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Environmental Hexachlorobenzene exposure and human male reproductive function

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Keywords

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Abbreviations: HCB, Hexachlorobenzene; SHBG, Sex hormone-binding globulin; FAI, Free Androgen Index; LH, luteinizing hormone; SCSA, sperm chromatin structure assay; FSH, follicle stimulation hormone; DFI, DNA fragmentation index; FCM, flow cytometry; LOD, lower limits of detection; BMI, body mass index; CV, coefficients of variation; WHO, World Health Organization; PCB, polychlorinated biphenyl; DDT, dichlorodiphenyltrichloroethane

Abstract

Hexachlorobenzene (HCB) is a persistent environmental fungicide that may disrupt androgen regulation. The aim of this study was to investigate associations between HCB levels and biomarkers of male reproductive function. 589 spouses of pregnant women from Greenland, Poland and Ukraine were enrolled between 2002 and 2004. The men provided semen and blood samples and were interviewed. HCB was measured in serum by gas chromatography. The mean serum concentrations of HCB were higher in Ukraine (182.3 ng/g lipid) and Greenland (79.0 ng/g lipid) compared to Poland (14.2 ng/g lipid). Sex Hormone Binding Globulin (SHBG) and Free Androgen Index (FAI) were associated with HCB in men from Ukraine and Poland.
This study spanning large differences in environmental HCB exposure levels shows a positive association for SHBG and negative association for FAI with high serum levels of HCB in fertile men, but without major consequences for semen quality and the Inuit study population.

Highlights

- Very high levels of HCB were found in men from Ukraine and Greenland.
- Free androgen index was negatively associated with HCB in men from Ukraine and Poland.
- Sex hormone binding globulin was positively associated with HCB in men from Ukraine and Poland.
- HCB serum levels seem to have no influence on semen quality.

1. Introduction

Hexachlorobenzene (HCB), a chlorinated aromatic hydrocarbon, was in the past commonly used as a fungicide (1). In 1978-1981 the estimated worldwide production of pure HCB was 10,000 tons per year. The half-life of HCB in humans is between 4 and 8 years (1) and because it bio-accumulates in fat, it is measurable in adipose tissue in a large percentage of investigated populations (2;3). HCB was listed for global ban under the Stockholm Convention on persistent organic pollutants (POPs) in 2001. Probably due to restrictions, the concentration of HCB is decreasing each year (4), but it is still found in the environment. Today, HCB is formed as a byproduct during the manufacture of other chemicals like solvents and pesticides (1).

HCB belongs to the chemical class of organochlorines that also includes polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) that have been associated with adverse reproductive outcomes and reduced fertility (5;6). HCB in low doses has been found to partially agonize androgen action, and in high doses to antagonize androgen action, in both *in vivo* and *in vitro* studies (7).
Studies of HCB exposure and human reproductive function are sparse. HCB levels in fatty tissue in children have been associated with cryptorchidism (8), but not when HCB was evaluated in breast milk or maternal blood (9;10). Also, hypospadias in sons has been associated with maternal levels of HCB (11), these findings pointing to anti-

androgenic *in vivo* effects at exposure concentrations encountered in the general population.

A study from 2013 investigating couple fecundity by time to pregnancy showed a significant negative fecundity odds ratio with serum HCB in females only, but the relation did not persist after adjustment for confounding factors (12). To our knowledge only two studies have investigated semen quality and HCB levels in humans (13;14). Both studies were based on assessment of HCB levels in infertility clients. The studies did not find higher levels of HCB among the men with poor semen quality compared to men with normal semen quality (13;14). Conflicting results have been obtained when associating serum levels of POPs, including HCB, and sex hormone levels in young and adult populations (15;16). The data on the link between HCB levels and male fertility and our knowledge on potential risk are limited. Therefore we aimed this study to investigate associations between HCB and biomarkers of reproductive function in a population of fertile men.

2. Material and methods

This cross-sectional investigation of HCB exposure was nested within the larger study by Toft *et al*, 2005, designed to evaluate environmental contaminants and reproductive effects.

The local ethics committees representing all participating populations approved the study. All subjects provided signed, informed consent.

Between May 2002 and February 2004, a total of 3,833 pregnant women and their male spouses were invited to participate in the study at their first antenatal care visit at 19 local settlements in Greenland and hospitals in Warsaw, Poland or Kharkiv, Ukraine. In total 1,710 women agreed to participate (44.6%). For the male fertility study, 440 men from Greenland, 287 men from Poland and 257 from Ukraine were interviewed and gave blood samples. Consecutively, some 200 men from each site were enrolled in the semen study; in Greenland (n=196), Warsaw, Poland (n=190), and Kharkiv, Ukraine (n=203) (17). The women were on average 24, 24 and 33 weeks pregnant in Greenland, Ukraine and Poland, respectively. During the pregnancy the men provided semen and blood samples and were interviewed regarding lifestyle,

occupation and reproductive history. Thirteen men (n=3 from Greenland, n=7 from Poland and n=3 from Ukraine) had missing HCB observations due to insufficient serum for analysis, and were excluded leaving a final study sample for analysis of 589 men. The 13 men excluded did not differ from the final study sample on regional demographics, hormones or semen characteristics. A detailed description of the parent study has previously been published (17).

2.1 Collection and analysis of semen samples

Participants were instructed to collect a semen sample by masturbation at their residence or in privacy in a room at the hospital after at least 48 hours of sexual abstinence. The sample was kept close to the body to maintain a temperature close to 37°C when transported to the laboratory immediately after collection. After liquefaction, the sperm concentration was determined on two aliquots of diluted semen samples (1:10 or 1:20) using an Improved Neubauer Hemacytometer (Paul Marienfeld, Bad Mergentheim, Germany). Sperm cell motility was determined by counting the proportion of *a*) fast progressive sperm; *b*) slowly progressive sperm; *c*) local motile sperm; and *d*) immotile sperm on 100 spermatozoa within each of two fresh drops of semen (18). Sperm morphology was determined for all samples at the Reproductive Medicine Centre, Skåne University Hospital Malmö, on Papanicolaou-stained smears. All staff involved locally in analyzing semen parameters participated in two quality control workshops (17). Sperm morphology was determined for all research sites at the Reproductive Medicine Centre, Skåne University Hospital Malmö, on Papanicolaou-stained smears. All semen analyses followed the recommendations by the World Health Organization (WHO) 1999.

One hundred and sixteen of the Greenlandic semen samples were collected up to one year after enrollment, where the interview and blood sampling occurred, since the laboratory was not available at the time of enrollment.

DNA damage was measured by sperm chromatin structure assay (SCSA) following a strictly standardized procedure described in (19). SCSA is a flow cytometric (FCM) technique which uses acridine orange as a DNA specific metachromatic fluorescent probe to discriminate between denaturated (red fluorescence, single stranded) and native (green fluorescence, double stranded) DNA regions. The sperm fraction with abnormal chromatin packaging and DNA breaks is characterized by an increased

susceptibility to acid-induced DNA denaturation *in situ* and is reported as the DFI (DNA Fragmentation Index). All SCSA analyses were performed at the Laboratory of Toxicology, ENEA Casaccia (Rome, Italy). A total of 20,000 sperm were analyzed for each sample. Inter-day SCSA coefficient of variation (CV) of the %DFI was 6%. The median inter-sample CV for %DFI was 1.5% (20). Further details are described elsewhere (21).

Strand breaks in DNA were determined using the terminal deoxynucleotidyl transferase-driven dUTP nick end labelling (TUNEL) assay and analyzed using an Epics XL FCM (Beckman Coulter, Fullerton, CA, USA) as described earlier (22). The intra-laboratory CV was under 5% (22). Frozen semen samples were shipped on dry ice to Polytechnic University of March, Ancona in Italy, for the TUNEL analysis. The pro- (Fas) and anti- (Bcl-xL) apoptotic markers on spermatozoa were measured at the same FCM facility in Ancona, Italy. To detect Fas on spermatozoa, incubation with anti-Fas primary monoclonal antibodies was followed by a goat anti-mouse IgG-FITC conjugated secondary antibodies treatment, an indirect immunofluorescence. Whereas for Bcl-xL assessment, the primary monoclonal antibody anti-Bcl-xL was detected by a goat anti-mouse IgG-PE conjugated. Details are described in depth elsewhere (22). 20,000 sperm cells were analyzed using flow cytometry. The intra-laboratory CV regarding the apoptotic markers was in the range from 6% for Fas and 9% for Bcl-xL (22).

2.2 Collection of blood samples

Venous blood samples were collected within one week of the semen collection for all men in Poland and Ukraine, except for 116 of the Greenlandic samples. The blood samples were centrifuged immediately after collection and sera were stored at −80°C for later analysis.

2.3 Determination of reproductive hormones

All assays were performed at Dept. of Clinical Chemistry, Laboratory Division, Skåne County, Malmö, Sweden. Serum concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and estradiol (E_2) were analyzed with immunofluorometric techniques using the UniCel DxI 800 Beckman Access Immunoassay system (Chaska, MN, USA). The total assay CV were 2.6%, 2.9% and 8.1%, respectively, with lower limits of detection (LODs) for the assays of 0.2 IU/L, 0.2 IU/L, and 8.0 pmol/L,

respectively. Serum testosterone levels were measured by means of a competitive immunoassay (Access; Beckman Coulter Inc., Fullerton, CA, USA) with a LOD of 0.35 nmol/L and total assay CV of 2.8% at 2.9 nmol/L and 3.2% at 8.1 nmol/L. SHBG concentrations were measured using a fluoro-immunoassay (Immulite 2000; Diagnostic Products Corporation, Los Angeles, CA, USA). The LOD was 0.02 nmol/L. The total assay CVs were 5.5% and 4.6%, respectively. Inhibin B levels were assessed using a specific immunometric method, as previously described, with a detection limit of 15 ng/L and intra-assay and total assay CVs < 7% (23). Free Androgen Index (FAI) was calculated by dividing total testosterone by SHBG, multiplied by 100. We converted estradiol pmol/L to estradiol pg/L, and testosterone nmol/L to ng/dL for calculation of the testosterone estradiol ratio (T/E_2). Finally, we calculated the testosterone/LH ratio (T/LH).

2.4 Determination of HCB

The analyses of HCB were performed as described by Rignell-Hydbom *et al*. (2012). Briefly, the HCB was extracted from the serum by solid phase extraction (Isolute ENV+) using on-column degradation of the lipids and analysis by gas chromatography mass spectrometry. C13-labeled HCB was used as an internal standard. The LOD was defined as the concentration corresponding to three times the standard deviation of the ratio between the blank area and the area of the internal standard and found to be 0.01 ng/mL. The reproducibility was determined by analyses of an in-house quality control (QC) sample containing 0.12 ng/mL. The QC sample was analyzed in each sample batch and the coefficient of variation was found to be 11% (24). The laboratory participates in the Erlangen Round Robin inter-laboratory control program for HCB with results within the tolerance limit.

2.5 Statistical methods

We used general linear models to analyze associations between serum concentrations of HCB and those of reproductive hormones as well as with measures of semen quality. The exposure, HCB, was considered as continuous variable, but also categorized according to three percentiles ($<25^{th}$, $25\text{-}75^{th}$ $>75^{th}$). HCB values <LOD were imputed by the maximum likelihood single imputation method (25).

The outcome variables, %DFI, %TUNEL, Fas, Bcl-xL, total sperm count, sperm

concentration, volume, motility, morphology, testosterone, estradiol, inhibin B, FSH, LH, FAI, T/E_2 ratio, T/LH ratio and SHBG were analyzed using continuous scales.

The concentrations of HCB differed significantly in the three study regions, Greenland, Poland and Ukraine, and therefore we stratified the analyses on regions. In multivariate models investigating reproductive hormones, we included serum lipids, season of blood collection, body mass index (BMI), age and cotinine in serum as potential confounders. In models investigating semen parameters we furthermore adjusted for spillage during semen sampling, genital infections and testicular disorders as well as sexual abstinence time. The confounders were selected based on *a priori* considerations of their role as known or potential risk factors.

To present an overview of crude associations we used Spearman's correlation to establish correlation between HCB and biomarkers of reproductive function, including the outcomes mentioned above as well as age, BMI and total serum lipids.

To improve normality and homogeneity of variance we transformed all outcome variables and HCB by the natural logarithm since data was not normally distributed. For the linear regression models, the beta coefficient can be interpreted as the percentage change in the dependent variable by one percent increase in the independent variable.

Due to significant heterogeneity in different regions of Greenland, we performed sub-analysis where we divided the participating cities in Greenland into four areas: Capital city (Nuuk), North (Ilulissat, Qeqertarsuag, Sisimut, Maniitsoq), South (Nanortalik, Qaqortoq, Narsaq, Paarmiut) and East (Tasiilaq and Kuummiut) and extracted data from the two areas in Greenland (East and North Greenland) with the highest HCB concentrations and investigated HCB according to the outcomes.

Statistical analysis of the data was performed with SAS software, version 9.3 for Windows (SAS Institute Inc., Cary, NC, USA).

3. Results

In Poland 20.5% (n=39) of the HCB concentrations were below LOD; all serum

samples from Greenland and Ukraine had HCB concentrations above the LOD.

Demographics, seminal characteristics, reproductive hormones and concentrations of HCB are shown in Table 1.
The mean concentration of HCB was significantly lower in Poland (14.2 ng/g lipid) compared to Ukraine (182.3 ng/g lipid) and Greenland (79.0 ng/g lipid) (Table 1). Within Greenland the Eastern part had the highest HCB concentration (n=23, mean= 140.2 (18.6-862.6) ng/g lipid) followed by the North (n=79, mean=84.7 (14.7-255.0) ng/g lipid), the capital (n=58, mean=60.6 (2.5-257.1) ng/g lipid) and the Southern part (n=34, mean=56.5 (16.4-145.2) ng/g lipid), data not shown.

3.1 Bivariate relationships for HCB

We found significant negative correlations between HCB and semen volume in men from Greenland (ρ=-0.15), HCB and estradiol and FAI in men from Ukraine (ρ=-0.16 and ρ=-0.21, respectively) and HCB and inhibin B in Polish men (ρ=-0.20) (Table 2). The Spearman correlations also revealed a negative correlation between HCB and BMI and estradiol in Ukrainian men only, whereas we found positive correlations between HCB and total serum lipids in men from Ukraine and Poland. Age was positively correlated to HCB in Greenland and Poland but not in the younger Ukraine population (Table 2).

3.2 Results from multiple regression analysis

HCB was not associated with any of the measured outcomes when analyzed as categorized parameter (data not shown). The anti-apoptotic marker Bcl-xL was positively associated with HCB, but only in men from Poland and only in the adjusted model (logß (95%CI) = 0.80(0.12;1.47), p=0.02). FAI was negatively associated with HCB in men from Ukraine and Poland, corresponding to decreases in FAI of 0.09% (p=0.01) and 0.10% (p=0.08) when serum HCB levels increased one percent, respectively. In Ukrainian and Polish men SHBG was positively associated with HCB serum levels. In men from Ukraine or Poland, one percent increase in HCB was associated with a 0.07% (p=0.04) or 0.11% (p=0.09) increase in SHBG, respectively. No associations emerged in the adjusted model for other reproductive hormones or semen quality measures (Table 3).

We did not observe associations between HCB and any of the outcomes in the two areas in Greenland with the highest HCB concentrations (East and North, n=102, mean HCB=96.5, median=67.0 ng/g lipid) (data not shown).

4. Discussion

To the best of our knowledge, this is the first population-based study of relatively high environmental HCB exposure and male reproductive function including semen quality and reproductive hormones. Men from Ukraine, which were the men with the highest HCB serum levels, had lower estradiol at higher HCB serum levels, but this finding did not persist after adjustment for lipids (p=0.08). However, in adjusted models, SHBG was positively associated with HCB in men from Ukraine and was borderline significant (logβ=0.11, p=0.09) in men from Poland, but not significant in men from Greenland. There can be several reasons for a positive association between HCB and SHBG including anti-androgenic, estrogenic, or thyroid hormone effects or it could be due to high BMI which has previously been reported to be associated with low SHBG (26). Both BMI and estradiol were correlated to HCB in Ukrainian men. To investigate if the association was due to BMI or estradiol we adjusted for these two covariates in the SHBG and HCB analysis and the association remained, suggesting the association was probably not driven by BMI or estradiol. Previously, we have investigated serum levels of the PCB congener CB-153 and the main DDT metabolite, 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p´-DDE) in the same study populations (22;27). They both belong to the same chemical class of organochlorines as HCB and might have similar mode of action. Like for HCB, CB-153 was positively associated with SHBG in Europeans only (ß=2.1, 95%CI=0.7;35). CB-153 was also found to be negatively associated with free testosterone in Europeans (ß=-0.05, 95% CI=-0.08;-0.02) (27). FAI, which is a proxy for unbound testosterone in serum (28), was negatively associated with HCB in our study. This fits with the SHBG finding, since high SHBG is associated with low free testosterone. Thyroid hormones increase hepatic SHBG production (29). A study of 341 men recruited from an infertility clinic reported an inverse association between T_3 and HCB (30), whereas a study of 110 Swedish fishermen did not show association between thyroid hormones and HCB (31). HCB has been associated with cryptorchidism (8) and hypospadias (11), which points

to anti-androgenic effects, therefor the positive association between SHBG and HCB found in our study might be an anti-androgenic effect. Therefore, the results might indicate hormone-like activity of some POPs in Caucasian populations.

Men from Poland had the lowest HCB concentration of the three study sites, but they exhibited higher levels of the anti-apoptotic marker Bcl-xL at higher levels of serum HCB after adjustment for potential confounders. On the other hand, sperm apoptotic markers were not associated with HCB for the Greenland and Ukraine populations, so the finding for Polish men was likely a chance finding due to multiple comparisons. Our population-based findings in male spouses of pregnant women are inconsistent with some but not all previous studies. Ferguson *et al.* (2012) investigated 341 male partners, aged 18–51, in subfertile couples seeking infertility evaluation and treatment (median HCB level was 14.9 ng/g lipid). These authors observed lower SHBG, testosterone and free testosterone at higher serum HCB levels, but only when unadjusted for lipids. In contrast, Dhooge *et al*. (2011), in a population of male adolescents (median age of 14.8 years), found positive associations between serum HCB (standardized by lipid) and estradiol, testosterone, free testosterone and the T/E_2 ratio (median HCB level was 22.8 ng/g lipid). These contradicting results can be due to different study populations. Most studies investigating HCB and semen quality have largely shown no significant associations, consistent with our results (12-14;16;31;32).

The median exposure level of men from Poland in our study was similar or slightly lower compared to other studies that have investigated lipid standardized HCB (15;16;30;32). The Polish men had the lowest serum HCB in our study, almost 13 fold lower than the Ukrainian men, which might be the reason for the borderline significant results compared to the significant results in the highly exposed Ukrainian men. We did not observe relationships between SHBG, FAI and HCB in men from Greenland, although they were the study population with the second highest HCB serum levels. Due to genetic differences, Inuit might be affected differently than the two European study populations.

HCB is highly lipophilic and serum lipid levels vary between fasting and non-fasting status, a correction for serum lipids is needed for the valid interpretation of serum HCB levels. Since other studies have reported HCB levels after standardizing for lipids (HCB ng/g lipids) we did the same, as shown in Table 1, to allow our results to be

comparable with those from other studies. In a simulation study, Schisterman *et al*. (2005) evaluated four statistical models showing that the lipophilic compound PCB, standardized by serum lipids as an independent variable, was highly prone to bias (weaker associations due to measurement error), a result also shown by others in the analysis of HCB (16;30;33). Therefore, we adjusted for total serum lipids as a covariate instead of standardizing the HCB by lipids.

As expected, total serum lipids and age were positively correlated with HCB in our study, which is in agreement with results from others (13;14). Because HCB has a long half-life, is lipophilic, accumulates in fat and the production of HCB has decreased with time, it has been found at higher concentrations in elderly and/or obese populations (34;35). We found a negative correlation between HCB and BMI in men from Ukraine, which did not fit expectations and might be a chance finding.

In this study HCB serum levels were highest in men from Ukraine, probably due to an excessive use of pesticides in the former Soviet Union (36), followed by men from Greenland. HCB levels have previously been found to be higher in Canadian Inuit compared to other inhabitants in the Arctic Canada, possibly because of their higher marine food intake especially from fatty fish and mammals (37). In a study investigating dietary contaminants in Northern Greenland in 1976 and 2004, the levels of HCB among Inuit consuming traditional food was measured (38). Even though today's Inuit consumption of traditional food is less than in the 1970's, the levels of HCB were higher in the samples from 2004. This indicates that the relative contamination levels in the local food products have increased from 1976 to 2004, probably due to the long half-life and lifelong bioaccumulation (38). Looking at the four Greenland sub-regions, we observed higher serum concentrations of HCB in men from Eastern and Northern Greenland compared to the capital and the Southern region. One reason might be the different life styles in different areas of Greenland. In the East and the North Greenland, the inhabitants live a more traditional life with high marine food consumption, especially seals, whereas in Nuuk, the capital city, the population, in particular the youn nger population, consumes more imported food (39;40).

The participation rate of the parent study was low with only 44.6%pregnant women participating. This was a consequence of the recruitment procedure in Ukraine, where

the contact between the participants and the project team was handled by many medical doctors. A sub-sample of 605 non-participating Ukrainian women was interviewed regarding demographic and reproductive information. Only age differed between participating and non-participating women with the latter being of slightly lower age. The men who did not accept the invitation did not differ from the participating men with regard to their wives' time to pregnancy (17).

The HCB concentrations varied substantially between the three study sites. HCB in serum was measured at the same laboratory by the same person, thus the HCB concentrations would not be expected to be biased because of inter-observer or – laboratory variation. Further, samples from the three countries were analyzed randomly across time, thus systematic differences related to season, equipment or batches are not expected.

In the Greenlandic study population 116 out of the 196 semen samples were collected up to one year after enrollment, whereas blood samples were drawn at enrollment. We do not believe this to bias the associations since no seasonal variations were found for HCB in Greenland, and due to the long half-life of HCB (4 to 8 years), a measurement on serum sample can be considered a good proxy of the exposure level within a year. If bias did occur, the estimate would most likely be biased toward the null hypothesis since the misclassification of HCB would have been non-differential with respect to the outcome.

5. Conclusions

Our findings indicate that SHBG and FAI might be associated with high environmental exposure levels of HCB in proven fertile men of European populations, whereas relatively high HCB serum levels seem to have no influence on semen quality.

Conflict of interest statement

The authors have no financial interest or other conflicts of interest in the publication of these results.

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Environmental Hexachlorobenzene exposure and human male reproductive function

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Abbreviations: HCB, Hexachlorobenzene; SHBG, Sex hormone-binding globulin; FAI, Free Androgen Index; LH, luteinizing hormone; SCSA, sperm chromatin structure assay; FSH, follicle stimulation hormone; DFI, DNA fragmentation index; FCM, flow cytometry; LOD, lower limits of detection; BMI, body mass index; CV, coefficients of variation; WHO, World Health Organization; PCB, polychlorinated

biphenyl; DDT, dichlorodiphenyltrichloroethane

1. Introduction

Hexachlorobenzene (HCB), a chlorinated aromatic hydrocarbon, was in the past commonly used as a fungicide (1). In 1978-1981 the estimated worldwide production of pure HCB was 10,000 tons per year. The half-life of HCB in humans is between 4 and 8 years (1) and because it bio-accumulates in fat, it is measurable in adipose tissue in a large percentage of investigated populations (2;3). HCB was listed for global ban under the Stockholm Convention on persistent organic pollutants (POPs) in 2001. Probably due to restrictions, the concentration of HCB is decreasing each year (4), but it is still found in the environment. Today, HCB is formed as a byproduct during the manufacture of other chemicals like solvents and pesticides (1).

HCB belongs to the chemical class of organochlorines that also includes polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) that have been associated with adverse reproductive outcomes and reduced fertility (5;6). HCB in low doses has been found to partially agonize androgen action, and in high doses to antagonize androgen action, in both *in vivo* and *in vitro* studies (7).
Studies of HCB exposure and human reproductive function are sparse. HCB levels in fatty tissue in children have been associated with cryptorchidism (8), but not when HCB was evaluated in breast milk or maternal blood (9;10). Also, hypospadias in sons has been associated with maternal levels of HCB (11), these findings pointing to anti-androgenic *in vivo* effects at exposure concentrations encountered in the general population.
A study from 2013 investigating couple fecundity by time to pregnancy showed a significant negative fecundity odds ratio with serum HCB in females only, but the relation did not persist after adjustment for confounding factors (12). To our knowledge only two studies have investigated semen quality and HCB levels in humans (13;14). Both studies were based on assessment of HCB levels in infertility clients. The studies did not find higher levels of HCB among the men with poor semen quality compared to men with normal semen quality (13;14). Conflicting results have been obtained when associating serum levels of POPs, including HCB, and sex hormone levels in young and adult populations (15;16). The data on the link between HCB levels and male fertility and our knowledge on potential risk are limited. Therefore we

aimed this study to investigate associations between HCB and biomarkers of reproductive function in a population of fertile men.

2. Material and methods

This cross-sectional investigation of HCB exposure was nested within the larger study by Toft *et al*, 2005, designed to evaluate environmental contaminants and reproductive effects.

The local ethics committees representing all participating populations approved the study. All subjects provided signed, informed consent.

Between May 2002 and February 2004, a total of 3,833 pregnant women and their male spouses were invited to participate in the study at their first antenatal care visit at 19 local settlements in Greenland and hospitals in Warsaw, Poland or Kharkiv, Ukraine. In total 1,710 women agreed to participate (44.6%). For the male fertility study, 440 men from Greenland, 287 men from Poland and 257 from Ukraine were interviewed and gave blood samples. Consecutively, some 200 men from each site were enrolled in the semen study; in Greenland (n=196), Warsaw, Poland (n=190), and Kharkiv, Ukraine (n=203) (17). The women were on average 24, 24 and 33 weeks pregnant in Greenland, Ukraine and Poland, respectively. During the pregnancy the men provided semen and blood samples and were interviewed regarding lifestyle, occupation and reproductive history. Thirteen men (n=3 from Greenland, n=7 from Poland and n=3 from Ukraine) had missing HCB observations due to insufficient serum for analysis, and were excluded leaving a final study sample for analysis of 589 men. The 13 men excluded did not differ from the final study sample on regional demographics, hormones or semen characteristics. A detailed description of the parent study has previously been published (17).

2.1 Collection and analysis of semen samples

Participants were instructed to collect a semen sample by masturbation at their residence or in privacy in a room at the hospital after at least 48 hours of sexual abstinence. The sample was kept close to the body to maintain a temperature close to 37°C when transported to the laboratory immediately after collection. After

liquefaction, the sperm concentration was determined on two aliquots of diluted semen samples (1:10 or 1:20) using an Improved Neubauer Hemacytometer (Paul Marienfeld, Bad Mergentheim, Germany). Sperm cell motility was determined by counting the proportion of *a*) fast progressive sperm; *b*) slowly progressive sperm; *c*) local motile sperm; and *d*) immotile sperm on 100 spermatozoa within each of two fresh drops of semen (18). Sperm morphology was determined for all samples at the Reproductive Medicine Centre, Skåne University Hospital Malmö, on Papanicolaou-stained smears. All staff involved locally in analyzing semen parameters participated in two quality control workshops (17). Sperm morphology was determined for all research sites at the Reproductive Medicine Centre, Skåne University Hospital Malmö, on Papanicolaou-stained smears. All semen analyses followed the recommendations by the World Health Organization (WHO) 1999.
One hundred and sixteen of the Greenlandic semen samples were collected up to one year after enrollment, where the interview and blood sampling occurred, since the laboratory was not available at the time of enrollment.

DNA damage was measured by sperm chromatin structure assay (SCSA) following a strictly standardized procedure described in (19). SCSA is a flow cytometric (FCM) technique which uses acridine orange as a DNA specific metachromatic fluorescent probe to discriminate between denaturated (red fluorescence, single stranded) and native (green fluorescence, double stranded) DNA regions. The sperm fraction with abnormal chromatin packaging and DNA breaks is characterized by an increased susceptibility to acid-induced DNA denaturation *in situ* and is reported as the DFI (DNA Fragmentation Index). All SCSA analyses were performed at the Laboratory of Toxicology, ENEA Casaccia (Rome, Italy). A total of 20,000 sperm were analyzed for each sample. Inter-day SCSA coefficient of variation (CV) of the %DFI was 6%. The median inter-sample CV for %DFI was 1.5% (20). Further details are described elsewhere (21).
Strand breaks in DNA were determined using the terminal deoxynucleotidyl transferase-driven dUTP nick end labelling (TUNEL) assay and analyzed using an Epics XL FCM (Beckman Coulter, Fullerton, CA, USA) as described earlier (22). The intra-laboratory CV was under 5% (22). Frozen semen samples were shipped on dry ice to Polytechnic University of March, Ancona in Italy, for the TUNEL analysis. The pro- (Fas) and anti- (Bcl-xL) apoptotic markers on spermatozoa were measured at the

same FCM facility in Ancona, Italy. To detect Fas on spermatozoa, incubation with anti-Fas primary monoclonal antibodies was followed by a goat anti-mouse IgG-FITC conjugated secondary antibodies treatment, an indirect immunofluorescence. Whereas for Bcl-xL assessment, the primary monoclonal antibody anti-Bcl-xL was detected by a goat anti-mouse IgG-PE conjugated. Details are described in depth elsewhere (22). 20,000 sperm cells were analyzed using flow cytometry. The intra-laboratory CV regarding the apoptotic markers was in the range from 6% for Fas and 9% for Bcl-xL (22).

2.2 Collection of blood samples

Venous blood samples were collected within one week of the semen collection for all men in Poland and Ukraine, except for 116 of the Greenlandic samples. The blood samples were centrifuged immediately after collection and sera were stored at −80°C for later analysis.

2.3 Determination of reproductive hormones

All assays were performed at Dept. of Clinical Chemistry, Laboratory Division, Skåne County, Malmö, Sweden. Serum concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and estradiol (E_2) were analyzed with immunofluorometric techniques using the UniCel DxI 800 Beckman Access Immunoassay system (Chaska, MN, USA). The total assay CV were 2.6%, 2.9% and 8.1%, respectively, with lower limits of detection (LODs) for the assays of 0.2 IU/L, 0.2 IU/L, and 8.0 pmol/L, respectively. Serum testosterone levels were measured by means of a competitive immunoassay (Access; Beckman Coulter Inc., Fullerton, CA, USA) with a LOD of 0.35 nmol/L and total assay CV of 2.8% at 2.9 nmol/L and 3.2% at 8.1 nmol/L. SHBG concentrations were measured using a fluoro-immunoassay (Immulite 2000; Diagnostic Products Corporation, Los Angeles, CA, USA). The LOD was 0.02 nmol/L. The total assay CVs were 5.5% and 4.6%, respectively. Inhibin B levels were assessed using a specific immunometric method, as previously described, with a detection limit of 15 ng/L and intra-assay and total assay CVs < 7% (23). Free Androgen Index (FAI) was calculated by dividing total testosterone by SHBG, multiplied by 100. We converted estradiol pmol/L to estradiol pg/L, and testosterone nmol/L to ng/dL for calculation of the testosterone estradiol ratio (T/E_2). Finally, we

calculated the testosterone/LH ratio (T/LH).

2.4 Determination of HCB

The analyses of HCB were performed as described by Rignell-Hydbom *et al*. (2012). Briefly, the HCB was extracted from the serum by solid phase extraction (Isolute ENV+) using on-column degradation of the lipids and analysis by gas chromatography mass spectrometry. C13-labeled HCB was used as an internal standard. The LOD was defined as the concentration corresponding to three times the standard deviation of the ratio between the blank area and the area of the internal standard and found to be 0.01 ng/mL. The reproducibility was determined by analyses of an in-house quality control (QC) sample containing 0.12 ng/mL. The QC sample was analyzed in each sample batch and the coefficient of variation was found to be 11% (24). The laboratory participates in the Erlangen Round Robin inter-laboratory control program for HCB with results within the tolerance limit.

2.5 Statistical methods

We used general linear models to analyze associations between serum concentrations of HCB and those of reproductive hormones as well as with measures of semen quality. The exposure, HCB, was considered as continuous variable, but also categorized according to three percentiles ($<25^{th}$, $25\text{-}75^{th}$ $>75^{th}$). HCB values <LOD were imputed by the maximum likelihood single imputation method (25).

The outcome variables, %DFI, %TUNEL, Fas, Bcl-xL, total sperm count, sperm concentration, volume, motility, morphology, testosterone, estradiol, inhibin B, FSH, LH, FAI, T/E_2 ratio, T/LH ratio and SHBG were analyzed using continuous scales.

The concentrations of HCB differed significantly in the three study regions, Greenland, Poland and Ukraine, and therefore we stratified the analyses on regions. In multivariate models investigating reproductive hormones, we included serum lipids, season of blood collection, body mass index (BMI), age and cotinine in serum as potential confounders. In models investigating semen parameters we furthermore adjusted for spillage during semen sampling, genital infections and testicular disorders as well as sexual abstinence time. The confounders were selected based on *a priori* considerations of their role as known or potential risk factors.

To present an overview of crude associations we used Spearman's correlation to establish correlation between HCB and biomarkers of reproductive function, including the outcomes mentioned above as well as age, BMI and total serum lipids.

To improve normality and homogeneity of variance we transformed all outcome variables and HCB by the natural logarithm since data was not normally distributed. For the linear regression models, the beta coefficient can be interpreted as the percentage change in the dependent variable by one percent increase in the independent variable.

Due to significant heterogeneity in different regions of Greenland, we performed sub-analysis where we divided the participating cities in Greenland into four areas: Capital city (Nuuk), North (Ilulissat, Qeqertarsuag, Sisimut, Maniitsoq), South (Nanortalik, Qaqortoq, Narsaq, Paarmiut) and East (Tasiilaq and Kuummiut) and extracted data from the two areas in Greenland (East and North Greenland) with the highest HCB concentrations and investigated HCB according to the outcomes.

Statistical analysis of the data was performed with SAS software, version 9.3 for Windows (SAS Institute Inc., Cary, NC, USA).

3. Results

In Poland 20.5% (n=39) of the HCB concentrations were below LOD; all serum samples from Greenland and Ukraine had HCB concentrations above the LOD.

Demographics, seminal characteristics, reproductive hormones and concentrations of HCB are shown in Table 1.
The mean concentration of HCB was significantly lower in Poland (14.2 ng/g lipid) compared to Ukraine (182.3 ng/g lipid) and Greenland (79.0 ng/g lipid) (Table 1). Within Greenland the Eastern part had the highest HCB concentration (n=23, mean= 140.2 (18.6-862.6) ng/g lipid) followed by the North (n=79, mean=84.7 (14.7-255.0) ng/g lipid), the capital (n=58, mean=60.6 (2.5-257.1) ng/g lipid) and the Southern part (n=34, mean=56.5 (16.4-145.2) ng/g lipid), data not shown.

3.1 Bivariate relationships for HCB

We found significant negative correlations between HCB and semen volume in men from Greenland (ρ=-0.15), HCB and estradiol and FAI in men from Ukraine (ρ=-0.16 and ρ=-0.21, respectively) and HCB and inhibin B in Polish men (ρ=-0.20) (Table 2). The Spearman correlations also revealed a negative correlation between HCB and BMI and estradiol in Ukrainian men only, whereas we found positive correlations between HCB and total serum lipids in men from Ukraine and Poland. Age was positively correlated to HCB in Greenland and Poland but not in the younger Ukraine population (Table 2).

3.2 Results from multiple regression analysis

HCB was not associated with any of the measured outcomes when analyzed as categorized parameter (data not shown). The anti-apoptotic marker Bcl-xL was positively associated with HCB, but only in men from Poland and only in the adjusted model (logß (95%CI) = 0.80(0.12;1.47), p=0.02). FAI was negatively associated with HCB in men from Ukraine and Poland, corresponding to decreases in FAI of 0.09% (p=0.01) and 0.10% (p=0.08) when serum HCB levels increased one percent, respectively. In Ukrainian and Polish men SHBG was positively associated with HCB serum levels. In men from Ukraine or Poland, one percent increase in HCB was associated with a 0.07% (p=0.04) or 0.11% (p=0.09) increase in SHBG, respectively. No associations emerged in the adjusted model for other reproductive hormones or semen quality measures (Table 3).

We did not observe associations between HCB and any of the outcomes in the two areas in Greenland with the highest HCB concentrations (East and North, n=102, mean HCB=96.5, median=67.0 ng/g lipid) (data not shown).

4. Discussion

To the best of our knowledge, this is the first population-based study of relatively high environmental HCB exposure and male reproductive function including semen quality and reproductive hormones. Men from Ukraine, which were the men with the highest HCB serum levels, had lower estradiol at higher HCB serum levels, but this finding did not persist after adjustment for lipids (p=0.08). However, in adjusted models, SHBG

was positively associated with HCB in men from Ukraine and was borderline significant (logβ=0.11, p=0.09) in men from Poland, but not significant in men from Greenland. There can be several reasons for a positive association between HCB and SHBG including anti-androgenic, estrogenic, or thyroid hormone effects or it could be due to high BMI which has previously been reported to be associated with low SHBG (26). Both BMI and estradiol were correlated to HCB in Ukrainian men. To investigate if the association was due to BMI or estradiol we adjusted for these two covariates in the SHBG and HCB analysis and the association remained, suggesting the association was probably not driven by BMI or estradiol. Previously, we have investigated serum levels of the PCB congener CB-153 and the main DDT metabolite, 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p´-DDE) in the same study populations (22;27). They both belong to the same chemical class of organochlorines as HCB and might have similar mode of action. Like for HCB, CB-153 was positively associated with SHBG in Europeans only (ß=2.1, 95%CI=0.7;35). CB-153 was also found to be negatively associated with free testosterone in Europeans (ß=-0.05, 95% CI=-0.08;-0.02) (27). FAI, which is a proxy for unbound testosterone in serum (28), was negatively associated with HCB in our study. This fits with the SHBG finding, since high SHBG is associated with low free testosterone. Thyroid hormones increase hepatic SHBG production (29). A study of 341 men recruited from an infertility clinic reported an inverse association between T_3 and HCB (30), whereas a study of 110 Swedish fishermen did not show association between thyroid hormones and HCB (31). HCB has been associated with cryptorchidism (8) and hypospadias (11), which points to anti-androgenic effects, therefor the positive association between SHBG and HCB found in our study might be an anti-androgenic effect. Therefore, the results might indicate hormone-like activity of some POPs in Caucasian populations.

Men from Poland had the lowest HCB concentration of the three study sites, but they exhibited higher levels of the anti-apoptotic marker Bcl-xL at higher levels of serum HCB after adjustment for potential confounders. On the other hand, sperm apoptotic markers were not associated with HCB for the Greenland and Ukraine populations, so the finding for Polish men was likely a chance finding due to multiple comparisons. Our population-based findings in male spouses of pregnant women are inconsistent with some but not all previous studies. Ferguson *et al.* (2012) investigated 341 male partners, aged 18–51, in subfertile couples seeking infertility evaluation and treatment

(median HCB level was 14.9 ng/g lipid). These authors observed lower SHBG, testosterone and free testosterone at higher serum HCB levels, but only when unadjusted for lipids. In contrast, Dhooge *et al*. (2011), in a population of male adolescents (median age of 14.8 years), found positive associations between serum HCB (standardized by lipid) and estradiol, testosterone, free testosterone and the T/E_2 ratio (median HCB level was 22.8 ng/g lipid). These contradicting results can be due to different study populations. Most studies investigating HCB and semen quality have largely shown no significant associations, consistent with our results (12-14;16;31;32).

The median exposure level of men from Poland in our study was similar or slightly lower compared to other studies that have investigated lipid standardized HCB (15;16;30;32). The Polish men had the lowest serum HCB in our study, almost 13 fold lower than the Ukrainian men, which might be the reason for the borderline significant results compared to the significant results in the highly exposed Ukrainian men. We did not observe relationships between SHBG, FAI and HCB in men from Greenland, although they were the study population with the second highest HCB serum levels. Due to genetic differences, Inuit might be affected differently than the two European study populations.

HCB is highly lipophilic and serum lipid levels vary between fasting and non-fasting status, a correction for serum lipids is needed for the valid interpretation of serum HCB levels. Since other studies have reported HCB levels after standardizing for lipids (HCB ng/g lipids) we did the same, as shown in Table 1, to allow our results to be comparable with those from other studies. In a simulation study, Schisterman *et al*. (2005) evaluated four statistical models showing that the lipophilic compound PCB, standardized by serum lipids as an independent variable, was highly prone to bias (weaker associations due to measurement error), a result also shown by others in the analysis of HCB (16;30;33). Therefore, we adjusted for total serum lipids as a covariate instead of standardizing the HCB by lipids.

As expected, total serum lipids and age were positively correlated with HCB in our study, which is in agreement with results from others (13;14). Because HCB has a long half-life, is lipophilic, accumulates in fat and the production of HCB has decreased with time, it has been found at higher concentrations in elderly and/or obese populations (34;35). We found a negative correlation between HCB and BMI in men from

Ukraine, which did not fit expectations and might be a chance finding.

In this study HCB serum levels were highest in men from Ukraine, probably due to an excessive use of pesticides in the former Soviet Union (36), followed by men from Greenland. HCB levels have previously been found to be higher in Canadian Inuit compared to other inhabitants in the Arctic Canada, possibly because of their higher marine food intake especially from fatty fish and mammals (37). In a study investigating dietary contaminants in Northern Greenland in 1976 and 2004, the levels of HCB among Inuit consuming traditional food was measured (38). Even though today's Inuit consumption of traditional food is less than in the 1970's, the levels of HCB were higher in the samples from 2004. This indicates that the relative contamination levels in the local food products have increased from 1976 to 2004, probably due to the long half-life and lifelong bioaccumulation (38). Looking at the four Greenland sub-regions, we observed higher serum concentrations of HCB in men from Eastern and Northern Greenland compared to the capital and the Southern region. One reason might be the different life styles in different areas of Greenland. In the East and the North Greenland, the inhabitants live a more traditional life with high marine food consumption, especially seals, whereas in Nuuk, the capital city, the population, in particular the youn nger population, consumes more imported food (39;40).

The participation rate of the parent study was low with only 44.6%pregnant women participating. This was a consequence of the recruitment procedure in Ukraine, where the contact between the participants and the project team was handled by many medical doctors. A sub-sample of 605 non-participating Ukrainian women was interviewed regarding demographic and reproductive information. Only age differed between participating and non-participating women with the latter being of slightly lower age. The men who did not accept the invitation did not differ from the participating men with regard to their wives' time to pregnancy (17).

The HCB concentrations varied substantially between the three study sites. HCB in serum was measured at the same laboratory by the same person, thus the HCB concentrations would not be expected to be biased because of inter-observer or – laboratory variation. Further, samples from the three countries were analyzed randomly across time, thus systematic differences related to season, equipment or batches are not

expected.

In the Greenlandic study population 116 out of the 196 semen samples were collected up to one year after enrollment, whereas blood samples were drawn at enrollment. We do not believe this to bias the associations since no seasonal variations were found for HCB in Greenland, and due to the long half-life of HCB (4 to 8 years), a measurement on serum sample can be considered a good proxy of the exposure level within a year. If bias did occur, the estimate would most likely be biased toward the null hypothesis since the misclassification of HCB would have been non-differential with respect to the outcome.

5. Conclusions

Our findings indicate that SHBG and FAI might be associated with high environmental exposure levels of HCB in proven fertile men of European populations, whereas relatively high HCB serum levels seem to have no influence on semen quality.

Conflict of interest statement

The authors have no financial interest or other conflicts of interest in the publication of these results.

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Table 1. Demographic, seminal and hormonal characteristics and HCB exposure of the study populations

	Greenland (N=196)			Poland (N=190)			Ukraine (N=20		
Demographic characteristics:	Mean	Median	Range	Mean	Median	Range	Mean	Median	
Age (years)	31.0	30.6	18.5–51.3	30.3	29.6	20.4–46.3	26.5	25.0	
Body Mass Index (kg/m^2)	26.1	25.5	14.0–57.8	25.8	25.4	18.5–38.1	24.2	24.0	
Cotinine (ng/ml)	165.1	143.5	0.4–570.0	40.0	0.4	0.0–445.7	153.5	119.7	
Period of abstinence (days)	5.3	3.0	0.5–240.0	7.7	4.0	0.1–90.0	3.9	3.0	
Seminal characteristic :									
Total sperm count (x10^6)	245.7	184.4	2.4–1496.0	345.2	197.2	3.7–2071.0	250.4	178.9	
Sperm Concentration (mill/mL)	72.0	52.6	0.6–374.0	89.2	64.0	2.1–419.0	73.3	59.4	
Volume (g)	3.5	3.2	0.7–9.7	3.8	3.5	0.5–10.5	3.5	3.1	
Normal morphology	7.0	6.0	0.0–20.0	6.7	6.0	0.0–16.0	7.4	7.0	
Motility A+B (%)	54.9	60.0	1.0–87.0	60.0	63.5	0.0–92.0	54.1	55.5	
DFI (%)	9.3	7.5	1.3–37.8	12.2	9.7	2.9–49.4	13.3	10.5	
TUNEL (%)	4.5	2.9	0.3–44.7	15.3	13.2	0.0–79.8	9.3	6.6	
Bcl-xL (%)	26.1	10.5	0.0–97.2	18.1	9.3	0.0–89.6	67.4	87.1	
Fas (%)	25.1	19.0	0.0–90.8	48.8	42.8	0.0–98.3	28.0	17.3	
Hormonal characteristics:									
Testosterone (nmol/L)	14.8	14.2	3.2–26.9	13.0	12.7	4.8–24.0	18.0	17.6	
LH (IU/L)	4.4	4.1	1.4–13.2	4.1	3.7	1.3–8.9	4.2	4.0	
FSH (IU/L)	4.9	4.3	0.0–14.6	4.0	3.6	0.7–16.7	4.2	3.4	
Free Androgen Index	55.3	52.4	22.3–106.3	60.7	55.9	24.3–164.0	69.8	66.5	
Estradiol (pmol/L)	65.5	65.3	32.8–112.6	75.8	68.9	37.5–297.3	84.0	78.9	
Inhibin B (ng/L)	184.2	181.0	48.0–470.0	158.0	153.0	27.0–338.0	194.6	186.0	
SHBG (nmol/L)	28.4	27.9	11.1–55.0	23.6	21.6	5.9–63.7	27.7	26.8	
T/E2 ratio	24.6	24.7	10.4–40.8	23.6	22.7	7.6–46.1	19.6	18.9	
Environmental chemical:									
Hexochlorobenzene (ng/g total lipid)	79.0	55.1	9.5–869.6	14.2	12.6	2.6–43.1	182.3	118.5	

[b]Genital infections: epididymitis, gonorrhea, Chlamydia or mumps in adulthood.
[c]Testicular disorders: treatment for retracted testis, surgery for varicose veins, torsio testis or testis cancer.

Table 2. Correlation between HCB concentrations in serum and biomarkers of reproductive function in fertile men from Greenland, Poland and Ukraine

Biomarkers of reproductive function	**Greenland (n=196)**	**Poland (n=190)**	**Ukraine (n= 203)**
DFI (%)	0.05	0.13	-0.00
TUNEL(%)	0.09	0.06	-0.03
Fas-positive(%)	0.07	-0.08	0.04
Bcl-xL(%)	0.07	0.15	-0.23
Semen volume (g)	**-0.15***	0.00	0.04
Total sperm count (x10^6)	-0.05	0.02	0.05
Sperm concentration (mill/mL)	0.04	0.01	0.01
Normal morphology (%)	0.01	-0.02	-0.08
Motility A+B (%)	-0.09	0.06	0.03
Immature sperms (%)	-0.04	0.05	0.02
Testosterone (nmol/L)	-0.04	-0.03	-0.10
Estradiol (pmol/L)	-0.04	-0.04	**-0.16***
LH (IU/L)	0.01	-0.04	0.00
FSH (IU/L)	0.11	0.12	0.07
Inhibin B (ng/L)	0.10	**-0.20***	-0.02
SHBG (nmol/L)	-0.01	0.05	0.11
Free androgen index	-0.05	-0.15	**-0.21***
T/E2 ratio	0.01	-0.05	0.01
Body Mass Index (kg/m^2)	0.06	0.01	**-0.20***
Total serum lipids	0.13	**0.24****	**0.18***
Age at interview (years)	**0.37****	**0.24****	0.02

*=p<0.05 **=p<0.001

Table 3. Adjusted associations between biomarkers of reproductive function and HCB. The log(ß) corresponds to the percentage cha dependent variable by one percent increase in the independent variable.

	Greenland (n=196)			**Poland (n=190)**			**Ukraine (n=203)**	
Biomarkers of reproductive function	**Log(ß)**	**95%CI**	***p*-value**	**Log(ß)**	**95%CI**	***p*-value**	**Log(ß)**	**95%CI**
DFI (%)	0.07	-0.05;0.19	0.25	0.12	-0.10;0.33	0.30	-0.04	-0.17;0.09
TUNEL(%)	0.00	-0.17;0.18	0.99	0.13	-0.19;0.45	0.42	-0.12	-0.39;0.14
Fas-positive(%)	0.15	-0.07;0.37	0.18	0.06	-0.40;0.52	0.79	0.03	-0.33;0.38
Bcl-xL(%)	0.08	-0.33;0.49	0.70	**0.80**	**0.12;1.47**	**0.02**	-0.09	-0.89;0.69
Semen volume (g)	-0.09	-0.20;0.02	0.11	0.02	-0.13;0.17	0.77	0.03	-0.08;0.13
Total sperm count ($x10^6$)	-0.06	-0.27;0.15	0.57	0.13	-0.21;0.46	0.46	-0.01	-0.23;0.23
Sperm concentration (mill/mL)	0.03	-0.12;0.22	0.77	0.10	-0.21;0.42	0.51	-0.03	-0.24;0.17
Normal morphology (%)	-0.02	-0.15;0.11	0.72	-0.00	-0.20;0.19	0.96	-0.09	-0.23;0.06
Motility A+B (%)	-0.07	-0.19;0.06	0.30	-0.05	-0.20;0.11	0.56	-0.04	-0.18;0.09
Immature sperms (%)	0.07	-0.16;0.30	0.52	0.06	-0.17;0.29	0.62	-0.07	-0.27;0.13
Testosterone (nmol/L)	-0.02	-0.191.19	0.99	0.63	-0.66;1.91	0.33	-0.19	-1.30;0.93
Estradiol (pmol/L)	-0.02	-0.08;0.04	0.55	-0.001	-0.11;0.11	0.98	-0.06	-0.12;0.00
LH (IU/L)	-0.00	-0.11;0.10	0.94	-0.00	-0.14;0.14	0.99	0.02	-0.07;0.12
FSH (IU/L)	0.00	-0.12;0.13	0.96	0.04	-0.13;0.22	0.62	0.09	-0.03;0.26
Inhibin B (ng/L)	0.05	-0.05;0.15	0.29	-0.06	-0.19;0.08;	0.40	-0.05	-0.13;0.02
SHBG (nmol/L)	-0.03	-0.11;0.05	0.46	0.11	-0.02;0.24	0.09	**0.07**	**0.00;0.14**
Free androgen index	0.03	-0.05;0.11	0.47	-0.10	-0.20;0.01	0.08	**-0.09**	**-0.16;-0.02**
T/E2 ratio	0.02	-0.05;0.09	0.62	0.02	-0.10;0.14	0.79	0.04	-0.03;0.10

Seminal biomarkers were adjusted for serum lipids, season of blood collection, BMI, age, cotinine in serum, spillage during semen sa infections, testicular disorders as well as sexual abstinence time.
Hormone levels were adjusted for serum lipids, season of blood collection, BMI, age and cotinine in serum.