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Abbreviations

AhR: Aryl hydrocarbon receptor

AhR-TEQ: TCDD toxic equivalence

AR: androgen receptor

CALUX: chemical activated luciferase gene expression

CI: confidence interval

CV: coefficient of variation

DDT: dichlorodiphenyl trichloroethane

E2: 17β-estradiol

ER: estrogen receptor

ERE: estrogen-response-element

HPLC: high performance liquid chromatography

p,p´- DDE: dichlorodiphenyl dichloro-ethylene

PCB: polychlorinated biphenyl

POP: persistent organochlorine pollutant

R1881: methyltrienolone

RLU: relative light units

SHBG: sex hormone binding globulin

SPE: solid phase extraction

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

XAR:xenoandrogen receptor transactivity

XER: xenoestrogen receptor transactivity

XER-EEQ: estradiol equivalence

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Abstract

Background: Semen quality in humans may be influenced by exposure to endocrine disrupting compounds.

Objectives: We analyzed associations between semen characteristics and serum xenoestrogen receptor (XER), xenoandrogen receptor (XAR), and aryl hydrocarbon receptor (AhR) transactivity. XER- and XAR activity were measured in serum samples cleared for endogenous steroid hormones and AhR activity in raw lipophilic serum extracts free of proteins.

Results: All together, 319 men from Warsaw (Poland), Greenland, Kharkiv (Ukraine) and Sweden provided semen and blood samples. No strong and consistent associations between xenobiotic activity and semen quality measures were observed in the four populations. However, when combining the data, across populations sperm concentration increased 40% per unit increase in XER activity (95% Confidence Interval: 1 to 79%) in the subgroup with XER activity below the reference level. Among subjects with XER activity above the reference level an increase of 14% (95% CI: 2 to 28%) was found. Furthermore, an increase of 10 % motile sperm per unit increase in XER activity below reference level (95% CI: 0.2 to 20) was found. We are unable to exclude that the associations are chance findings.

Conclusion: Alteration of XER, XAR or AhR transactivity within the range found in serum from the general European and Inuit population seems not to markedly deteriorate sperm cell concentration, motility or morphology in adult men.

Introduction

Human serum is contaminated with numerous manmade chemicals released into the environment during past decades (AMAP 2003). Several xenobiotic compounds have weak agonistic or antagonistic actions on steroid receptors in *in-vitro* assays (Bonefeld-Jorgensen 2004; Sohoni and Sumpter 1998) and in animal models (Gray, Jr. 1998). The estrogen receptor (ER), androgen receptor (AR) and aryl hydrocarbon receptor (AhR) are expressed throughout the male genital tract and sex hormone signaling plays a pivotal role for development and regulation of male reproductive function (Hess 2003; Holdcraft and Braun 2004; Schultz et al. 2003). Effects on male reproductive organs or reproductive function in adult rats have been observed following exposure to weak xenohormones such as polychlorinated biphenyl (PCB) (Hsu et al. 2003), dichlorodiphenyl trichloroethane (DDT) (Ben Rhouma et al. 2001) and polychlorinated dibenzo-*p*-dioxins (Chahoud et al. 1992; Gray, Jr. et al. 1995; Simanainen et al. 2004). Also in humans, exposure to chemical compounds with endocrine disrupting properties has been suggested to be related to the apparent decline in semen quality (Swan et al. 2003). However, it remains unknown whether the low-level exposure to xenohormones that virtually all humans are exposed to, have implications for reproductive health (Safe 2000; Sharpe and Irvine 2004; Storgaard et al. 2006; Toft et al. 2004).

In large-scale epidemiological studies addressing health risks related to hormonal active xenobiotics, it is practically and economically unfeasible to measure serum levels of more than a few compounds. It is of considerable interest that techniques have been developed to examine the xenobiotic activity of sex hormone receptors in serum fractions free of endogenous hormones (Fernandez et al. 2004; Hjelmborg et al. 2006; Rasmussen et al. 2003). Hereby it has become possible to examine human health outcomes in relation to the integrated receptor activity of hundreds of xenobiotics that are found in human serum.

This paper is to our knowledge the first report of semen quality in relation to xenohormone and AhR activity. We report cross-sectional relations of serum xenobiotic receptor activities and semen quantity and quality in four geographical different study groups that were selected to obtain high contrast in body burdens of PCBs and the main DDT metabolite dichlorodiphenyl dichloro-ethylene (*p,p´*- DDE) (Toft et al. 2005a).

Materials and methods

We addressed pregnant couples in 19 towns and settlements all over Greenland, in Warsaw (Poland) and in Kharkiv (Ukraine). Pregnant women and their partners were consecutively enrolled during antenatal visits. Moreover, Swedish fishermen were enrolled separately and independent of current pregnancy. Altogether 798 men provided fresh semen sample with a ratio of semen providers relative to all men that were encouraged to deliver a semen sample of 79% in Greenland (201 men), 7% in Sweden (191 men), 29% in Warsaw (198 men) and 33% in Kharkiv (208 men). Blood samples were collected from the participating men within one week of the semen sample collection, except for a subgroup of 116 men from Greenland which had their blood sample collected up to one year in advance. Venous blood samples were collected in 10 ml vacuum tubes and after centrifugation the serum samples were stored at –80 °C until analysis. The local ethical committee in each of the four participating countries approved the study, and each participants gave a written informed consent prior to the study. Details of study design, selection of populations and data collection are given in (Toft et al. 2005a).

Receptor mediated chemical activated luciferase gene expression (CALUX) assays of serum extracts were performed in a subset of men who provided semen samples. There were altogether 365 men available with one, two or all three receptor activity values spanning from 262 subjects for the AR assay, 338 for the AhR assay and 358 for the ER assay. Due to limited amount of

serum available all receptor assays were not performed on each sample. Data on both xenobiotic activity and semen quality were available from in total 319 men. Characteristics of the study groups are provided in Table 1. The 319 men did not differ significantly from the remaining 479 men delivering a semen sample regarding sperm concentration, motility, morphology, age or period of abstinence prior to collection of the semen sample (data not shown).

Measurements of xenobiotic receptor activity in serum

For estrogenic and androgenic activity determinations the serum fraction (F1) containing persistent organochlorine pollutants (POPs) and free of endogenous estrogens and androgens was obtained by SPE-HPLC extraction. Solid phase extraction (SPE) was carried out using Oasis HLB (6ml, 500mg) extraction cartridges (Waters, Milford, MA, US). Extracted compounds were collected using a VAC ELUT SPS 24 vacuum manifold (Varian, Harbor City, CA, USA). The HPLC system consisted of an Alliance 2695 separations module with a 300 µl injection loop, equipped with a 2996 Photodiode Array Detector and a Fraction Collector II (Waters, Milford, MA, US). Separation was performed on a Spherisorb Si 60 analytical column 250 × 4.6 mm i.d., 5 µm particle size (Waters, Milford, MA, USA) as described in (Hjelmborg et al. 2006).

Extraction of lipophilic POPs from serum to be tested for AhR activity was performed by ethanol and hexane followed by cleaning on Florisil + Na_2SO_4 column (Ayotte et al. 2005), at Le Centre de Toxicologie, Sante Foy, Quebec, Canada.

Measurements of the xenobiotic induced receptor activities are described in detail in (Bonefeld-Jorgensen et al. 2006; Krüger et al. unpublished data; Long et al. 2006). For the estrogenic, the androgenic and the AhR activity assays all samples were tested in triplicate in two sets of tests

designed to test the basal response on the receptor assay and the response when a physiological level of the respective ligand is present, respectively. The test of basal xenobiotic activity of the serum extract alone (termed XER-, XAR- and AhR activity) was designed to test primarily for agonistic effects, but if the response on the assay was below the reference level (response of the solvent control) an antagonistic effect is indicated. On the other hand, the test for activity when the active ligands [17β-estradiol (E2), methyltrienolone (R1881), or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)] was present in a concentration giving 40-50% of maximum induction (termed XERcomp XARcomp and AhRcomp) was designed to test primarily for antagonistic effects but if response on the assay higher than reference values was observed an additive or synergistic effect is indicated. Solvent controls and/or samples from a pooled serum sample of Danish men and women were run in parallel for each assay. The coefficient of variations (CVs) between the 3 to 5 aliquots from the same serum samples, between solvent control samples with and without ligand added in a concentration of 40-50% maximum activity and the interassay variability between pooled serum samples are given in Table 2.

The estrogenic, androgenic and AhR mediated dioxin-like activities were determined using receptor mediated CALUX assays. For each assay, a cell line with the respective receptor and the luciferase reporter vector was employed as described in (Bonefeld-Jorgensen et al. 2005; Bonefeld-Jorgensen et al. 2006; Krüger et al. unpublished data; Long et al. 2006). Briefly, the estrogen response was measured in the stable transfected MVLN human breast cancer cell line carrying the estrogen-response-element (ERE)-luciferase reporter vector (kindly provided by M. Pons, France) (Bonefeld-Jorgensen et al. 2006). The androgen receptor activity was determined in the Chinese Hamster Ovary cells (CHO-K1) transiently co-transfected with the MMTV-LUC reporter vector (kindly provided by Dr. Ronald M. Evans, Howard Huges Medical Institute, CA) and the AR expression plasmid pSVAR0 (kindly provided by Dr. A.O. Brinkmann, Erasmus

University, Rotterdam) (Krüger et al. unpublished data). The AhR-activity was determined in stable transfected mouse hepatoma cell line Hepa1.12cR carrying the AhR-luciferase reporter vector provided by M.S. Denison (University of California, USA) (Long et al. 2006). The luciferase activity was measured in a LUMIstar luminometer (Ramcon, Denmark) in 96 well plates. The luciferase activity was calculated as relative light units (RLU) per μg cell protein (relative to the respective solvent control) and finally presented as RLU per ml serum. The reference values for the solvent control activity corresponding to the amount of serum extract added for the ER and AR assay was 3.14 RLU/ml serum, and 6.67 RLU/ml serum for the AhR assay. The estradiol equivalence (XER-EEQ) and TCDD toxic equivalence (AhR-TEQ) was calculated on samples that show values significantly higher than solvent control using a standard dose response curve of 17β-estradiol and TCDD, respectively. For details see (Bonefeld-Jorgensen et al. 2006; Long et al. 2006). The validity of these assays have been confirmed by testing the agonistic or antagonistic response of natural hormones and a series of chemicals with endocrine disrupting activity (Bonefeld-Jorgensen et al. 2001; Bonefeld-Jorgensen et al. 2006; Fernandez et al. 2004; Hjelmborg et al. 2006; Krüger et al. unpublished data; Long et al. 2006; Pliskova et al. 2005; Rasmussen et al. 2003).

Collection and analysis of semen samples

Semen samples were collected by masturbation at the residence or in privacy in a room at the hospital. The subjects were asked to abstain from sexual activities for at least two days before collecting the sample, and to note the actual abstinence time. If collected at home, the sample was kept close to the body to maintain a temperature close to 37 °C when transporting it to the laboratory immediately after collection.

The samples were analysed for motility and concentration according to the WHO (1999) manual for basic semen analysis (WHO 1999). Briefly, for each sperm sample, the sperm concentration was determined on two aliquots of diluted semen samples (1:10 or 1:20) using an Improved Neubauer Hemacytometer (Paul Marienfeld, Bad Mergentheim, Germany). If the difference between the counts on the two aliquots exceeded 10% of the sum, two new assessments were made. Using a microscope mounted with a heated stage (37C°), the sperm cell motility was determined by counting the proportion of a) fast progressive sperm; b) slowly progressive sperm; c) local motile sperm; and d) immotile sperm on 100 sperm within each of two fresh drops of semen, placed on a preheated (37C°) clean glass slide, and covered with a cover slip. All semen samples were analysed by one researcher in each country and all semen analysers had been trained in a series of three workshops held before and during the sample collection at the Fertility Centre, Malmö University Hospital, Sweden. This centre is accredited by the European Academy of Andrology and participates in the Nordic Association of Andrology and the European Society of Human Reproduction and Embryology (ESHRE) quality control programme. The median inter-individual coefficient of variation (CV) was 8.1% for sperm concentration assessment and 11.1% for motility (grade a+b) assessment (Toft et al. 2005b).

The morphology of the sperm from all the countries in this project were determined centrally by two technicians at the Fertility Centre, Malmö University Hospital, on Papanicolaou stained smears using the WHO (1999) criteria.

Statistical analyses

The effects of serum xenobiotic activities on semen quality are not easy to predict due to the highly interconnected hormonal regulation of spermatogenesis. Thus, a broad strategy for statistical analysis was employed. Visual inspection of scatter plots of the crude associations

between exposure and outcome did not indicate any threshold effect for any of the associations. However, to allow for analysis of non-monotonic response across the whole range of receptor activity, multiple linear regression analysis were performed in two receptor activity subgroups showing 1) lower or 2) higher receptor activity compared to the reference value of their respective solvent controls, representing agonistic and antagonistic responses, respectively. If the homogeneity test did not indicate different associations across the study populations, we computed common estimates across all four study populations. When significant associations or heterogeneity was indicated the associations in the single populations were evaluated by Spearman's correlation analysis.

Before inclusion in the linear regression models sperm concentration, percentages of sperm cells with normal morphology, age and abstinence time were transformed by the natural logarithmic function, which improved normality and homogeneity of variance, as indicated by inspection of Q-Q plots. The percentage of motile sperm cells was not transformed. Sperm concentration was, *a priori* decided to be adjusted for duration of sexual abstinence before delivery of the semen sample and age. Analyses of motility were restricted to the 95 % of samples for which the analysis was initiated within 60 minutes after collection. To decide which of the potential confounders, listed in Table 1, to adjust for in the multivariate models we used the change in estimate method suggested by (Greenland 1989). None of the β-coefficients were changed more than 10% when these potential confounders were included one by one in the models except for high alcohol consumption (>21 drinks/week). Due to the limited number of participants with high alcohol consumption and lacking information on all the Swedish participants, we made alternative analysis excluding high alcohol consumers and achieved similar results as without considering alcohol. Therefore, the results are presented without adjustment or restriction

regarding alcohol consumption. All statistical analyses were performed using SAS 9.1.3 software.

Results

The test of homogeneity across populations indicated that there were country differences in the association of sperm concentration to XARcomp activity, AhR activity and AhR-TEQ, and furthermore the proportion of normal sperms seemed to differ between countries in the subgroup with XARcomp activity < 3.14 (table 3). Geographical differences with significant associations between the continuous exposure markers and semen outcomes were only found for XARcomp activity and AhR activity which showed negative and positive associations to sperm concentration in Warsaw, respectively, but no significant associations in the other populations (see supplemental Table 1). In all other tested associations no indication of heterogeneity across populations was found and therefore combined analysis could be performed. When the data from the four study groups were combined (Table 3) a statistical significant positive association of sperm concentration and XER activity was found both below (40% increase per unit increase in XER activity; 95% Confidence Interval (CI) 1 to 79%) and above the reference level (14%; 95% CI 2 to 28%), but not across the whole range of activity (9%; 95% CI –1 to 20%). The correlation analysis in the individual study groups indicated that the association below reference level was mainly driven by a positive association in Warsaw, and the association above reference level was mainly driven by a positive association in Sweden (see supplemental Table 1). A scatter plot of the association is given in Figure 1 A. Furthermore, sperm motility was positively associated to XER activity in the subgroup below the reference level with an increase of 10 % motile sperm per unit increase in XER activity. This association seemed also to be

driven by a positive association in Warsaw. A scatter plot of sperm motility and XER activity is given in Figure 1 B.

In the groups stratified at the reference level, no statistical significant difference in sperm count, motility or morphology was observed between the groups with low or high receptor activity.

Discussion

The present study suggested a positive association of serum xenoestrogen activity and sperm concentration and motility across the four study groups. Furthermore, we found geographical differences in some of the tested associations, with the most marked effects in the population from Warsaw. Clearly, the statistically significant but rather weak associations need to be confirmed in future studies before any strong conclusion about associations can be made.

The positive association of sperm concentration and XER activity found at XER activity <3.14 could be due to adverse effects of antiestrogenic compounds on sperm count, which is plausible because of the known essential role of estrogen receptor function in male reproduction (Hess 2003). However, the assay specifically designed to test for competitive effects did not confirm an association between effects on this assay and sperm count across study populations. The positive association of sperm concentration and XER activity > 3.14 indicating a stimulating effect of exogenous estrogenic compounds on sperm counts are contrary to the expected, but could be hypothesized to be caused by an antiapoptotic role of estrogens on germ cells (Pentikainen et al. 2000) or a direct stimulatory effect of estrogens on spermatogenesis, since ERs are present in male germ cells (Lambard and Carreau 2005).

It is well known that there is a cross talk between ER, AR and AhR (Morrow et al. 2004; Pascussi et al. 2004) which may lead to other responses on the receptors *in vivo*, compared to the

ex vivo tests used in the present study, where the response on the single receptors are tested. This further complicates the interpretation of the associations of the semen quality and receptor activities. However, by evaluating the combined responses on the different receptors we might get closer to net effects *in vivo*.

For the group from Warsaw a predominantly net estrogenic serum activity was observed where 21% of the samples had induced estrogenic effects and antiestogenic effects where only found on 7% of the samples compared to 1-14% agonistic activity and 19-71% antagonistic activity in the other study groups (Bonefeld-Jorgensen et al. 2006). This may explain why the associations of XER activity and sperm count and motility were mainly found in Warsaw. Whether the negative association between XARcomp and semen concentration in Warsaw reflects an increase of the androgenic activity affecting the sperm concentration negatively can only be hypothesized. Similarly, whether the positive correlation between the AhR activity (and AhR-TEQ) and semen concentration for the Warsaw study group can be explained by an increased metabolism of chemicals with adverse effects on semen concentration can at this step only be a theory.

Only three of the 50 associations (6%) tested by linear regression did significantly differ from unity, which is close to the 5% positive findings, which were expected to occur under the null hypothesis of no difference (Table 3). Therefore, most, if not all, of the observed associations might be chance findings.

In the present study we included populations with both a large within and between population exposure contrast to POPs, which are known to interfere with ERs, ARs and AhRs (Bonefeld-Jorgensen et al. 2001; Pliskova et al. 2005). The study groups from Greenland and Sweden

represent highly exposed populations, whereas the other populations reflect the exposure situation generally found in different parts of Europe. Except for the Swedish fishermen, the included populations were selected to reflect the general population in the regions. People with occupational exposure or people living in accidentally polluted areas may have higher levels of exposure to POPs or other compounds potentially interfering with the ER, AR or AhR.

In the present study, the difference in net RLU activity in the assays was only 2-3 fold between samples with low activity (p5) and high activity (p95), indicating that the actual contrast in biological response was limited. However, the exposure contrast between the samples with low level (p5) and high level of calculated ER-EEQ and AhR-TEQ was in the range of 5 to 10 fold, indicating substantial differences in the amount of chemicals present in the samples with low and high activity (Bonefeld-Jorgensen et al. 2006; Long et al. 2006). The limited contrast in biological activity may be one of the reasons for the lack of consistent associations found in the present study.

Agonistic or antagonistic ER and AR activity was found in some individuals from each population, but with large variation between countries. For example, 71% of the samples from Greenland showed antagonistic XERcomp activity, whereas antagonistic XERcomp activity was only found in 7% of the samples from Warsaw (Bonefeld-Jorgensen et al. 2006). However, agonistic AhR activity was found in almost all of the serum samples from the included populations in the present study [97%, (Long et al. 2006)], and therefore we would especially expect to detect effects of this exposure marker across populations if it was associated with adverse effects on semen quality. In rats, administration of a single dose of TCCD in a concentration of 0.05 μg/kg during gestation reduced offspring sperm count by 25% (Gray et al. 1997), but adult exposure to TCDD required a dose of 3 μg/kg to have effect on male testis

(Chahoud et al. 1992). Thus, it seems that male reproductive function may be affected by AhR inducing agents, but it is likely that the most sensitive period to reproductive disturbances is the fetal period where small alterations of receptor activity may be of crucial importance for development of reproductive organs. In the present study we were not able to determine whether fetal exposure to compounds with effects on xenobiotic activity is affecting adult semen quality, but we found no indication that xenobiotic AhR agonistic activity was related to reduced sperm counts or impairment of other semen characteristics in adult men.

The overall number of subjects included in the present study was expected to be sufficient to detect associations between xenobiotic activity and semen quantity and quality even though some misclassification on both exposure and outcome may appear. However, the statistical power to detect effects on semen quality within populations may be limited, in particular for sperm concentration which is known to show considerable intra- and inter-individual variation (Bonde et al. 1996).

If sub-fertile men with low or high receptor activities were selectively declining to participate, the findings would be biased. Although the participation rate was low in three of the four regions, selection bias is unlikely, because the men had no knowledge about either semen quality or xenobiotic serum receptor activity. Furthermore, the data did not indicate markedly different associations in the study group from Greenland, where the participation rate was high, compared to the other study groups.

The *ex vivo* xenohormone assays have been validated thoroughly (Bonefeld-Jorgensen et al. 2006; Hjelmborg et al. 2006; Krüger et al. unpublished material) and since no consistent associations between xenohormone activity and natural estrogen or testosterone levels were

found, it is unlikely that the xenohormone activities were influenced by contamination of endogenous steroid hormones (Bonefeld-Jorgensen et al. 2006; Hjelmborg et al. 2006; Krüger et al. unpublished data).

Negative findings may be biologically plausible since most xenobiotic hormonal actions of single compounds are weak in comparison with endogenous hormones (Safe 2000; Sharpe and Irvine 2004). In the present study the median estimated estrogen equivalents (XER-EEQ) among subjects with agonistic estrogenic serum activity (13%) was on average 0.7 pg/ml serum, which is 4 % of the median estradiol value (19 pg/ml) measured in the males in the present project (Giwercman et al. 2006). Only about 2 % of the total estradiol in men is free and not bound to SHBG or albumin (Van Pottelbergh et al. 2004), whereas xenohormones has been indicated to bind to SHBG or albumin with much lower affinity, and therefore to be bioavailable to a larger extent (Crain et al. 1998). A more direct measure of the response of the xenoestrogens can be seen as the further increase in the XERcomp assay, which has been determined to be 21% (Bonefeld-Jorgensen et al. 2006). The activity of xenoestrogens is thus considerable, but also the natural variation between men in estrogen level is high (Giwercman et al. 2006) and therefore it is likely that the homeostatic processes of the body can compensate for these changes in estrogenic activity and therefore the production and maturation of sperms will not be affected. However, the xenobiotic serum estrogenicity was in the percentage range of physiological levels in males and far higher than expected when considering the measured concentrations and the weak estrogenic potency of individual POPs – some five to six orders of magnitude lower than 17 β-estradiol (Bolger et al. 1998). Since only the serum fraction including the POPs and not containing endogenous estrogens was used for the analysis, this may indicate that mixtures of several estrogenic compounds may cause actions much higher than simple summation of effects (Rajapakse et al. 2002).

In conclusion, in the present study we found that *ex vivo* estrogenic, androgenic or dioxin like activity in serum samples from the general population in Europe and among Inuits was not consistently and strongly associated to adult semen quality. Future analysis should be aimed at investigating if disturbances of fetal ER, AR or AhR activity cause more severe reproductive effects.

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Table 1. Characteristics of samples with measures of semen quality and at least one of the xenohormone assays.

	Warsaw, Poland n=83	Greenland n=54	Kharkiv, Ukraine n=86	Fishermen, Sweden n=96
OUTCOMES				
Sperm concentration x10^6/ml, median (p5-p95)	59 (8-241)	48 (11-152)	56 (17-159)	53 (9-192)
Total sperm count x10^6/ml, median (p5-p95)	190 (19-1310)	160 (25-650)	170 (35-750)	160 (15-480)
Volume ml, median (p5-p95)	3.8 (1.3-7.2)	3.0 (1.2-7.2)	3.1 (1.3-7.9)	2.8 (0.8-7.2)
A+B motile %, mean (p5-p95)	56 (0-85)	50 (19-79)	57 (19-88)	57 (15-89)
Normal morphology %, median (p5-p95)	6 (2-12)	6(2-14)	8 (1-16)	7 (1-16)
EXPOSURE MARKERS				
XER activity RLU/ml serum median (p5-p95)[a]	3.1 (2.6-5.5)	2.9 (2.3-3.4)	3.2 (2.4-4.0)	3.0 (2.6-4.1)
XER-EEQ pg/g lipid median (p5-p95)[b]	130 (44-520)	-[c]	140 (80-580)	84 (50-360)
XER competitive activity RLU/ml serum median (p5-p95)[d]	3.0 (2.5-5.8)	2.7 (2.2-3.3)	2.9 (2.2-3.5)	2.9 (2.0-3.5)
XAR activity RLU/ml serum median (p5-p95)[e]	3.5 (2.2-6.4)	3.9 (2.6-5.6)	3.6 (2.3-5.0)	3.7 (2.4-5.8)
XAR competitive activity RLU/ml serum median (p5-p95)[f]	3.0 (1.9-4.1)	4.0 (3.2-6.0)	2.2 (1.3-3.3)	2.9 (2.0-4.6)
AhR activity RLU/ml serum median (p5-p95)[g]	36 (15-74)	18 (8-62)	27 (15-45)	34 (12-75)
AhR-TEQ pg/g lipid median a (p5-p95)[b]	320 (130-360)	190 (100-600)	330 (180-630)	460 (220-920)
AhR competitive activity RLU/ml serum median (p5-p95)[h]	6.5 (4.6-8.3)	7.8 (5.8-10.5)	6.7 (1.8-9.8)	6.1 (4.7-8.3)
POTENTIAL CONFOUNDERS				
Abstinence period days, median (p5-p95)	3.0 (1-30)	2.5 (0.5-7.0)	3.0 (2.0-7.0)	3.0 (1.0-10.0)
Age, mean (p5-p95)	30 (26-38)	30 (20-40)	28 (20-38)	46 (32-62)
Time to analysis minutes, mean (p5-p95)	51 (40-65)	34 (30-50)	36 (25-63)	46 (25-65)
Body mass index kg/m^2, mean (p5-p95)	26 (20-32)	26 (20-32)	24 (20-30)	26 (22-31)
Season for sperm collection				
Spring (%)	5	46	0	53
Summer (%)	24	0	3	28
Fall (%)	30	54	57	19
Winter (%)	41	0	40	0
Fever last three months (%)	11	11	8	4
Urogenital infections (%)	6	85	1	22
Urogenital surgery (%)	2	0	0	1
Spillage yes (%)	10	13	14	16
Current smoking (%)	30	81	71	23
Alcohol consumption (>21 drinks/week) (%)	4	8	0	n.a.

[a] & [d] frequency of samples with ER agonistic and antagonistic effect 21% and 7% (Warsaw), 1% and 71% (Greenland), 14% and 30% (Kharkiv) and 12% and 19% (Sweden), respectively.

[b] Calculated on data from samples with agonistic effects only. Agonistic activity calculated as samples (triplicates) that differed significantly from the solvent control values using student T test. For details see Bonefeld-Jorgensen et al. 2006, Long et al.2006. The equivalence factor for AR (XAR-TEQ) could not be calculated since the weak agonistic responses did not reach the linear range of the R1881 (positive control) dose-response curve.

[c] One subject only, data not presented.

[e] & [f] : frequency of samples with AR agonistic and antagonistic effect 25% and 21% (Warsaw), 35% and 3% (Greenland), 26% and 50% (Kharkiv) and 34% and 8% (Sweden), respectively.
[g] & [h] : frequency of samples with AhR agonistic and antagonistic effect 100% and 8% (Warsaw), 92 and 3% (Greenland), 100% and 34% (Kharkiv) and 95% and 12% (Sweden), respectively.

Table 2. Coefficient of variation in the different xenobiotic assays.

	Intra assay CV (between aliquots from the same sample)	Solvent control inter-assay CV	Solvent control +ligand (-comp) Inter-assay CV	Inter assay CV (Pooled serum samples run in each assay)
XER	5	1	2	13
XAR	11	11	13	31
AhR	11	17	18	[a]

[a] Not analyzed in this assay

Table 3. Analysis of homogeneity across populations and results of multiple linear regressions of semen characteristics on xenobiotic serum activity in the aggregated dataset adjusted for study population (if applicable). Furthermore adjusted mean semen quality in the activity groups is given.

	Log sperm concentration [a]			Motile sperm [b]			Normal sperm cells		
	P (homo-geneity)	β and 95% CI	Geo mean sperm conc. 95%CI	P (homo-geneity)	β and 95% CI	Mean motility 95%CI	P (homo-geneity)	β and 95% CI	Geo mean normal sperm cell (95%CI)
XER activity	0.53	0.09 (-0.01;0.20)	53 (48;58)	0.86	1.60 (-1.31;4.52)	56(54;58)	0.91	0.05 (-0.03;0.13)	7.0 (6.5;7.5)
XER activity < 3.14	0.17	0.40 (0.01;0.79)[c]	55 (49;62)	0.15	10.2 (0.2;20.2)[c]	56 (52;59)	0.61	-0.06 (-0.36;0.24)	6.6 (6.0;7.2)
XER activity ≥ 3.14	0.24	0.14 (0.02;0.28)[c]	51 (43;59)	0.70	1.6 (-2.1;5.3)	54 (50;58)	0.87	0.03 (-0.07;0.12)	7.6 (6.8;8.5)
XER-EEQ pg/g lipid	0.56	<0.001(-0.002;0.002)	57 (43;75)	0.63	0.02 (-0.03;0.07)	54 (47;61)	0.60	0.0006 (-0.0009;0.0021)	6.7 (5.5;8.1)
XER competitive activity	0.98	-0.08 (-0.22;0.05)	54 (49;59)	0.45	-1.7 (-5.4;2.0)	56 (54;59)	0.25	-0.009 (-0.11;0.09)	7.0 (6.6;7.5)
XERcomp. activity < 3.14	0.78	-0.15 (-0.48;0.18)	54 (49;60)	0.93	-6.4 (-15.3;2.5)	55 (52;58)	0.74	-0.18 (-0.43;0.07)	7.1 (6.6;7.7)
XERcomp. activity ≥ 3.14	0.92	-0.10 (-0.32;0.12)	52 (44;63)	0.53	-4.6 (-10.1;0.8)	58 (53;63)	0.99	0.05 (-0.11;0.22)	6.9 (6.0;7.9)
XAR activity	0.10	-0.06 (-0.14;0.02)	58 (52;64)	0.82	-0.5 (-2.8;1.8)	56 (54;59)	0.76	0.01 (-0.05;0.07)	7.1 (6.6;7.7)
XAR activity <3.14	0.62	0.11 (-0.34;0.57)	60 (50;73)	0.15	9.4 (-5.2;23.9)	59 (53;65)	0.86	0.18 (-0.27;0.62)	6.9 (6.0;8.1)
XAR activity ≥3.14	0.31	-0.07 (-0.17;0.03)	58 (52;65)	0.45	0.2 (-2.7;3.1)	55 (51;58)	0.51	0.01 (-0.06;0.08)	7.1 (6.5;7.8)
XAR competitive activity	0.03	-	58 (52;64)	0.96	0.7 (-3.4;4.7)	56 (54;59)	0.62	0.00 (-0.11;0.11)	7.1 (6.6;7.7)
XARcomp. activity < 3.14	0.39	-0.12 (-0.40;0.16)	64 (55;75)	0.74	-3.0 (-11.6;5.6)	54 (50;59)	0.03	-	6.9 (6.1;7.7)
XARcomp. activity ≥ 3.14	0.92	0.04 (-0.21;0.28)	53 (45;63)	0.54	1.9 (-5.4;9.1)	58 (52;62)	0.67	0.04 (-0.16;0.25)	7.3 (6.4;8.4)
AhR activity	0.03	-	55 (50;61)	0.85	0.07 (-0.07;0.20)	56 (53;58)	0.76	0.004 (-0.002;0.009)	7.0 (6.6;7.5)
AhR-TEQ pg/g lipid	0.001	-	55 (50;61)	0.08	-0.004 (-0.02;0.01)	56 (53;59)	0.19	0.0002 (-0.0002;0.0005)	7.0 (6.5;7.5)
AhR competitive activity	0.94	-0.01 (-0.07;0.05)	55(50;61)	0.77	-0.2 (-1.7;1.3)	56 (53;58)	0.98	0.007 (-0.03; 0.05)	7.0 (6.6;7.5)
AHRcomp activity <6.67	0.27	-0.04 (-0.17;0.09)	54 (47;63)	0.88	0.7 (-8.4;9.7)	58 (54;62)	0.43	-0.007 (-0.11;0.09)	6.8 (6.2;7.6)
AHRcomp activity ≥ 6.67	0.26	-0.05 (-0.17;0.06)	55 (48;63)	0.34	2.2 (-1.0;5.3)	53 (50;57)	0.95	-0.03 (-0.30;0.23)	7.2 (6.5;7.9)

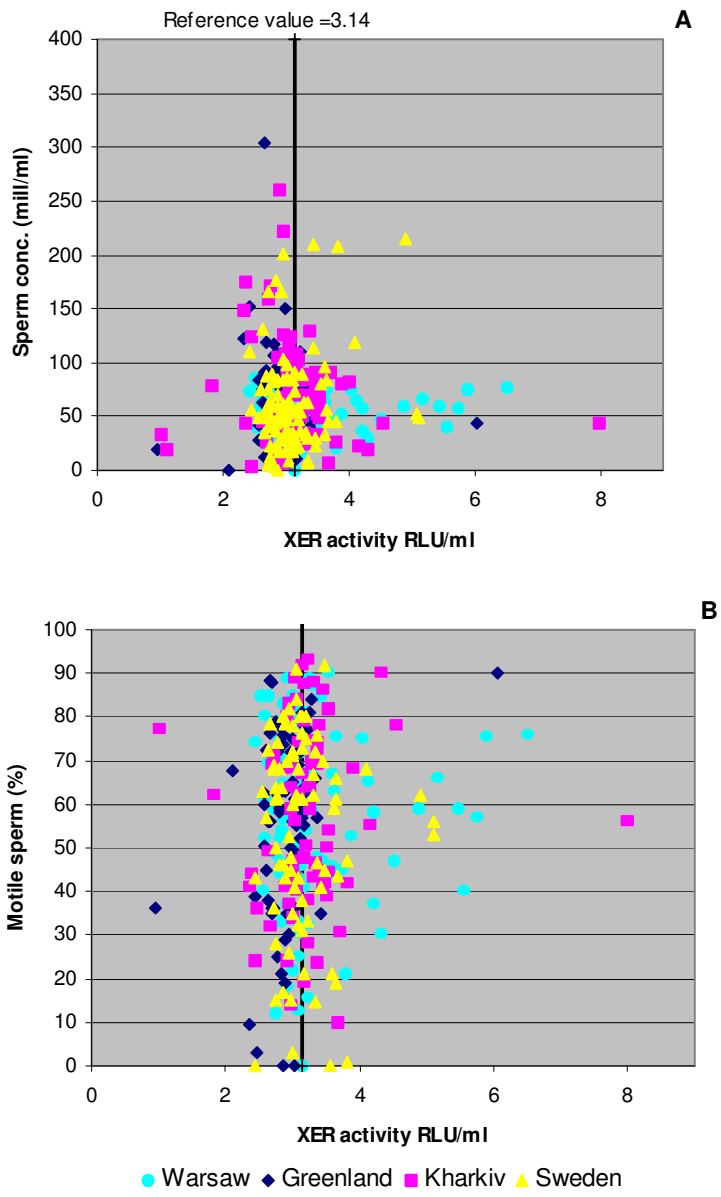
a Adjusted for age, abstinence time.

b Restricted to samples analyzed within one hour after collection.

c Significant associations ($p<0.05$).

Figure legend

Figure 1: Scatter plot of sperm concentration (A) and sperm motility (B) in relation to XER activity in the four populations.


Reference value =3.14
A
Sperm conc. (mill/ml)
400
350
300
250
200
150
100
50
0
0
2
4
6
8
XER activity RLU/ml
B
Motile sperm (%)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
XER activity RLU/ml
Warsaw
Greenland
Kharkiv
Sweden