Xenoandrogenic Activity in Serum Differs across European and Inuit Populations

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Background: Animal and *in vitro* studies have indicated that human male reproductive disorders can arise as a result of disrupted androgen receptor (AR) signalling by persistent organic pollutants (POPs). Our aim in the present study was to compare serum xenoandrogenic activity between study groups with different POP exposures and to evaluate correlations to the POP proxy markers 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB-153) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethylene (*p,p′*-DDE).

Methods: We determined xenoandrogenic activity in the serum fraction containing the lipophilic POPs but free of endogenous hormones. Adult male serum (n = 261) from Greenland, Sweden, Warsaw (Poland), and Kharkiv (Ukraine) was analyzed. Xenoandrogenic activity was determined as the effect of serum extract alone (XAR) and in the presence of the synthetic AR agonist R1881 (XARcomp) on AR transactivated luciferase activity.

Results: The study groups differed significantly with respect to XARcomp activity, which was increased in the Inuits and decreased in the European study groups; we observed no difference for XAR activity. We found the highest level of the AR antagonist *p,p′*-DDE in Kharkiv, and accordingly, this study group showed the highest percent of serum samples with decreased XARcomp activities. Furthermore, the percentage of serum samples with decreased XARcomp activities followed the *p,p′*-DDE serum level for the European study groups. No correlations between serum XAR or XARcomp activities and the two POP markers were revealed.

Conclusions: The differences in XARcomp serum activity between the study groups suggest differences in chemical exposure profiles, genetics, and/or lifestyle factors.

Key words: AR activity, CB-153, human serum, polychlorinated biphenyls, *p,p′*-DDE. *Environ Health Perspect* 115(suppl 1):21–27 (2007). doi:10.1289/ehp.9353 available via *http://dx.doi.org/* [Online 8 June 2007]

The endocrine-disrupting potential of persistent organic pollutants (POPs) such as organochlorine pesticides, polychlorinated biphenyls (PCBs), and polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDDs/PCDFs) has received significant attention since the early 1990s. These endocrine-disrupting chemicals (EDCs) have the potential to mimic, enhance, or antagonize the biological activity of endogenous hormones and thereby interfere with the development and function of the immune, reproductive, and central nervous systems, and thus the cognitive development in animals and humans (Ahmed 2000; Bonefeld-Jørgensen 2004; Bonefeld-Jørgensen and Ayotte 2003; Hotchkiss et al. 2002; Jacobson and Jacobson 1996; McLachlan et al. 2001; Patandin et al. 1999). The use of many organochlorine pesticides and PCBs has been restricted or banned since the 1970s in most industrialized countries, but their lipophilic and persistent nature cause bioaccumulation and magnification in the marine food web and piscivorous birds (Dewailly et al. 1993), and thus continue to be a potential health threat to wildlife and humans. POPs are globally ubiquitous due to long-range transport by atmospheric and oceanic currents and are found in, for example, Arctic areas (Barrie et al. 1992; Macdonal et al. 2000). Many PCB congeners, PCDDs/PCDFs, and 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)] have been found in human blood, adipose tissue, and breast milk (Archibeque-Engle et al. 1997; Bonefeld-Jørgensen and Ayotte 2003; Turusov et al. 2002).

The androgen receptor (AR) is the key regulatory element of androgen cell signaling and is essential for male reproductive functions and development, including spermatogenesis (Sharpe 2006). Antagonistic effects on the AR activity *in vitro* have been reported for several pesticides including *p,p′*-DDE (Kelce et al. 1995), dieldrin (Andersen et al. 2002; Roy et al. 2004), fenarimol (Andersen et al. 2002), methiocarb (Andersen et al. 2002; Birkhoj et al. 2004), prochloraz (Andersen et al. 2002; Birkhoj et al. 2004; Vinggaard et al. 2002), and vinclozolin (Kelce et al. 1994). Furthermore, several PCB congeners (CBs 49, 66, 74, 77, 105, 118, 126, 138, 153, and 156) have been reported to act as AR antagonists (Bonefeld-Jørgensen et al. 2001; Endo et al. 2003; Schrader and Cooke 2003).

During recent years there has been a concern regarding a possible time-related deterioration of human sperm production and a concomitant increase in the incidence of testicular germ cell cancer and congenital abnormalities of male reproductive organs, such as hypospadias and cryptorchidism (Huyghe et al. 2003; Manson and Carr 2003; Skakkebaek 2003; Toppari 1996; Vidaeff and Sever 2005). Virtanen et al. (2005) suggested that the so-called testicular dysgenesis syndrome (poor sperm counts, testicular cancer, hypospadias, and cryptorchidism) is the result of genetic predisposition combined with certain environmental- and lifestyle–related exposures, including EDCs. Many POPs have shown hormone-disrupting activities in experimental studies, and prenatal animal exposures to pesticides, PCBs, and *p,p′*-DDE clearly have shown reproductive disruptive effects, including decreased sperm quality and malformations (Gray et al. 1999, 2000; Kelce et al. 1997; Kitamura et al. 2003; Shono et al. 2004).

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The combined effects of the large number of environmental contaminants present in human and wildlife are unexplored. Studies on the combined effects of some estrogenic and antiandrogenic chemicals performed *in vitro* and in rats (Birkhoj et al. 2004; Gray et al. 2001, 2004; Hotchkiss et al. 2004; Nellemann et al. 2003; Payne et al. 2001) have shown that the combined effects of single compounds of low potency cannot be ignored. In fact, Rajapakse et al. (2002) showed *in vitro* that xenoestrogen mixtures below their no observed effect concentration caused a dramatic additive enhancement of hormone actions.

The present study is part of a large collaborative research project, INUENDO, funded by the European Union Commission with the overall aim to estimate the impact of POP exposure on human fertility. Cohorts were established in three European countries—Sweden, Poland, and Ukraine—together with cohorts of Inuits from Greenland. 2,2′,4,4′,5,5′-Hexachlorobiphenyl (CB-153) and *p,p′*-DDE were used as proxy biomarkers of exposure to POPs. The specific aim of the present study was to determine the actual levels of xenoandrogenic activity in the serum fractions containing lipophilic POPs but free of endogenous hormones, and to evaluate whether xenoandrogenic activity was associated with the two POP proxy markers. We used the AR-mediated chemically activated luciferase expression (AR-CALUX) bioassay to assess the integrated biological activity of AR-active lipophilic POP compounds in their actual mixture in serum.

Xenoestrogenic activity in human adipose tissue and the serum POP fraction free of endogenous hormones have previously been described (Bonefeld-Jørgensen et al. 2006; Fernandez et al. 2004; Ibarluzea et al. 2004; Rasmussen et al. 2003; Rivas et al. 2001), and total androgenic bioactivity has been measured in whole human serum, including endogenous hormones (Paris et al. 2002; Raivio et al. 2001; Roy et al. 2006). However, the present study is, to our knowledge, the first report of the actual xenoandrogenic activity of the lipophilic POP faction of human serum. Our hypothesis was that xenoandrogenic activity would differ between study groups because of different exposure profiles and that the selected POP proxy markers would correlate to the xenoandrogenic activities.

Methods

Study population and collection of blood samples. Subjects for the main INUENDO study were recruited among pregnant women and their male spouses from May 2002 through February 2004 in 19 cities and settlements in Greenland, in Warsaw (Poland), and in Kharkiv (Ukraine) (Toft et al. 2005). In addition to the pregnant couples, an already established cohort of fishermen from Sweden (Rignell-Hydbom et al. 2005) was included. Altogether, 798 men provided semen and blood samples. In the present study, we analyzed serum samples from a subset of the INUENDO study groups, consisting of 261 adult males (37 Inuits from Greenland, 83 men from Warsaw, 59 from Sweden, and 82 from Kharkiv). We had intended to analyze 400 serum samples of high/low POP exposure; however, in some cases too little blood was collected for the analysis. Because of time limitations, the serum samples were randomly selected before POP determination. The study was approved by the local ethical committees representing all participating populations, and all subjects gave written or oral informed consent. Venous blood samples were collected into 10-mL vacuum tubes; after centrifugation, serum samples were transferred to brown glass bottles (Termometerfabriken, Gothenburgh, Sweden).

Determination of CB-153 and p,p′-DDE. Serum concentrations of CB-153 and *p,p′*-DDE were determined using solid phase extraction (SPE) and on-column degradation of lipids, followed by analysis with gas chromatography–mass spectrometry (Rignell-Hydbom et al. 2004). The relative standard deviations, calculated from samples analyzed in duplicate on different days, was < 18% for CB-153 and < 11% for *p,p′*-DDE. The detection limits were 0.05 ng/mL for CB-153 and 0.1 ng/mL for *p,p′*-DDE. The analyses of CB-153 and *p,p′*-DDE were part of the Round Robin intercomparison program (H. Drexler, Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine, University of Erlangen-Nuremberg, Germany) with analysis results within the tolerance limits. The POP concentrations were adjusted for serum lipids analyzed with enzymatic methods, and the interassay coefficients of variation (CVs) were 1.5–2.0% (Jonsson et al. 2005).

SPE-HPLC fractionation of the serum samples. To obtain the serum fraction containing the actual mixture of bioaccumulated lipophilic POPs, we performed an SPE followed by high performance liquid chromatography (HPLC) fractionation on 3.6 mL serum (Bonefeld-Jørgensen et al. 2006; Hjelmborg et al. 2006). The first HPLC fraction (F1: 0.00–5.30 min, protected from light in brown tubes), which included most POPs while leaving out the endogenous hormones (Hjelmborg et al. 2006), was evaporated to near dryness and frozen for later AR-CALUX analysis. To verify that the endogenous hormones were separated from F1 but present in F2.1 (5.30–12.0 min), we performed AR-CALUX analyses of the F1 and F2.1 SPE-HPLC fractions of different male serum.

Batches of serum from the blood bank of Aarhus Sygehus (Denmark) were combined and distributed into 3.6-mL portions and stored at –80°C. This was done for male (KHM) and female (KHF) serum; on a weekly basis, one sample from each sex was processed by the SPE-HPLC method in parallel with the project samples; these samples served as serum controls for this cleanup procedure. The mean intra-CV (coefficient of variation within the same assay) of these serum controls was < 11%, and the inter-CV (day to day) was < 31% ($n = 12$).

On the day of analyses, the SPE-HPLC F1 fractions (project samples and serum controls) were dissolved as previously described (Bonefeld-Jørgensen et al. 2006; Hjelmborg et al. 2006). The serum samples were analyzed randomly, and we attempted to analyze serum from different study groups in each independent assay.

AR-CALUX assay. AR transactivity was determined in Chinese Hamster ovary cells (CHO-K1) by transient cotransfection with the *MMTV-LUC* reporter vector (kindly provided by R.M. Evans, The Salk Institute for Biological Studies, La Jolla, CA, USA) and the AR expression plasmid *pSVAR0* (kindly provided by A.O. Brinkmann, Erasmus University, Rotterdam, the Netherlands).

The synthetic AR agonist methyltrienolone (R1881) and the antagonist hydroxyflutamide (HF; Mikromol GmbH, Luckenwalde, Germany), both protected from the light, were used as dose–response controls in each assay. We determined the EC_{50} (median effective concentration) of R1881 (0.025 nM) and the IC_{50} (median inhibitory concentration) of HF (58.5 nM) using the Chapman, 4 parameters equation in Sigma Plot (SPSS Inc., Chicago, IL, USA). The R1881 EC_{50} served as a positive control on each separate plate and was used to determine the competitive effect of HF and the F1 serum fraction. The solvent controls (± R1881 EC_{50}) consisted of samples treated the same as the SPE-HPLC F1 fractions, but without the serum. The solvent control and the R1881 EC_{50} solvent control had inter-CVs of ≤ 11% ($n = 13$) and ≤ 13% ($n = 13$), respectively. The xeno-AR activity was determined in the F1 serum fraction alone (XAR), and the competitive xeno-AR activity (XARcomp) was determined upon co-treatment of cells with F1 and R1881 EC_{50}.

We performed the reporter gene assay as described by Birkhoj et al. (2004) with minor modifications: The transfection was carried out with 100 ng DNA, and the transfection reagent Fugene was not removed before application of the samples; the dissolved SPE-HPLC F1 samples ± R1881 EC_{50}, in triplicate, were added to the cells in parallel with the described controls (KHM, KHF, R1881 EC_{50}, and solvent ± R1881 EC_{50}). Cell lysis,

followed by luciferase activity and protein measurements, were performed as described by Bonefeld-Jørgensen et al. (2006).

The luciferase activity was corrected to the cell protein content and expressed in relative luciferase units per microgram protein (RLU/protein). If one of the triplicate values deviated > 30% from the other two values, the mean was calculated from two wells only. To standardize the data for transfection efficiency, the XAR and XARcomp activity data was normalized to the respective solvent controls, which were set to 1. Finally, the data was given as activity per milliliters of serum, and the values of the solvent controls were 3.13 RLU/mL serum.

Statistical analysis. We checked data distribution using Q-Q plots. The XAR and XARcomp activities and lipid-adjusted CB-153 and *p,p′*-DDE were natural-log (ln) transformed to improve normality, and the statistical analyses were performed on the continuous ln-transformed data.

We performed the comparisons of means of XAR and XARcomp among the different study groups using one-way analysis of variance (ANOVA). If significant differences were observed between the groups, multiple comparison ad hoc tests were performed using the least-significant difference (LSD) pair-wise multiple comparison test for variables with equal variance ($p > 0.05$) and Dunnett's T3 test for variables with an unequal variance ($p \leq 0.05$). We tested for homogeneity of variance using Levene's test.

We used Spearman's rank correlation to evaluate associations of the xenoandrogenic activity both to POP markers and to endogenous serum estradiol and testosterone (total and free), which served as method verification that the sex hormones were excluded from the F1 fraction. The overall association between POP markers and xenoandrogenic activity across the study groups were evaluated by comparing the regression lines for each study group using multiple linear regression analysis. In the first step, we investigated whether the associations across the study groups are homogenous. If this hypothesis was accepted, we used a model with parallel regression lines to analyze common slopes and intercepts of the regression lines for each study group. If both the hypothesis of a common slope and the hypothesis of a common intercept were accepted, indicating that the regression lines are equal, the data for the study groups could be combined. However, if one or both of the hypotheses were rejected, the regression lines are not equal and the data cannot be combined.

Our hypothesis was that a potential determinant of POP bioaccumulation might also be a potential determinant for serum xenoandrogenic activity. From the literature and from an assessment of the total INUENDO study populations (Jonsson et al. 2005; Toft et al. 2005), age and seafood intake are known determinants affecting the POP serum level. We also evaluated lifestyle characteristics (Table 1) as potential determinants of XAR and XARcomp activities. Using multivariate linear regression to evaluate the impact of POP biomarkers and potential determinants on XAR/XARcomp, blocks of variables, together with either CB-153 or *p,p′*-DDE, were entered as follows: In block I, age and seafood intake were included in the model; and in block II, age, seafood, smoking status (smoked ever, yes/no), body mass index (BMI), and alcohol and coffee intake were included. Because many values of the potential determinants were missing, the number of available observations in the confounder analyses are much smaller than in the unadjusted analysis on the full data set (full data set, $n = 250$; block I of confounders, $n = 192$; block II, $n = 104$). Therefore, we performed the analysis only on the block II data set ($n = 104$) containing information about all the determinants. The statistical analysis was performed in SPSS 10.0 (SPSS Inc, Chicago, IL, USA). We considered a p-value ≤ 0.05 to be statistically significant.

Results

Correlations between xenoandrogenic serum activity and endogenous hormones: method verification. AR-CALUX analysis of the SPE-HPLC serum fractions F1 and F2.1 showed that the F2.1 fraction (containing androsterone and testosterone,) elicited significantly higher XAR activity than the F1 fraction (free of endogenous hormones) (Figure 1A). This indicates that the endogenous androgens were excluded from the F1 fraction. The XAR activity of the KHM and KHF F1 serum controls did not differ significantly from the solvent control, whereas the XARcomp activity of KHM was significantly lower than the R1881 EC_{50} solvent control (Figure 1B).

We found no correlation between XAR and endogenous blood testosterone (free and total) and estradiol for the combined data of the study groups (data not shown).

Basic characteristics of the study groups. Demographic and lifestyle factors (Table 1) that may potentially influence the AR-mediated activities of the study groups were similar with those obtained for the total INUENDO study population (Toft et al. 2005). The levels of CB-153 and *p,p′*-DDE (Table 2) were in the same range as for the main study groups

Table 1. Characteristics of the men in the study groups.

	Greenland	Warsaw	Sweden	Kharkiv	All
Age (years)					
No.	35	81	58	79	253
Median (SD)	30 (6.0)	30 (4.1)	44 (9.8)	25 (5.4)	30 (9.6)
Min–max	18–46	24–46	24–68	16–45	16–68
BMI (kg/m^2)					
No.	36	80	59	80	255
Median (SD)	26 (3.7)	25 (3.4)	26 (2.8)	23 (3.1)	25 (3.3)
Min–max	19–35	19–38	22–36	19–36	19–38
Alcohol (drinks/week)					
No.	23	71		61	155
Median (SD)	2.8 (4.6)	4.0 (6.5)	NA	3.0 (2.9)	3.0 (5.2)
Min–max	0–31	0–30		0.5–15	0–31
Smoking					
Percent (No.)	86 (35)	49 (81)	64 (59)	82 (81)	68 (256)
Seafood (days/week)					
No.	36	76		80	192
Median (SD)	1.5 (1.5)	1.0 (1.2)	NA	4.0 (1.2)	2.0 (1.6)
Min–max	0–7	0–9		1–9	0–9
Coffee (cups/day)					
No.	34	70		35	139
Median (SD)	3.0 (4.0)	1.3 (1.2)	NA	2.0 (1.2)	2.0 (2.5)
Min–max	0–12	0–6		1–7	0–12
Total T (nmol/Ll)					
No.	11	69	58	82	220
Median (SD)	15 (4.1)	13 (4.3)	11 (5.0)	18 (4.5)	15 (5.3)
Min–max	10–23	6–23	4–28	8–31	4–31
Free T (nmol/L)					
No.	11	69	58	82	220
Median (SD)	0.32 (0.07)	0.30 (0.08)	0.24 (0.08)	0.39 (0.10)	0.30 (0.11)
Min-max	0.11–0.49	0.15–0.51	0.09–0.50	0.20–0.64	0.09–0.64
Estradiol (nmol/L)					
No.	11	69	58	82	220
Median (SD)	59 (16)	70 (32)	64 (21)	79 (24)	71 (27)
Min–max	31–85	45–297	25–154	33–144	25–297

Abbreviations: max, maximum; Min, minimum; NA, not available; T, testosterone. No. is the number of individuals with data for the specific demographic and lifestyle characteristics.

(Jonsson et al. 2005) as were the intercorrelations between serum concentration of CB-153 and *p,p′*-DDE (Greenland: r_S = 0.93; Sweden: r_S = 0.68; Kharkiv: r_S = 0.52; Warsaw: r_S = 0.35).

The XAR activity of the study groups did not differ significantly (ANOVA, p = 0.51), reflecting that the percentage of serum extracts with increased XAR activity (Table 2) was similar for the four study groups (25–35%).

One-way ANOVA (p < 0.001) followed by multiple comparison of means showed that the XARcomp activity was significantly different between the study groups (LSD test, p < 0.001) except for Sweden and Warsaw (LSD test, p = 0.91). The four study groups also differed with respect to the percentage of serum samples with increased or decreased XARcomp activity (Table 2): in Greenland 22% and 3%, and in Kharkiv 0% and 50%, respectively. For the Warsaw and Swedish study groups, 21% and 8%, respectively, of the serum samples showed a decreased XARcomp activity. For the European study groups, the highest level of *p,p′*-DDE was found in Kharkiv, and this study group had the highest percentage of serum samples (50%) with decreased XARcomp activity. The lowest level of *p,p′*-DDE was observed in the Swedish study group, the group in which we found the lowest percentage of serum samples (8%) with decreased XARcomp activity.

Statistical analysis of xenoandrogenic activity and POP markers. We found no correlations between the POP markers and either XAR or XARcomp in the four study groups (data not shown). Adjusting for seafood and age (block I) and for seafood, age, smoking status, BMI, alcohol, and coffee (block II) in a multiple linear regression model did not change the results (data not shown).

Multiple regression analysis of both response variables (XAR and XARcomp) showed homogeneity of slopes between study groups and the POP markers (Table 3). Furthermore, in the model with parallel regression lines, we found common slopes and intercepts for XAR against CB-153 and *p,p′*-DDE (Table 3), which support the ANOVA analysis showing no difference between the study groups. However, no significant correlations were observed when data were combined (data not shown). For XARcomp against CB-153 and *p,p′*-DDE, we found common slopes but different intercepts (Table 3). Thus, the difference in XARcomp activity between the study groups (ANOVA, p < 0.001) still existed after adjustment for CB-153 and *p,p′*-DDE.

In the European study groups, the percentage of serum samples with decreased XARcomp activity followed the level of *p,p′*-DDE (Kharkiv > Warsaw > Sweden); therefore, we tried to analyze the combined European data. We observed a significant negative association (r_S = –0.21; p = 0.001) between XARcomp and *p,p′*-DDE across the combined European study groups. In the multiple regression analysis of the combined European study groups, the hypothesis of a common intercept was rejected (data not shown).

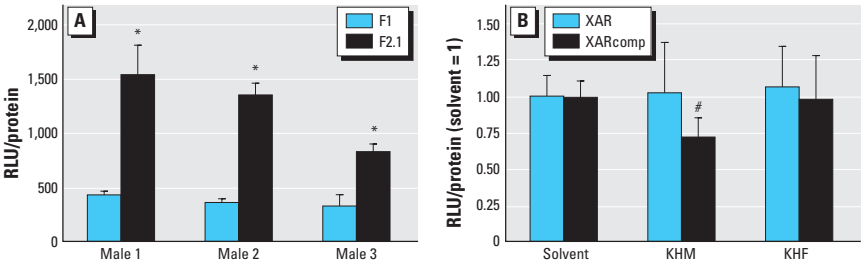

Figure 1. Verification of the HPLC procedure. (*A*) XAR activity of the HPLC fractions F1 (0.00–5.30 min; without endogenous hormones) and F2.1 (5.30–12.0 min; with endogenous hormones) from three different males were determined in the AR-CALUX assay. (*B*) Mean ± SE of the F1 fraction of KHM and KHF serum controls alone (XAR; n = 12) or in the presence of R1881 EC_{50} (XARcomp; n = 9).
*XAR activity of the F2.1 fraction significantly higher than for the F1 fraction. #Significantly lower than the R1881 EC_{50} solvent control.

Table 2. Xenoandrogenic serum activities and lipid-adjusted serum levels of CB-153 and *p,p′*-DDE.

	Greenland	Warsaw	Sweden	Kharkiv	All
XAR RLU/mL serum					
No.	37	83	59	82	261
Mean ± SE	3.85 ± 0.14	3.93 ± 0.21	3.83 ± 0.13	3.57 ± 0.09	3.78 ± 0.08
Percent increased	35	25	34	26	28
Percent decreased	3	5	5	2	4
XARcomp RLU/mL serum					
No.	37	83	59	82	261
Mean ± SE	4.05 ± 0.18	2.99 ± 0.07	3.01 ± 0.10	2.25 ± 0.06	2.91 ± 0.06
Percent increased	22	7	10	0	8
Percent decreased	3	21	8	50	26
CB-153 (ng/g lipid)					
No.	35	83	58	76	252
Mean ± SE	262 ± 34	20 ± 2	238 ± 27	58 ± 5	115 ± 10
p,p′-DDE (ng/g lipid)					
No.	35	83	58	76	252
Mean ± SE	678 ± 83	653 ± 37	299 ± 39	1,130 ± 77	718 ± 35

The percent increased and percent decreased indicate the percentage of serum samples (of the total number of samples) that elicited a significant increase or decrease in XAR/XARcomp activity, respectively. In each independent assay, the significant activity differences between the triple F1 fraction determinations and their respective solvent controls (percent increased and percent decreased) were determined by the Student's *t*-test and *t*-test using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) ($p \le 0.05$).

Table 3. Multiple regression of the combined study group data.

Response variable	Exposure variable	Homogeneity of slopes (*p*-value)	Common slope estimate (SE); *p*-value	Common intercept	Adjusted R^2 (*p*-value)
XAR (n = 252)	CB-153	0.63	0.003 (0.02); 0.91	0.45	0.00
	p,p′-DDE	0.96	–0.001 (0.03); 0.98	0.54	0.00
XARcomp (n = 252)	CB-153	0.92	0.01 (0.02); 0.80	< 0.001*	0.36
	p,p′-DDE	0.56	0.05 (0.03); 0.10	< 0.001*	0.37

Response and exposure variables are ln-transformed. Test for homogeneity of association between exposure variable and outcome variable across study groups, p > 0.05; hypothesis of homogeneity accepted. Estimated common slope across study groups assuming homogeneity of slopes, p > 0.05; hypothesis of a common slope accepted. Test for a common intercept across study groups assuming a common slope, p > 0.05; hypothesis of a common intercept accepted. Adjusted R^2 assuming a common slope, adjusted for degrees of freedom.
*Statistically significant ($p \le 0.05$).

Discussion

In the present study we found that *a*) the XARcomp activity in the Inuits was significantly different from that in the European study groups; *b*) the percentage of serum samples with decreased XARcomp activity in the European study groups [Kharkiv (50%) > Warsaw (21%) > Sweden (8%)] positively followed the *p,p′*-DDE serum level; *c*) the XAR activity was not significantly different between the study groups; and *d*) there were no significant correlations between xeno-AR activities and any of the two POP markers.

It is well documented, both *in vitro* and *in vivo*, that *p,p′*-DDE is an AR antagonist (Araki et al. 2005; Gray et al. 1999; Roy et al. 2004; Vinggaard et al. 1999), which is consistent with the observed decreased XARcomp activities in the European groups. Interestingly, in a parallel INUENDO study, the fecundability among couples in Kharkiv

was lower than among couples from the other study groups (Toft et al. 2005). Furthermore, the lowest sperm motility in semen samples was found in the Kharkiv samples, compared with the three other study groups (Toft et al. 2005). However, in the present study, we found high *p,p′*-DDE levels in serum in the Inuits, who also had the highest percentage of serum samples (22%) with increased XARcomp activity. These data from the different ethnic groups could be caused by a different composition of serum POPs, despite the high amounts of *p,p′*-DDE in Inuits. In parallel INUENDO studies that investigated the xenoestrogenic (Bonefeld-Jørgensen et al. 2006) and dioxin-like (Long et al. 2006) activities in human serum samples, significant differences between Inuits and the European study groups were also observed. In addition to a different exposure pattern, dietary habits and/or lifestyle factors and the genetic difference between Inuits and Caucasians (de Maat et al. 1999) may also be taken into account when evaluating the observed differences.

We found no correlations between the xeno-AR activities and the two POP markers. This is in accordance with previous studies in human adipose tissue or serum that reported the lack of correlation between several suspected endocrine disruptors and estrogenic activities (Fernandez et al. 2004; Ibarluzea et al. 2004; Rasmussen et al. 2003; Rivas et al. 2001). Furthermore, CB-153 might not be optimal as a global exposure marker for POPs because the pattern of distribution of noncoplanar and coplanar CBs can differ geographically depending on occupational and/or nonoccupational exposures (Dewailly et al. 1994).

We rejected the hypothesis of a common intercept for XARcomp activities in the multiple linear regression model. However, when we assumed a common intercept, a significant negative correlation was found between the XARcomp activity and *p,p′*-DDE for the combined European study groups. This suggests that *p,p′*-DDE might be involved in the decreased XARcomp activity in a concentration-dependent manner in the European study groups.

The total androgenic bioactivity in whole human serum samples has been investigated in cell-based reporter gene assays (Paris et al. 2002; Raivio et al. 2001; Roy et al. 2006); these assays represented reliable methods for determining androgenic activities in biological samples. However, these studies determined the total androgenic bioactivity in whole human serum, which contained the endogenous hormones. In contrast, our assay determines the xenoandrogenic activity of the serum fraction of bioaccumulated POPs only.

The inter-CV was ≤ 13% for the solvent controls and < 31% for the serum controls (KHM and KHF). However, our CVs are within the range observed in other *in vitro* studies (Korner et al. 2004) using reporter gene assays (the CV for the androgen control was 22% in stably transfected human prostate adenocarcinoma PC-3 cells, 30% in transiently transfected CHO-K1 cells, and 57% in stably transfected human breast carcinoma MDA-kb2 cells).The higher CV of the serum controls compared with the solvent controls in our study can be explained by further variations introduced by the SPE-HPLC extraction.

The R1881 EC_{50} value of 0.025 nM in our assay was somewhat lower than that seen in other reporter gene assays using other cell lines (Blankvoort et al. 2001; Christiaens et al. 2005; Korner et al. 2004; Lemaire et al. 2004). In stable transfected PC3 cells (PALM), Lemaire et al. (2004) found an EC_{50} for R1881 of 0.066 nM in one study, whereas Korner et al. (2004), who also used PALM cells, reported an EC_{50} of 0.11 nM. Thus, even in the same cell line, the EC_{50} value can vary between laboratories.

Hjelmborg et al. (2006) previously showed that the F1 fraction, analyzed for xenoandrogenic effects, included PCB-180, PCB-126, PCB-81, endosulfan, PCB-153, PCB-138, bisphenol A dimethacrylate, *o,p′*-DDT, 4n-nonylphenol, 4-octylphenol, 4-OH-PCB-121, vinclozolin, bisphenol A, butyl hydroxy anisole, and methiocarb. The F2.1 fraction contained the endogenous hormones pregnenolone, androsterone, progesterone, estrone, and testosterone, as well as the pesticides fenarimol and prochloraz (Hjelmborg et al. 2006). To verify our SPE-HPLC AR-CALUX method, we analyzed the F2.1 serum fraction in parallel with the F1 serum fraction free of endogenous hormones. The significantly higher XAR activity of the F2.1 fraction compared with the F1 fraction indicates that the endogenous hormones, which are capable of activating the AR, are present in F2.1 but not in F1 fractions. Furthermore, the XAR activity of the KHM F1 fraction did not differ from the solvent control or KHF. Because total androgenic bioactivity is higher in men than in women (Paris et al. 2002), a significantly higher XAR activity for KHM, compared with KHF, would have been expected if the endogenous hormones were present in the F1 serum fraction. Vinggaard et al. (1999) previously showed that estradiol is able to compete with R1881 for transactivation of AR in an AR-CALUX assay similar to the one used in the present study. Therefore, we investigated correlations between XAR and the sex hormones testosterone (total and free) and estradiol. We found no correlations between XAR and the sex hormones, confirming optimal separation of the endogenous hormones from the SPE-HPLC F1 fraction. In investigations of total androgen bioactivity in whole human serum samples, including endogenous hormones, Raivio et al. (2001) and Roy et al. (2006) found significant correlations between serum testosterone levels and androgen bioactivity. In a parallel study using the same SPE-HPLC approach, Bonefeld-Jørgensen (et al. 2006) observed no correlations between the endogenous hormones in serum and the xenoestrogenic activity of the F1 serum fraction; this further confirms the separation of the endogenous hormones.

We used the serum (KHM, KHF) from Danish individuals as controls for the SPE-HPLC cleanup procedure. The XAR data of the serum controls from 12 independent assays were not significantly different from the solvent control, indicating that no compounds present in the serum sample were able to activate AR activity. However, compared with the XARcomp activity of the R1881 EC_{50} solvent control, the XARcomp activity of KHM was significantly lower but that of KHF was not. Thus the SPE-HPLC F1 fraction seems to contain a mixture of bioaccumulated lipophilic POPs that differ between samples and are able to interfere with the R1881-induced AR activity in the CALUX assay.

To our knowledge, this is the first study to determine the integrated biological activity of AR-active lipophilic compounds in their actual mixture in serum; therefore, potential determinants for the activity were unknown. We hypothesized seafood, age, smoking status, BMI, alcohol, and coffee to be potential determinants of AR activity. However, the data did not change upon adjustment for these potential determinants. A different possibility is that other exogenous chemicals might confound the obtained data and that the two selected POP markers do not adequately reflect and/or represent the chemicals primarily responsible for the determined xenoandrogenic effects.

Conclusions

We found a significantly higher percentage of serum samples with decreased XARcomp activity for the Kharkiv study group, which had the highest *p,p′*-DDE level of the four study groups. Moreover, in the European study groups, the percentage of serum samples with decreased XARcomp activity followed the *p,p′*-DDE serum levels. In the Inuit study group, we found a significantly higher percentage of serum samples with increased XARcomp activity compared with the European study groups. No consistent correlations were found between XAR/XARcomp and the two POP proxy markers for the individual study groups or across study groups, suggesting that neither CB-153 nor *p,p′*-DDE alone can predict the xenoandrogenic activity of the serum samples included in the present study.

Our hypothesis was that xenoandrogenic activity would differ between the study groups with different POP profiles, and that the two selected POP markers would correlate to the xenoandrogenic activities. The first hypothesis was accepted: The differences in XARcomp serum activity between the study groups seem to reflect differences in chemical exposure profiles, genetics, and/or lifestyle factors. However, the second hypothesis was rejected; therefore, the selected POP markers could not be used as biomarkers of exposure to determine the xenoandrogenic effects.

The present *ex vivo* biomarker AR-CALUX activity assay may contribute to the insight of the impact of the total bioaccumulated EDCs geographically, which is difficult to predict on the basis of chemical analysis alone. However, the determined XAR/XARcomp activities of the serum fraction containing lipophilic POPs may fail to detect effects accomplished by hydrophilic compounds or effects through other receptors or other mechanisms.

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