ORIGINAL ARTICLE

Phthalates, perfluoroalkyl acids, metals and organochlorines and reproductive function: a multipollutant assessment in Greenlandic, Polish and Ukrainian men

Virissa Lenters,1 Lützen Portengen,1 Lidwien A M Smit,1 Bo A G Jönsson,2 Aleksander Giwercman,3 Lars Rylander,2 Christian H Lindh,2 Marcello Spanò,4 Henning S Pedersen,5 Jan K Ludwicki,6 Lyubov Chumak,7 Aldert H Piersma,8 Gunnar Toft,9 Jens Peter Bonde,10 Dick Heederik,1 Roel Vermeulen1

ABSTRACT

Objectives Numerous environmental contaminants have been linked to adverse reproductive health outcomes. However, the complex correlation structure of exposures and multiple testing issues limit the interpretation of existing evidence. Our objective was to identify, from a large set of contaminant exposures, exposure profiles associated with biomarkers of male reproductive function.

Methods In this cross-sectional study (n=602), male partners of pregnant women were enrolled between 2002 and 2004 during antenatal care visits in Greenland, Poland and Ukraine. Fifteen contaminants were detected in more than 70% of blood samples, including metabolites of di(2-ethylhexyl) and diisononyl phthalates (DEHP, DiNP), perfluoroalkyl acids, metals and organochlorines. Twenty-two reproductive biomarkers were assessed, including serum levels of reproductive hormones, markers of semen quality, sperm chromatin integrity, epididymal and accessory sex gland function, and Y:X chromosome ratio. We evaluated multipollutant models with sparse partial least squares regression, a simultaneous dimension reduction and variable selection approach which accommodates joint modelling of correlated exposures.

Results Of the over 300 exposure–outcome associations tested in sPLS models, we detected 10 associations encompassing 8 outcomes. Several associations were notably consistent in direction across the three study populations: positive associations between mercury and inhibin B, and between cadmium and testosterone; and inverse associations between DiNP metabolites and testosterone, between polychlorinated biphenyl-153 and progressive sperm motility, and between a DEHP metabolite and neutral α-glucosidase, a marker of epididymal function.

Conclusions This global assessment of a mixture of environmental contaminants provides further indications that some organochlorines and phthalates adversely affect some parameters of male reproductive health.

INTRODUCTION

There has been considerable scientific and public concern, particularly over the past two decades, that exposure to environmental contaminants may impede development in utero, perturb hormonal homoeostasis and regulation, and contribute to subfertility in humans. This concern stems in part from evidence of geographic variability in semen quality,1 secular trends of decreasing testosterone2 and reports, although inconsistent and contested, of secular declines in sperm concentrations and semen quality.3–4

Though the surge in studies on environmental contaminants and male reproductive health, interpretation of the evidence base is hampered by the piecemeal approach to many investigations. In observational epidemiology, often one class of compounds is correlated with a subset of outcomes, disregarding correlations among background low-level exposures. We chose to simultaneously evaluate a large set of environmental contaminant exposures and biomarkers of male reproductive function in a pregnancy-based study of men from Greenland, Poland and Ukraine.5 6 The set of contaminants comprises high priority7 legacy and emerging contaminants that some organochlorines and phthalates adversely affect some parameters of male reproductive health.
contaminants of concern, suspected to interfere with the endocrine system: high-molecular-weight phthalates, plasticisers used in a wide variety of consumer products, including polyvinyl chloride; perfluorooalkyl acids (PFAs), surfactants used in many applications, such as water and stain-repellent coatings; three non-essential metals; one polychlorinated biphenyl and two organochlorine pesticides. These leach from products or are deposited directly into the environment, leading to near-ubiquitous human exposure, and all, except phthalates, are persistent and bioaccumulate.

We sought an approach to identify exposure–outcome associations between the 19 exposures, representing four classes of xenobiotic compounds, and 22 biomarker outcomes, reflecting various aspects of male reproductive function. Given the relatively weak prior information for the many possible associations, we decided to take a more agnostic, data-driven approach. That the exposures are highly correlated represented a methodological challenge. For this reason, we used sparse partial least squares (sPLS) regression modelling, which relates linear combinations of a subset of the most predictive exposures to an outcome via linear regression. Unlike conventional ordinary least squares (OLS) regression, this dimension reduction approach does not suffer from instability in estimates and failure to converge due to multicollinearity, and enabled a joint assessment of the full set of measured biomarkers of low-level environmental contaminant exposures and reproductive function.

**MATERIALS AND METHODS**

**Study populations**

Study design and data collection procedures have been described previously. Briefly, pregnant women and their male partners were recruited during routine antenatal care visits from 2002 through 2004 inclusive at a large central hospital in Warsaw, Poland, at three hospitals and eight antenatal clinics in Kharkiv, Ukraine, and at local hospitals in 19 municipalities and settlements across Greenland. Of the 1710 couples enrolled (45% participation rate), male partners were consecutively invited to participate in a semen study until approximately 200 men at each of the three locations had agreed (38% participation rate). Participants provided a semen sample, a venous blood sample; cadmium (Cd) and lead (Pb) by cold vapour atom fluorescence spectrophotometry, 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153) and the dichlorodiphenyltrichloroethane metabolite, 1,1-dichloro-2,2-bis(p-chlorophenyl)-ethylene (p,p'-DDE), were analysed by gas chromatography-mass spectrometry, as was hexachlorobenzene (HCB) according to a modified method by Otero et al. Twenty-two markers of reproductive function (listed in table 1) were assessed following standardised protocols: reproductive hormones, conventional semen characteristics, markers of sperm chromatin integrity and apoptosis, markers of epididymal and accessory sex gland function, and the proportion of Y:X chromosome-bearing sperm. Analytical details are provided in the online supplementary methods and table S1.

**Statistical analysis**

We used sPLS regression modelling to assess associations between the exposure profiles and each outcome. In PLS regression, an X-matrix of exposures and Y outcome vector are simultaneously decomposed into latent variables and regressed in a way that maximises the covariance between X and Y. These latent variables, called components, are orthogonal, linear combinations of the full set of input variables. Chun and Keles proposed sPLS, embedding variable selection into the PLS algorithm by eliminating ‘uninformative’ X-variables via penalisation. A penalty term (L1) is applied during dimension reduction, and regression coefficients are obtained via PLS for the reduced set of X-variables (see online supplementary methods). Model complexity is a function of the number of components used to construct the model (K) and the degree of sparsity (η). To determine the optimal model (avoid overfitting), we performed Monte Carlo cross-validation, and added a null (intercept only) model in the cross-validation loop to assess significance. We tested models with K values 1–5 and η values between 0.01 and 0.99 (more sparse) in steps of 0.01, using a fivefold cross-validation, repeated (with different random partitions of the data) 500 times. We selected the most parsimonious model within one SE of the overall minimum mean squared error of prediction (MSEP).

We took several data pretreatment and exploration steps before undertaking sPLS analyses. We imputed exposure data below the limit of detection (LOD) (0–18%) and values missing-at-random in the matrix of exposures (2–16%). Values were imputed from a log-normal probability distribution via single conditional imputation, dependent on the population, and detected values for the other exposures. In the primary analyses, HCB, PCB-153 and p,p'-DDE were lipid adjusted (see online supplementary methods), and four contaminants for which <70% of the samples had concentrations above the LOD were excluded: MEOHP, MOiNP, PFUnDA and PFDoDA. To approximate normality, we used natural logarithm (ln)-transformed values for all exposure variables and for 14 of the 22 outcome variables.

To address potential confounding, we employed a two-stage regression approach: first, each outcome and each exposure were separately regressed on potential confounders, and second, sPLS regression models were fit inputting the residuals. We a priori selected the set of potential confounders, including study population and serum cotinine level, and variably age, body mass index (BMI), abstinence period and time of blood sampling, depending on the outcome (as specified in table 1). We imputed missing covariate data (0.8–16%; as elaborated in the online supplementary methods). To assess the potential for...
between X-scaling by log-transformation reduced the variance differences outcomes. Mean centring also removed baseline offsets, while dimension reduction.23

Lenters V.,

As an additional screening approach, we tested 330 single-

BMI, body mass index; GM, geometric mean; LOD, limit of detection; P, percentile, SCSA, sperm chromatin structure assay; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labelling.

overadjustment, in a secondary analysis we evaluated sPLS models with exposure and outcome variables only prestandardised (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in diseased (centred) for study population. This was considered the minimal adjustment, necessary due to the large differences in disease...
mean outcome level. Statistical analyses were performed using R V3.0.1 (R Foundation for Statistical Computing, Vienna, Austria), and specifically the spls package for sPLS modelling.25

RESULTS

Descriptive characteristics of the study populations are shown in table 1. Participants from Kharkiv were slightly younger than their counterparts from Greenland and Warsaw, and fewer participants from Warsaw smoked. In exploring the structure of the exposure data, we observed large variations within and between the three study populations (figure 1). Levels differed across study populations for all contaminant exposures (analysis of variance p value <0.05, online supplementary table S2), except for MOiCP. Levels were higher in samples from Greenland compared to Warsaw and Kharkiv for many exposures, especially for Hg and PCB-153. Correlations within the X-matrix ranged from $r = -0.48$ to 0.91 (figure 2). We also observed distributional differences for many outcomes (table 1), although distributions for the three populations were generally more similar for the outcomes than for the exposures.

Of the 22 sPLS regression models tested in the primary analysis (n=286–600 complete cases), the X-matrix significantly improved the prediction error beyond the null model for 8 outcomes: luteinising hormone (LH), inhibin B, total testosterone, free testosterone, semen volume, progressive sperm motility, terminal deoxynucleotidyl transferase dUTP nick end-labelling assay (TUNEL) DNA fragmentation index (DFI), and neutral $\alpha$-glucosidase (NAG). Within each of these significant sPLS regression models, 1 to 3 exposures within the X-matrix contributed to a total of 10 significant exposure–outcome associations (table 2). The optimal models were all K=1 component models and quite sparse ($\eta$ between 0.64 and 0.99).

In the secondary analysis with exposure and outcome variables only prestandardised for study population but not for other potential confounders, sex hormone-binding globulin (SHBG) was additionally selected (the inclusion of the X-matrix improved the MSEP beyond the null model), and the LH model was no longer selected (see online supplementary table S3). Extra exposures were also selected, for a total of 24 associations.

In the covariate-adjusted OLS regression analyses, 8 overlapping exposure–outcome associations, comprising the same 8 outcomes but fewer exposures (8 of 10) compared to the primary sPLS analyses, were identified as significant (FDR <10%). Two associations, between MHiNP and total testosterone and TUNEL DFI, were significant at an FDR <5% significance threshold (see online supplementary figure S2). An additional association between MEHHP and TUNEL DFI, not selected in the sPLS model, was significant in OLS analyses (see online supplementary table S3). Consistent with the sPLS modelling results, a greater number of associations was significant in the secondary analysis with OLS models only adjusted for study population compared to the primary analysis with further adjusted models (15 compared to 9; 14 of which overlapped with those selected in sPLS secondary analysis only prestandardised for study population).

No additional associations were detected in the sensitivity analysis testing contaminants with <70% detection frequency inputted as binary detect versus non-detect variables. In a reanalysis using only the complete (non-imputed) exposure and covariate data, 2 associations—$p,p'$-DDE and LH, and MHiNP and total testosterone—were selected in both the sPLS and OLS modelling (with n=321–480 and 328–586 complete cases, respectively, except for Bcl-xL positivity models which had 192–252 complete cases). This analysis had reduced power, and while CIs were wider, coefficients for the complete case analysis were remarkably similar to the primary analysis with imputed data (data not shown). Coefficients for lipid unadjusted organochlorines, adjusted for total lipids, were only slightly different in magnitude (see footnotes of table 2).

---

**Figure 1** Box plots of the distributions of exposure biomarker concentrations per study population. MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; MHdNP, mono(4-methyl-7-hydroxyoctyl) phthalate; MOiCP, mono(4-methyl-7-carboxyheptyl) phthalate; Hg, mercury; PFHxS, perfluorhexane sulfonic acid; PFOS, perfluorooctane sulfonic acid; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFDA, perfluorodecanoic acid; HCB, hexachlorobenzene; PCB, polychlorinated biphenyl; DDE, dichlorodiphenyldichloroethylene.
**DISCUSSION**

Using a multipollutant modelling approach, we identified 10 associations between biomarkers of environmental contaminant exposures and outcomes. For 5 of the 10 sPLS-selected associations, there was a significant interaction between exposure and study population in the OLS sensitivity analysis (table 2). It was apparent that 8 associations were consistent in slope direction across study populations. Poland exhibited a slope in the opposite direction for LH and p,p'-DDE, and for the oxidative phthalates and TUNEL DFI. Associations with consistent slope directions were consistent in slope across study populations in regression models stratified by population. Table 2 shows the associations between biomarkers of environmental contaminant exposures and outcomes, with corresponding regression coefficients selected from multipollutant sPLS regression modelling.

**Table 2** Exposure–outcome associations and regression coefficients selected from multipollutant sPLS regression modelling and corresponding single-pollutant OLS regression coefficients

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Exposure*</th>
<th>sPLS K, η†</th>
<th>β_sPLS</th>
<th>OLS β_OLS</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH* (IU/L)</td>
<td>p,p'-DDE (ng/g)</td>
<td>1, 0.99</td>
<td>0.083</td>
<td>0.083††,§ (0.031 to 0.135)</td>
<td>0.002¶</td>
<td></td>
</tr>
<tr>
<td>Inhibin B (ng/mL)</td>
<td>Hg (ng/mL)</td>
<td>1, 0.99</td>
<td>10.79</td>
<td>10.82 (3.90 to 17.73)</td>
<td>0.002‡</td>
<td></td>
</tr>
<tr>
<td>Total testosterone (nmol/L)</td>
<td>MHiNP (ng/mL)</td>
<td>1, 0.90</td>
<td>−1.14</td>
<td>−1.15†† (−1.74 to −0.57)</td>
<td>0.0001§</td>
<td></td>
</tr>
<tr>
<td>Free testosterone (nmol/L)</td>
<td>MOiCP (ng/mL)</td>
<td>1, 0.64</td>
<td>−0.0113</td>
<td>−0.019†† (−0.032 to −0.007)</td>
<td>0.002¶</td>
<td></td>
</tr>
<tr>
<td>Cd (ng/mL)</td>
<td>0.0091</td>
<td>0.0012 (0.001 to 0.023)</td>
<td>0.029</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semen volume* (mL)</td>
<td>MEHHP (ng/mL)</td>
<td>1, 0.99</td>
<td>−0.106</td>
<td>−0.106 (−0.167 to −0.045)</td>
<td>0.0008§</td>
<td></td>
</tr>
<tr>
<td>Progressive sperm (%)</td>
<td>PCB-153 (ng/mL)</td>
<td>1, 0.99</td>
<td>−3.37</td>
<td>−3.37†† (−5.48 to −2.15)</td>
<td>0.002¶</td>
<td></td>
</tr>
<tr>
<td>TUNEL DFI* (%)</td>
<td>MHiNP (ng/mL)</td>
<td>1, 0.99</td>
<td>−0.218††</td>
<td>−0.218†† (−0.332 to −0.104)</td>
<td>0.0002‡</td>
<td></td>
</tr>
<tr>
<td>NAG* (μl/ejaculate)</td>
<td>MEHHP (ng/mL)</td>
<td>1, 0.77</td>
<td>−0.163</td>
<td>−0.164 (−0.255 to −0.073)</td>
<td>0.0005§</td>
<td></td>
</tr>
</tbody>
</table>

**Regression coefficients (β) are expressed per ln-unit change in exposure. Refer to online supplementary table S3 for per cent changes and coefficients from models only adjusted for covariates (residuals).**

*Variable ln-transformed in statistical analyses.
†sPLS tuning parameters: K, the number of components used and η, the degree of sparsity.
‡Interaction p value <0.10 for the cross-product term between exposure and study population (see online supplementary figure S3). For 5 of the 10 sPLS-selected associations, there was a significant interaction between exposure and study population in the OLS sensitivity analysis. For 8 associations, consistency in slope direction across study populations were consistent in slope across study populations in regression models stratified by population. Poland exhibited a slope in the opposite direction for LH and p,p'-DDE, and for the oxidative phthalates and TUNEL DFI. Associations with consistent slope directions were consistent in slope across study populations in regression models stratified by population. Table 2 shows the associations between biomarkers of environmental contaminant exposures and outcomes, with corresponding regression coefficients selected from multipollutant sPLS regression modelling.

**Table 2** Exposure–outcome associations and regression coefficients selected from multipollutant sPLS regression modelling and corresponding single-pollutant OLS regression coefficients

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Exposure*</th>
<th>sPLS K, η†</th>
<th>β_sPLS</th>
<th>OLS β_OLS</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH* (IU/L)</td>
<td>p,p'-DDE (ng/g)</td>
<td>1, 0.99</td>
<td>0.083</td>
<td>0.083††,§ (0.031 to 0.135)</td>
<td>0.002¶</td>
<td></td>
</tr>
<tr>
<td>Inhibin B (ng/mL)</td>
<td>Hg (ng/mL)</td>
<td>1, 0.99</td>
<td>10.79</td>
<td>10.82 (3.90 to 17.73)</td>
<td>0.002‡</td>
<td></td>
</tr>
<tr>
<td>Total testosterone (nmol/L)</td>
<td>MHiNP (ng/mL)</td>
<td>1, 0.90</td>
<td>−1.14</td>
<td>−1.15†† (−1.74 to −0.57)</td>
<td>0.0001§</td>
<td></td>
</tr>
<tr>
<td>Free testosterone (nmol/L)</td>
<td>MOiCP (ng/mL)</td>
<td>1, 0.64</td>
<td>−0.0113</td>
<td>−0.019†† (−0.032 to −0.007)</td>
<td>0.002¶</td>
<td></td>
</tr>
<tr>
<td>Cd (ng/mL)</td>
<td>0.0091</td>
<td>0.0012 (0.001 to 0.023)</td>
<td>0.029</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semen volume* (mL)</td>
<td>MEHHP (ng/mL)</td>
<td>1, 0.99</td>
<td>−0.106</td>
<td>−0.106 (−0.167 to −0.045)</td>
<td>0.0008§</td>
<td></td>
</tr>
<tr>
<td>Progressive sperm (%)</td>
<td>PCB-153 (ng/mL)</td>
<td>1, 0.99</td>
<td>−3.37</td>
<td>−3.37†† (−5.48 to −2.15)</td>
<td>0.002¶</td>
<td></td>
</tr>
<tr>
<td>TUNEL DFI* (%)</td>
<td>MHiNP (ng/mL)</td>
<td>1, 0.99</td>
<td>−0.218††</td>
<td>−0.218†† (−0.332 to −0.104)</td>
<td>0.0002‡</td>
<td></td>
</tr>
<tr>
<td>NAG* (μl/ejaculate)</td>
<td>MEHHP (ng/mL)</td>
<td>1, 0.77</td>
<td>−0.163</td>
<td>−0.164 (−0.255 to −0.073)</td>
<td>0.0005§</td>
<td></td>
</tr>
</tbody>
</table>

**Regression coefficients (β) are expressed per ln-unit change in exposure. Refer to online supplementary table S3 for per cent changes and coefficients from models only adjusted for covariates (residuals).**

*Variable ln-transformed in statistical analyses.
†sPLS tuning parameters: K, the number of components used and η, the degree of sparsity.
‡Interaction p value <0.10 for the cross-product term between exposure and study population (see online supplementary figure S3). For 5 of the 10 sPLS-selected associations, there was a significant interaction between exposure and study population in the OLS sensitivity analysis. For 8 associations, consistency in slope direction across study populations were consistent in slope across study populations in regression models stratified by population. Poland exhibited a slope in the opposite direction for LH and p,p'-DDE, and for the oxidative phthalates and TUNEL DFI. Associations with consistent slope directions were consistent in slope across study populations in regression models stratified by population. Table 2 shows the associations between biomarkers of environmental contaminant exposures and outcomes, with corresponding regression coefficients selected from multipollutant sPLS regression modelling.

**Table 2** Exposure–outcome associations and regression coefficients selected from multipollutant sPLS regression modelling and corresponding single-pollutant OLS regression coefficients

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Exposure*</th>
<th>sPLS K, η†</th>
<th>β_sPLS</th>
<th>OLS β_OLS</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH* (IU/L)</td>
<td>p,p'-DDE (ng/g)</td>
<td>1, 0.99</td>
<td>0.083</td>
<td>0.083††,§ (0.031 to 0.135)</td>
<td>0.002¶</td>
<td></td>
</tr>
<tr>
<td>Inhibin B (ng/mL)</td>
<td>Hg (ng/mL)</td>
<td>1, 0.99</td>
<td>10.79</td>
<td>10.82 (3.90 to 17.73)</td>
<td>0.002‡</td>
<td></td>
</tr>
<tr>
<td>Total testosterone (nmol/L)</td>
<td>MHiNP (ng/mL)</td>
<td>1, 0.90</td>
<td>−1.14</td>
<td>−1.15†† (−1.74 to −0.57)</td>
<td>0.0001§</td>
<td></td>
</tr>
<tr>
<td>Free testosterone (nmol/L)</td>
<td>MOiCP (ng/mL)</td>
<td>1, 0.64</td>
<td>−0.0113</td>
<td>−0.019†† (−0.032 to −0.007)</td>
<td>0.002¶</td>
<td></td>
</tr>
<tr>
<td>Cd (ng/mL)</td>
<td>0.0091</td>
<td>0.0012 (0.001 to 0.023)</td>
<td>0.029</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semen volume* (mL)</td>
<td>MEHHP (ng/mL)</td>
<td>1, 0.99</td>
<td>−0.106</td>
<td>−0.106 (−0.167 to −0.045)</td>
<td>0.0008§</td>
<td></td>
</tr>
<tr>
<td>Progressive sperm (%)</td>
<td>PCB-153 (ng/mL)</td>
<td>1, 0.99</td>
<td>−3.37</td>
<td>−3.37†† (−5.48 to −2.15)</td>
<td>0.002¶</td>
<td></td>
</tr>
<tr>
<td>TUNEL DFI* (%)</td>
<td>MHiNP (ng/mL)</td>
<td>1, 0.99</td>
<td>−0.218††</td>
<td>−0.218†† (−0.332 to −0.104)</td>
<td>0.0002‡</td>
<td></td>
</tr>
<tr>
<td>NAG* (μl/ejaculate)</td>
<td>MEHHP (ng/mL)</td>
<td>1, 0.77</td>
<td>−0.163</td>
<td>−0.164 (−0.255 to −0.073)</td>
<td>0.0005§</td>
<td></td>
</tr>
</tbody>
</table>

**Regression coefficients (β) are expressed per ln-unit change in exposure. Refer to online supplementary table S3 for per cent changes and coefficients from models only adjusted for covariates (residuals).**

*Variable ln-transformed in statistical analyses.
†sPLS tuning parameters: K, the number of components used and η, the degree of sparsity.
‡Interaction p value <0.10 for the cross-product term between exposure and study population (see online supplementary figure S3). For 5 of the 10 sPLS-selected associations, there was a significant interaction between exposure and study population in the OLS sensitivity analysis. For 8 associations, consistency in slope direction across study populations were consistent in slope across study populations in regression models stratified by population. Poland exhibited a slope in the opposite direction for LH and p,p'-DDE, and for the oxidative phthalates and TUNEL DFI. Associations with consistent slope directions were consistent in slope across study populations in regression models stratified by population. Table 2 shows the associations between biomarkers of environmental contaminant exposures and outcomes, with corresponding regression coefficients selected from multipollutant sPLS regression modelling.
exposure and biomarkers of male reproductive function from several hundred associations tested in (sub) fertile men from Greenland, Warsaw and Kharkiv. This is the first time several of the relationships have been assessed in an epidemiological study, or simultaneously.

One of the most statistically robust and consistent associations was between DiNP metabolites and decreasing free (bioactive) testosterone and total testosterone. DEHP metabolites did not exceed the significance threshold in confounder-adjusted analyses but exhibited inverse relationships. DiNP exposure is out-pacing DEHP exposure in Europe, where DiNP use is less restricted than that of DEHP. These inverse associations are consistent with an antiandrogenic effect, which has more often been observed compared to pro-androgenic effects in experimental studies. Phthalates are generally considered to act by inhibiting Leydig cell synthesis of testosterone. While most experimental studies have focused on in vitro exposure, DEHP and its primary metabolite MEHP were recently shown to suppress steroidogenesis of adult human testis explants in vitro, without affecting production of the hormone insulin-like factor 3 (INSL3). Several epidemiological studies have found inverse associations between (urinary) MEHP and testosterone in men; in 74 occupationally exposed workers and 63 controls in China, and in a pooled analysis of 425 men of infertile couples and 425 men recruited through a US infertility clinic. No association was found in 234 young Swedish men sampled during routine medical conscript examinations. Joensen et al considered another exposure metric, the proportion of the summed urinary metabolites excreted as the primary metabolite, as a marker of DEHP and DiNP metabolism; %MEHP and %MiNP were associated (significant at p<0.05 or near-significant) with total testosterone and free androgen index in 881 healthy young men from Denmark around 19 years of age. However, no significant associations were reported for the sum of primary and secondary metabolites. Primary monoester phthalate metabolites were not measured in the current study—only secondary oxidative metabolites—due to the lipase activity of serum and risk of contamination from the diesters from the sampling devices.

We found a clear dose-dependent decrease in serum testosterone levels with increasing levels of MHiNP. We recently discovered, however, that the measurement of MHiNP was possibly confounded by an unknown co-eluting agent. Nevertheless, in an additional analysis using the molar sum of the other two DiNP metabolites versus all three oxidative metabolites tested in relation to total testosterone, the magnitude of the regression coefficients was similar: β_{OLS} = −0.713 (p value=0.0037) for ΣDiNP{MONT+MOCF} versus −0.929 (0.0007) for ΣDiNPom. Moreover, that other phthalate metabolites were significantly inversely associated with testosterone lends weight to the evidence that one or more phthalates is indeed related to decreasing testosterone levels.

We observed a positive association between cadmium and free testosterone in the sPLS analyses, although this association was not significant in the OLS analyses adjusted for potential confounders, notably cotinine. Disentangling the effect of smoking and cadmium is difficult as smoking is an important source of cadmium exposure. A cadmium—testosterone association has previously been reported in 219 men recruited from two US infertility clinics, and in a representative sample of 1262 US men, although this latter association did not sustain statistical significance after adjustment for smoking. An experimental study of chronic low-dose oral exposure in rats reported cadmium-related elevated testosterone; however, the underlying mechanism remains unknown.

We observed decreasing NAG levels with increasing MEHHP. Sperm undergo maturation in the epididymis, and NAG is a marker of epididymal function. A smaller study (n=234) of urinary phthalates and NAG in Swedish conscripts was null. In a study of 40 rats administered di-n-butyl phthalate, epididymal weight and NAG activity decreased dose dependently, in line with our finding.

We found decreasing sperm DNA damage with increasing levels of the secondary hydroxylated metabolite of DiNP, counter to the hypothesis of a deleterious effect of exposure. However, the exposure—outcome relationship was inconsistent in direction, exhibiting a positive slope in the Polish study population. We detected associations for DNA fragmentation assessed with TUNEL, but not the sperm chromatin structure assay (SCSA). TUNEL represents DNA strand breaks measured directly in the ejaculated spermatozooa, whereas SCSA is a measure of susceptibility to acid denaturation. Sperm DNA damage might be mediated by testicular oxidative stress. In rats, DEHP decreased antioxidant enzymes and concomitantly increased reactive oxygen species. In 379 US men seeking infertility treatment, urinary monoethyl phthalate and MEHP were positively associated with neutral Comet assay parameters. MEHP was more strongly associated when adjusted for oxidative metabolites (MEHHP and MEOHP), and similar to our finding for MEHHP, the metabolites were inversely associated with DNA damage. Hauser et al suggested that an increased ratio of oxidative metabolites to MEHHP could represent interindividual differences in rates of DEHP metabolism to ‘less toxic’ metabolites, or it may reflect inhibition of phase 1 enzymes involved in xenobiotic metabolism.

Some of the exposure—outcome associations tested in the current analysis have previously been tested in the same or overlapping study populations in analyses considering a single-pollutant or a single class of chemicals (refer to refs. 39–41; previous investigations of PCB-153 and p,p’-DDE were reviewed by Bonde et al). In addition to identifying several novel associations, we confirmed, using a more conservative significance threshold and joint modelling approach, several previously reported associations, including an unfavourable inverse association between PCB-153 and the proportion of progressively motile sperm. Other smaller studies have reported comparable findings, for instance, for PCB-153 in 305 Swedish men, and for PCB-138 but not for PCB-153 and the odds of <50% progressive motility in 212 US men recruited at an infertility clinic (uncorrected for multiple comparisons).

We also confirm a previously reported strong positive association between mercury and inhibin B. As inhibin B, predominantly excreted by Sertoli cells, is correlated with sperm concentration and counts and serves as a marker of spermatogenesis, this positive association is contrary to a deleterious effect of exposure. The authors suggest that this association may be driven by a higher consumption of seafood—particularly relevant for Greenlandic Inuit—and the resultant concomitant exposure to mercury and α-3 polyunsaturated fatty acids. There are indications that the latter may beneficially impact certain semen quality parameters. This mercury—inhibin B association was not observed in 219 US men recruited from infertility clinics.

We observed an association between MEHHP and a decline in semen volume, as previously reported for this study population. A null finding for MEHP was reported for a study of
234 young Swedish men, and a statistically significant increase in semen volume for the highest versus lowest %MiNP quartile in 881 Danish men was interpreted by the authors as a chance finding. We also observed a positive, yet inconsistent-across-populations, association between \textit{p,p'}-DDE and increasing LH, as previously reported. As LH regulates testosterone production by Leydig cells, this is in line with an antiandioogenic effect of \textit{p,p'}-DDE. A null finding was reported for 341 men from a US infertility clinic with lower \textit{p,p'}-DDE levels (geometric mean 236 ng/g lipids). We found indications of associations between phthalates, metals and organochlorines and SHBG, which did not sustain significance on adjustment for confounders, specifically BMI. These non-significant perturbations in SHBG, together with the other associations with hormones, might be indicative of effects of exposure profiles on hypothalamo–pituitary–gonadal axis regulation. Similar to the potential (over)adjustment for cotinine, adjustment for BMI may introduce overadjustment bias rather than (only) prevent confounding bias, given that some exposures may causally influence BMI. A limitation of this assessment is the cross-sectional nature of this study, although, besides phthalates, all measured contaminants are relatively stable over time and as such were most likely reasonable proxies of recent past exposure as related to the measured outcomes. However, while blood and semen samples were collected on the same day for nearly all of the participants from Poland and Ukraine, for around 60% of the participants from Greenland, samples were collected more than 3 days apart (elaborated in the online supplementary methods), contributing to misclassification of exposure, especially for the phthalates. Analyses excluding these participants yielded generally consistent results.

Recent advances in molecular epidemiology (high-throughput screening techniques and the introduction of the exposome concept) imply that epidemiological researchers will be confronted more and more with the challenging task of analysing data with high numbers of measured analytes with often complex correlation structures. Single-pollutant modelling may result in a high potential for false positives (type I errors), although multiple testing corrections and the recently proposed environment-wide association study design address this. Using multipollutant OLS regression modelling may lead to inflated variances and flipped signs of parameter estimates, thus making it difficult to disentangle the independent effects of these variables. Methods which project data onto a lower dimensional space, like PLS regression, can better cope with collinear variables and, as a corollary, reduce the number of models tested. While PLS-based methods are well established in chemometrics and bioinformatics, they are only recently gaining traction in genomics, metabolomics and epidemiology. In the current analysis, the associations selected using sPLS rather closely matched those we would have selected using single-pollutant OLS models. However, if we had screened for associations using multipollutant OLS models, accounting for correlations between exposures, these estimates would have suffered from multicollinearity and been unreliable (variance inflation factors exceeded 3 for 8 to 9 of the 15 exposure terms across multipollutant OLS models).

As sPLS captures the complementary contribution of X-variables, it has the power to detect multiple pollutants which might not be detected in single-pollutant modelling. This can be explained by additivity, and/or consistency at large, in that noise from measurement error and biological variation can be averaged out when a larger number of X-variables inform the latent variable. This was evident for the free testosterone model, and for several models in the sensitivity analysis with inputs only prestandardised for study population, in which up to 8 exposures were selected. Further, in constructing latent variables, sPLS regression delineates groupings in the X-matrix of exposures, which is a step forward in tackling the long-standing challenge of assessing mixture effects of contaminants.

Some aspects of (s)PLS modelling implementation are underdeveloped, such as quantifying uncertainty (stability of the selection), and explicitly correcting for multiple testing, beyond the implicit sparseness step. We estimated an empirical null distribution using a Monte Carlo approach to establish a significance threshold. A caveat of sPLS is that penalisation methods optimise prediction and will tend to select one of two highly correlated exposures with nearly equal coefficient sizes. Competing multipollutant modelling approaches exist (eg, tree-based models, elastic net penalised regression); however, simulation and validation assessments, specifically for data structures relevant for environmental epidemiology (often low dimensional), are lacking.

There is no straightforward way to adjust for potential confounders in (s)PLS analyses. Adjusting for confounders in sPLS models by inputting residuals in a two-step approach should, as with OLS modelling, yield unbiased regression coefficients, although with a slight loss of efficiency under certain (sample size, correlation) scenarios. We observed large contrasts in exposures across the three study populations, and some differences in outcomes such as TUNEL DFI. To reduce the risk of ecological bias due to potential between-population confounding, in all analyses we prestandardised (mean centred) the exposure and outcome data by population (sPLS models) or adjusted by population (OLS models). This may have artificially reduced true contrast in exposure and outcome data between study populations, and negatively affected our power to detect some associations.

While (s)PLS modelling has some power to detect non-linear relationships (weighted over components), the PLS and OLS regression coefficients reflect linear relationships. In an exploratory analysis with generalised additive modelling, this assumption of linearity was met for most exposure–outcome relationships (<2% deviated from linearity, with an estimated degree of freedom >1.0). Further research is needed to compare the efficacy of sPLS regression with other multipollutant modelling and variable selection approaches, including non-parametric techniques.

CONCLUSIONS

We used a systematic, multipollutant modelling approach to perform a global assessment of biomarkers of contaminant exposure and male reproduction function in one of the largest studies to date. We identified several environmentally perturbed outcomes, including a robust inverse association between DiNP phthalates and circulating testosterone, and between PCB-153 and sperm motility.

Author affiliations

1 Division of Environmental Epidemiology, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
2 Division of Occupational and Environmental Medicine, Lund University, Lund, Sweden
3 Reproductive Medicine Centre, Skåne University Hospital Malmö, Lund University, Malmö, Sweden
4 Laboratory of Toxicology, Unit of Radiation Biology and Human Health, ENEA Casaccia Research Center, Rome, Italy
5 Centre for Arctic Environmental Medicine, Nuuk, Greenland
**Environment**

**Contributors** VL, LP, DH and RV conceived and designed the current data analyses. JPB, GT, AG, LR, BAGI, MS, HSP, IKL and LC designed and collected data for the cohort study. BAGI and CHL performed chemical analyses. VL analysed the data and drafted the manuscript, with supervision from LP and RV. All authors, particularly LP, RV, DH, LAMS, AHP, JPB, GT, AG and MS, participated in the interpretation of results and revision of the manuscript. All authors approved the final version of the manuscript.

**Funding** This work was supported by the European Commission’s 7th and 5th Framework Programmes (grants FP7-ENV-2008-1-226217 and QLK4-CT-2001-00202).

**Competing interests** None.

**Patient consent** Obtained.

**Ethics approval** Polish Bioethical Committee, Ethical Committee for Human Research Protection.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**REFERENCES**


Phthalates, perfluoroalkyl acids, metals and organochlorines and reproductive function: a multipollutant assessment in Greenlandic, Polish and Ukrainian men


*Occup Environ Med* 2015 72: 385-393 originally published online
September 10, 2014
doi: 10.1136/oemed-2014-102264

Updated information and services can be found at:
http://oem.bmj.com/content/72/6/385

These include:

**Supplementary Material**
Supplementary material can be found at:
http://oem.bmj.com/content/suppl/2014/09/10/oemed-2014-102264.DC1

**References**
This article cites 45 articles, 9 of which you can access for free at:
http://oem.bmj.com/content/72/6/385#BIBL

**Email alerting service**
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

**Topic Collections**
Articles on similar topics can be found in the following collections

Editor's choice (120)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/