Persistent organochlorine pollutants and human reproductive health

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The thesis is based on the following 10 papers:

1. Toft G, Hagmar L, Giwercman A, Bonde JP. Epidemiological evidence on reproductive effects of persistent organochlorines in humans. Reprod Toxicol 2004,19:5-26.
2. Toft G, Rignell-Hydbom A, Tyrkiel E, Shvets M, Giwercman A. Quality control workshops in standardization of sperm concentration and motility assessment in multicentre studies. Int J Androl 2005, 28:144-149.
3. Toft G, Axmon A, Giwercman A, Thulstrup AM, Rignell-Hydbom A, Pedersen HS, Ludwicki JK, Zvyezday V, Zinchuk A, Spano M, Manicardi GC, Bonefeld-Jørgensen EC, Hagmar L, Bonde JP; INUENDO. Fertility in four regions spanning large contrasts in serum levels of widespread persistent organochlorines: a cross-sectional study. Environ Health 2005, 4:26.
4. Toft G, Pedersen HS, Bonde JP; INUENDO Research Team. Semen quality in Greenland. Int J Circumpolar Health, 2004, 63:174-178.
5. Toft G, Rignell-Hydbom A, Tyrkiel E, Shvets M, Giwercman A, Lindh CH, Pedersen HS, Ludwicki JK, Lesovoy V, Hagmar L, Spanó M, Manicardi GC, Bonefeld-Jorgensen EC, Thulstrup AM, Bonde JP. Semen quality and exposure to persistent organochlorine pollutants. Epidemiology. 2006, 17:450-458.
6. Toft G, Long M, Krüger T, Hjelmborg PS, Bonde JP, Rignell-Hydbom A, Tyrkiel E, Hagmar L, Giwercman A, Spanó M, Bizzaro D, Pedersen HS, Lesovoy V, Ludwicki JK, Bonefeld-Jørgensen EC. Semen quality in relation to xenohormone and dioxin-like serum activity among Inuits and three European populations. Environ Health Perspect 2007, 115:15-20.
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impact on human sperm chromatin integrity. Hum Reprod 2005, 20:3488-3499.
8. Toft G, Axmon A, Lindh CH, Giwercman A, Bonde JP. Menstrual cycle characteristics in European and Inuit women exposed to persistent organochlorine pollutants. Hum Reprod 2008, 23:193-200.
9. Toft G, Thulstrup AM, Jönsson BA, Pedersen HS, Ludwicki JK, Zvezday V, Bonde JP. Fetal loss and maternal serum levels of 2,2',4,4',5,5'-hexachlorbiphenyl (CB-153) and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE) exposure: a cohort study in Greenland and two European populations. Environ Health. 2010, 9:22.
10. Bonde JP, Toft G, Rylander L, Rignell-Hydbom A, Giwercman A, Spano M, Manicardi GC, Bizzaro D, Ludwicki JK, Zvyezday V, Bonefeld-Jørgensen EC, Pedersen HS, Jönsson BA, Thulstrup AM; INUENDO. Fertility and markers of male reproductive function in Inuit and European populations spanning large contrasts in blood levels of persistent organochlorines. Environ Health Perspect 2008, 116:269-277.

INTRODUCTION

A considerable proportion of apparently healthy young couples are experiencing problems achieving pregnancy. Approximately 15% of European couples have to wait more than one year after the cessation of contraception until pregnancy is achieved [1] and more than 6% of all children born in Denmark are conceived after the use of assisted reproduction [2]. Whether or not the quality of human semen is decreasing has been under debate [3;4], but it is evident that a considerable proportion of young men have suboptimal semen quality [5].

Several potential causes of suboptimal human semen quality have been indicated, including lifestyle factors such as smoking, obesity, alcohol and stress [6], physical factors such as heat [7], biological factors such as sexually transmitted diseases [8] and chemical factors including exposure to pesticides and industrial chemicals [9]. Also, female reproductive function may be seriously affected by most of the abovementioned factors [10].

The possible effects on human reproduction after exposure to environmental contaminants, within the range experienced by the general population, have been addressed in some studies but often with conflicting results [11-13].

However, there are considerable numbers of animal studies that point towards adverse effects of environmental toxicants on male and female reproductive health in both experimental and wildlife

settings [14-17], although the exposure levels that caused reproductive toxicity in most of these studies were several times higher than the human exposure level observed in the general population.

Of particular concern regarding the adverse effects on human fertility is the exposure to compounds with endocrine-disrupting capabilities, which are suspected to interfere with the development and functioning of sex hormone-regulated processes such as sperm production and ovulation [18;19]. Due to interference with hormone receptors or steroid producing or degrading enzymes, even low concentrations of these compounds could disturb endocrine-regulated processes.

Several environmental pollutants can be measured in human biological samples from almost all people worldwide. Some of the environmental pollutants detected at the highest concentrations in human serum belong to the group of organochlorine pollutants. Among the organochlorine pollutants the polychlorinated biphenyl (PCB) congener PCB-153 and the dichlorodiphenyltrichloroethane (DDT) degrading product dichlorodiphenyldichloroethylene (DDE) are generally the most commonly detected compounds in human serum samples [20]. The chemical structures of PCB and DDE resemble steroid hormones (Table 1) and endocrine properties have been demonstrated for these compounds [21;22]. Studying the effects of selected organochlorine pollutants on human reproductive health may provide an insight into whether or not exposure to a low level of environmental chemicals affects human reproductive health.

Table 1. Structural formula of the most potent natural oestrogen and androgen compounds in humans and classes of selected endocrine-disrupting compounds

Name	Structural formula	Oestro-genic[a]	Andro-genic[a]	Reference
17β-oestradiol		+	+	[23]
Dihydro-testosterone		+	+	[23]
PCB-153		-	0	[22]
p,p'-DDT		+	-	[23;24]
p,p'-DDE		0	-	[25]
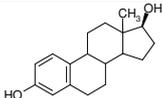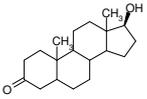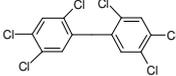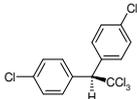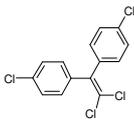
[a] Indicates whether or not the selected compounds showed endocrine activity when assessed in in vitro assays: + = agonistic effect; - = antagonistic effect, 0 = no (anti)oestrogenic or (anti)androgenic effects.

Persistent organic pollutants (POPs) are organic compounds that are highly resistant to environmental degradation, with half-lives in the environment and in living organisms in the range of several years. Persistent organic pollutants bioaccumulate in fatty tissues in organisms and become biomagnified through the food chain from lower organisms to top predators, including humans. An important class of POPs are organochlorines, which include a number of anthropogenic compounds that were manufactured on a large scale since the 1930s. Concentrations of the most common PCB congeners and DDE and several other organochlorines such as chlordane, aldrin, dieldrin, hexachlorobenzene, toxaphene and dioxins were found to be highly correlated with serum samples from the general human population, and exposure to the most common PCB-congener (CB-153) has therefore been suggested as an indicator of the overall level of exposure to persistent organochlorines [26;27]. However, the congener profile of PCBs and correlations with other organochlorine compounds can differ considerably between populations due to differences in the sources of exposure and the time since peak exposure [28], and therefore different exposure profiles between countries may induce heterogeneity in exposure-response associations based on single contaminant measurements.

The production of PCBs and DDTs has been limited or completely banned since the 1970s in most developed countries. The last PCB production facility in Russia was shut down in 1993. Despite this, organochlorine compounds, including PCBs, are still being released into the environment from previously produced material or through combustion processes. Polychlorinated biphenyls have been used in a number of industrial applications including hydraulic fluids and insulating fluid in electrical transformers and capacitors, and also in building construction as an additive to caulking, grouts and paints.

In particular, their use in window and door-sealing materials in buildings still confers a direct exposure route to humans, especially the low-level chlorinated more volatile PCB congeners [29]. However, dietary exposure is still the main route of exposure of the majority of PCB congeners.

The average concentration of PCBs in human tissues has decreased since the ban on the use of these compounds in most countries. During the past 10 years, this decrease has levelled off. Compounds are still detected in blood and milk samples in the vast majority of humans worldwide [30]. The level of DDE has also decreased since the ban on the use of DDT in the 1970s in most developed countries, but due to its low costs and high efficiency as a pesticide against mosquitoes carrying malaria, DDT is still produced and used, mainly in India and several African countries, with an annual production of 4-5000 tonnes, and it was used until the year 2000 in South America and until 2003 in China [31].

In addition to experimental studies on animals, two episodes of accidental contamination of food with PCBs in Taiwan and Japan provided evidence regarding the effects of high levels of human exposure on reproduction. Also, observational studies have addressed the effects of more moderate exposure levels to POPs on human reproductive health. These studies indicated that high concentrations of persistent organochlorines can cause spontaneous abortions, delayed pregnancy and reduced birth weight and that they can induce a skewed sex ratio and alter the age of puberty onset [11;12;32]. Most of the effects were demonstrated

at exposure levels above the present exposure level in European populations. However, it is not clear whether the present day low levels of POP exposure found in European populations can affect human reproductive health or not. Several reviews pointed out that additional research is needed to elucidate whether or not low levels of persistent organochlorine pollutants affect human reproductive health [12;13;32;33].

Methods of studying male fertility
Several methods have been developed in order to study male fertility. Semen quality studies are frequently used as a marker of male fertility. The probability of achieving pregnancy (fecundability) has been demonstrated to be highly dependent on concentrations of sperm of up to about 40 million/ml [34-36]. In 2010, the World Health Organization (WHO) reference value for a "normal" sperm concentration was set at 15 million/ml based on the 5th percentile of sperm concentrations among fertile men with a time to pregnancy of less than one year [37]. The new guidelines have been widely discussed. Skakkebaek [38] suggested multiple cut-off points, with the new WHO level (15 million/ml) indicating a low sperm count and a level of less than 40 million/ml indicating a suboptimal sperm concentration, whereas Joffe [39] pointed out that it would be impossible to dichotomise sperm concentrations into levels indicating whether or not people need medical treatment for infertility since the association between sperm concentration and male fecundability can best be described as linear (up until a level of 40 million/ml).

In addition to sperm concentration, sperm morphology and motility are part of the standard evaluation of semen quality, and these measures have also been shown to be associated with fecundability, independently of sperm concentration [34]. Other attempts have been made to develop new measures of semen quality, including the development of the sperm chromatine structure assay, which identifies the proportion of single-stranded to double-stranded DNA after mild acid denaturation as a measure of DNA integrity. This measure has also been demonstrated to be associated with fecundability independent of sperm concentration [40].

Methods of studying female fertility
Time to pregnancy after the discontinuation of contraception has often been used as a measure of female fertility in studies on the effects of environmental or lifestyle exposures on female fertility. However, it should be noted that time to pregnancy is a measure of the couples fecundability and not the female partner alone [41]. Attempts have been made to use other measures associated with female fertility as markers of female fertility. These include studies of menstrual cycle characteristics and the risk of spontaneous abortions [42]. Furthermore, in addition to previously used hormonal markers such as follicle stimulating hormone (FSH), oestradiol, progesterone and inhibin B, recent advances in predicting ovarian reserve have been obtained by measuring anti-müllerian hormone (AMH), mainly among infertile women [43]. In a recent study, the AMH was also indicated as a predictor of fecundability among the general population [44].

Update on the epidemiological evidence regarding the effects of persistent organochlorines on human reproduction
A thorough review on the epidemiological evidence up to 2004 regarding the effects of persistent organochlorines on human reproduction was presented in [13] as part of the dissertation (discussed in detail later). Since 2004, a number of studies have been published in addition to the papers included in the present thesis. We published an updated review on the epidemiological evidence of impaired reproductive health and cancer related to endocrine-disrupting chemicals in 2009 [45], including papers published within the period from 2002-2007. The outcomes studied included cryptorchidism, hypospadia, semen quality, menstrual cycle, endometriosis, time to pregnancy, testis cancer and prostate cancer. The published studies indicated slightly and not statistically significant increased risk of cryptorchidism and hypospadias at higher DDE levels [46;47], whereas semen quality can be affected in highly exposed populations and sperm cell motility can be reduced at increasing PCB exposure levels (discussed in detail in the semen quality section later). Studies on menstrual cycles disturbances suggest that both PCB and DDE may affect this outcome, although not entirely consistent between studies (discussed in detail later). Associations between PCB exposure and endometriosis have been observed, although only in relative small studies [48;49]. Slightly increased waiting time to pregnancy has also been associated to PCB or DDE exposure [50;51]. A few smaller case-control studies indicates increased risk of testicular and prostate cancer at high PCB exposure level [52;53]. In general, the associations between organochlorine exposure and reproductive outcomes were weak, but the majority of the associations pointed towards adverse effects, although most of the associations studied were not statistically significant.

After 2007, the majority of the published studies evaluated reproductive outcomes measured at birth such as birth anthropometrics and the presence of genital malformations in relation to in utero organochlorine exposure. Table 2 presents an overview of the reproductive outcomes in recent studies after in utero exposure to persistent organochlorines, published from 2007-2010.

The results of most of the recent studies pointed towards negative effects of PCB exposure on birth weight [54-61], whereas the associations between exposure to other POPs (DDT, DDE, HCB, PCDD, PCDF) and birth weight were less consistent [56;58-62]. Other birth outcomes studied include genital malformations, and recent studies on these outcomes confirmed previous findings of weak associations at most between the POP exposure studied (PCB, DDT, lindane, mirex , HCE, beta-HCCH) and cryptorchidism and hypospadia [63-65]. A few studies addressed other issues such as foetal loss [60;66;67] and sex ratio [68;69]. No conclusive evidence of adverse effects of the POPs studied were found on these outcomes.

The majority of the studies have been performed on European or North American birth cohorts. Results from European birth cohorts on data regarding the association between PCB or DDE exposure and birth weight/gestational age were summarized in a recent meta-analysis confirming a negative effect of PCB on birth weight across populations [70].

Table 2. Studies on birth outcomes after in utero exposure to persistent organochlorine pollutants published from 2007-2010.

Reference Country (Study Acronym)	Design	N	Exposure & timing	Outcome	Summary of the results
[64] Spain. (INMA)	Nested case control	50 cases 114 controls	DDT, lindane, mirex in placenta samples	Cryptorchidism or hypospadias	Increased odds of urogenital malformations at higher exposure
[54] Denmark (DNBC)	Cohort	100	PCB measured in serum from pregnant women	Birth weight: Placenta weight	Reduced birth weight and placenta weight
[63] France	Case control	151 125	PCB, DDE in cord blood PCB, DDE in colostrum	Cryptorchidism	Higher DDE and PCB in colostrum of mothers of cryptorchid boys
[55] Slovakia (PCB-cohort)	Cohort	1057	PCB in maternal serum	Birth weight	Only association among Romani boys
[71] Germany, (Duisburg)	Cohort	104	PCDD, PCB in maternal blood	Testosterone and oestradiol in cord blood	Reduced foetal oestradiol and testosterone at higher PCDD; not PCB
[62] Norway, (HUMIS)	Case based	326	HCB in breast milk	Birth weight	Moderate associations between HCB foetal growth among smokers
[67] Greenland, Poland, Ukraine. (INUENDO)	Cohort	678	PCB, DDE in maternal serum	Foetal loss	Increased odds of foetal loss at high PCB and DDE
[57] USA	Cohort	899	PCB, PBB in maternal serum	Birth weight, gestational age	No significant associations
[65] USA	Nested case control	219 cases 564 controls	HCE, HCB, beta-HCCH in maternal serum	Cryptorchidism	No significant associations
[72] USA	Cohort	529 women; 1344 pregnancies	PCB, PBB in maternal serum	Spontaneous abortions	No association
[58] USA	cohort	404	DDE, PCB in maternal plasma	Birth weight, head circumference, birth length, Ponderal index, gestational age	DDE associated with lower birth weight and head circumference
[59] USA	cohort	722	PCB, DDE, HCB in cord serum	Birth weight, crown-heel length and head circumference	Small negative association between PCB and birth weight
[68] USA	Cohort	399	PCB (11 congeners) in maternal serum	Sex ratio	Decreased males at high PCB
[73] Japan (Yusho)	Cohort	512 pregnancies	PCB, PCDF in maternal serum Accidental exposure	Pregnancy loss, preterm delivery.	Preterm delivery higher after exposure. Pregnancy loss tended to increase.
[74] USA	Nested case control	230 cryptochidism 201 hypospadias 593 control	PCB measured in serum from pregnant women	cryptorchidism hypospadia	Risk of hypospadia slightly increased at higher PCB – no effect on cryptorchidism
[60] Japan	Population based	514	PCDD, PCDF and PCBs in maternal blood	Birth weight	PCDD and PCDF TEQ associated with reduced birth weight (boys only)
[69] USA	Cohort	865	PCB, PBB before giving birth	Sex ratio	Suggestions of increased males; no significant associations.
[61] Singapore	Population based	41	Organochlorine pesticides, PCB, PBDE in cord blood	Foetal growth; Apgar score.	Chlordane and PCB negatively associated with foetal growth

Main purposes of the thesis
The main purpose of the series of studies included in the present dissertation was to evaluate whether or not the present level of PCB and DDE exposure affects human reproductive functioning. In addition to a review of existing studies, this was evaluated in a series of empirical studies based on the INUENDO study cohort (see details later in the methods section).

The following main specific research questions were addressed through a series of empirical studies [67;75-80]:
1) Can semen quality be assessed accurately across populations?
2) Does human fertility vary between and within selected European countries showing large contrasts in exposure levels to PCB and DDE?
3) Does exposure to PCB and DDE and markers of xenobiotic activity interfere with semen quantity and quality after exposure in adulthood?
4) Does exposure to PCB and DDE affect female reproductive functioning measured as menstrual cycle irregularities and risk of spontaneous abortions?

METHODS
Study design and recruitment to the INUENDO study
The INUENDO study was a multi-centre study of male and female fertility in relation to biopersistent organic pollutants supported by the European Union 5th framework programme (contract no. QLK4-CT-2001-00202), www.inuendo.dk. This study was designed as a pregnancy-based cross-sectional study on male and female fertility, with the secondary aim of establishing a cohort available for future follow-up studies.
Pregnant couples were recruited for the study from Greenland, Ukraine and Poland, whereas only a period of attempting to get pregnant was required for the inclusion of Swedish fishermen's wives. The females filled in a standardized questionnaire on lifestyle and reproductive health and had a blood sample drawn. Male partners of the pregnant couples were included, if possible, and filled in a questionnaire and had a blood sample drawn. The Swedish fishermen were recruited independently of the included cohort of fishermen's wives. In all four populations a sub-sample of men delivered a semen sample. In total, 2269 females were recruited for the study and were interviewed about reproduction and lifestyle factors, whereas 798 men participated in a semen study. The participation rates varied somewhat between populations. Among the women, the largest participation rate of 90% was achieved in Greenland whereas only 26% in Ukraine agreed to participate. The participation rate in the semen study varied from 79% in Greenland to 7% in Sweden (Table 3).

The serum samples were analysed for the presence of CB-153 and p,p'-DDE [81]: CB-153 was detected in 96.4% of the samples whereas p,p'-DDE was detected in 99.7%. Serum samples that contained amounts of these organochlorines that were lower than the detection limit was assigned a value of half the detection limit before inclusion in the epidemiological analyses. Furthermore, serum triglyceride and cholesterol levels were determined and the values were adjusted for lipids based on the formulae: Men: Total = 0.96 + 1.28 x (triglycerides + cholesterol); Women: Total = 1.13 + 1.31x (triglycerides + cholesterol) [82], and the lipid-adjusted values were used in all of the epidemiological analyses.

Table 3 Number of people approached for the INUENDO study and participation in different aspects of the study

	Poland Warsaw	Greenland Inuits	Ukraine Kharkiv	Sweden Fishermen
Eligible female target population, n	690	665	2478	1439
TTP interviews, n	472	598	640	559
Participation rate %	68	90	26	39
Female blood samples, n	261	572	612	544
Participation rate %	38	87	25	38
Male interviews, n	472	576	637	195
Male blood samples, n	257	439	287	195
Eligible men approached for the semen study, n	690	256	640	2783
Semen samples, n	198	201	208	191
Participation rate %	29	79	33	7

The xenobiotic responses of oestrogen- (ER), androgen- (AR) and Aryl hydrocarbon (Ahr) -receptor mediated activity were measured in serum samples from a total of 262 men for the AR assay, 338 for the AhR assay and 358 for the ER assay. These were measured as agonistic or antagonistic responses in cell lines co-transfected with the ER, AR or Ahr receptors. In order to eliminate interference from endogenous hormones, the serum samples were fractionated before analysis and only the fraction containing persistent organochlorines and the majority of other endocrine-disrupting compounds, but not endogenous oestrogens or androgens, were analysed [83-85].

Implications of the study design and epidemiological analyses
Basically, the studies were designed as four separate studies of exposure to environmental contaminants and effects on human fertility, performed using standardized methods in the four populations.

Since the study involved a number of statistical evaluations of associations between exposures and outcomes, the risk of chance findings in the separate populations was high, but including four separate populations in the design allowed us to look for consistency between populations and dose-response associations over a larger range of exposures than would have been possible if the study was only carried out on a single population. Therefore, all of the results presented herein include evaluations in single countries and – if homogeneity tests allowed it – a combined estimate across populations was also presented.

Discussion of the results
Review of the epidemiological evidence of adverse effects of organochlorines on reproductive health
A systematic review of the epidemiological evidence of the effects of persistent organochlorines on human reproductive health [13] found that exposure to high levels of persistent organochlorine pollutants may have severe human reproductive health effects, including adverse effects on semen quality and increased risk of testicular cancer in males, and menstrual cycle abnormalities, spontaneous abortions, increased waiting time to pregnancy, reduced birth weight, skewed sex ratio and altered age at sexual development in females. However, most of these effects were

demonstrated at higher levels of exposure than the present day exposure levels in Europe and North America.

After the main INUENDO studies were completed we published a review on the epidemiological evidence of impaired reproductive health and cancer related to endocrine-disrupting chemicals [45], and also other authors have recently published reviews on the reproductive toxicity of persistent organochlorine pollutants [9;11;86-88]. The main conclusions from these reviews established the fact that there were indeed some associations between POP exposure and reproductive outcomes. For example, an association between PCB exposure and sperm cell motility across a wide range of exposure levels have now been sufficiently documented [77], and DDT/DDE exposure seems to only affect human semen quality at a high level of exposure, as found among workers spraying DDT or people who lived in recently DDT-sprayed houses. However, as pointed out by several of the reviews, large uncertainties still exist on the potential long-term adverse effects of POP exposure during the foetal period of life.

Quality control and standardization of semen analyses
Previous studies showed considerable variations in semen quality assessment between technicians before attempts were made to standardize the process [89;90].

In order to assure comparable data in the four countries included in the INUENDO study, a series of quality control workshops were organized. We demonstrated how repeated quality control workshops can reduce and achieve low variance between semen analysers in the assessment of sperm concentration and motility [75]. For the sperm concentration assessment, the variation decreased from an initial coefficient of variation of 27.7% in the first workshop to 8.1% in the third workshop on data collection. This method was less effective for reducing the coefficient of variation (CV) in the assessment of motile sperm proportions, which decreased from 16.5 to 11%. The results of the trained semen analysers were also compared to those of "experts" and similar results of sperm concentration and motility were found comparing trained semen analysers and "experts" at the third workshop. The CV for sperm concentration assessment in the third workshop was considerably lower than the average CV of 15.2% obtained in an American multi-centre study from the same time period [91]. Also, sperm motility varied slightly less in our study than in the American study (13.6%). In that study quality control was assessed based on a shipment of conserved semen samples and videotaped motility sequences after initial training of the laboratory technicians who performed the study.

Our study [75] indicates that repeated training on fresh samples results in low levels of variation, which is of the utmost importance when performing a multi-centre study such as the INUENDO study, especially when semen quality values are compared between countries, where systematic differences in the assessment of semen quality can easily produce false differences between countries if the technicians have not been thoroughly trained before data collection.

Fertility across and within the countries studied
Previous studies demonstrated considerable variations in semen quality and time to pregnancy between countries [1;5]. Fertility levels in the populations included in the INUENDO study have not previously been compared, but considerable genetic variation between the populations, ranging from the Inuits in Greenland to north and eastern European populations, making observations of differences likely. We compared fertility levels in the four regions by including comparisons of waiting time to pregnancy and semen quality between the four populations [92].

Differences in fertility between countries were observed, with the lowest fecundability ratio in Kharkiv – in between in Warsaw and Greenland – and the highest fecundability ratio among the Swedish fishermen. Although these countries varied in the levels of exposure to organochlorine pollutants, differences in the methods of contraception used between the countries could also have been a likely cause of the differences observed. The semen quality was remarkable similar between the countries, with no statistically significant differences in adjusted sperm concentration or morphology. However, sperm motility differed somewhat between the countries, with a slightly lower proportion of motile sperm in Kharkiv (54%) and Greenland (55%) compared to Warsaw (60%), which was statistically similar to Sweden (56%). Based on the results from the quality control assessment presented above, these differences are not likely to be due to sampling differences between countries.

Also, within Greenland, the sperm cell concentration did not seem to markedly differ between regions, but sperm cell motility differed between regions with an average of 47% motile sperm in east Greenland compared to 54 to 59% among men from the west coast [76]. The study further suggested that the median sperm cell concentration found in Greenland was within a low range compared to most of the other previously studied European, American and Asian populations [5;93;94]. However, this comparison should be interpreted with caution due to a lack of control for known, strong determinants of semen concentration, such as sexual abstinence time. Furthermore, the study demonstrated that a semen study can be performed with a high participation rate (78%), as compared to previous studies with participation rates ranging from 15% to 54% [5;93;94]. The high participation rate in Greenland was probably due to detailed personal information to potential participants about the project and a high level of awareness of the Greenlandic population about the potential adverse effects of organochlorine pollutants.

Persistent organochlorine pollutants and semen quality
We evaluated the effects of CB-153 and p,p'-DDE on sperm concentration, motility and morphology and found no association between CB-153 or p,p'-DDE exposure and sperm concentration or morphology [77]. However, in all of the four countries studied, sperm cell motility tended to decrease with increasing CB-153 exposure, and this association was statistically significant in the Greenlandic and Swedish populations, and in the pooled analysis where sperm cell motility decreased by 3.6% CI (1.7 to 5.6) per log unit increase in CB-153. When we analysed the data separately for the four exposure groups it seemed that only the high exposure group (>400 ng/g lipid) had significantly reduced sperm motility compared to the low exposure group (0-50 ng/g lipid CB-153). Furthermore, p,p'-DDE also seemed to be related to sperm motility in the Greenlandic population, but not significantly so in any of the remaining populations, including Kharkiv, where the highest level of exposure was observed, suggesting that the effects observed for DDE could have been caused by the high correlation between PCB and DDE in the Greenlandic population. In conclusion, PCB and DDE are unlikely to have an effect on sperm cell concentration or morphology at the exposure levels found in the general population in Europe, whereas sperm cell motility

could be affected among Europeans and Greenlanders with the highest levels of exposure to PCB. Our results on decreased sperm motility in relation to PCB exposure are in line with the results of previous studies on smaller populations [95-98], and by adding our study to this series of studies, it appears to be likely that semen motility can be affected by PCB exposure.

In populations with present or recent DDT usage, the levels of exposure to this compound are several times higher than the level measured in the INUENDO study. In a study that included 311 South African men who used DDT indoors to protect against malaria, the authors found that the total sperm count and sperm cell motility was negatively associated with DDT or DDE exposure [99]. Also, in a study of 116 Mexican men, sperm cell motility, morphology and sperm chromatin condensation were negatively affected in the group with the highest exposure level [100], and a negative association between DDT exposure and sperm concentration was observed among 60 South African men working in a malaria control centre that used DDT [101]. These findings on DDT/DDE exposures thus indicates that DDT/DDE exposure in highly exposed populations can be associated to semen quality. However, the level of exposure to these compounds observed in the general population in countries without present or recent use is probably not causing any effect on male reproductive function.

In addition to direct chemical measurements of PCB-153 and p,p'-DDE, xenohormone and dioxin-like activity were measured to explore potential integrated endocrine-active effects of organochlorines and other chemicals in human serum samples on the reproductive outcomes studied [84;85;102]. Xenobiotic receptor activities were determined in 319 men using a chemically activated luciferase gene expression (CALUX) assay. We found no strong or consistent associations between xeno-oestrogenic, -androgenic or dioxin-like activity measured as both agonistic and antagonistic responses in receptor assays [78]. However, the results suggested that sperm concentration and motility could be related to antioestrogenic effects, although this was only found in one of the two assays designed to test this. Thus, it seemed that exposure to the concentrations experienced in the present study, for a mixture of compounds, did not interfere with the oestrogen-, androgen- or dioxin receptors in a way that causes effects on human semen quality. Furthermore, we were not able to demonstrate that the effects on sperm cell motility were due to xenohormone effects of the persistent organochlorine pollutants, although we cannot exclude possible effects of endocrine-disrupting compounds on semen quality that were not captured by the receptor assays performed.

Associations between xenohormone activity and sperm DNA damage and markers of apoptosis were also described in the INUENDO population [103;104]. In these studies, we found no consistent associations in the xenobiotic-induced receptor activities between the populations studied. So far, no studies have attempted to replicate the findings of xenobiotic-induced receptor activity and the different aspects of semen quality, and, until additional studies are performed, no firm conclusion regarding a possible association can be made.

We did find a strong and dose-dependent association between the DNA fragmentation index (DFI) measured by the sperm chromatine structure assay (SCSA) and CB-153 exposure among the European populations, but not in the Greenlandic population, even though the highest exposure level was found there [79].

Another outcome measured by the SCSA: high DNA satiability (HDS), which is a measure of altered chromatin condensation, was not associated with CB-153. Exposure to p,p'-DDE was not associated with either DFI or HDS in any of the populations studied.

The average level of DFI was lower in Greenland compared to the European populations, and it was suggested that the Inuits from Greenland may be more resistant to CB-153 exposure, probably due marked differences in the genetic makeup that were partly elucidated in other studies on this population [105;106].

Markers of female fertility
The time from cessation of the use of contraception until the women in the INUENDO study got pregnant (time to pregnancy) was shorter among Greenlandic couples exposed to high levels of CB-153 and p,p'-DDE [50]. Increased time to pregnancy may be caused by either male or female reproductive problems.

We did not find consistent associations between CB-153 or p,p'-DDE exposure and menstrual cycle characteristics across the populations [80]. However, some associations were found within populations, including an increased risk of short cycles (<= 24 days) at high levels of CB-153 exposure in the population of Swedish fishermen's wives, and an increased risk of longer cycles after high levels of DDE exposure among the Polish women, but the opposite effect was found among Greenlandic women, who also seemed to be protected against long cycles at high levels of CB-153 exposure.

Associations between organochlorine exposure and menstrual cycle characteristics were previously evaluated in a number of studies [107-114]. However, the majority of these studies had small sample sizes and/or very imprecise exposure estimations based on, for example, the consumption of fish. Previous studies that included a considerable sample size and had more accurate exposure estimations include a study from the Collaborative Perinatal Project in the USA, which included 2314 women [111] and indicated increased cycle length and irregularity with increasing levels of PCB and DDE exposure. Another study on 466 Chinese textile workers showed increased risk of short menstrual cycles at high levels of DDT/DDE exposure [112]. The PCB exposure level in the American study was within the same range as found in our study, but the DDE levels in the abovementioned studies were considerably higher. Thus, our study, in combination with these previous studies, suggests that exposure to present day levels of PCB or DDE in Europe has only minor effects on menstrual cycles, but more severe effects in populations with present or recent DDT usage cannot be excluded.

Spontaneous foetal loss is an outcome that is frequently occurring in humans. About 1/3 of all pregnancies terminate before the foetus is fully developed. About 2/3 of these are lost early in pregnancy at the embryonic stage, and thus may not be recognised by the woman [115]. Previous studies indicated that POP exposure may be associated with the increased risk of spontaneous abortion [73;108;116-121], although other studies were not able to demonstrate such an increased risk after POP exposure [122-126]. In particular, the effects of POP exposure on spontaneous abortion at relatively low exposure levels have been contradictory, and additional studies in this area are needed. Our results indicated an increased risk of ever experiencing foetal loss in the groups with the highest levels of PCB and DDE exposure

compared to the groups with the lowest exposure levels (odds ratio 2.4; CI 1.1-5.5 for PCB-153 and OR 2.5; CI 0.9-6.6 for p,p'-DDE) [67]. However, no clear dose-response associations were observed and some differences between countries were observed, limiting the generalizability of the results. Furthermore, it should be noted that the associations observed were based on the retrospective recording of spontaneous abortions and that the levels of POP exposure measured at the time of the study were used as estimates of previous exposure levels.

The data on male and female reproductive health in the INUENDO project were reanalysed and summarized in [127]. We found similar effects to those reported above on the main male and female reproductive outcomes.

DISCUSSION OF METHODS
The participation rate in the INUENDO study varied somewhat between the populations included, ranging from 25% to 87% among the women and from 7% to 79% among the men who gave a semen sample (Table 3). The low participation rate in some of the populations increases the risk of selection bias. Previous studies demonstrated that participants in semen quality studies tend to be overrepresented by people with previous reproductive health problems [128]. Therefore, the comparison of fertility between countries [75] may have been biased due to differential selection in the different populations. However, the majority of the analyses [67;76-80] compared male or female fertility to their measured levels of xenobiotic exposure, which were unknown at the time when the potential participants decided whether or not to participate. Therefore, differential selection is not considered a main problem in these studies.

The misclassification of exposure level or an outcome represents another potential source of bias. Although the level of exposure was only measured once during pregnancy in the women or close to the time of providing a semen sample in the males, it is reasonable to believe that this exposure level represents a good approximation of the level of exposure during the relevant time period in relation to male sperm production and the female menstrual cycle and spontaneous abortions due to the slow degradation of the measured compounds in humans with a half-life of 9 years for DDE [129] and 14 years for PCB-153 [130]. In addition, some misclassification of the level of exposure due to variability in the results from day-to-day chemical assessments is expected, although strict quality control measures were followed and all of the quality control measurements showed levels within the tolerance levels [81].

Regarding the outcomes, some misclassification is also likely. Male semen quality is known to be highly variable from day to day and a single estimate may only provide a rough estimate of the average sperm concentration over a longer time period [131]. However, the between-subject variability is considerably larger than within-subject variability, and it has been estimated that samples from additional men would provide more information to epidemiological semen studies than additional samples from the same men [132].

Also the female fertility outcomes studied may not have been accurately determined. Both the menstrual cycle characteristics and incidence of previous spontaneous abortions were based on self-reported information, and some misclassification is therefore

likely. However, the women were unaware of their exposure levels at the time of reporting these data, which made recall bias unlikely.

Since the potential misclassification of exposure is not expected to be related to the outcomes studied and misclassification of the outcome is not expected to be related to exposure, the possible misclassification could attenuate the studied associations but it is not likely to produce false associations.

In observational studies such as this, confounding factors can affect the results. In each paper we carefully evaluated potential confounders and, when relevant, included these in the analysis. However, the fact that the results might have been affected by unmeasured confounders cannot be excluded, although we have no reason to believe we did not include any strong confounding factors in the associations studied.

Validity
Considerable effort was put into quality control assurance throughout the project. For semen quality, [75] describes the variability in semen quality measures between the semen analysers. Particularly after training, we obtained a between-observer coefficient of variation of less than 11% on the semen quality outcomes studied, and this variation was at the same level as found between technicians from the reference laboratory.

Spontaneous abortion and menstrual cycle characteristics were retrospectively obtained from questionnaires. The validity of this information was not directly assessed since it was not possible to get supplementary sources of information on these outcomes. However, the questionnaire used was based on a validated questionnaire developed for the European Infertility and Subfecundity study group [1]. A master version was prepared in English and translated into the native language for the study populations included. Subsequently, back translation into English was performed and any ambiguity in the wording was corrected. Although this ensures standardized data collection, differences in the concept of definitions of spontaneous abortion and irregularities in the menstrual cycle may exist between countries, and this may partly explain the fairly large differences between countries in the reporting of these outcomes. Furthermore, this limits the ability to perform a combined analysis across countries. Due to a non-homogeneous association between exposure levels and outcomes in relation to the menstrual cycle characteristics, we did not attempt to perform a combined analysis for this outcome, and the analysis on spontaneous abortions across populations should be interpreted with caution.

Including diverse populations in a study can, however, be considered a strength regarding the external validity of the study, where similar associations across countries, as demonstrated for the relationship between PCB-153 and sperm cell motility, indicate that the results may also be applicable to other populations. In addition, the dissimilar results observed between the populations for some of the outcomes, for example menstrual cycle characteristics, makes it less likely that the effects observed were causally associated with the levels of exposure measured. However, some of the dissimilarities between the countries could be explained by the differences in exposure level; furthermore, the differences in genetic makeup in the different countries may make some populations more susceptible to organochlorine exposure than others. As part of the INUENDO study, we obtained results indicating that

differences in the CAG repeat length of the androgen receptor makes people with a short androgen receptor length more susceptible to adverse effects on semen quality following PCB-153 exposure [133], and considerable differences between countries exist regarding the prevalence of short CAG repeat lengths [105]. In our population we also studied the GGN repeat of the androgen receptor and found no difference in the levels of susceptibility between different polymorphisms [133]. Whether or not other genetic polymorphisms in genes related to reproductive functioning or contaminant degradation might explain some of the differences observed between the populations is currently unknown.

Also, differences in food consumption may modify the associations observed and explain some of the differences found between the populations, since PCB and DDE exposure mainly occurs through the consumption of food and we observed the consumption of seafood to be a determinant of organochlorine exposure in Greenland and Sweden [81]. However, we only had limited information on food consumption in the present study and thus were not able to analyse in detail the potential modifying effect of specific food items on the exposure-response association observed.

Main conclusions
- The evaluation of published epidemiological studies [13] on the effects of persistent organochlorines on human reproductive health indicated that high levels of exposure to persistent organochlorines may affect human reproductive health. However inconsistent effects were found at lower levels of exposure, as encountered in the general European and North American population, although some of these inconsistencies might be explained by limited study sizes and methodological issues. Recent reviews showed limited evidence of adverse effects after adult exposure to persistent organochlorines, although foetal growth may be affected after PCB- exposure, indicating that increased attention should be paid to delayed effects after foetal exposure to PCB.
- Although time to pregnancy differed markedly between the populations studied, differences in the contraceptive methods used are more likely to explain these differences than intrinsic differences between the populations. Semen quality was remarkably similar between the countries studied, where only a slightly higher proportion of motile sperm were found in the Polish population compared to the other populations studied.
- Within Greenland sperm concentration was also similar between regions, but sperm cell motility differed, with a lower motility observed in eastern Greenland.
- Sperm cell motility was consistently associated with PCB-153 exposure across the countries, whereas sperm concentration and morphology was unrelated to PCB-153 or DDE, within the exposure range measured.
- The alterations measured in xeno-oestrogenic, -androgenic and dioxin-like activity in the serum samples did not seem to markedly deteriorate sperm cell concentration, motility or morphology in adult men.
- Sperm chromatin structure alterations were associated with PCB-153 exposure among the European men but not among the Inuit men from Greenland.
- PCB-153 and DDE were not strongly related to menstrual cycle characteristics, but different associations were observed between PCB and DDE exposure levels and menstrual characteristics between countries.
- The chance of ever reporting spontaneous abortions was increased among women with high levels of PCB or DDE exposure.

However, due to lack of a clear dose response and differences between the countries, these results should be interpreted with caution.
- The summary of the INUENDO results [127] concluded that organochlorine pollutants may interfere with male reproductive functioning without major effects on fertility.
- Overall, the results included in the present dissertation demonstrate that several aspects of male and female fertility may be affected by organochlorine exposure. Most of the observed associations were related to PCB-153 exposure, whereas DDE exposure did not seem to cause independent effects at the concentrations measured. The associations observed did not indicate major effects on human fertility and only a minor part of human fertility problems are likely to be explained by exposure to organochlorines in adulthood.

Suggestions for future research
Although the present series of studies only suggest limited effects of organochlorine exposure on the selected outcomes, we were not able to exclude the possibility that exposure to other substances may affect human reproductive functioning. We have therefore initiated additional analyses on stored serum samples from the INUENDO study in order to measure the concentrations of a number of potential reproductive toxicants known to be present in human serum samples in considerable amounts, including: perflourinated compounds, phthalate metabolites, bisphenol A, brominated flame retardants (PBDEs), hexachlor benzene, lead, cadmium and mercury.

Furthermore, animal studies have indicated that developing reproductive organs and subsequent reproductive functioning may be more sensitive to exposure to reproductive toxicants than fully developed reproductive organs [134]. So far, this has only been poorly elucidated in human studies, but studies on maternal smoking indicates that, at least for this type of xenobiotic exposure, foetal exposure is more important than adult exposure [135]. This also suggests that other environmental contaminants may more seriously affect reproductive functioning when foetuses are exposed. The effects of foetal exposure on later reproductive health should therefore be evaluated before we can conclude whether or not low level of exposure to persistent organochlorines has substantial effects on human reproductive functioning.

Additional genetic analyses are also warranted in order to identify possible gene-environment interactions that may help identify susceptible individuals. These genetic analyses should not only focus on hormone receptor functioning but also on the genes involved in steroidogenesis and the metabolism of steroids and chemicals with the potential to disturb reproductive functioning.

SUMMARY
The present dissertation focuses on the reproductive health effects in humans from four diverse populations, including an Inuit population from Greenland, a Swedish population of fishermen and fishermen's wives, and urban populations from the cities of Warsaw in Poland and Kharkiv in Ukraine, representing populations with considerable variations in organochlorine exposure levels due to differences in the consumption of contaminated food items and the period since banning the use of the organochlorines selected in the present study. Due to bioaccumula-

tion and their long half-lives in humans, these compounds are still ubiquitously detected in humans.

The study included a total of 2269 women who provided information via detailed questionnaires and 798 men who provided semen samples. Time to pregnancy varied between the populations included, whereas semen quality was remarkably similar with only minor differences in motility between countries and within regions in Greenland. An extensive quality control programme ensured a low level of variation between analysers in the evaluation of semen quality during semen sample collection. Sperm concentration and morphology were not associated with PCB-153 or DDE exposure, but sperm motility was consistently associated with PCB-153 exposure across populations. Xenoestrogen, -androgen and dioxin-like activity in serum samples were not consistently associated with semen quality measures, indicating that the associations observed with sperm motility were not caused via direct effects on these receptors. The sperm chromatin structure assay showed a higher level of DNA fragmentation under higher PCB-153 exposure levels in the European populations, but not in the Greenlandic population.

Disturbances in the female menstrual cycle were not consistently associated with PCB-153 or DDE exposure across the countries, but our results suggested a higher probability of ever having a spontaneous abortion among women with high PCB-153 or DDE exposure levels.

Overall, the results suggest that PCB-153, but probably not DDE, may affect aspects of male and female reproductive functioning in European and Arctic populations at the levels of exposure currently experienced in these populations, although the associations observed did not seem to be a major cause of reduced human fertility.

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