β-coefficient (95% CI) of 0.06 (0.01; 0.11)) for men with 21-23 CAG repeats.

Generally, associations between *in utero* exposure to PFOA and CASA results supported the results from the conventional semen analysis, except for no significant inverse trend between PFOA and CASA progressive motility for the sons with less than 21 CAG repeats.

7. Discussion

7.1 Methodological considerations

7.1.1 Selection bias

Selection bias is present if the association between the exposure and the outcome is different between participants and non-participants in a study.

Overall, the participation rate for the studies I-IV was 38%, which is not particularly high. Thus, selection bias was a potential concern. Men having fertility problems are more willing to participate in semen quality studies according to previous studies [102-104]. However, participants in this cohort were young and most had no reproductive experience. It is therefore unlikely that the majority of the participants were aware of potential fertility problems, and thus improbable that participation in this study was related to fecundity. Other studies suggest that fertility state does not affect the rate of participation but rather suggest an influence of personal factors related to male reproductive health - for instance history of reproductive tract disease [105]. Fifteen percent of the sons who attended the physical examination had a history of reproductive tract disease compared to only 8% for the sons who did not participate. There was, however, no reason to suppose that potential over-sampling of men with a history of reproductive tract disease would be related to exposure. Hence, it is unlikely that this could have biased the results.

There were no differences in exposure levels between the sons who participated in the physical examination, those who only completed questionnaires, and those who did not participate in the follow-up study (Table 2). Hence, losses to follow-up were not associated with maternal pregnancy week 30 serum levels of PFOA, PFOS, ∑PCB, ∑DL-PCB, or p,p'-DDE.

We identified a tendency towards less maternal smoking during pregnancy for the sons who participated in the follow-up study compared to non-participants. Since smoking is usually associated with SES, one could expect that those with high SES would have been more willing to participate. However, as seen in Table 2, there were no differences between the proportions of high SES between those who participated and those who did not. A better subdivision of the SES variable (for example divided into more groups or combined with another socioeconomic variable) would have been more informative in this regard. However,

due to the relatively limited number of study participants, further subdivisions of the socioeconomic variable would have reduced the statistical power.

The participants were not aware of the specific hypotheses of the study before they participated, and sons had no knowledge of their intrauterine POP exposures.

Thus, even if slightly more sons with suboptimal semen quality participated in the study, we have no reason to believe that associations between POP exposure and semen quality markers would be different between participants and non-participants. Hence, in spite of the moderate participation rate, we do not consider selection bias to be a major concern for the studies included in the thesis.

7.1.2 Information bias

Information bias is a systematic under- or overestimation of measurement or error in the classification of exposures, outcomes, and/or confounders – misclassification-, leading to bias. Misclassification can be either non-differential, where the misclassification of either outcome or exposures is unrelated to each other or it can be differential, where the misclassification of exposure is related to the outcome or vice versa. Non-differential misclassification blurs the ability to identify contrasts between exposure groups, thereby increasing the risk of type II errors (bias towards the null). Differential misclassification is characterized by differences in the quality of information between the groups for comparison and can lead to bias in either direction (toward or away from the null). Generally, measuring biological markers reduces misclassification, and allows for more accurate assessments of exposure or outcome variables.

All exposure measurements were performed blinded to the outcomes, and exposures were measured using the appropriate techniques. Thus, none were estimated from questionnaires or other sources, where information on the exposure could be prone to recall bias. We cannot exclude the presence of measurement bias e.g. caused by incorrect calibration of the measurement equipment for some batches, but it is unlikely that any measurement error would be related to the outcomes, and thus a potential misclassification of the exposure would be non-differential.

All semen and blood sample analyses were performed blinded to the participants' prenatal POP levels and according to standardized methods. However, as indicated in study I, we cannot exclude the presence of misclassification of the semen variables, for example in

distinguishing rapidly progressive spermatozoa from the slowly progressive spermatozoa, as this is a subjective measure which is prone to inter- and intra-observer variability. In order to reduce this potential misclassification, we included CASA which is less prone to variability, and chose to report both conventional assessments and CASA results in the studies II-IV, since the two methods did not produce interchangeable results.
Although self-measurement of testicular volume using a Prader orchidometer has been shown to be comparable to measures obtained by an experienced examiner [94], it cannot be excluded that there would be some degree of misclassifications in the estimations of testicular size by the participants (Study II-III). Additionally, for the reproductive hormone measurements we are also not able to exclude possible measurement bias. Nevertheless, it seems unlikely that possible outcome misclassifications in the present studies could be related to the exposures. Thus, potential misclassifications would be of non-differential character.

We received additional information on one of our participants with low gonadotrophin levels (below detection limit) and low sperm concentration and total sperm count, in the time between Study II and III. This study participant reported abuse of anabolic steroids, and consequently, he was excluded from all further analyses. A subanalysis where this participant was not included in the analyses on associations between *in utero* exposure to PFOA and the investigated outcomes in study II did not change the estimates, significances levels, or directions of the associations.

We used self-reported information from the questionnaires for confounder control in the multivariable models. Participants were asked to provide retrospective information regarding history of sexually transmitted diseases (epididymitis, chlamydia, or gonorrhea), cryptorchidism, hypospadias, or other diseases related to the reproductive organs. This could potentially be associated with risk of recall bias. However, misclassification in this regard would only lead to recall bias if the misclassification was related to the outcome, which is unlikely due to the young age and lack of reproductive experience of the participants.

Information on covariates related to the participants' mothers (smoking during pregnancy and SES) was collected during pregnancy. Hence, the risk of recall bias is limited. However, since smoking information was based on self-reports instead of e.g. cotinine measurements with respect to the smoking variable, we cannot exclude that some of the women might have under-reported smoking during pregnancy. Yet, smoking during pregnancy was socially

accepted in the late 1980s in Denmark. Therefore misclassification of mothers' smoking during pregnancy is not expected to play a major role in these studies.

With respect to SES, one could expect those with low annual incomes to be more reluctant to answer the question regarding total household income. This would result in an underestimation of women with lower SES compared with the source population. It is however highly unlikely that this underestimation would be related to their sons' semen quality or reproductive hormone levels.

7.1.3 Confounding

The result of confounding - confusion of effects - leads to bias. Consequently, confounding is a central concern in epidemiological studies. A confounding variable is an independent risk factor of the outcome, which is not on the causal path between exposure and outcome (e.g. an intermediate step), and is causally or non-causally associated with the exposure.
Abstinence time, maternal smoking during pregnancy [106], the participants' own smoking status [107], and sons' BMI [108], are all variables that have been suggested to be risk factors for semen quality measures. They were chosen as possible confounders because they potentially could be unevenly distributed in the exposure groups, which could lead to bias if they were not taken into consideration. If for example, men exposed to the highest POP levels *in utero* also were the ones being most exposed to maternal smoking during pregnancy, higher sperm concentrations among the men less exposed to POPs *in utero* could then be misinterpreted as an inverse effect on sperm concentration solely attributed to POPs rather than smoking if we did not adjust for smoking in the model.

In study IV, the number of participants was reduced to 155, which limited our opportunities for confounder adjustment. Hence, adjustment for the participants' own smoking status and BMI were not included in the model, although these potential confounder variables were included in the models in study II and III, where associations were investigated by multivariable regression analyses.

Initially it was discussed, whether or not to include parity as a potential confounder in the models for study II-IV. Parity could potentially be a risk factor for the outcome if you would think that multiparous women have a high ability to reproduce, which they would genetically pass on to their offspring. Additionally, parity is related to prenatal exposure, as substantial

amounts of POPs are transferred from the mother to the infant by breastfeeding. Hence, maternal POP levels during pregnancy would be dependent on previous breastfeeding.
However, parity and measuring POP levels directly in maternal serum samples taken during pregnancy actually provide similar information – namely a proxy for prenatal POP exposure. Thus, an inclusion of parity in the model could possibly lead to over adjustment of the exposure and because parity precedes prenatal exposure in time, and we have a measure for prenatal exposure from the maternal serum samples from pregnancy week 30, we chose not to adjust for parity in our models. A sub-analysis adjusting MI ∑PCBs, ∑DL-PCBs, and p,p'-DDE analyses for parity (nulliparous yes/no) did not change the estimates or directions of the associations.

We cannot exclude the risk of confounding caused by factors that we did not or were not able to adjust for. In addition, residual confounding, resulting from too gross covariate classifications or subdivisions, cannot be excluded. However, we have no specific reason to suppose that our results were confounded by unknown factors or residual confounding.

7.2 Main findings in light of other studies

The method for conventional semen analysis has been standardised and by following the WHO recommendations on the examination and processing of human semen, results should be comparable between laboratories worldwide. The conventional assessment, however, is still limited due to the subjectivity of the assessments, which can introduce considerable variation over time, between examiners, and between laboratories – especially if examiners have not been properly trained and quality control is not monitored. The CASA method provides a quick and more objective alternative to the conventional assessment, yet several different CASA systems using different algorithms exist, which complicates standardization between CASA methods. Here we found sperm concentration and motility category medians to differ substantially between the CASA assessment using CRISMAS Clinical software Version 4.6 and the conventional method for semen analysis, in addition to a high ICC for sperm concentration assessments and low ICCs between the motility categories. As both methods might be associated with measurement error, we do not know the true value of the semen analysis results, but the conventional method is generally considered the gold standard. Another study comparing sperm concentration assessed by conventional semen analysis and CRISMAS CASA (Version 1) found a less pronounced overestimation of sperm concentration by CASA as compared to our study [109]. This could be due to differential

performance of the CASA systems used in the two studies (Version 1 versus Version 4.6) or to differences in the assessments of conventional semen analysis. Discrepancies similar to ours between CASA and the conventional method have been described by others [110]. Yet, more recent studies have shown good compliance between different CASA systems and the conventional method for semen analysis [111-113], and several have suggested that CASA estimates of sperm concentration and sperm motility are significantly associated to probability of conception [109,114-115].

The latest WHO manual on semen analysis [116] highlights that using CASA systems for routine diagnostic applications should be subject to great care in terms of use and settings of the instrument ensuring optimal performance in relation to repeatability and reliability. Also, quality control procedures should be carefully followed in order to establish high standards of CASA measurement [116].

Due to the reported discrepancies between the two methods for semen analyses in our study, we chose to report both conventionally assessed and CASA results in all the manuscripts included in the thesis.

Our studies were the first longitudinal studies to report on long-term associations between *in utero* exposure to PFAAs, PCBs, and p,p'-DDE and markers of semen quality and reproductive hormone levels in young adulthood. The longitudinal design, chosen for the studies in the present thesis, is advantageous to cross-sectional studies attempting to evaluate on the same hypotheses. This longitudinal setting allowed us to investigate the hypothesis that exposures to POPs during critical phases of fetal development are associated with long-term effects on male reproductive health in adulthood.

We found inverse associations between prenatal exposure to PFOA and sperm concentration, total sperm count, and CASA percentage progressive motility in addition to positive associations to LH and FSH levels in young adulthood. No associations between *in utero* exposure to PFOA and semen volume or sperm morphology or any of the remaining reproductive hormones were identified. Additionally, there were no statistically significant associations between any of the measures of semen quality or reproductive hormone levels and *in utero* exposure to PFOS. The few experimental studies having investigated this hypothesis in rats developmentally exposed to ammonium PFOA were not suggestive of adverse effects on the male reproductive system [75,76]. Human cross-sectional studies, however, have suggested inverse associations between serum levels of PFOA and PFOS in adulthood and sperm morphology [117,118]. Additionally, adult life plasma levels of PFOA showed positive correlations with LH and free testosterone levels and serum PFOS levels

were inversely associated with free and total testosterone [119,120]. Differences in the associations between *in utero* exposure and adult life exposure to PFOA and PFOS on semen quality measures could possibly be explained by different vulnerability towards external exposures during prenatal life and adulthood. For example, sperm morphology might be most sensitive to exposures taking place in adult life during sperm maturation, while sperm concentration and total sperm counts would be most affected by exposures taking place during fetal reproductive organ development and Sertoli cell proliferation and differentiation.

We observed positive associations between PFOA exposure during fetal life and LH and FSH levels in adulthood. This is in agreement with our findings of inverse associations between PFOA and sperm concentration and total sperm count, since gonadotrophin levels are generally inversely associated with sperm concentration and total sperm count as suggested by several studies of the general population and subfertile men [121-123]. Furthermore, because proliferation of Sertoli cells during fetal life is a determinant for sperm production capacity later in life, the inverse association between prenatal PFOA exposure and sperm concentration and total sperm count could also be an indication of PFOA acting on Sertoli cells *in utero*, but the mechanism remains unclear.

Although *in utero* exposure to PFOA was associated with higher LH levels, exposure was not associated with reduced testosterone levels in adulthood. However, a subanalysis showed testosterone/LH ratio to be inversely associated with *in utero* exposure to PFOA, indicating that prenatal PFOA exposure may also have long-term consequences on Leydig cell function in adult life.

One study has suggested that postnatal dioxin exposure via breastfeeding, rather than prenatal exposure, is associated with reduced semen quality [82]. PFAAs are transferred from mother to child via breastfeeding resulting in postnatal exposure correlated with prenatal exposure [31]. Hence, it cannot be excluded that the associations reported here are partly explained by postnatal exposure.

We found no associations between *in utero* exposure to ∑PCBs, ∑DL-PCBs, and p,p'-DDE and semen quality or reproductive hormone levels when evaluating results from the main multiple imputation models. However, complete case analyses and the MI model based on the best transformations of the outcomes, in terms of achieving a normal distribution of residuals, suggested a positive association between prenatal p,p'-DDE exposure and sperm concentration. However, under the assumption that our data were missing at random and given that all assumptions for the outcome variables imputed on the continuous scale were met, we expect MI results to be closer to the "true" values compared with complete case data.

Thus, we did not observe adverse associations between prenatal exposure to PCBs and p,p'-DDE and semen quality, testicular size, or reproductive hormone levels in young adulthood.
In contrast to our findings, animal studies on gestational and lactational exposure to PCB-118 and PCB-101, covering a span between slightly elevated levels and orders of magnitude higher PCB levels than what humans are exposed to, have indicated reduced testis weights, in both rat and mice offspring [66,67]. Decreased daily sperm production and sperm counts were found in rats [66] and reduced sperm viability but not reduced sperm concentration was observed in developmentally exposed mice [67] suggesting associations between prenatal PCB exposure and male reproductive capacity in rodents.
In line with our results, *in utero* exposure to PCB and p,p'-DDE has been found not to be associated with increased risks of cryptorchidism and hypospadias in the offspring [60,63-64], whereas some are indicative of small increased risks [59,61-62]. Studies on the reproductive health of the young men exposed to high levels of PCBs *in utero* as a result of the Yu-Cheng oil-disease in 1979 and the above mentioned study on perinatal exposures to dioxin, resulting from the Seveso accident in 1976, are suggestive of adverse effects of pre- and perinatal exposures on the male reproductive system [81,82]. This is contradictory to the findings of our study on PCBs and dioxin-like PCBs but may be explained by differences in exposure levels between the studies.

Associations between PCBs and p,p'-DDE and sperm quality markers have been suggested to be modified by *AR* CAG repeat lengths in a cross-sectional study of 680 men from four regions (Greenland, Sweden, Warsaw (Poland), and Kharkiv (Ukraine) representing different exposure levels to POPs [90].
As an extension to our pervious findings we further examined whether the associations identified between *in utero* exposure to PFOA and semen quality and reproductive hormone levels were modified by offspring *AR* CAG repeat lengths. This study suggested a tendency towards shorter (<21) or longer (>23) *AR* CAG repeats associated with increased vulnerability towards exposure to PFOA *in utero* with respect to measures of male reproductive health in early adulthood.
In the study by Giwercman et al. 2007, they found that differences between below and above median exposure to PCB-153 and p,p'-DDE levels for sperm concentration, total sperm count, and DFI were most pronounced for men with short CAG repeats (<22) [90]. This is in concordance with our results except that we also found indications of increased vulnerability towards *in utero* exposure to PFOA for participants with long CAG repeats (>23). Also, 22 CAG repeats compared with 16 and 28, respectively, is associated with optimal AR receptor

activity *in vitro* and increased odds of infertility for men with shorter (<22) or longer (>23) CAG repeats compared with the reference 22-23 repeats [86,87], indicating that that CAG repeat lengths are associated with AR activity in a non-linear manner, which supports the non-linear vulnerability relationship between PFOA and number of CAG repeats suggested in our study.

8. Conclusions

The two methods – conventional semen analysis and CRISMAS CASA – used for assessment of sperm concentration and sperm motility classifications in the present thesis were not compatible. The most pronounced differences between the methods were classification of rapidly- and slowly progressive spermatozoa. Consequently, sperm analysis results from both methods were reported in the manuscripts included in the thesis.

Results from the follow-up studies suggested that *in utero* exposure to PFOA was associated with long-term consequences on male reproductive health, and thus was in support of the endocrine disruptor hypothesis. In extension of these results, including sons' number of *AR* CAG repeats in the model suggested that the number of CAG repeats appeared to modify individual susceptibility towards prenatal PFOA exposure with respect to male reproductive health measures, suggesting a most pronounced effect for those with CAG repeats shorter or longer than 21-23.

Associations between *in utero* exposure to PFOS, ∑PCBs, ∑DL-PCBs, and p,p'-DDE and semen quality, testicular size, or reproductive hormone levels were not suggestive of long-term effects on male reproductive health.

9. Perspectives and future research

With our findings suggesting long-term consequences of prenatal PFOA exposure on male reproductive health and no apparent effects of *in utero* exposure to PFOS, PCBs, and p,p-'DDE, we may have provided a small piece of the puzzle trying to explain how come a large proportion of young med in western countries have reduced or suboptimal semen quality - but the puzzle is far from complete. There is still a lot we do not know with respect to *in utero* exposure and adverse effects on the male reproductive system – also with respect to compounds that are not persistent in the environment. Also, even though exposure to many of the well known POPs has decreased significantly during the past decade, exposures levels of

some of the newer compounds are increasing. Thus, persistent pollutants in the environment will probably be a cause for concern for many years to come.

A limitation to our study is that we only assessed one exposure at a time, which has been tradition in epidemiological studies. Since we are not solely exposed to a single chemical at a time, but rather a cocktail of many different potentially harmful substances, that may be able to produce adverse effects even when each individual exposure level is low, this is probably not the most optimal approach. Recent advances in methods for chemical exposure assessments are now allowing us to measure a multitude of chemicals in relatively small volumes of biomaterial. Thus, a significant future challenge in the field of reproductive epidemiology is to find the most optimal method to evaluate associations between a variety of different compounds and reproductive health outcomes simultaneously.

It is important that we take up this challenge and seize the opportunity to be able to bring the puzzles together with respect to potential endocrine modulating agents and other exposures that are potentially hazardous to reproductive health.

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Published manuscripts:

1. **A comparison of conventional and computer-assisted semen analysis (CRISMAS software) using samples from 166 young Danish men.** A. Vested, C. H. Ramlau-Hansen, J. P. Bonde, A. M. Thulstrup, S. L. Kristensen, and G. Toft. *Asian Journal of Andrology*, 2011, 13 (3): 453-8. doi: 10.1038/aja.2011.14

2. **Associations of in Utero Exposure to Perfluorinated Alkyl Acids with Human Semen Quality and Reproductive Hormones in Adult Men.** A. Vested, C. H. Ramlau-Hansen, S. F. Olsen, J. P. Bonde, S. L. Kristensen, T. I. Halldorsson, G. Becher, L. S. Haug, E. H. Ernst, and G. Toft. *Environmental Health Perspectives*, 2013, 121 (4):453-8 doi: 10.1289/ehp.1205118

3. **In utero exposure to persistent organochlorine pollutants and reproductive health in the human male.** A. Vested, C. H. Ramlau-Hansen, S. F. Olsen, J. P. Bonde, H. Støvring, S. L. Kristensen, T. I. Halldorsson, P. Rantakokko, H. Kiviranta, E. H. Ernst, G. Toft. *Reproduction*, 2014, 148(6): 635-646 doi: 10.1530/REP-13-0488

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