Xenoestrogenic activity in blood of European and Inuit populations.

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ABSTRACT

Background. Human exposure to persistent organic pollutants (POPs) such as polychlorinated dibenzo-*p*-dioxins:furans, polychlorinated biphenyls and organochlorine pesticide residues e.g. 1,1-dichloro-2,2-bis (*p*-chlorophenyl)-ethylene (*p,p'*-DDE) is ubiquitous and found in all individuals. Studies have documented endocrine disrupting effects of POPs including impact on reproduction. The aim of the present study was to compare the integrated level of xenoestrogenic activity in serum of groups with varying POP exposure, and to evaluate possible correlations to the POP biomarkers, 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) and *p,p'*-DDE.

Methods. The study included 358 men: Greenlandic Inuit's, Swedish fishermen, and Warsaw (Poland) and Kharkiv (Ukraine) inhabitants. Xenoestrogenicity of serum extracts alone (XER) and XER competitive (XERcomp) effect on 17β-estradiol induced ER transactivity were assessed in the hormone free, lipophilic serum fraction containing the POPs using the MVLN human breast cancer cells.

Results. Inuit XER activity differed from the European groups, which did not mutually differ. Similar results were obtained for XERcomp. XER activity of Inuit samples were negatively associated to levels of CB-153 and *p,p*'-DDE. For the Warsaw samples XER was positively associated to *p,p'*-DDE level, and the estimated estradiol equivalence level decreased with increasing CB-153 levels. No interaction between XER/XERcomp and CB-153/*p,p'*-DDE levels was observed across the study groups.

Conclusion. The results suggested that the selected POP markers alone can not predict the integrated xenoestrogenic serum activity, and that the variation in xenoestrogenic serum activity reflects differences in chemical exposure patterns, genetic factors and /or life style factors and can contribute to the assessment of chemical body burdens.

1. INTRODUCTION

Human exposure to environmental contaminants is ubiquitous and affects also individuals living far away from the source of contaminants such as industries, combustion and waste disposal sites. Everyone carries a burden of persistent organochlorine pollutants (POPs) in their body, a burden mainly caused by dietary exposure. POPs, such as polychlorinated dibenzo-*p*-dioxin:furans (PCDD:PCDF), polychlorinated biphenyls (PCBs) and certain organochlorine pesticides e.g. 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane (DDT) and its main metabolite 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p*'-DDE), and toxaphenes accumulate in body fat tissues of humans and animals world wide due to their resistance to environmental and biotic degradation. Although most of the POPs have been restricted or prohibited for use already in the 1970's, and that the tissue levels in humans were reduced from the 1960s to the 1980s, the temporal trend of exposure decline has been less obvious the last twenty years. Today, e.g. PCBs can still be released into the environment from poorly maintained hazardous waste sites and illegal or improper dumping of PCB wastes such as leaks from old electrical transformers. DDT is still used in some areas for malaria mosquito control [1].

The burden of POPs in the Arctic has been monitored for more than ten years and some of the highest body burden levels have been determined in Arctic Inuit's mainly because of their high intake of marine mammals. In several districts in Greenland the PCB body burden levels exceed Canadian guidelines and at the East Coast districts Ittoqqortoormiit (Scoresbysund) the level of action [2, 3]. The burden of POPs in Swedish fishermen's family has been reported [4-6] but the burdens for the general populations in Central and Eastern Europe is less systematically examined [7-10]. Many POPs, including PCBs, PCDD:PCDF and pesticides can mimic hormone activities and are, therefore, potential endocrine disrupters suspected to increase the risk of cancer, birth defects, reproductive and neuro-immune disorders [11-13]. To date, no clear-cut evidence for adverse endocrine-related human health

effects of POPs have been obtained at the individual or population level. However, data from studies on wild-life species, laboratory animals and biomarker effects *in vitro* have strengthened the need for further research to address the concern.

The sex steroid receptors such as the estrogen receptor α (ERα) and ERβ and the androgen receptor (AR) belongs to the nuclear receptor family and are generally ligand-dependent transcription factors [14-17]. Their genomic mediated pathway include steps as binding of ligand to receptor, translocation into nucleus and binding of the receptor-ligand complex to a specific DNA response element causing a gene response. Androgens and estrogens are not male or female hormones only. Both hormones are important in both sexes and an absolute necessity for reproductive development including male fertility [18]. The steroid androgen hormones, testosterone and dihydrotestosterone, are necessary for the normal male phenotype and the presence of ERα and ERβ in human fetal testis and epididymis cells indicates that estrogens play a physiological role in regulations of spermatogenesis in mammals [19-21]. There is extensive data of *in vitro* mechanism based models on endocrine-related POPs effects on sex hormonal systems [22-25]. Although much less potent compared to 17β-estradiol (E2) the three PCB congeners CB-138, CB-153 and CB-180, comprising up to 50% of the bio-accumulated sum of PCBs, elicit a receptor mediated antiestrogen and/or antiandrogen *in vitro* activity [26] and similarly some hydroxylated PCBs exert low potency estrogenic and/or antiestrogenic effects [27, 28]. Generally PCBs do not bind to the sex hormone binding globulin (SHBG) or bind with very low affinity [29], which may cause a higher bio-availability of the compounds. Several POPs including pesticides, TCDD and dioxin-like PCBs (CB-126, CB-28, CB-105) were reported to affect *in vitro* and *in vivo* the aromatase which converts testosterone to estrogens and thus influence the ratio between the two sex steroid hormones [22, 30, 31]. Animal studies have indicated that prenatal exposure to POPs as PCBs, PCDD and *p,p'*-DDE is associated with reduced male fertility [32-38], whereas the effect of POPs on human fertility is still controversial [39-44]. There is a need for

an integrated risk assessment of endocrine disrupters (EDs) [45]. *In vitro* studies of xenoestrogen mixtures present at or below their no-observed-effect concentration (NOEC) or sub-NOEC was demonstrated to cause a dramatic additive enhancement of hormone actions [46, 47]. Thus the epidemiological documentation of POPs as EDs is complicated by several factors e.g. chemical determined may also act with other chemicals not being determined in the study. In addition influences of dietary, lifestyle and genetic differences must be taken into consideration. Rather than monitoring the level of identified xenoestrogens, the integrated biological activity was used to estimate the xenobiotic burden of serum samples using the E-screen MCF-7 cell proliferation *in vitro* assay [28, 48-50]. To avoid false positive data the xenoestrogens was extracted from serum samples and separated from the endogenous steroid hormones. Bio- accumulating xenoestrogens are generally lipophilic and separation based on lipophilicity is a feasible method using high-performance liquid chromatography (HPLC).

The present study was a part of the EU supported research project Inuendo (www.inuendo.dk) with the overall aim to estimate the impact of POP exposure on human fertility. The specific aim of the present study was to compare xenoestrogenic activities between the study groups and to evaluate whether the serum levels of POP biomarkers were associated to the integrated sum of xenoestrogenic activity in blood. The serum level of CB-153 and *p,p*'-DDE was used as a proxy markers for the body burden of total PCBs and POPs in general, respectively.

2. SUBJECTS AND METHODS

2.1 Study Population and collection of blood samples

Adult male were recruited during 2002-2004 as spouses to pregnant women in Greenland (GRL) (n=72; Sisimiut, West Coast n=50, and Tasiilaq, East Coast n=22), in Warsaw, Poland (n = 99) and in Kharkiv, Ukraine (n = 88) [44]. Earlier studies have reported differences in POP levels between habitants of the East and West Coast of Greenland [3, 6, 51], and therefore the GRL data was evaluated as the sum and as separate districts. A subgroup (n = 100) of an already established cohort of fishermen from Sweden [52] was also included in the study. The men included in this study were selected randomly between all men of the total Inuendo study groups. Information about demographic and lifestyle factors as age, BMI, intake of seafood, coffee, smoking habits, and alcohol consumption was collected by interviews (Table 1) [6, 53]. Venous blood samples were collected into 10 ml vacuum tubes and after centrifugation the serum was transferred to Nunc tubes and stored at -80º C for later analysis [6]. The study was approved by the local ethical committees representing all participating populations and all subjects signed an informed consent.

2.2 Determination of PCB-153 and p,p'-DDE in serum

Serum concentrations of PCB-153 and *p,p'*-DDE were determined using solid phase extraction and on-column degradation of lipids followed by analysis with gas chromatography mass spectrometry. Levels of detection, coefficients of variation and participation in quality control programs have been described in detail elsewhere. PCB-153 and *p,p´*-DDE levels were adjusted for serum lipids.[6, 54].

2.3 SPE-HPLC fractionation of the serum samples

To obtain the serum fraction containing the actual mixture of bio-accumulated POPs a solid phase extraction (SPE) followed by high performance liquid chromatography (HPLC)

fractionation was performed on 3.6 ml serum (Hjelmborg et al. 2005, manuscript in prep.). Similar to the described methods [28, 48, 55] POPs were extracted from the serum samples by SPE using Oasis HLB cartridges (Waters, Milford, MA, USA). This crude serum extract was then further processed using HPLC in order to separate the POPs from the endogenous hormones to avoid a false response in the ER mediated chemical activated luciferase gene expression (ER-CALUX) assay. The first fraction (F1: 0.00–5.30 min, protected from light in brown tubes) was defined to include most POPs while leaving out all endogenous hormones. This F1 SPE-HPLC fraction of the serum samples was evaporated and frozen for later ER-CALUX analysis.

Two pools of blood bank serum (Aarhus Sygehus, Denmark) one male (KHM) and one female (KHF) were distributed into tubes with 3.6 ml and stored at -80° C. One sample from each sex was frequently processed by the SPE-HPLC method in parallel with the project samples serving as serum controls for the cleanup procedure. The day to day inter assay coefficient of variation (CV) of the SPE-HPLC + ER-CALUX analyses of these control blood samples were 13%.

2.3.1 Dissolving the SPE-HPLC samples

The SPE-HPLC extracts samples (project samples and controls) were thawed and protected from light during handling. Sample solvent, 20 µl EtOH:H_2O:DMSO (50:40:10), was added to each sample tube and the samples were placed in an Eppendorf Thermomixer Comfort at 550 rpm and 37° C for 15 minutes. Then 200 µl growth media [0.5% Dextran-Charcoal treated foetal calf serum (DC-FCS, Hyclone, Bie & Berntsen, Aarhus, DK) in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Invitrogen, Taastrup, DK) supplemented with 4 mM glutamine (Sigma-Aldrich, Vallensbaek Strand, DK) and 1.28 mg/ml garamycin (Schering-Plough, Dassel, Germany)] was added to each sample and the shaking at 550 rpm and 37° C was continued for another 15 minutes. Then each sample was

transferred to two new test tubes (100 µl/tube) each containing 400 µl growth media with or without the normal ER ligand 17β-estradiol (E2), respectively. These samples were in triplicate (100 µl/well) used for ER-CALUX determinations.

2.3.2 Controls for the xenoestrogenic determinations in cell cultures

An E2 dose-response control in the concentration range from 0.05 pM to 500 pM (prepared from a 10 mM E2 stock solution in 96% ethanol with the final concentration less than 0.1%) was performed in parallel each analysis day as described [25, 56] (Figure 1). The maximal effective concentration EC_{100} of E2 was 150 pM. The half maximum effective concentration (EC_{50}) of E2 was calculated to 33 pM by fitting the dose-response data into Chapman, 4 parameter sigmoid equation curve using Sigma Plot 8.0 (SPSS, Chicago, IL, USA). The E2 concentration of 25 pM exerting 40% of the maximal effective concentration ($EC_{40)}$ was used to determine the competitive xenoestrogenic (XERcomp) effect of SPE-HPLC serum extracts upon co-exposure of the MVLN cells (see section 2.4).

The E2-EC_{100} and the E2-EC_{40} were used as parallel positive controls in each assay. Furthermore, the solvent controls (+/- E2-EC_{40}) consisted of sample solvent treated like the SPE-HPLC samples but without the serum extracts.

2.4 Estrogen receptor chemically activated luciferase expression (ER-CALUX) assay

The stable transfected MVLN human breast cancer cell line (kindly provided by M. Pons, France) carrying the endogenous ERα and ERβ genes, and the introduced estrogen-response-element (ERE)-luciferase reporter vector [57, 58] was used for determination of the sum of ER trans-activation responses. Xenoestrogenic activity (XER) was determined by exposure of the cells to serum extracts alone and the competitive XER (XERcomp) activity upon co-exposure of the cells to serum extract + 25 pM E2 (EC_{40}). The ER-CALUX assay can be described in short as follows: Cells were seeded in 96 well plates at a density of $3x10^4$

cells/well and cultured in growth media containing 1% DC-FCS for 24 h, and after media removal exposed to the test sample in 0.5% DC-FCS DMEM, 100 µl/well (see 2.3.1). After incubation for another 24 h and media removal, cells were added 50 µl lysis buffer (Roche, Basel, Switzerland). Luciferase (luc) activity was measured in a LUMIstar luminometer (BMG Lumistar, RAMCON, Denmark) directly in the 96 well plates according to a standard curve of recombinant luciferase (Bie & Berntsen, Denmark) as described [56]. Protein (internal standard) content was determined by adding 50 µl flourescamin (0.5 g/l) diluted in acetonitrile to each well followed by fluorometric measurements in the WALLAC VICTOR2 (PerkinElmer, USA) at 355/460 nm wavelength according to a standard curve of bovine serum albumin (BSA) (Promega, Madison WI, US). The measured luc data per well was then corrected for the well cell density using the protein measurements and expressed in relative light units per microgram protein (RLUs/µg protein). All controls and SPE-HPLC F1 extracts were analysed in triplicate. The solvent controls of XER and XERcomp activity were sample solvent only and the sample solvent plus 25 pM E2 (EC_{40}), respectively (see 2.3.1 and 2.3.2 for handling procedure). Significant activity differences compared to the solvent control (% agonists (additive/synergistic) and % antagonists, Table 2A) were tested by the Student's t-test (Microsoft Office Excel, significance level $p \leq 0.05$). If one of the triplicate values deviated more than 30% the mean was calculated from two wells only. For XER and XERcomp activity calculation the respective solvent controls (RLU/µg protein) were set to 1 [56]. Finally, the data was given as activity per ml serum and the values of the solvent controls were 3.13 RLU / ml serum.

The estradiol equivalence (XER-EEQ) value of the agonistic XER activities was obtained by interpolation of data onto the Chapman 4 parameter sigmoid curve using the Sigma Plot curve shown in Figure 1.

No cell toxicity on MVLN determined by CellTiter 96 Cell Proliferation assay from Promega (Madison WI, US) [56] was observed after exposure to SPE-HPLC extracts. Both the average

ER-CALUX inter-CV of the solvent controls and intra-individual CV for serum sample extracts were below 5%.

2.5 Statistical analysis

The statistical analysis was performed in SPSS 10.0 (SPSS Inc, Chicago, IL). The term statistically significant level is used to denote a p-value ≤ 0.05. Normal distribution was assessed by Q-Q plots. To improve normality and homogeneity of variance, the XER and XERcomp activities as well as lipid adjusted CB-153 (CB-153lip) and *p,p'*-DDE (DDElip) were natural logarithmic transformed and the statistical analyses were performed on the ln-transformed data. The POP variables were treated as continuous variables.

The comparisons of means between XER, XERcomp and XER-EEQ were performed by the Oneway-ANOVA test. When ANOVA indicated significant group difference complementary multiple comparison *ad hoc* tests were used. The test for homogeneity of variance was performed with Levene's test. The least significant difference (LSD) pair wise multiple comparison test was used for the variables with equal variance ($p \geq 0.05$) and Dunnett's T3 test was used for the variables with an unequal variance ($p \leq 0.05$).

The association in each study group between POP marker and xenoestrogen activity was evaluated by means of Spearman's rank correlation. The overall association between the POP marker and xenoestrogen activity across the study groups (combined data) were evaluated by comparing the regression lines for each study group (multiple regression analysis).

Currently, few studies of xenoestrogenic activity in human blood have been reported [28, 48, 55, 59] and thus knowledge is limited about which dietary or other life-style determinants, excep POPs and some other xenobiotics, that might affect xenoestrogenic activity. Our hypothesis is that a potential determinant of POP bioaccumulation might also be a potential determinant for serum xenoestrogenic activity. As known from the literatures and also from an assessment of the total Inuendo study populations [6, 44] age and seafood are such

determinants. Moreover, lifestyle characteristics (Table 1) were evaluated as potential determinants of XER and XERcomp levels of combined data and the separate study groups. Using multivariate linear regression analyses, assessing the impact of POP biomarkers on XER and XERcomp, the impact of potential confounders were evaluated by entering blocks of variables together with either CB-153 or *p,p'*-DDE as follows: In the first step age and seafood intake (continuous variables) were included in the model, and in the second step additionally smoking status (smoked ever yes/no), and body mass index (BMI) and coffee intake was included as continuous variables. Alcohol consumption was only recorded for limited number (n = 117) of subject and not for the Swedish study group. Therefore, the second step was carried out with and without alcohol consumption in the potential confounder model, which did not change the overall pattern of non-adjusted to adjusted data. Due to many missing values on the potential confounders the number of available observations in the confounder analyses are much smaller than in the unadjusted analysis on the full dataset (full dataset: N=348, first step of confounders: N=231; second step: N=172). A reduction of the number of observations with 50% might introduce serious selection problems, and hence the confounder analyses might lack greater validity.

The XER and XERcomp activities were determined in protein free serum extracts free of endogenous estrogens and testosterone. As a method verification Spearman's rank correlation analyses of XER and XERcomp against determined blood levels of estradiol and testosterone (total and free) [60] were performed on the combined study group data.

3. Results

3.1 Basic characteristics and serum CB-153 and p,p'-DDE levels of the study groups

The distribution of demographic and lifestyle factors that may potentially influence the ER-mediated activities of the 358 adult males in this study (Table 1) were similar with that obtained for the total Inuendo study population [44]. The serum lipid adjusted CB-153 and *p,p'*-DDE median levels of the study groups in the present sub study (Table 2A) were in the same range as the main study groups [6, 44]. The order of serum CB-153 level was Tasiilaq (GR) > Sweden ≥ Sisimiut (GR) > Kharkiv > Warsaw, and for the *p,p'*-DDE serum level Tasiilaq (GR) ≥ Kharkiv > Warsaw ≥ Sisimiut (GR) > Swedish fishermen (Tables 2A). As for the main Inuendo study populations [6] a correlation between serum concentration of lipid-adjusted CB-153 and *p,p'*-DDE was found for the sub groups of this study. Higher correlations were found in Greenland, (Sisimiut, and Tasiilaq), and Sweden while relatively lower correlation was observed for the study group of Kharkiv and Warsaw (Table 3).

3.2 Xenoestrogenic serum activity levels in the four study groups

The levels of XER activities with respect to the study groups are given in Table 2A and Figure 2. Since the CB-153 and *p,p'*-DDE serum levels determined in the two Greenlandic districts, Sisimiut (West Coast) and Tasiilaq (East Coast), differed these two groups were evaluated separately in parallel with the combined Greenlandic data (GR-sum). The median of XER of the study groups was in the order Kharkiv ≥ Warsaw ≥ Sweden > Sisimiut ≥ Tasiilaq, where the lower and higher medians reflects the frequency of antagonists and agonistic samples, respectively (Table 2A, Fig. 2A). The lowest and highest level of serum XER activity was measured in the Greenlandic Tasiilaq district and the Swedish group, respectively. The maximum antagonistic impact of GR samples decreased the XER activity approximately 3.3 fold below the background solvent control activity level. In contrast, the highest agonistic XER activity measured in Sweden reached a 4 fold increase above

background level. The Greenlandic serum extracts elicited predominantly antagonistic effect on XER and XERcomp, 35% and 71% of the samples, respectively, whereas the European samples elicited relatively higher frequency of XER agonistic activity (12-21%), (Table 2A). The median of XERcomp activity of serum extracts was in the order Warsaw > Sweden ≥ Kharkiv ≥ Tasiilaq ≥ Sisimiut (Table 2A and Fig. 2B). The most competitive (XERcomp) antagonistic effect on E2 induced ER activity was observed for a Swedish serum exerting a 3-fold activity decrease, and the highest synergistic and/or additive XERcomp effect was observed for a Warsaw serum extract being 2.2 fold above the E2-EC_{40} induced reference level. The serum extracts of the Warsaw study group elicited the highest frequency of XERcomp additive/synergistic ER effects, whereas the highest frequency of XERcomp antagonistic ER effects were observed for Greenland > Kharkiv > Sweden (Table 2A and Fig 2B).

This indicates that the net XER and XERcomp measured in serum extracts of the Warsaw study group had the potential to activate ER and further increase E2-EC_{40} induced reference level, and that the general high net XERcomp antagonistic activity determined for Greenland > Kharkiv > Swedish serum extracts even more clearly decreased the E2-EC_{40} reference level.

ANOVA analyses showed clearly significant differences in XER and XERcomp between the study groups (Table 2B). Furthermore, multiple comparisons of means showed significantly different XER activities between the Inuit’s (GR-sum) and each of the three European study groups. For the GR-subgroups, Sisimiut XER data differed from the Warsaw and the Swedish groups, whereas the Tasiilaq data did not differ from any of the other study groups. The mean XER activity and XER-EEQ of the European study groups did not mutually differ (Table 2B). Because only one GR sample elicited XER agonistic activity no median XER-EEQ value is given for the Greenlandic group (Table 2A and B). Concerning the XERcomp activity the

GR-sum data differed from the Warsaw group, which in turn differed from all other study groups (Table 2B).

3.3 Associations between xeno-estrogenic activity and CB-153 and p,p'-DDE

XER activity for GR-sum showed an inverse correlation to the *p,p'*-DDE levels, and for the Sisimiut XER data (but not for Tasiilaq data) an inverse correlation to both POP markers were found (Table 3, Fig. 3A and B).The XERcomp activity of the Greenlandic data was not associated with any of the POP markers. The XER and XERcomp activities were significantly inter-correlated for the Sisimiut and the GR-sum data, but not for the Tasiilaq data (Table 3). In summary, the Sisimiut data seems to have a relatively high impact on the combined GR-sum data.

For the Warsaw study group the XER activity showed a significant positive correlation to the *p,p'*-DDE data, and for XER-EEQ a significant negative correlation to CB-153 was observed (Table 3, Fig. 3C and D). The Warsaw group showed a strong positive inter-correlation between the corresponding XER and XERcomp activity (Table 3).

No correlations between XER, XERcomp or XER-EEQ and any of the two POP markers were found for the Swedish fishermen study group, whereas a positive and significant inter-correlation between the corresponding XER and XERcomp activities was observed (Table 3).

Neither for the Kharkiv study group were any correlations of the XER, the XERcomp or the XER-EEQ data with the POP markers observed (Table 3), nor any inter-correlation between the corresponding XER and XERcomp data.

Adjustment for the potential confounders in the multivariate regression model did not give any different results for the impact of the POP biomarkers on XER and XERcomp as compared with the unadjusted models, neither for the combined data or each study group (data not shown).

3.4 Multiple regression of xeno-estrogenic activity on POP data across the study groups

Scatter plots of XER or XERcomp against CB-153 or *p,p'*-DDE for the four study groups are shown in Figure 4. Multiple regression analysis of both response variables (XER and XERcomp) showed no interaction between study groups and CB-153/*p,p'*-DDE (Table 4), i.e. parallel regression lines among study groups. Furthermore, a model with parallel regression lines showed significant differences between the intercepts of the study groups (Table 4), thus the differences in XER/XERcomp between study groups shown in Table 2B still exist after adjustment for CB-153 or DDE. Finally, we note that although correlations was observed between XERs and the POP markers for the Greenlandic and Warsaw study groups (Table 3), for the combined data the associations between XER/XERcomp and CB-153/DDE, as measured by the common slopes, are very weak and not statistically significant (Table 4).

3.5 Correlations between xeno-hormone activities and endogenous hormone levels

To verify the exclusion of endogenous hormones from the SPE-HPLC- F1 serum extracts used for CALUX activity measurements the XER, XER-EEQ and XERcomp results were evaluated for possible correlation to blood estradiol (pmol/L) and testosterone (nmol/L) (free and total) levels. No correlations were found between the serum XER/XERcomp activities and blood sex hormone levels neither for the combined data nor for the separate study groups.

4. DISCUSSION

Humans are exposed to a mixture of environmental chemicals, which vary depending mainly on dietary intake but might also be influenced by proximity to the emission sources. The chemicals which bio-accumulate in animal and human tissues are of special concern since the exposure is lifelong beginning already during the embryonal and fetal periods, and continuing through breast feeding and intake of food. An array of POPs like PCBs, pesticides and PCDD:PCDF have been demonstrated to interfere with endogenous hormones and specific nuclear receptors *in vitro* and in animal studies [13, 22, 56, 61, 62]; actions which may alter the cellular hormone homeostasis *in vivo.* The xenohormone burden in humans consisting of many different chemicals acts either as agonists and/or antagonists of the various nuclear receptors, which also mutually interacts [63-66]. Thus, it is impossible to predict the sum of integrated xenohormone activity of the complex chemical mixture found in human blood.

In the present study we determined the actual integrated xenoestrogenic activity in serum of adult men from Greenland, Sweden, Warsaw (Poland) and Kharkiv (Ukraine), representing populations with different POP exposure patterns [3-6, 51].

The XER and XERcomp activities were significantly different between the groups. The Inuit clearly differed from the European study groups eliciting no agonistic but high frequency of the samples (71%) elicited antagonistic action. In contrast, the European group serum samples exerted both agonistic (12 - 21% of the samples) and antagonistic effects (7 - 30% of the samples). Thus XER-EEQ could not be calculated for Inuit's samples, whereas the XER-EEQ level of the three European groups did not differ significantly. In concordance with the present results, we did previously determine high XER/XERcomp antagonistic effects in Tasiillaq serum samples (Bonefeld-Jorgensen et al. 2005, in prep.), which might result in a relative decreased ratio of serum xenoestrogenecity/xenoandrogenecity for Inuit's as supported by parallel xenoandrogenic serum activity measurement for the same group of men (Krüger et al. 2005; in prep). The differences in xenoestrogenic action between the Inuit and

the European groups might be explained by different exposure pattern and/or life style or genetic background and deserve further research.

The high inter-correlation between the CB-153 and *p,p'*DDE in the Inuit's and the Swedish group and the relatively lower inter-correlation for the Warsaw and Kharkiv groups indicates that there are regional variations in exposure sources for these two compounds. This variation in POP marker burdens between the populations (Table 2 and 3) might also suggest differences in body burdens of other POPs not determined in this study, which might be elicited by differences of the integrated xenobiotic serum activities.

Significant correlations between XER activity and the POP markers were only observed for the Greenlandic and the Warsaw groups, whereas no association to the POP markers were found for XERcomp for any of the study groups. We did evaluate the Inuit study group districts, Tasiilaq (East Coast) and Sisimiut (West Coast), separately because of different CB-153 and *p,p'*-DDE exposure burdens, which also were reflected in the correlation pattern between xenoetrogenic activity and the POP markers. The Sisimiut group, with the lower POP marker burden compared to Tasiilaq, showed a relatively strong inverse linear association between XER activity and both CB-153 and *p,p'*-DDE, whereas no correlations were found for the Tasiilaq group. For the combined Inuit study group a significant negative correlation between XER and *p,p'*-DDE and borderline ($p = 0.07$) for XER versus CB-153 was found. Because of the high inter-correlation between the two POP index-markers in Inuit's it can not be assessed which one dominates the impact on XER activity. In contrast, the Warsaw study group, eliciting the highest incidence of agonistic XER activity, showed a positive correlation for XER and *p,p'*-DDE. Interestingly, although relatively low level burdens of CB-153 for the Warsaw study group, a strong ($r_s = -0.45$) inverse correlation to the XER-EEQ (pg/g lipid) level was observed. Future studies might support these observations or elucidate whether our observations are chance findings between multiple comparisons.

The use of CB-153 and *p,p´*-DDE in serum as index biomarkers of POP exposure has been supported by several previous studies [67-70]. However, it must be taken into consideration that the relative concentrations of non-coplanar and co-planar PCBs can differ between regions depending on varying exposure sources. Studies of Inuit populations from Canada support in general the use of CB-153 as a surrogate marker of exposure to non-dioxin-like PCBs present in the Arctic food-chain [71]. However, the ratio between serum levels of CB-153 (and other non-coplanar congeners) and co-planar PCBs was higher in Canadian Inuit's than in Canadian Caucasians from the Arctic area [72], which calls for caution using CB-153 as a global exposure marker for POP as well as for xenobiotic serum activities. Therefore, it might be expected that the ratio between non-dioxin-like, non-coplanar PCBs (e.g. CB-153) and co-planar PCB (e.g. CB-126) also is higher for the Greenlandic Inuit's analyzed in the present study compared to the three European study groups. In support of this assumption serum CALUX-TCDD equivalence (TEQ) determined in the same four study groups was higher for the three European groups than in Inuit's (Long et al. 2005, manuscript in prep.). No correlations were found between the blood sex hormone levels and xenoestrogenic serum activities, which support an optimal separation of endogenous hormones from the analyzed serum extracts.

In summary, a heterogeneous pattern of correlations between the POP-markers and XER activities were observed for the study groups. The only significant results were an inverse association of XER activity to CB-153 level in the Greenlandic group, dominated by the Sisimiut data, and a positive and a negative association of XER to *p,p'*-DDEand XER-EEQ to CB-153 for the Warsaw group, respectively. For the Inuit's it can not be assessed which of the two POPs might be the main involved factor, however, for the Warsaw group the very low concentration of CB-153 and low inter-correlation of marker POPs indicates that *p,p'*-DDE might have a positive effect on XER activity and CB-153 an inverse impact on XER as

elicited by XER-EEQ. Overall, these data might be explained by the high level of PCBs in Greenland with predominant anti-estrogenic actions [26, 28, 59, 73-75] and relatively high level of *p,p'*-DDE in the Warsaw group having weak estrogenic action [28, 46, 76, 77]. However, that does not explain why no correlations were observed for the Swedish and Kharkiv groups since they have a PCB and *p,p'*-DDE marker profile similar to Greenland and Warsaw, respectively. Furthermore, multiple regression analyses showed, that the determined differences of XER and XERcomp activity between the study groups could not be explained by the two selected POP proxy markers alone or in combination with the measured potential confounders in the present study.

The heterogeneous pattern of xenoestrogenic correlations to CB-153/*p,p'*-DDE levels and also the differences in xenoestrogenic activity among the study groups might be explained by different exposure patterns; i) the estrogenic effects elicited by ER transactivity is a consequence of the combined sum effects of many POPs having either ER agonistic or ER antagonistic actions; ii) the two selected POP markers do not reflect and/or represent the chemicals primarily responsible for the determined xenoestrogenic effects; and iii) the combined response of the serum mixture is significantly affected although the concentration of the single compounds found in human serum is lower than that needed to elicit a response of the compound on its own in an *in vitro* system. Furthermore, the concerted action of exposure pattern, genetic background and life style factors may contribute to the xenoestrogenic activity level.

Some of the bio-accumulated POPs have been characterized either as weak estrogens or antiestrogens [26, 28, 46, 59, 73-80]. Certainly it is impossible to depict the total cumulative xeno-estrogenic impact *in vivo* as well as in the *ex vivo* XER measurements in the present biomarker activity analyses. Studies have shown *in vitro* that the combined effect of several xenoestrogens including POPs at sub-NOEC exerts an additive effect, which led to dramatic change in estradiol *in vitro* action [46, 47]. To elucidate potential human and wildlife

responses to the accumulated and combined impact of xenoestrogens like POPs it remains to be demonstrated mechanistically how environmental chemicals with antiestrogen effects can modify the response of chemicals acting estrogenic in a concerted action.

Four studies using similar approach as the present one have been reported. In contrast to our determination of the integrated xenoestrogenic serum activity by the more specific impact on ER transactivation, the other studies used the E-screen MCF-7 cell proliferation as end point to determine the estrogenic action of the non-polar serum fraction containing bio-accumulated lipophilic POPs [28, 48-50]. Moreover, we analyzed the xenoestrogenic action in male blood samples whereas the other four studies were focused on female adipose tissues [48-50] and female serum from pregnant and non-pregnant women [28]. These studies determined a higher frequency (~ 60-70%) of subjects with significantly increased estrogenic activity in the non-polar serum fraction of Spanish and Faroese women. For non-pregnant Danish women the frequency of subjects exceeding the background level was 22.7%, similarly to the frequency determined for the European males in the present study. In contrast to our study no correlation between neither CB-153 nor *p,p'*-DDE and xeno-estrogenicity determined in the bioassay were found in these studies. Although using another approach it was recently reported that high levels of PCBs in Slovakia male serum samples were associated with a decreased ER mediated activity and increased AhR mediated activity [59]. It can be argued that xenoestrogenic equivalents contribute only a few percent to the endogenous hormone activity – even when possible higher bioavailability of the xenobiotics is taken into consideration. However, considering the further increase of XERcomp, we calculated (using the data of the E2 dose-response curve, Figure 1) for this subgroup of men (n = 86) a further mean activity increase of 47% and 21% in relation to the minimum (34 pM) and mean (70 pM) endogenous estradiol level, respectively. Since the level of endogenous estrogens is much lower in male than in female the xenoestrogenic activity might have higher impact on health risk in males.

4.1 Conclusions and perspectives

The four country based study groups showed a clear difference in serum xeno-estrogenicity as well as differences in correlation to the CB-153 and *p,p'*-DDE proxy markers. The antiestrogenic action was predominant in Inuit serum samples and higher frequency of agonistic and thus XER-EEQ levels were observed for the European study groups. The CB153 and *p,p'*-DDE alone seems not to be optimal global POP markers of the integrated xenoestrogenic serum activity. Thus in future studies the present *ex vivo* biomarker activity assay may be useful in assessment of geographical surveys for the impact of environmental xenoestrogens, which can reflect differences in the exposure pattern of chemicals and contribute to chemical state of art body burden assessment.

Because of the complex interaction between chemicals present in human serum and their potential to interfere with endogenous hormones and the natural ligand-receptor interactions the actual *ex vivo* assay for determination of the total integrated xenoestrogenicity may provide more insight into the endocrine disrupting impact of environmental compounds than monitoring compounds by chemical analyses alone. The combined additive/synergistic and/or inhibiting interaction of chemicals complicates the prediction of endocrine disruption based on chemical analyses only. On the other hand, exposure assessment based upon measurements of xenohormonal activity of the isolated SPE-HPLC, F1 POP serum fraction may fail to detect effects accomplished1) by other non-lipophillic compounds in serum, 2) through other receptors or 3) other mechanisms. More work still needs to be done to fully understand to which extent *ex vivo* measurements of receptor-transactivation reflects the *in vivo* situation. Ibarluzea et al. 2004 [50] found that estrogenicity of adipose tissue extracts including bio-accumulated xenoestrogens was associated with higher risk of breast cancer in leaner women. Currently we are evaluating possible associations between xenoestrogen action in the Inuendo

male blood and the impact on male fertility factors such as traditional semen quality markers and specific markers of DNA damage and seminal apoptosis. These future studies are likely to more accurately elucidate possible adverse endocrine disrupting effects of the combined xenoestrogen activity in human serum, compared to the traditional analysis of single or few chemical compounds.

Competing interests

Nothing to declare

Authors' contributions

ECB-J drafted the work and was the main responsible for design, performance and data evaluation of the specific project; PHJ, TSR and BSA performed the mechanistic work and data evaluation; CHL performed POP determinations in blood; ME was the main responsible for the statistical work; JPB, AG and LH designed the overall Inuendo project. JPB and GT coordinated the execution of the project and GT had main responsibility for creating the joint database. All authors, including C-GM and MS, participated in the design of the study, commented on the draft, and have read and approved the final manuscript.

Acknowledgements

Thanks to all CMT group members; Inger Sørensen for technical assistance, Tanja Krüger, Manhai Long and Mandana Ghisari for scientific support. A special thanks to Jan K. Ludwicki and Vladimir Lesovoy for coordination of the blood sampling in Warsaw and Kharkiv, respectively. Also a special thanks to Anna Rignell-Hydbom, Katarzyna Goralczyk, Henning S. Pedersen for taking the blood samples in Sweden, Warswa and Greenland, respectively. Thanks to Héléne Åkesson, Berit Holmskov and Christina Held for performing chemical analyses in a skillful way. This study is part of the Project "INUENDO - Biopersistent organochlorines in diet and human fertility. Epidemiological studies of time to pregnancy and semen quality in Inuit and European populations", supported by The European Commission to the 5th Framework Programme Quality of Life and Management of Living Resources, Key Action 4 on Environment and Health (Contract no. QLK4-CT-2001-00202) and INTAS (contract no 2001-2005). http://www.inuendo.dk. The work has also been funded

by the Danish Environmental Protection Agency, the Swedish Research Council and the Swedish Council for Environment, Agricultural Sciences and Spatial Planning.

References

1. Turusov V, Rakitsky V, Tomatis L: **Dichlorodiphenyltrichloroethane (DDT): ubiquity, persistence, and risks**. *Environ Health Perspect*, 2002. **110**(2): p. 125-8.

2. Deutch B,Hansen JC: **High human plasma levels of organochlorine compounds in Greenland. Regional differences and lifestyle effects**. *Dan Med Bull*, 2000. **47**(2): p. 132-7.

3. AMAP: **AMAP Assessment 2002, kap5: Human Health in the Arctic**. *Arctic Monitoring and Assessment Programme (AMAP), Oslo, Norway.*, 2003. (**http://www.amap.no**) **- Publications online: (DEFINITIVE_POPS6.pdf)**(ISBN 82-7971-016-7): p. xiv+137pp (Chapter 5).

4. Svensson BG, Nilsson A, Jonsson E, Schutz A, Akesson B, Hagmar L: **Fish consumption and exposure to persistent organochlorine compounds, mercury, selenium and methylamines among Swedish fishermen**. *Scand J Work Environ Health*, 1995. **21**(2): p. 96-105.

5. Rylander L,Hagmar L: **Mortality and cancer incidence among women with a high consumption of fatty fish contaminated with persistent organochlorine compounds**. *Scand J Work Environ Health*, 1995. **21**(6): p. 419-26.

6. Jonsson BA, Rylander L, Lindh C, Rignell-Hydbom A, Giwercman A, Toft G, Pedersen HS, Ludwicki JK, Goralczyk K, Zvyezday V, Spano M, Bizzaro D, Bonefeld-Jorgensen EC, Manicardi GC, Bonde JP, Hagmar L, Toft G, Axmon A, Giwercman A, Thulstrup A, Rignell-Hydbom A, Pedersen HS, Ludwicki J, Zvyesday

V, Zhinchuk A, Spano M, Manicardi G, Bonefeld-Jorgensen E, Hagmar L, Bonde JP: **Inter-population variations in concentrations, determinants of and correlations between 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) and 1,1-dichloro-2,2-bis (p-chlorophenyl)-ethylene (p,p'-DDE): a cross-sectional study of 3161 men and women from Inuit and European populations**
Fertility in four regions spanning large contrasts in serum levels of widespread persistent organochlorines: a cross-sectional study. *Environ Health*, 2005. **4**(1): p. 27.

7. Langer P, Kocan A, Tajtakova M, Petrik J, Chovancova J, Drobna B, Jursa S, Pavuk M, Koska J, Trnovec T, Sebokova E, Klimes I: **Possible effects of polychlorinated biphenyls and organochlorinated pesticides on the thyroid after long-term exposure to heavy environmental pollution**. *J Occup Environ Med*, 2003. **45**(5): p. 526-32.
8. Langer P, Tajtakova M, Guretzki HJ, Kocan A, Petrik J, Chovancova J, Drobna B, Jursa S, Pavuk M, Trnovec T, Sebokova E, Klimes I: **High prevalence of anti-glutamic acid decarboxylase (anti-GAD) antibodies in employees at a polychlorinated biphenyl production factory**. *Arch Environ Health*, 2002. **57**(5): p. 412-5.
9. Czaja K, Ludwicki JK, Goralczyk K, Strucinski P: **Organochlorine pesticides, HCB, and PCBs in human milk in Poland**. *Bull Environ Contam Toxicol*, 1997. **58**(5): p. 769-75.
10. Gladen BC, Monaghan SC, Lukyanova EM, Hulchiy OP, Shkyryak-Nyzhnyk ZA, Sericano JL, Little RE: **Organochlorines in breast milk from two cities in Ukraine**. *Environ Health Perspect*, 1999. **107**(6): p. 459-62.

11. Carpenter DO, Arcaro KF, Bush B, Niemi WD, Pang S, Vakharia DD: **Human health and chemical mixtures: an overview**. *Environ Health Perspect*, 1998. **106 Suppl 6**: p. 1263-70.

12. Bonefeld-Jorgensen EC: **The Human Health Effect Programme in Greenland, a review**. *Sci Total Environ*, 2004. **331**(1-3): p. 215-31.

13. Bonefeld-Jorgensen EC,Ayotte P: **Toxicological Properties of POPs and Related Health Effects of Concern for the Arctic Populations (Chapter 6)**, in *AMAP assessment 2002: Human Health in the Arctic, Vol.* 2003, Arctic Monitoring and Assessment Programme (AMAP): Oslo, Norway. p. xiv + 137.

14. Fu M, Wang C, Zhang X, Pestell R: **Nuclear receptor modifications and endocrine cell proliferation**. *J Steroid Biochem Mol Biol*, 2003. **85**(2-5): p. 133-8.

15. Verrijdt G, Haelens A, Claessens F: **Selective DNA recognition by the androgen receptor as a mechanism for hormone-specific regulation of gene expression**. *Mol Genet Metab*, 2003. **78**(3): p. 175-85.

16. Schwabe JW,Teichmann SA: **Nuclear receptors: the evolution of diversity**. *Sci STKE*, 2004. **2004**(217): p. pe4.

17. Bjornstrom L,Sjoberg M: **Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes**. *Mol Endocrinol*, 2005. **19**(4): p. 833-42.

18. Hess RA: **Estrogen in the adult male reproductive tract: a review**. *Reprod Biol Endocrinol*, 2003. **1**(1): p. 52.

19. Shapiro E, Huang H, Masch RJ, McFadden DE, Wu XR, Ostrer H: **Immunolocalization of androgen receptor and estrogen receptors alpha and beta in human fetal testis and epididymis**. *J Urol*, 2005. **174**(4 Pt 2): p. 1695-8; discussion 1698.

20. Carreau S: **Estrogens--male hormones?** *Folia Histochem Cytobiol*, 2003. **41**(3): p. 107-11.

21. Couse JE, Mahato D, Eddy EM, Korach KS: **Molecular mechanism of estrogen action in the male: insights from the estrogen receptor null mice**. *Reprod Fertil Dev*, 2001. **13**(4): p. 211-9.

22. Andersen HR, Vinggaard AM, Rasmussen TH, Gjermandsen IM, Bonefeld-Jorgensen EC: **Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro**. *Toxicol Appl Pharmacol*, 2002. **179**(1): p. 1-12.

23. Long M, Laier P, Vinggaard AM, Andersen HR, Lynggaard J, Bonefeld-Jorgensen EC: **Effects of currently used pesticides in the AhR-CALUX assay: comparison between the human TV101L and the rat H4IIE cell line**. *Toxicology*, 2003. **194**(1-2): p. 77-93.

24. Hofmeister MV,Bonefeld-Jorgensen EC: **Effects of the pesticides prochloraz and methiocarb on human estrogen receptor alpha and beta mRNA levels analyzed by on-line RT-PCR**. *Toxicol In Vitro*, 2004. **18**(4): p. 427-33.

25. Grunfeld HT,Bonefeld-Jorgensen EC: **Effect of in vitro estrogenic pesticides on human oestrogen receptor alpha and beta mRNA levels**. *Toxicol Lett*, 2004. **151**(3): p. 467-80.

26. Bonefeld-Jorgensen EC, Andersen HR, Rasmussen TH, Vinggaard AM: **Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity**. *Toxicology*, 2001. **158**(3): p. 141-53.

27. Brouwer A, Longnecker MP, Birnbaum LS, Cogliano J, Kostyniak P, Moore J, Schantz S, Winneke G: **Characterization of potential endocrine-related health effects at low- dose levels of exposure to PCBs**. *Environ Health Perspect*, 1999. **107 Suppl 4**: p. 639-49.

28. Rasmussen TH, Nielsen F, Andersen HR, Nielsen JB, Weihe P, Grandjean P: **Assessment of xenoestrogenic exposure by a biomarker approach: application of the E-Screen bioassay to determine estrogenic response of serum extracts**. *Environ Health*, 2003. **2**(1): p. 12.

29. Jury HH, Zacharewski TR, Hammond GL: **Interaction between human plasma sex hormone-binding globulin and xenobiotic ligands**. *Journal of Steroid Biochemistry & Molecular Biology*, 2000. **75**: p. 167 - 176.

30. Lai KP, Wong MH, Wong CK: **Effects of TCDD in modulating the expression of Sertoli cell secretory products and markers for cell-cell interaction**. *Toxicology*, 2005. **206**(1): p. 111-23.

31. Gerstenberger SL, Heimler I, Smies R, Hutz RJ, Dasmahapatra AK, Tripoli V, Dellinger JA: **Minimal endocrine alterations in rodents after consumption of lake trout (Salvelinus namaycush)**. *Arch Environ Contam Toxicol*, 2000. **38**(3): p. 371-6.

32. Anbalagan J, Kanagaraj P, Srinivasan N, Aruldhas MM, Arunakaran J: **Effect of polychlorinated biphenyl, Aroclor 1254 on rat epididymis**. *Indian J Med Res*, 2003. **118**: p. 236-42.

33. Fielden MR, Halgren RG, Tashiro CH, Yeo BR, Chittim B, Chou K, Zacharewski TR: **Effects of gestational and lactational exposure to Aroclor 1242 on sperm quality and in vitro fertility in early adult and middle-aged mice**. *Reprod Toxicol*, 2001. **15**(3): p. 281-92.

34. Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA, Wilson EM: **Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist**. *Nature*, 1995. **375**(6532): p. 581-5.

35. Nixon A, Benghuzzi H, Cason Z: **Morphometric evaluation of ovarian tissue exposed to PCB conventionally and in a sustained manner**. *Biomed Sci Instrum*, 2003. **39**: p. 434-9.

36. Peterson RE, Theobald HM, Kimmel GL: **Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons**. *Crit Rev Toxicol*, 1993. **23**(3): p. 283-335.

37. Sager DB, Shih-Schroeder W, Girard D: **Effect of early postnatal exposure to polychlorinated biphenyls (PCBs) on fertility in male rats**. *Bull Environ Contam Toxicol*, 1987. **38**(6): p. 946-53.

38. Steinhardt GF: **Endocrine disruption and hypospadias**. *Adv Exp Med Biol*, 2004. **545**: p. 203-15.

39. Axmon A, Rylander L, Stromberg U, Dyremark E, Hagmar L: **Polychlorinated biphenyls in blood plasma among Swedish female fish consumers in relation to time to pregnancy**. *J Toxicol Environ Health A*, 2001. **64**(6): p. 485-98.

40. Axmon A, Rylander L, Stromberg U, Jonsson B, Nilsson-Ehle P, Hagmar L: **Polychlorinated biphenyls in serum and time to pregnancy**. *Environ Res*, 2004. **96**(2): p. 186-95.

41. Dallinga JW, Moonen EJ, Dumoulin JC, Evers JL, Geraedts JP, Kleinjans JC: **Decreased human semen quality and organochlorine compounds in blood**. *Hum Reprod*, 2002. **17**(8): p. 1973-9.

42. Younglai EV, Foster WG, Hughes EG, Trim K, Jarrell JF: **Levels of environmental contaminants in human follicular fluid, serum, and seminal plasma of couples undergoing in vitro fertilization**. *Arch Environ Contam Toxicol*, 2002. **43**(1): p. 121-6.

43. Yu ML, Guo YL, Hsu CC, Rogan WJ: **Menstruation and reproduction in women with polychlorinated biphenyl (PCB) poisoning: long-term follow-up interviews of the women from the Taiwan Yucheng cohort**. *Int J Epidemiol*, 2000. **29**(4): p. 672-7.

44. Toft G, Axmon A, Giwercman A, Thulstrup A, Rignell-Hydbom A, Pedersen HS, Ludwicki J, Zvyesday V, Zhinchuk A, Spano M, Manicardi G, Bonefeld-Jorgensen E, Hagmar L, Bonde JP: **Fertility in four regions spanning large contrasts in serum levels of widespread persistent organochlorines: a cross-sectional study**. *Environ Health*, 2005. **4**(1): p. 26.

45. Bridges JW,Bridges O: **Integrated risk assessment and endocrine disrupters**. *Toxicology*, 2004. **205**(1-2): p. 11-5.

46. Payne J, Scholze M, Kortenkamp A: **Mixtures of four organochlorines enhance human breast cancer cell proliferation**. *Environ Health Perspect*, 2001. **109**(4): p. 391-7.

47. Rajapakse N, Silva E, Kortenkamp A: **Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action.** *Environ Health Perspect*, 2002. **110**(9): p. 917-21.

48. Rivas A, Fernandez MF, Cerrillo I, Ibarluzea J, Olea-Serrano MF, Pedraza V, Olea N: **Human exposure to endocrine disrupters: standardisation of a marker of estrogenic exposure in adipose tissue**. *Apmis*, 2001. **109**(3): p. 185-97.

49. Fernandez MF, Rivas A, Olea-Serrano F, Cerrillo I, Molina-Molina JM, Araque P, Martinez-Vidal JL, Olea N: **Assessment of total effective xenoestrogen burden in adipose tissue and identification of chemicals responsible for the combined estrogenic effect**. *Anal Bioanal Chem*, 2004. **379**(1): p. 163-70.

50. Ibarluzea Jm J, Fernandez MF, Santa-Marina L, Olea-Serrano MF, Rivas AM, Aurrekoetxea JJ, Exposito J, Lorenzo M, Torne P, Villalobos M, Pedraza V, Sasco AJ, Olea N: **Breast cancer risk and the combined effect of environmental estrogens**. *Cancer Causes Control*, 2004. **15**(6): p. 591-600.

51. Deutch B, Pedersen HS, Hansen JC: **Dietary composition in Greenland 2000, plasma fatty acids and persistent organic pollutants**. *Sci Total Environ*, 2004. **331**(1-3): p. 177-88.

52. Rignell-Hydbom A, Rylander L, Giwercman A, Jonsson BA, Lindh C, Eleuteri P, Rescia M, Leter G, Cordelli E, Spano M, Hagmar L: **Exposure to PCBs and p,p'-DDE and human sperm chromatin integrity**. *Environ Health Perspect*, 2005. **113**(2): p. 175-9.

53. Toft G AA, Giwercman A, Thulstrup A.M., Rignell-Hydbom A, Sloth Pedersen H, Ludwicki J, Zvyezday V, Spano M, Manicardi G.C., Bonefeld-Jørgensen E.C., Hagmar L, Bonde JP: **Fertility in four regions spaning large contrasts in serum levels of widespread persistent organochlorines**. *Environmental Health 2005, 4, 26.*, 2005.

54. Rignell-Hydbom A, Rylander L, Giwercman A, Jonsson BA, Nilsson-Ehle P, Hagmar L: **Exposure to CB-153 and p,p'-DDE and male reproductive function**. *Hum Reprod*, 2004. **19**(9): p. 2066-75.

55. Sonnenschein C, Soto AM, Fernandez MF, Olea N, Olea-Serrano MF, Ruiz-Lopez MD: **Development of a marker of estrogenic exposure in human serum**. *Clin Chem*, 1995. **41**(12 Pt 2): p. 1888-95.

56. Bonefeld-Jorgensen EC, Grünfeld HT, Gjermandsen IM: **Effect of pesticides on estrogen receptor transactivation in vitro: A comparison of stable transfected MVLN and transient transfected MCF-7 cells.** *Mol Cell Endocrinol*, 2005. **In press**.

57. Demirpence E, Duchesne MJ, Badia E, Gagne D, Pons M: **MVLN cells: a bioluminescent MCE-7-derived cell line to study the modulation of estrogenic activity**. *J Steroid Biochem Mol Biol*, 1993. **46**(3): p. 355-64.

58. Pons M, Gagne D, Nicolas JC, Mehtali M: **A new cellular model of response to estrogens: a bioluminescent test to characterize (anti) estrogen molecules**. *Biotechniques*, 1990. **9**(4): p. 450-9.

59. Pliskova M, Vondracek J, Canton RF, Nera J, Kocan A, Petrik J, Trnovec T, Sanderson T, van den Berg M, Machala M: **Impact of polychlorinated biphenyls contamination on estrogenic activity in human male serum**. *Environ Health Perspect*, 2005. **113**(10): p. 1277-84.

60. Giwercman Aleskander AR-H, Bo Jonsson, Herning S Pedersen, Jan K. Ludwicki, Maryna Shevets, Valentyna Zvyezday, Gunnar Toft, Lars Rylander, Lars Hagmar and Jens Peter Bonde: **Reproductive hormone levels in men exposed to persistent organohalogen polutions: a study of Inuits and three European cohorts**. *submitted*, 2005.

61. Nilsson S,Gustafsson JA: **Estrogen receptor action**. *Crit Rev Eukaryot Gene Expr*, 2002. **12**(4): p. 237-57.

62. Nilsson S,Gustafsson JA: **Biological role of estrogen and estrogen receptors**. *Crit Rev Biochem Mol Biol*, 2002. **37**(1): p. 1-28.

63. Thomsen JS, Wang X, Hines RN, Safe S: **Restoration of aryl hydrocarbon (Ah) responsiveness in MDA-MB-231 human breast cancer cells by transient expression of the estrogen receptor**. *Carcinogenesis*, 1994. **15**(5): p. 933-7.

64. Kharat I,Saatcioglu F: **Antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin are mediated by direct transcriptional interference with the liganded estrogen receptor. Cross-talk between aryl hydrocarbon- and estrogen-mediated signaling**. *J Biol Chem*, 1996. **271**(18): p. 10533-7.

65. Safe S,Krishnan V: **Chlorinated hydrocarbons: estrogens and antiestrogens**. *Toxicol Lett*, 1995. **82-83**: p. 731-6.

66. Thomsen JS, Wang X, Hines RN, Safe S: **Restoration of aryl hydrocarbon (Ah) responsiveness in MDA-MB-231 human breast cancer cells by transient expression of the estrogen receptor**. *Carcinogenesis*, 1994. **15**(5): p. 933-7.

67. Grimvall E, Rylander L, Nilsson-Ehle P, Nilsson U, Stromberg U, Hagmar L, Ostman C: **Monitoring of polychlorinated biphenyls in human blood plasma: methodological developments and influence of age, lactation, and fish consumption**. *Arch Environ Contam Toxicol*, 1997. **32**(3): p. 329-36.

68. Glynn AW, Wolk A, Aune M, Atuma S, Zettermark S, Maehle-Schmid M, Darnerud PO, Becker W, Vessby B, Adami HO: **Serum concentrations of organochlorines in men: a search for markers of exposure**. *Sci Total Environ*, 2000. **263**(1-3): p. 197-208.

69. Deutch B, Pedersen HS, Jorgensen EC, Hansen JC: **Smoking as a determinant of high organochlorine levels in Greenland**. *Arch Environ Health*, 2003. **58**(1): p. 30-6.

70. Gladen BC, Schecter AJ, Papke O, Shkyryak-Nyzhnyk ZA, Hryhorczuk DO, Little RE: **Polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans, and coplanar polychlorinated biphenyls in breast milk from two cities in Ukraine**. *J Toxicol Environ Health A*, 1999. **58**(3): p. 119-27.

71. Muckle G, Ayotte P, Dewailly EE, Jacobson SW, Jacobson JL: **Prenatal exposure of the northern Quebec Inuit infants to environmental contaminants**. *Environ Health Perspect*, 2001. **109**(12): p. 1291-9.

72. Dewailly E, Ayotte P, Brisson J, Dodin S: **Breast cancer and organochlorines**. *Lancet*, 1994. **344**(8938): p. 1707-8.

73. Gregoraszczuk EL, Grochowalski A, Chrzaszcz R, Wegiel M: **Congener-specific accumulation of polychlorinated biphenyls in ovarian follicular wall follows repeated exposure to PCB 126 and PCB 153. Comparison of tissue levels of PCB and biological changes**. *Chemosphere*, 2003. **50**(4): p. 481-8.

74. Drenth HJ, Bouwman CA, Seinen W, Van den Berg M: **Effects of some persistent halogenated environmental contaminants on aromatase (CYP19) activity in the human choriocarcinoma cell line JEG-3**. *Toxicol Appl Pharmacol*, 1998. **148**(1): p. 50-5.

75. Connor K, Ramamoorthy K, Moore M, Mustain M, Chen I, Safe S, Zacharewski T, Gillesby B, Joyeux A, Balaguer P: **Hydroxylated polychlorinated biphenyls (PCBs) as estrogens and antiestrogens: structure-activity relationships**. *Toxicol Appl Pharmacol*, 1997. **145**(1): p. 111-23.

76. Andersen HR, Andersson AM, Arnold SF, Autrup H, Barfoed M, Beresford NA, Bjerregaard P, Christiansen LB, Gissel B, Hummel R, Jorgensen EB, Korsgaard B, Le Guevel R, Leffers H, McLachlan J, Moller A, Nielsen JB, Olea N, Oles-Karasko A, Pakdel F, Pedersen KL, Perez P, Skakkeboek NE, Sonnenschein C, Soto AM, et al.: **Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals**. *Environ Health Perspect*, 1999. **107**(Suppl 1): p. 89-108.

77. Chen CW, Hurd C, Vorojeikina DP, Arnold SF, Notides AC: **Transcriptional activation of the human estrogen receptor by DDT isomers and metabolites in yeast and MCF-7 cells**. *Biochem Pharmacol*, 1997. **53**(8): p. 1161-72.

78. Fischer LJ, Seegal RF, Ganey PE, Pessah IN, Kodavanti PR: **Symposium overview: toxicity of non-coplanar PCBs**. *Toxicol Sci*, 1998. **41**(1): p. 49-61.

79. Arcaro KF, Yi L, Seegal RF, Vakharia DD, Yang Y, Spink DC, Brosch K, Gierthy JF: **2,2',6,6'-Tetrachlorobiphenyl is estrogenic in vitro and in vivo**. *J Cell Biochem*, 1999. **72**(1): p. 94-102.

80. Gierthy JF, Arcaro KF, Floyd M: **Assessment of PCB estrogenicity in a human breast cancer cell line**. *Chemosphere*, 1997. **34**(5-7): p. 1495-505.

Figure legends

Figure 1. 17β-Estradiol (E2) dose-response in MVLN cells. The MVLN cells were seeded in 96 well plates at a density at $3x10^4$ cells/well and exposed to E2 in the concentration range 0.05 pM to 500 pM for 24 hours. Solvent control (EtOH) was set to 100%. Mean values ± standard deviations are shown, n=10. RLU: relative luciferase unit per µg cell protein. The EC_{50}, the EC_{40} and the EEQ values were calculated by interpolation to the curve using the Sigma Plot program. For further description see the method section.

Figure 2. The xenoestrogenic CALUX activity of study groups. (A) Agonistic activity of serum extracts alone (XER) and (B) competitive XER activity upon coexposure with 25 pM E2 (EC_{40}) (median, quartiles (25% and 75%) and extreme variables). For the Swedish study group an extreme agonistic (A) RLU value of 12.02 was determined (not shown). The reference line of the respective solvent controls ± SD (3.13 ± 0.16) are given as stiplet lines.

Figure 3. Scatter plot of the relation between xenoestrogenic activity and CB-153 and *p,p'*-DDE. Scatter plot of selected correlations between XER activity, RLU/ml serum, and CB-153, (A, Sisimiut) and XER activity, RLU/ml serum, and *p,p'*-DDE (B, Sisimiut; D, Warsaw) and XER-EEQ level, ng/g lipid, and CB-153 (C, Warsaw), (see Table 3). For definitions see legend to Table 2. The values are given as ln transformed data. RLU: relative luciferase units.

Figure 4. Schematic illustration of xenoestrogenic activities related to the POP markers Xeno-estrogenic (XER) and competitive XER (XERcomp) serum activities related to CB153 and *p,p'*-DDE levels for the four country based study groups as a relation between (A) XER and *p,p'*-DDE, (B) XER and CB153, (C) XERcomp and *p,p'*-DDE, and (d) XERcomp and *p,p'*-DDE. The values are given as ln transformed data.

Greenland: ■ – – – ; Warsaw: ● ——— ; Sweden: ▼ ·········· ; Kharkiv: * – · – · –

Table 1. Demographic and life style characteristics of men in the study groups

		Greenland GR, sum n=75	*Sisimiut GR n=50*	*Tasiilaq GR n=25*	***Warsaw PL n=100***	***Sweden SE n=100***	***Kharkiv UA n=88***	***All n=363***
Age	median	30	30	28	30	46	26	32
(year)	min-max	23-47	18-46	20-43	18-46	24-67	16-45	16-67
BMI	median	26	25	26	26	26	24	25
(Kg/m^2)	min-max	19-38	12-36	20-58	12-58	22-37	19-36	12-58
Alcohol	median	2.0	2.0	2.0	3.5	n.a	2.5	3.0
(drink/week)	min-max	0-35	0-35	0-21	0-30		0.2-15	0-35
Smoking	%	87	86	88	49	40	82	68
Seafood	median	2.0	2.0	2.0	1.0	n.a	4.0	2.0
(days/week)	min-max	0-9.0	0-9.0	0-7.0	0-9.0		1.0-9.0	0-9.0
Coffee	median	3.0	3.0	4.0	2.0	n.a	2.0	2.0
(cups/day)	min-max	0-20	0-20	0-10	0-6.0		1.0-7.0	0-20
Testoserone	median	14	16	13	13	12	18	14
(nmol/l)	min-max	3.2-75	10-23	3.2-25	6.5-23	4.2-28	8.4-32	3.2-32
Estradiol	median	59	61	58	72	67	81	71
(nmol/l)	min-max	31-88	31-85	38-88	45-296	25-155	33-144	25-296
SHBG	median	27	31	24	22	32	26	27
(nmol/l)	min-max	11-62	19-62	11-48	5.9-79	7.8-68	9.5-54	5.9-79
FSH	median	4.6	4.3	4.7	3.6	5.1	3.5	4.3
(IU/l)	min-max	0.7-25	1.9-25	0.7-11	0.7-17	1.8-20	1.2-12	0.7-25
LH	median	4.6	4.0	4.6	3.7	3.9	4.0	4.0
(IU/l)	min-max	1.6-13	2.6-8	1.6-13	1.8-8.9	1.5-11	1.4-9.5	1.4-13
Inhibin	median	204	209	194	149	169	181	170
(ng/l)	min-max	57-367	71-367	57-361	22-338	67-427	60-364	22-427

2A		Greenland GR, sum	Sisimiut GR	Tasiilaq GR	Warsaw PL	Sweden SE	Kharkiv UA	ALL
XER[*1] **RLU/ml serum**	N	72	50	22	98	100	88	358
	Median	**2.9**	**3.0**	**2.8**	**3.1**	**3.0**	**3.2**	**3.0**
	Min	*1.0*	*2.4*	*1.0*	*2.4*	*2.4*	*1.0*	*1.1*
	Max	*6.0*	*3.4*	*6.0*	*6.5*	*12*	*8.0*	*12*
	% agonist	*1*	*0*	*4*	*21*	*12*	*14*	-
	% antagonist	*35*	*36*	*32*	*5*	*12*	*17*	-
XER-EEQ pg/g lipid[*2]	**N**	1	0	1[♣]	21	10	11	43
	Median	-	-	-	**103**	**76**[♥]	**139**	**114**
	Min	-	-	-	*44*	*50*	*809*	*44*
	Max	-	-	-	*516*	*364*	*580*	*580*
XER comp[*3] **RLU/ml serum**	N	72	50	22	94	94	88	348
	Median	**2. 7**	**2.6**	**2.8**	**3.0**	**2.9**	**2.9**	**2.9**
	Min	*2.0*	*2.2*	*2.0*	*1.8*	*1.0*	*1.1*	*1.0*
	Max	*3.8*	*3.7*	*3.8*	*7.0*	*6.8*	*4.5*	*7.0*
	% add/syn	*1*	*2*	*0*	*13*	*3*	*1*	-
	% antagonist	*71*	*76*	*60*	*7*	*19*	*30*	-
CB153 ng/g lipid	N	74	49	25	100	98	82	354
	Median	**220**	**170**	**540**	**16**	**210**	**47**	**79**
	Min	*5.1*	*5.1*	*72*	*3.3*	*41*	*5.5*	*3.3*
	Max	*5500*	*780*	*5500*	*130*	*1500*	*200*	*5500*
DDE ng/g lipid	N	74	49	25	100	98	82	354
	Median	**630**	**490**	**1400**	**570**	**240**	**880**	**560**
	Min	*66*	*66*	*193*	*240*	*55*	*324*	*55*
	Max	*13000*	*2200*	*13000*	*2100*	*2300*	*12000*	*13000*

2B		*All study group data	Greenland GR- sum	Sisimiut GR	Warsaw PL	Sweden SE	Kharkiv UA
XER		**0.003**					
	Sisimiut		-	**-**	**<0.001**	**0.02**	0.42
	Tasiilaq		-	0.98	0.24	0.48	0.68
	Warsaw		**<0.001**		-	0.35	0.15
	Sweden		**0.005**			-	0.58
	Kharkiv		**0.03**				
XER-EEQ**		0.63					
	Sisimiut		-	-	nd	nd	nd
	Tasiilaq		-	-	nd	nd	nd
	Warsaw		nd		-	0.93	0.79
	Sweden		nd			-	0.76
XERcomp		**<0.001**					
	Sisimiut		-	-	**<0.001**	**0.03**	**0.01**
	Tasiilaq		-	0.59	**0.02**	1.00	1.00
	Warsaw		**<0.001**		**-**	**0.004**	**0.002**
	Sweden		0.11			-	1.00
	Kharkiv		0.06				

Table 2 Xenoestrogenic serum activities, estradiol equivalents and lipid adjusted CB153 and *p,p'*-DDE in serum of the study groups

A. Xenoestrogenic serum activities, estradiol equivalents and lipid adjusted CB153 and *p,p'*-DDE in serum of the study groups. GR: The study group in Greenland was evaluated both as sum of the combined data and as divided into the two involved districts (Sisimiut and Tasiilaq) because of different levels of exposure to POP markers. ***1**: XER: Xeno-estrogenic activity, agonistic or antagonistic activity of serum extract alone given as the relative luciferase activity (RLU) per ml serum as ratio to the solvent background control (3.13 RLU/ml serum), which was set to 1. The % agonistic and % antagonistic indicates the % of samples eliciting a significantly increase or decrease in XER activity compared to solvent background control. ***2**: XER–estradiol equivalents (XER-EEQ) of the samples eliciting significantly agonistic activity given as pg/g serum lipid, data calculated by interpolation to the E2 dose response curve using the sigma plot program (see figure 1). ♣: One serum sample only from Greenland had an agonistic action and thus no XER-EEQ value is given. ♥: One Swedish sample was too high to calculate the XER-EEQ. ***3**: XERcomp: XER competitive activity of serum extract + 25 pM 17β-estradiol (E2-EC_{40}) given as ratio to RLU/ml serum to the E2-EC_{40} solvent control (3.13 RLU/ml serum), which was set to 1; % add/syn: additive/synergistic and % antagonistic indicates the % of samples responding with a further increase or a decrease of the E2-EC_{40} induced activity, respectively.

B. Multiple comparisons of means of xenoestrogenic activity of serum extract between the study groups. Ln transformed data and lipid adjusted POP data was used for the Oneway ANOVA and Post Hoc statistical analyses. The data given are *p* values. *: ANOVA evaluation of the combined study group data except ** for XER-EEQ including only the European groups. Statistical significant results are given in bold.

Table 3. Spearman's correlation analyses between xenoestrogenic serum activities and the level of CB153 and *p,p'*-DDE.

	Greenland GR, sum			Sisimiut GR			Tasiilaq GR			**Warsaw** PL			**Sweden** SE			**Kharkiv** UA		
	n	r_s	p	*n*	r_s	p	*n*	r_s	p	*n*	r_s	p	*n*	r_s	p	*n*	r_s	p
CB153																		
XER	*70*	-.22	.07	*48*	**-.32**	**.03**	*22*	.23	.30	*98*	.02	.89	*98*	.007	.95	*82*	.13	.26
XER-EEQ*	-	-	-	-	-	-	-	-	-	*21*	**-.45**	**.04**	*10*	.007	.86	*11*	-.15	.67
XERcomp	*70*	.16	.18	*48*	.02	.88	*22*	-.07	.77	*94*	.07	.50	*92*	-.13	.21	*82*	.03	.81
***p,p'*-DDE**																		
XER	*70*	**-.29**	**.02**	*48*	**-.39**	**<.01**	*22*	.13	.57	*99*	**.20**	**.05**	*98*	-.04	.72	*82*	-.09	.42
XER-EEQ*	-	-	-	-	-	-	-	-	-	*21*	-.13	.57	*10*	.12	.75	*11*	-.09	.79
XERcomp	*70*	.11	.33	*48*	-.05	.74	*22*	*-.13*	.58	*94*	.13	.21	*92*	-.18	.08	*82*	.02	.85
XER/XERcomp[1]	*70*	**.23**	**.05**	*50*	**.28**	**.05**	*22*	.23	.34	*94*	**.52**	**<.001**	*94*	**.26**	**.01**	*88*	*.46*	.08
CB153/ DDE[2]	*74*	**.94**	**<.001**	*49*	**.90**	**<.001**	*25*	**.89**	**<.001**	*100*	**.27**	**<.01**	*98*	**.75**	**<.001**	*82*	**.45**	**<.001**

ln-transformed and POP lipid adjusted data was used. For definition of GR, XER, XERcomp and XER-EEQ see legend to Table 2. Statistical significant data is given in bold. [1] and [2]: Spearmans correlation between XER vs XERcomp and CB-153 and *p,p'*-DDE, respectively

Table 4: Multiple regression of the combined study group data

Response variable	Exposure variable	No interaction (p-value)	Common slope Estimate (SE), p-value	Common intercept (p-value)	Adjusted R-square
XER (N=348)	CB-153	0.86	-0.01 (0.01), 0.34	0.05	0.029
	p,p'- DDE	0.24	-0.03 (0.02), 0.12	0.005	0.034
XERcomp (N=338)					
	CB-153	0.20	0.01 (0.02), 0.45	<0.001	0.087
	p,p'- DDE	0.15	-0.006 (0.02), 0.71	<0.001	0.086

Response and exposure variables are ln-transformed in the analysis. No interaction: p-value for the test for no interaction between study group and exposure variable. Common slope: the estimated common slope across study groups assuming no interaction. Common intercept: p-value for the test of a common intercept across study groups assuming a common slope. Adjusted R-square assuming a common slope.

Figure 1. 17β-Estradiol (E2) dose-response in MVLN cells.

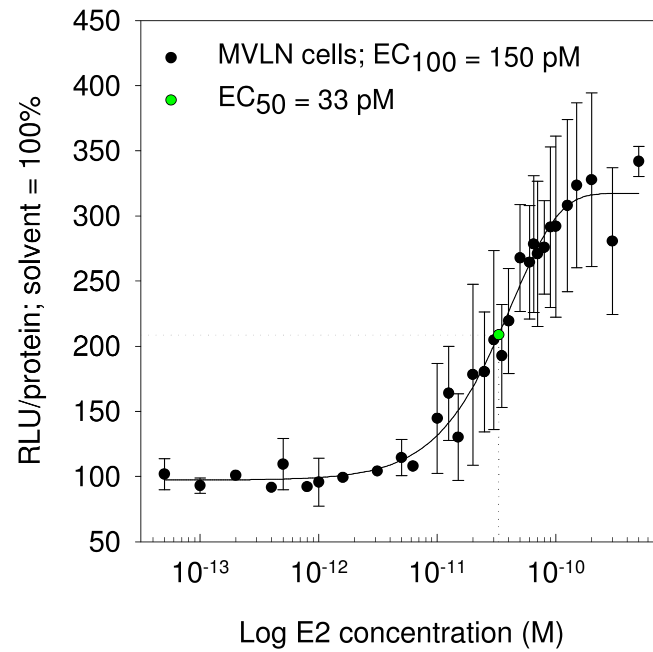

Figure 1

Figure 2. The xenoestrogenic serum activity of study groups.

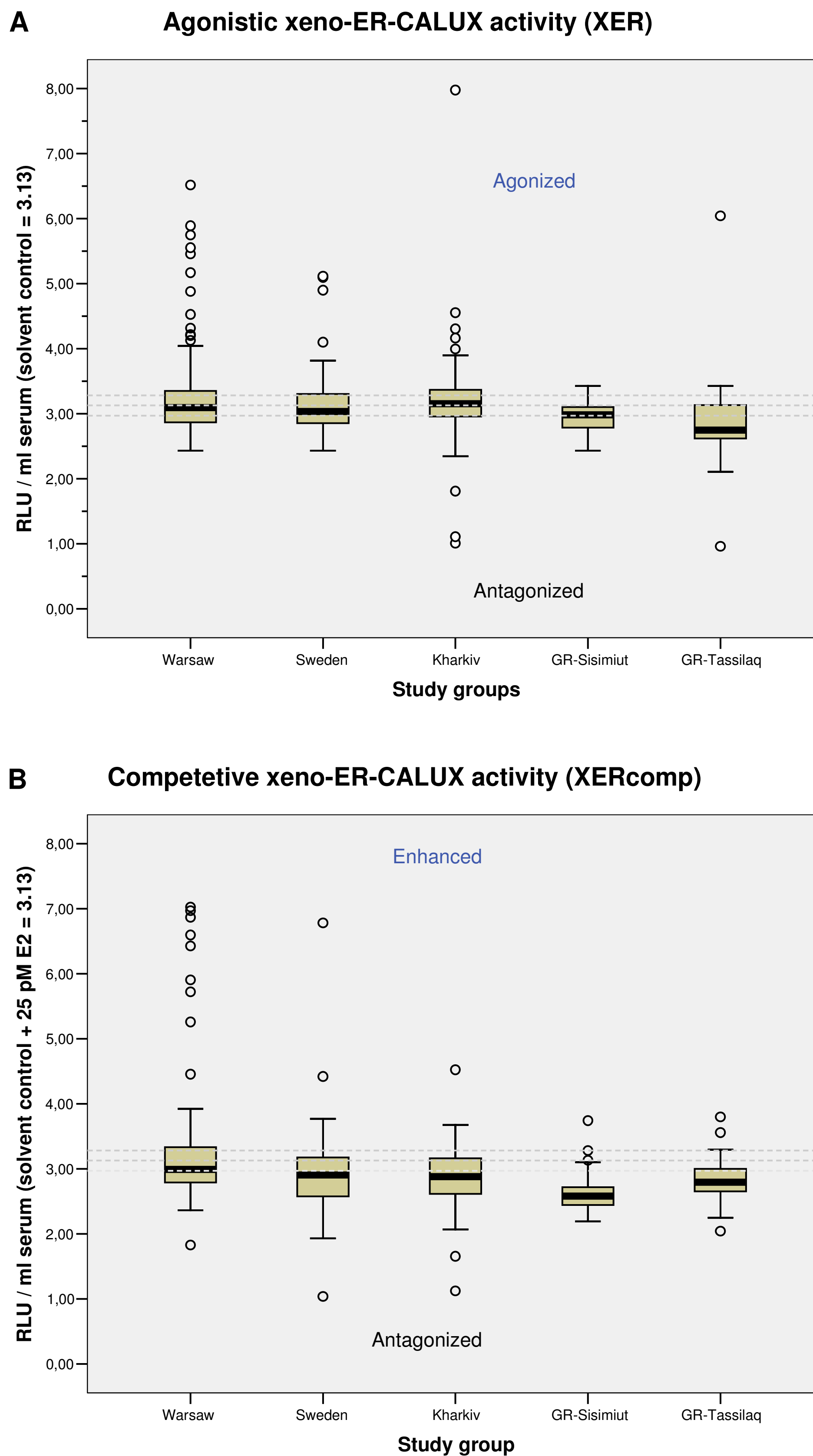

Figure 2

Figure 3. Scatter plot of the relation between xenoestrogenic activity and CB-153 and *p,p'*-DDE.

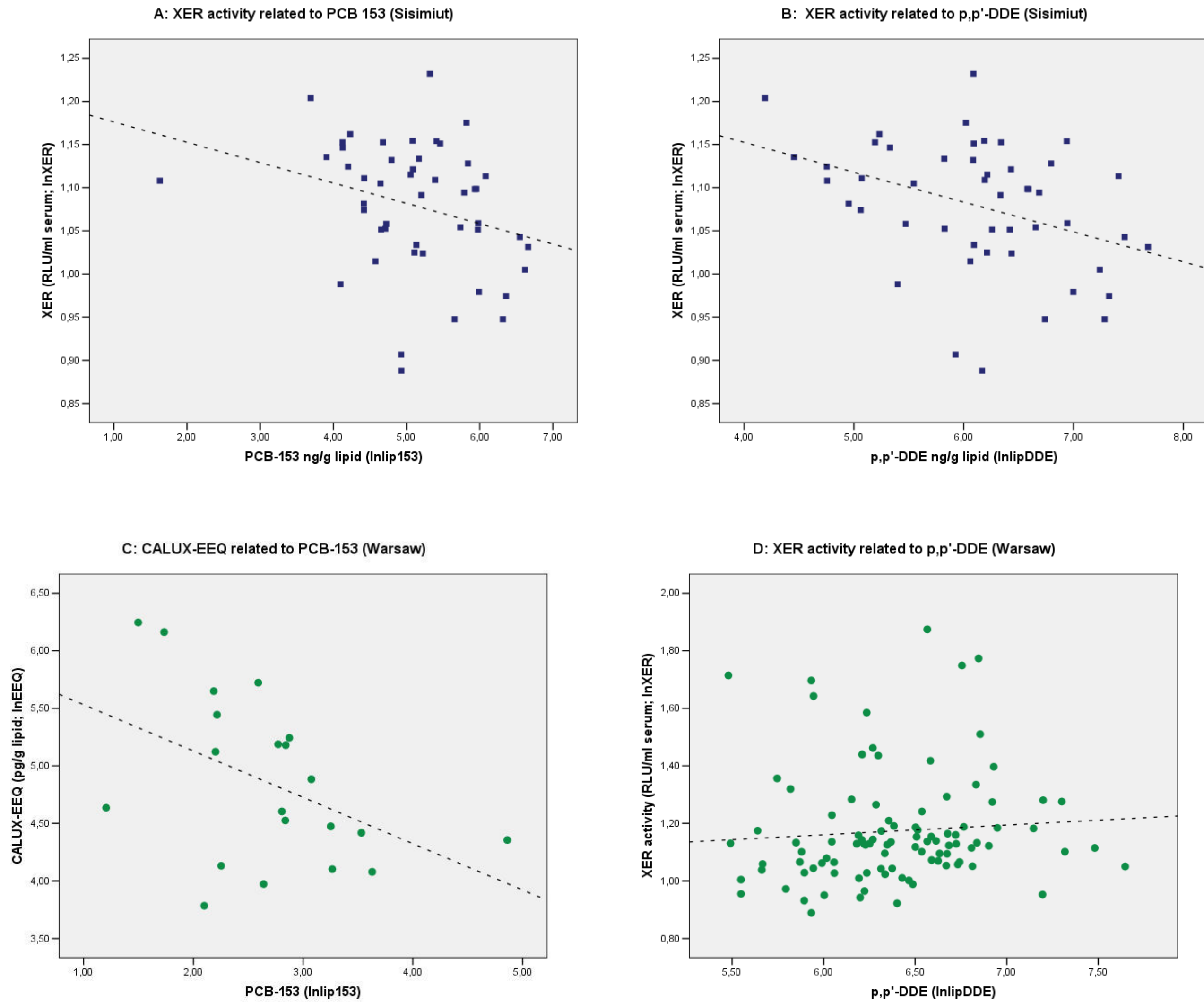

Figure 3

Figure 4. Schematic illustration of xenoestrogenic activities related to the POP markers

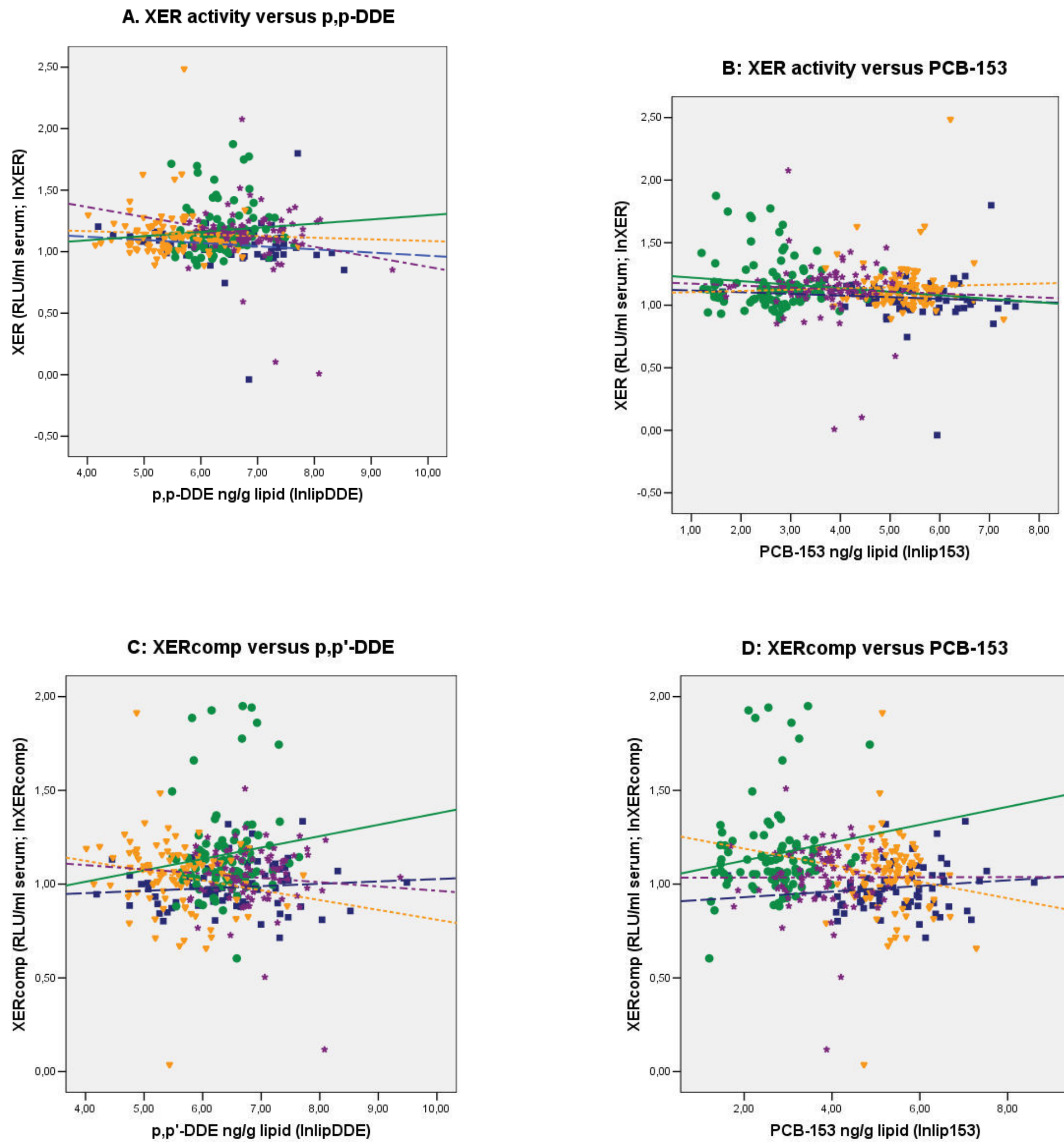

Figure 4