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Novel application of statistical methods for analysis of multiple toxicants identifies DDT as a risk factor for early child behavioral problems

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ABSTRACT

Background: The aim of this study was to assess the association between postnatal exposure to multiple persistent organic pollutants (POPs) measured in breast milk samples and early behavioral problems using statistical methods to deal with correlated exposure data.

Methods: We used data from the Norwegian HUMIS study. We measured concentrations of 24 different POPs in human milk from 612 mothers (median collection time: 32 days after delivery), including 13 polychlorinated biphenyls (PCB) congeners, 6 polybrominated diphenyl ethers (PBDE) congeners and five organochlorine compounds. We assessed child behavioral problems at 12 and 24 months using the infant toddler symptom checklist (ITSC). Higher score in ITSC corresponds to more behavioral problems. First we performed principal component analysis (PCA). Then two variable selection methods, elastic net (ENET) and Bayesian model averaging (BMA), were applied to select any toxicants associated with behavioral problems. Finally, the effect size of the selected toxicants was estimated using multivariate linear regression analyses.

Results: *p,p′*-DDT was associated with behavioral problems at 12 months in all the applied models. Specifically, the principal component composed of organochlorine pesticides was significantly associated with behavioral problems and both ENET and BMA identified *p,p′*-DDT as associated with behavioral problems. Using a multiple linear regression model an interquartile increase in *p,p′*-DDT was associated with a 0.62 unit increase in ITSC score (95% CI 0.45, 0.79) at 12 months, corresponding to more behavioral problems. The association was modified by maternal education: the effect of *p,p′*-DDT was strongest in women with lower education (β=0.59; 95%CI: 0.38, 0.81) compared to the mother with higher education (β=0.14; 95%CI: −0.05, 0.34) (*p*-value for interaction=0.089). At 24 months, neither selection method consistently identified any toxicant associated with behavioral problems.

Conclusion: Within a mixture of 24 toxicants measured in breast milk, *p,p′*-DDT was the single toxicant associated with behavioral problems at 12 months using different methods for handling numerous correlated exposures.

Abbreviations: POPs, persistent organic pollutants; PCBs, polychlorinated biphenyls; p,p′-DDT, 1,1,1-trichloro-2,2-bis (4chlorophenyl) ethane; p,p′DDE, 1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene; HCB, Hexachlorobenzene; ß-HCH, ß-hexachlorocyclohexane; PBDEs, polybrominateddiphenyl et; PCA, principal component analysis; ENET, elastic net; BMA, Bayesian model averaging; HUMIS, Norwegian Human Milk Study; NIPH, Norwegian Institute of Public Health; GC–MS, liquid-liquid extraction and gas chromatography – mass spectrometry; LOD/LOQ, limit of detection/quantification; ITSC, Infant/Toddler Symptoms Checklist; MBR, Medical Birth Registry of Norway; BMI, body mass index; SCL-5, Hopkins Symptom Checklist; IQR, interquartile range; DAG, directed acyclic graph; PCs, Principal Components; CI, Confidence Interval; PIP, posterior inclusion probability; MSE, mean-squared error

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1. Introduction

Persistent organic pollutants (POPs) are environmental chemicals with well-documented toxic potency. They are resistant to degradation in nature, accumulate in wildlife and humans and biomagnify through food chains. POPs encompass a wide variety of chemicals including organochlorines [polychlorinated biphenyls (PCBs), 1,1,1-trichloro-2,2-bis (4chlorophenyl) ethane (p,p′-DDT), and 1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene (p,p′DDE), hexachlorobenzene (HCB), and ß-hexachlorocyclohexane (ß-HCH)], brominated compounds [polybrominateddiphenyl ethers (PBDEs)]] and many other chemicals. Fetuses and newborns are exposed to POPs through placental transfer and breastfeeding (Bergonzi et al., 2009; Dingemans et al., 2011; Ribas-Fito et al., 2001). Many POPs have been banned across much of the world (Stockholm Convention on Persistent Organic Pollutants (POPs, 2009), and thus, concentrations of PCBs, *p,p′*-DDE and HCB in humans have decreased over time (Nickerson, 2006). However, most of these compounds are still detectable in the blood of current generations due to their persistence in the environment and their long half-lives in humans (Jönsson et al., 2005; Toms et al., 2009).

PCBs, PBDEs, *p,p′*-DDE, HCB are considered developmental neurotoxicants (Grandjean and Landrigan, 2014), mainly based on studies looking at the association between *in utero* exposure to single pollutants and neuropsychological development. However, children are exposed to multiple toxicants, which coexist in exposure sources and are, therefore, often highly correlated. To date, few studies have used a multipollutant approach to study the association between toxicants and health (Agay-Shay et al., 2015; Braun et al., 2014; Grandjean et al., 2012; Lee et al., 2007; Lenters et al., 2015b; Patel et al., 2010). In general, there is a lack of knowledge on the potential neurotoxicity of mixtures, and in particular that resulting from exposure through breastfeeding.

We aimed to assess the association between postnatal exposure to 24 different POPs measured in milk samples and early behavioral problems at 12 and at 24 months of age, using the longitudinal HUMIS birth cohort. We employed principal component analysis (PCA), elastic net (ENET) and Bayesian model averaging (BMA) in order to identify toxicants potentially associated with behavioral problems in early life. Then, we used traditional multivariate regression methods to estimate the association between selected toxicant(s) and early behavioral problems.

2. Methods

2.1. Study population

The “Norwegian Human Milk Study” (HUMIS) is a multi-center birth cohort of mother-child pairs recruited between 2002 and 2009. Within approximately two weeks of giving birth, mothers were recruited by public health nurses during a routine home visit to all new mothers in Norway, except in Østfold county where mothers were recruited at the maternity ward, two consecutive term births for every preterm. Participants were asked to collect a 25 ml breast milk sample each morning for eight consecutive days, preferably sampled between 2 weeks and two months in line with the WHO recommendation, but they were informed that milk sampled otherwise was also accepted, but changes in sampling protocol were noted. The milk was stored in a 250 ml container kept in the freezer. Date and time of collection were recorded for each sample, as well as whether a breast pump had been used. When the container had been filled, participants mailed it by regular mail, except in the county of Østfold where the milk samples were collected by study personnel and kept frozen during transport to the Norwegian Institute of Public Health (NIPH). Further details have been published elsewhere (Eggesbø et al., 2011).

Informed consent was obtained prior to the study and the study was approved by the Norwegian Data Inspectorate and Regional Ethics Committee for Medical Research. Overall, 36% of the invited women declined to participate in the study.

So far, among the 2.606 participants in the HUMIS study, 612 women have had their milk samples analyzed for the complete list of POPs (due to financial constraints not everyone could be analyzed at once): 498 were randomly selected; 54 were oversampled based on preterm status and 60 were oversampled due to rapid growth of their infant (Fig. 1). Among these 612 subjects 52 and 78 had not sent in the 12 and 24 month questionnaires, respectively. In addition, there were some with missing values on the specific questions needed for the neuropsychological assessments (12 and 6, for 12 and 24 month questionnaires, respectively) (Fig. 1). Therefore, the final subsets used in this study were 548 and 528, for 12 and 24 months respectively.

2.2. Exposure measurement

The POPs concentrations were determined in breast milk. The median age of child at start of sampling was 32 days after delivery (min 2 days, max 171 days). The chemical measurements were performed in two laboratories. Briefly, the concentrations of six PBDE congeners (PBDE 28, 47, 99, 100, 153 and 154) were determined in 612 samples at the Department of Exposure and Risk Assessment, NIPH using liquid-liquid extraction and gas chromatography (GC) – mass spectrometry (MS) with negative chemical ionization as described in detail elsewhere (Thomsen et al., 2010, 2007).

Concentrations of HCB, β-HCH, *p,p′*-DDE, *p,p′*-DDT, oxychlordane (oxyCD), seven non-dioxin-like polychlorinated biphenyls (ndl-PCBs) (IUPAC nos.: 74, 99, 138, 153, 170, 18, and 194), and six dioxin-like mono-ortho PCBs (mo-PCBs) (IUPAC nos.: 105, 114, 118, 156, 157 and 189) were measured at the Department of Exposure and Risk Assessment, NIPH (Thomsen et al., 2007) in 532 samples using liquid-liquid extraction and GC–MS with negative chemical ionization, and at the University of Life Science-NMBU in a further 80 samples using liquid/liquid extraction, gravimetrical lipid determination and clean-up with sulfuric acid (Eggesbø et al., 2011, 2009; Polder et al., 2009, 2008). All the methods used are described in more detail in supplemental material (Supplemental Methods S1). We replaced POP concentrations below the limit of detection/quantification (LOD/LOQ) with a randomly generated number between 0 and the analysis-specific LOQ.

2.3. Behavioral assessment

We assessed the behavioral problems at 12 and 24 months using a subset of items from the Infant/Toddler Symptoms Checklist (ITSC): long version (De Gangi and Poisson, 2000), which mothers completed. The ITSC is a questionnaire that assesses self-regulation and aspects of temperament, and identifies any regulatory problems that may be arising, such as fussiness, going quickly from a whimper to a loud cry, and sleeping and eating difficulties in children aged 7–30 months. For the present study, we included questions on self-regulation, attention, sleep, eating or feeding, dressing-bathing-touch, and listening-language-sound subscales. For each item, the child is rated as “never” or “sometimes” fits the description (0); “fitted the description in the past” (1); or “fits the description most of the time” (2). The scores for each item are summed obtaining a total score, with higher score indicating worse behavior. In the present study, the ITSC at 12 months included a total of 28 items (mean score = 2.73; SD = 3.41;

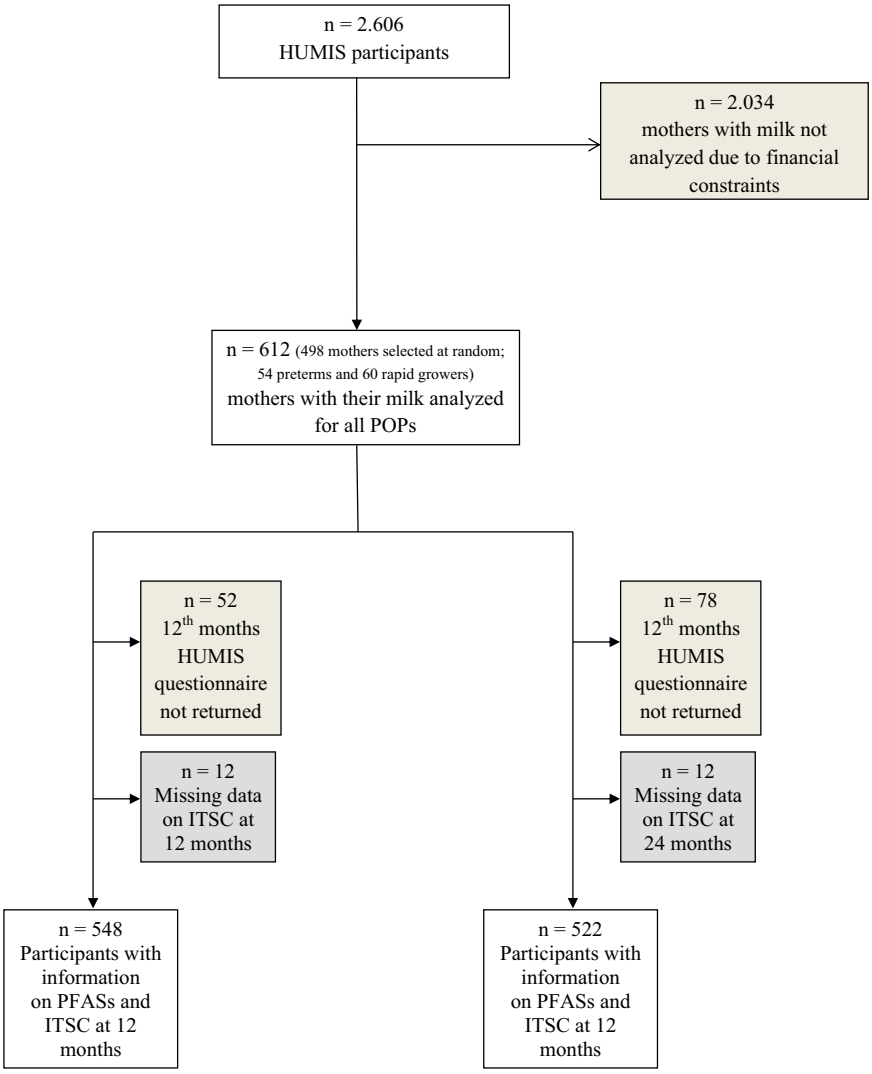

Fig. 1. Flowchart of participants in the study.

range=0 to 24), whereas the ITSC at 24 months included a total of 33 items (mean score=2.66; SD=2.67; range=0 to 22). The internal consistency in our study of ITSC at 12 and 24 months was adequate for research use (Cronbach alpha, 0.7) (Bland and Altman, 1997). In our study, the Spearman correlation between ITSC at 12 and at 24 months was 0.34 (p-value < 0.001).

2.4. Other variables

Information on child's sex, birth weight, gestational age and maternal smoking during pregnancy was obtained from the Medical Birth Registry of Norway (MBR) (Skjaerven et al., 2000). Maternal age at delivery was calculated based on birth date from the Norwegian personal identification number. Sociodemographic characteristics education, parity, interpregnancy interval, maternal smoking during pregnancy, duration of breastfeeding (exclusive and partial), maternal pre-pregnancy body mass index (BMI) and information on fish intake during pregnancy were obtained from questionnaires answered by the mother after delivery (median 6 weeks), or at 6, 12 or 24 months. Information on child's age at milk sample collection was collected by a separate form kept on the freezer during collection, as well as in the 6 months questionnaire. Finally, mothers' distress was measured by the Hopkins Symptom Checklist (SCL-5) in a questionnaire administered to mothers at 6 months.

3. Statistical analysis

3.1. Variable selection

First, we studied the determinants of behavioral problems at 12 and 24 months testing the bivariate association between outcomes and a set of covariates using t-test or ANOVA. Afterwards, we assessed the correlations (Spearman) between the selected 24 POPs: 13 PCB congeners (PCB 74, 99, 105, 114, 118, 138, 153, 156, 157, 170, 180, 189 and 194), 6 PBDE congeners (PBDE 28, 47, 99, 100, 153 and 154), *p,p′-DDT*, *p,p′-DDE*, HCB, β -HCH and oxyCD. Then, we performed principal component analysis (PCA), which is a dimensionality reduction method transforming correlated variables into a smaller number of uncorrelated principal components (Table 1). PCA components with an Eigenvalue > 1 were retained. We used the retained principal components in a regression analyses adjusting for covariates.

We then implemented two different variable selection techniques: elastic net (ENET) and Bayesian model averaging (BMA) (Table 1). ENET is a hybrid penalized regression method which combines a ridge and LASSO penalization that is robust to extreme correlations among the predictors (Friedman et al., 2010). It was proposed as a method for analyzing high dimensional data (i.e. analysis of highly correlated toxicants like POPs) which solved the instability of the lasso solution paths when predictors are highly

Table 1
Summary of the different statistical methods used in the present paper.

	Main purpose of this method	Strengths	Weaknesses	Why we used this method in the present study?
PCA	Variable selection based on explained variability	Useful in dimension reduction, minimal assumptions Principal components are independent	Lack of interpretability of components.	To identify groups of toxicants that explain variation in exposure
ENET	Penalized regression techniques have been used to ensure sparsity in obtained results under that assumption that among many predictors only few are causally related.	The elastic net procedure is especially valuable when the number of predictors (p) is much bigger than the number of observations (n). The elastic net supports grouping of variables, where strongly correlated predictors tend to be in (out) the model together	Method only considers linear associations and no interactions. May select groups of correlated variables as it does not handle collinearity Biased coefficients due to shrinkage estimation	To select toxicants most strongly related to the outcome.
BMA	Model selection based on posterior probabilities	Averages over entire model space consisting of all combination of variables Accounts for model uncertainty	Results may be complicated to interpret. Restricted to linear or logistic models	To select toxicants most strongly related to the outcome
Weighted multiple regression	Confirmatory analysis to elicit effects of selected variables	Well-known model for prediction Easy to interpret Inferential statistics are available	Sensitive to multicollinearity Less accurate for a non-linear relationship Sensitive to outliers	To quantify the association between toxicants selected by previous techniques

correlated (Zou and Hastie, 2005). A Lasso penalty (λ), which is the sum of the absolute values of the coefficients, shrinks the regression coefficients towards zero enforcing sparsity. Ridge regression penalizes the square of the regression coefficients, shrinking coefficients of correlated variables proportional towards zero, but never exactly to zero. Whereas lasso tends to select only one variable from a group of correlated variables, elastic net can select a group of collinear variables while still performing variable selection. A second tuning parameter (α) controls the balance between the lasso and ridge penalties; we optimized α and λ using cross-validation.

Next we performed BMA to deal with model uncertainty while selecting relevant variables (Sun et al., 2013). Compared to conventional variable selection methods which ignore model uncertainty, BMA has the advantage that it does not select for a single "best" model and instead makes inferences by averaging over a range of possible models. BMA-based confidence intervals are calibrated by taking account of both sampling variation within models and between-model uncertainty. The selection of models by BMA was done using the model with the highest posterior model probability. In addition, we also analyzed the results from posterior inclusion probability (PIP). PIP of a variable is the posterior probability that the variable should be included in a model averaged over all models, given the data (Hoeting et al., 1999).

3.2. Assessing the association between selected toxicant(s) and behavioral problems

After the variable selection process, we fitted weighted multiple linear regressions for behavioral problems at 12 and 24 months and the toxicants selected by ENET and BMA, and adjusting for selected confounders. We used weighted regression analysis to account for the oversampling of preterm and rapid grower infants. The main reasons for conducting a final weighted multivariate linear regression analysis were: 1) to estimate the joint effect of only the selected variables on the outcomes, while adjusting for covariates; 2) shrinkage based variable selection (as done in ENET) provides biased effect estimates and confidence intervals may be difficult to compute; and, 3) ordinary least squares parameters are easy to interpret (Lenters et al., 2015a). Regression analyses were conducted including the selected toxicant(s) both as continuous variables (as interquartile range (IQR) increases). We selected confounders based on a directed acyclic graph (DAG) representing associations reported in the existing literature: child's age at milk sample collection (days), fish consumption during pregnancy (servings per month), maternal age (years), maternal education ($\leq$ 12 years or less/ $>$ 12 years), duration of exclusive breastfeeding (weeks), duration of partial breastfeeding (weeks), parity (nulliparous/multiparous), pre-pregnancy BMI (continuous score), maternal smoking during pregnancy (never/former/current), preterm (yes/no) and child's sex (male/female). The minimally sufficient adjustment set was identified using DAGitty v2.0 (www.dagitty.net) (Supplemental Fig. S2). Multiple regression analyses were repeated including the selected toxicant(s) as categorical variables (in tertiles). In addition, we tested for potential effect modification by duration of exclusive breastfeeding (continuous), child's sex and maternal education ($\leq$ 12 years of education/ $>$ 12 years of education) including the product term between selected toxicants and effect modifiers, considering $p < 0.15$ as the cut-off for interaction. As sensitivity analyses, we repeated the main analysis restricting to subjects with milk sample collected within the first 30 days after birth. We also repeated the main analysis excluding preterm children. We repeated the main analysis adjusting by the method of milk collection (pump or hand). Finally, we also repeated the main analysis also adjusting for maternal distress at 6 months.

Statistical analyses and mathematical modeling were done using Stata 13 (Stata Corporation, College Station, Texas) and R (RCore Team, 2013). Results are per interquartile range (IQR) increase.

4. Results

Boys presented marginally more behavioral problems at 12 months than girls, but there were no differences at 24 months. Likewise, behavioral problems were higher among preterm than term children at 12 months, but not at 24 months. Children of younger mothers and less educated mothers had more behavioral problems at both 12 and 24 months (Table 2).

The univariate description of the 24 toxicants included in the analysis is presented in Table 3. The correlation matrix among the 24 different POPs included in the present study is presented in Supplemental Fig. S3. In general, we found statistical significant correlations between all the toxicants: the highest correlation was observed between organochlorine pesticides and different PCBs congeners and the lowest correlation between organochlorine pesticides and PBDEs.

4.1. Selection of variables

Results of PCA are presented in Supplemental Tables S4 and S5. Four Principal Components (PCs) were retained with an Eigenvalue > 1. Four PCs accounted for 80% of the total variance. PC1 is composed of PCBs, PC2 comprises all the PBDE congeners, PC3 is mainly composed of organochlorine pesticides and PC4 comprises HCB, PCB99 and PCB194. A positive association was observed between the PC3 component (organochlorine pesticides) and behavioral problems at 12 months (more behavioral problems) in the principal component regression analysis (β=0.50; 95% Confidence Interval (CI) [0.19; 0.81]) (Table 4). No associations were found between any of the four principal components and behavioral problems at 24 months.

Based on elastic net, *p,p′*-DDT, *p,p′*-DDE, HCB, PCB156, PBDE28 and PBDE47 were selected in the model with the minimal mean-squared error (MSE) as associated with behavioral problems at 12 months (see Fig. 2 for results and model performance details). However, the error curve was not conclusive because there is no unique tuning parameter that minimizes the validation of MSE. BMA selected the model including only *p,p′*-DDT, as the model with the highest posterior probability (Posterior model probability=0.66) (Fig. 3). The next model in terms of posterior model probability included only the intercept. Results from PIP confirmed that *p,p′*-DDT is the only variable related with behavioral problems at 12 months (PIP=0.749) (Supplementary Table S6).

The different variable selection methods used to select toxicants associated with behavioral problems at 24 months were not consistent (Supplemental Fig. S7a and S7b). ENET selected two PCB congeners (PCB170 and 194) while BMA selected the model including only the intercept as the model with the highest posterior model probability. Moreover, results from BMA also indicated that the variable with the highest PIP was PCB118, although the value was very low (PIP=0.042) (Supplementary Table S8). Such results suggest a lack of evidence for any of the toxicants being associated with the outcome at 24 months, although we did test both PCB170 and 194 in the final multivariate analysis.

4.1.1. Assessing the effect of selected toxicant(s) on behavioral problems

The ITSC at 12 months had a median score of 2 (IQR=4). The multiple linear regression analysis showed an increase of 0.62 points (95%CI [0.45, 0.79]) in the total score of ITSC at 12 months associated with an IQR (IQR=1.50 ng/g lipid adjusted) increase in *p,p′*-DDT (Table 5). We tested *p,p′*-DDT in tertiles and did not observe any exposure-response trend (*p*-trend=0.534) (Supplemental Table S9). The observed association between *p,p′*-DDT and behavioral problems at 12 months was modified by maternal education (*p*-value for interaction=0.098). The association was higher among the less educated mothers (β=0.59; 95%CI [0.38, 0.81]) than in the highest educated group (β=0.14; 95%CI [−0.05,

Table 2
Bivariate association between behavioral problems at 12 and 24 months, assessed by the infant toddler symptom checklist (ITSC), and covariates of interest, in Norwegian children.

	N	ITSC12			ITSC24		
		Mean	(SD)	*p*	Mean	(SD)	*p*
All	548	2.72	(3.28)	–	3.48	(2.67)	–
Sex							
Female	239	2.42	(3.06)	0.052	3.44	(2.45)	0.989
Male	308	2.96	(3.43)		3.51	(2.81)	
Preterm							
No	486	2.60	(3.20)	0.040	3.49	(2.66)	0.797
Yes	62	3.67	(3.75)		3.42	(2.76)	
Parity							
Nulliparous	212	2.80	(3.38)	0.896	3.69	(2.66)	0.186
Multiparous	336	2.67	(3.22)		3.35	(2.67)	
Maternal age							
< 25 years	72	3.36	(3.63)	0.081	4.52	(3.06)	0.004
25–35 years	386	2.55	(3.21)		3.31	(2.66)	
> 35 years	90	2.98	(3.27)		3.38	(2.19)	
Maternal education							
12 years or less	107	3.43	(4.02)	0.029	4.02	(3.07)	0.054
> 13 years	437	2.54	(3.06)		3.39	(2.58)	
Maternal distress at 6 months							
No	377	2.46	(3.28)	< 0.001	3.06	(2.34)	< 0.001
Mild distress	165	3.24	(3.48)		4.18	(2.80)	
High distress	12	6.09	(4.10)		6.17	(5.29)	
Maternal pre-pregnancy BMI							
Normal	336	2.70	(3.05)	0.929	3.50	(2.75)	0.979
Overweight	138	2.84	(3.44)		3.42	(2.62)	
Obese	55	2.87	(4.32)		3.44	(2.50)	
Maternal smoking							
No	333	2.62	(3.27)	0.311	3.30	(2.29)	0.203
Former	153	2.71	(3.13)		3.69	(3.40)	
Current	55	3.37	(3.68)		4.10	(2.75)	
Fish during pregnancy							
< 5 servings/month	173	2.98	(3.76)	0.247	3.61	(2.83)	0.586
> 5–9 servings/month	171	3.05	(3.38)		3.57	(2.84)	
> 9 servings/month	177	2.39	(2.76)		3.29	(2.40)	
Duration of exclusive breastfeeding							
0–1 month	55	2.71	(3.28)	0.662	3.85	(2.43)	0.408
> 1–3 months	69	3.06	(3.24)		3.49	(2.79)	
> 3 – 6 months	310	2.67	(3.32)		3.36	(2.53)	
> 6 months	114	2.68	(3.22)		3.66	(3.08)	
Duration of partial breastfeeding							
0–1 month	46	3.45	(4.00)	0.547	3.32	(2.23)	0.188
> 1–3 months	54	3.00	(3.23)		4.58	(3.99)	
> 3–6 months	150	2.74	(3.42)		3.55	(2.80)	
> 6 months	298	2.55	(3.09)		3.28	(2.31)	

(SD): Standard deviation. BMI: Body mass index.

Table 3
Univariate description of the POPs (ng/g lipid adjusted) analyzed in the present study.

	% > LOD	Mean	(SD)	Min	P10	P25	P50	P75	P90	Max
PBDE28	98	0.22	(0.37)	0.00	0.05	0.09	0.14	0.24	0.37	5.59
PBDE47	100	1.92	(4.70)	0.14	0.50	0.71	1.09	1.71	3.04	73.63
PBDE99	99	0.51	(1.47)	0.01	0.13	0.18	0.28	0.46	0.79	28.31
PBDE100	99	0.41	(0.85)	0.01	0.14	0.19	0.26	0.40	0.64	15.67
PBDE153	99	0.65	(0.79)	0.01	0.30	0.38	0.52	0.70	1.00	14.78
PBDE154	91	0.04	(0.08)	0.01	0.01	0.02	0.03	0.05	0.07	1.70
HCB	100	11.04	(6.48)	1.72	6.46	8.23	10.37	12.93	15.64	127.86
B-HCH	99	4.26	(5.45)	0.04	1.48	2.21	3.38	4.95	6.77	93.67
oxyCD	100	3.68	(2.19)	0.65	1.76	2.33	3.15	4.50	6.09	24.60
p,p′-DDT	100	2.55	(2.43)	0.69	1.10	1.44	2.02	2.94	4.16	35.16
p,p′-DDE	100	62.93	(51.09)	10.81	23.61	33.33	48.80	76.00	116.71	617.26
PCB74	100	3.73	(2.01)	0.91	1.91	2.44	3.37	4.47	5.89	22.00
PCB99	100	4.61	(2.26)	1.24	2.37	3.02	4.15	5.62	7.34	24.74
PCB105	100	1.52	(0.88)	0.41	0.75	0.98	1.37	1.82	2.40	12.72
PCB114	98	0.35	(0.10)	0.01	0.17	0.24	0.32	0.42	0.58	2.09
PCB118	100	7.06	(3.94)	1.66	3.71	4.69	6.41	8.43	10.63	62.23
PCB138	100	20.17	(10.40)	5.53	10.15	13.76	18.11	24.43	32.40	145.06
PCB153	100	35.46	(18.88)	8.75	18.6	24.57	31.71	42.24	55.39	296.03
PCB156	100	3.41	(1.89)	0.59	1.70	2.21	2.92	4.08	5.50	22.73
PCB157	99	0.65	(0.41)	0.01	0.31	0.41	0.55	0.80	1.10	4.87
PCB170	99	6.74	(4.16)	0.00	2.79	4.51	6.06	8.32	11.54	46.94
PCB180	100	18.25	(10.18)	4.33	9.51	12.08	15.93	21.33	29.59	142.46
PCB189	99	0.28	(0.17)	0.00	0.13	0.17	0.24	0.33	0.46	2.48
PCB194	99	1.46	(1.26)	0.00	0.68	0.89	1.26	1.69	2.44	22.72

LOD: Limit of Detection; (SD): Standard deviation.

Table 4
Principal component regression analysis with behavioral problems at 12 and 24 months assessed with the infant toddler symptom checklist (ITSC).

	PC1 (PCBs)			PC2 (PBDEs)			PC3 (Organochlorine Pesticides)			PC4 (HCB, PCB99 and PCB194)		
	Coef	95%CI		Coef	95%CI		Coef	95%CI		Coef	95%CI	
ITSC12	0.01	(−0.08,	0.09)	−0.03	(−0.15,	0.09)	0.50	(0.19,	0.81)	−0.07	(−0.26,	0.12)
ITSC24	−0.06	(−0.14,	0.01)	−0.02	(−0.14,	0.11)	0.08	(−0.09,	0.25)	−0.02	(−0.15,	0.11)

PC: Principal Component; Coef: Coeficient; 95%CI: 95% Confidence Interval.
PC1 (PCBs) is composed by the next PCB congeners: 114, 118, 138, 153, 156, 157, 180 and 189.
PC2 (PBDEs) is composed by the next PBDE congeners: 28, 47, 99, 100, 153 and 154.
PC3 (Organochlorine pesticides) is composed by: HCB, B-HCH, *p,p′*-DDE and *p,p′*-DDT.
PC4 (HCB, PCB99 and PCB194).
Models were adjusted for child's age at milk sample collection, maternal education, fish consumption during pregnancy, maternal age, duration of exclusive and partial breastfeeding, parity, maternal smoking during pregnancy, pre-pregnancy BMI, preterm and child's sex.

0.34]) (Table 5 and Supplementary Fig. S10). We detected no significant effect modification of the association between *p,p′*-DDT and behavioral problems at 12 months by child's sex (*p*-value for interaction=0.978) or duration of exclusive breastfeeding (*p*-value for interaction=0.408). Finally, when we included the six toxicants primarily selected by ENET, again, only *p,p′*-DDT increased the risk of behavioral problems at 12 months (β=0.35; 95%CI [0.08, 0.63]) (Supplemental Table S11).

The ITSC at 24 months had a median score of 3 (IQR=2). Although the selection methods did not suggest any clear toxicant associated with behavioral problems at 24 months, we performed multivariate linear regression analysis including the two variables more likely to be associated with this outcome: PCB170 and 194. There were no significant associations between exposure and behavioral problems at 24 months. Results were not modified by maternal education (Supplemental Table S12).

The negative association between *p,p′*-DDT and behavioral problems at 12 months remained when we restricted the main analysis to only subjects with milk sample collected within the first 30 days after birth (β=0.64; 95%CI [0.56, 0.72]). When we excluded preterm children, the same toxicants were selected, and the results of the multiple linear regression were identical (β=0.62; 95%CI [0.46, 0.89]). Results of the main analysis did not change after additional adjustment for method of milk collection or laboratory nor after adjustment for maternal distress (data not shown).

5. Discussion

This is the first study to evaluate the association between a mixture of POPs and early behavioral problems using classical methods such as PCA as well as modern ones such as ENET and BMA that deal appropriately with correlated exposure data, and we identified DDT as a risk factor for child behavioral problems. In the HUMIS cohort, and in general in Norway, environmental concentrations of POPs are low compared to other countries, although the long duration of breastfeeding increases overall infant exposure (Iszatt et al., 2015). PCA revealed that the principal component composed of organochlorine pesticides increased behavioral problems at 12 months but not at 24 months. *p,p′*-DDT was consistently selected by the different variable selection methods as associated with behavioral problems at 12 months. The final adjusted multivariate analysis further confirmed that postnatal exposure to *p,p′*-DDT was significantly associated with increased behavioral problems at 12 months. Moreover, maternal education had a modifying effect on the association between *p,p′*-DDT and behavioral problems at 12 months, with stronger

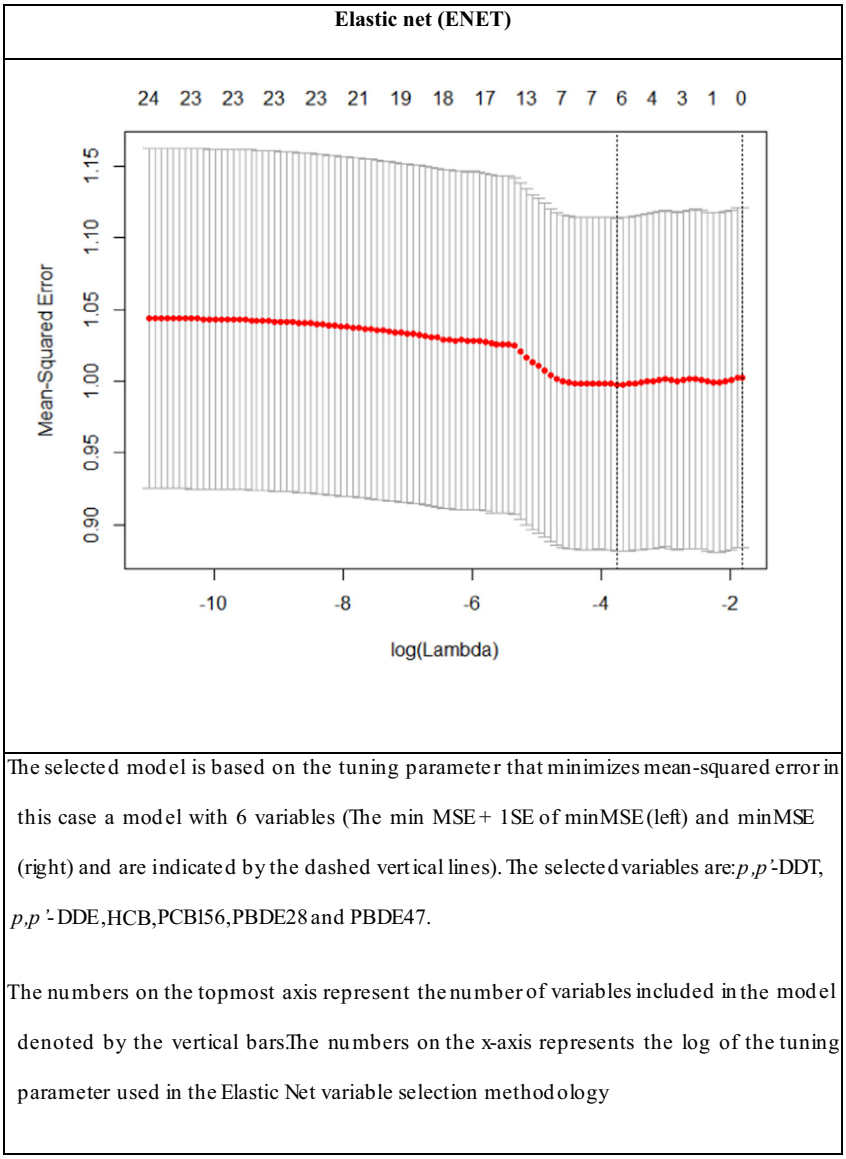

Fig. 2. Main results from selection methods ENET of toxicants associated with behavioral problems at 12 months assessed by the Infant Toddler Symptom Checklist (ITSC).

associations found in less educated mothers. At 24 months, we observed no clear associations between the 24 toxicants studied in the present study and behavioral problems.

As discussed in a recent study (Agay-Shay et al., 2015), PCA is a useful method to reduce dimensionality in correlated exposure matrix as well as to test the effect of exposure to mixtures of toxicants. In our study, we observed that three first components are composed of the three main group of POPs included in the present paper (PCBs, PBDEs and organochlorine pesticides). The inclusion of these factors in a regression analysis allowed us to evaluate the effect of these groups of POPs as mixtures. However, PC regression coefficients have no direct interpretation and the method cannot consider additive or interactive (synergistic or antagonistic) effects, particularly for exposures that are part of the same component. In addition to PCA, we used elastic net (ENET) which includes both the LASSO and ridge regression, to identify POPs potentially related to our outcomes (Lenters et al., 2015a; Zou and Hastie, 2005). ENET overcomes the limitations of LASSO, allowing for both high-dimensionality and highly correlated variables in the model. ENET permits a grouping effect during variable selection, such that a group of highly correlated variables tend to have coefficients of similar magnitude. This allows for the selection of groups of correlated features when groups are not known in advance. ENET is particularly valuable when the number of predictors is much higher than the number of observations ($p \gg n$). However, ENET has some weaknesses such as only considering linear associations and providing biased coefficients due to shrinkage estimation. Finally, we also used BMA to select toxicants related to early behavioral problems. BMA is a Bayesian model selection method based on posterior inclusion probabilities of explanatory variables in a linear model (Hoeting et al., 1999; Sun et al., 2013). BMA draws its strength from averaging over the entire model space consisting of all combinations of variables and also accounts for model uncertainty. Among the main limitations of BMA, we found that results may be difficult to interpret (based on posterior model probabilities) and that BMA is restricted to linear or logistic models. A further limitation of the three methods used (PCA, ENET and BMA) is that they do not explicitly allow for the evaluation of interactions between chemicals.

The present study is the first to find a negative association between *p,p'*-DDT concentrations in human milk and early child behavior, in the domain of self-regulation. We do not know however, what the critical window for exposure is, since breast milk concentrations are highly correlated with maternal

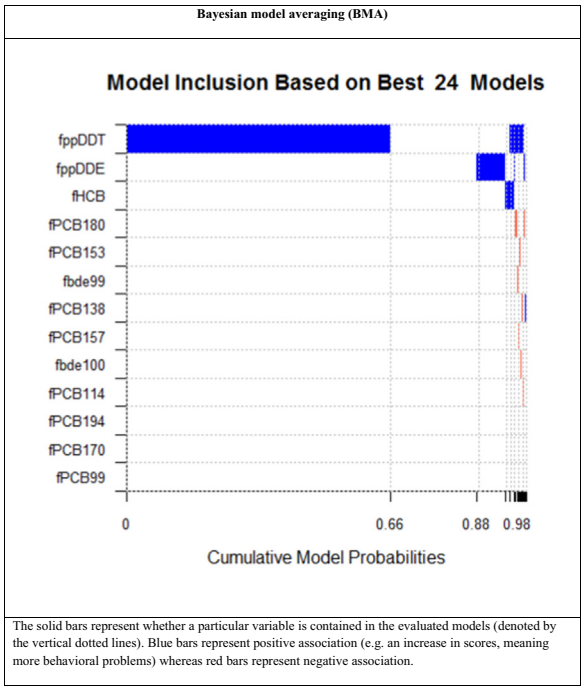

Fig. 3. Main results from selection methods BMA of toxicants associated with behavioral problems at 12 months assessed by the Infant Toddler Symptom Checklist (ITSC).

Table 5
Weighted multiple linear regression model with behavioral problems at 12 months as the outcome (assessed by the infant toddler symptom checklist (ITSC)) with inclusion of the specific POPs that were selected by elastic net and Bayesian model averaging.

	Unadjusted		Adjusted[a]	
	Coef	95%CI	Coef	95%CI
p,p′-DDT (per IQR increase)	0.63	(0.46, 0.81)	0.62	(0.45, 0.79)
Effects of an IQR increase in *p,p′*-DDT at different levels of maternal education				
12 or less years of education	0.63	(0.45, 0.81)	0.59	(0.38, 0.81)
> 12 years of education	0.10	(−0.06, 0.28)	0.14	(−0.05, 0.34)
p-value for interaction		*0.112*		*0.098*

Coef: Coeficient; 95%CI: 95% Confidence Interval; IQR: Interquartile range.
IQR increase in *p,p′*-DDT = 1.50 ng/g lipid adjusted.
[a] Adjusted for child's age at milk sample collection, maternal education, fish consumption during pregnancy, maternal age, duration of exclusive and partial breastfeeding, parity, maternal smoking during pregnancy, pre-pregnancy BMI, preterm and child's sex.

concentrations (Wang et al., 2004). Further studies need to address this question. Our results revealed an increase of 0.62 points in the total score of ITSC at 12 months (median in our study sample: 2 points) associated with an IQR increase in *p,p′*-DDT in our population (*p,p′*-DDT-IQR = 1.50 ng/g lipid adjusted). Such an increase may have public health consequences since self-regulation problems have been associated with adverse outcomes later in life, such as school achievement, attention-deficit and hyperactivity disorder (ADHD) and obesity (Choudhry et al., 2013; Sawyer et al., 2014). Although DDT is considered a developmental neurotoxicant (Grandjean and Landrigan, 2014), only three studies have tested its effects on human neuropsychological development (Eskenazi et al., 2006; Jusko et al., 2012; Ribas-Fitó et al., 2006) with most focusing on its metabolite DDE. Moreover, due to the strong correlation between *p,p′*-DDT and other POPs, previous studies investigating these compounds (and not *p,p′*-DDT) could theoretically attribute effects to these other compounds that are actually due to DDT exposure. This highlights the importance of studying substances that are highly correlated simultaneously in mixture models.

Experimental studies show that *p,p′*-DDT binds to and activates estrogen receptors (ERs) in both reproductive and other tissues including the brain of adult rodents (Mussi et al., 2005; Zhuang et al., 2012). However, *p,p′*-DDT and metabolites have much lower estrogenic potency than the endogenous hormone, 17β-estradiol (Chen et al., 1997; Lee et al., 2013) and may not affect endocrine functions at the background levels detected in humans. The occurrence of endocrine disruptors in the central nervous system (CNS) of developing individuals (fetus, children) and adults indicate that estrogenic signaling is central in the development and functioning of CNS (Schaub and Wood, 2009). Experimental data shows that DDT has the potential to cause abnormal sexual behavior (Mussi et al., 2005) and induce changes in aggression and learning abilities (Eriksson et al., 1992). Furthermore, DDT exposure during lactation induced estrogenic activity in the mice brain but not in the liver, implying that the brain is specifically sensitive to DDT exposure (Mussi et al., 2005).

Interestingly, the negative effect of *p,p′*-DDT on early behavioral problems in our study was markedly higher among children of lesser educated mothers. We speculate that better parenting qualities in higher educated mothers may compensate for minor deficits in their children. Indeed, maternal educational level, income and occupation have been found to be positively associated with better parenting (Santos et al., 2008). Stimulating materials and experiences mediate the relationship between socioeconomic status and family income and children's intellectual and academic achievement, from infancy to adolescence (Bradley et al., 2001; Emmett and Jones, 2014; Francis et al., 2002; Rosales et al., 2009). In addition, mothers with high educational level tend to avoid negative disciplinary practices, which is associated with less behavioral problems during childhood, particularly externalizing problems (Bøe et al., 2014). In contrast lower educated women may have fewer resources (i.e. less stimulating materials and experiences), to handle “difficult” children (i.e. such as with the traits we see associated with *p,p′*-DDT), which could aggravate symptoms.

There are several possible explanations for the lack of consistency between the results at 12 and at 24 months. Firstly, there could be other factors modifying the effect, apart from maternal education, that we have not taken into account but which could reduce our ability to detect an effect. Secondly, the differences observed could be explained by the questionnaire used to assess behavioral problems (the ITSC). Results from the bivariate analysis suggest that ITSC at 12 months is a more sensitive instrument in detecting abnormalities than ITSC at 24 months. For instance differences between preterm and term babies and between males and females were detected at 12 but not at 24 months. Although, the internal consistency of ITSC at 12 and 24 months was previously found to be adequate for research use (Bland and Altman, 1997), in the present study the Spearman correlation between the two measures was not high (Spearman Rho = 0.34). This suggests that our questionnaire does not measure the same trait consistently over time, which also could explain the lack of agreement over time. Finally, the lack of association at 24 months could be due to postnatal exposure misclassification. We used concentrations in breast milk, which is assumed to be a good proxy of

postnatal exposure but which does not capture exact postnatal exposure profiles, which differs depending on growth and breastfeeding practices (Verner et al., 2013). This misclassification might bias our results toward the null and this could be particularly important at 24 months because of the longer time between exposure and outcome. Although we adjusted the multivariate models by duration of exclusive and partial breastfeeding to account for this, this would not totally eliminate this potential bias. In addition, we repeated our main analysis restricted to subjects with milk samples collected within the first 30 days after birth to avoid substantial changes in concentrations of lipophilic compounds in milk and this did not alter our results.

The present study is affected by a number of limitations. One of the main limitations is that we indirectly assessed behavior development by a parent-completed questionnaire. This may induce misclassification in our study, nevertheless it is very unlikely that it is differential misclassification because the probability of being misclassified in rating higher (or lower) behavioral problems is independent of exposure status (which is unknown to the mothers). The absence of a complete neuropsychological development battery administered by an expert neuropsychologist might also cause misclassification of our outcome of interest. The oversampling of preterm children included in this study might have generated a selection bias as this group has increased risk of neuropsychological problems. However, their influence on our findings was immaterial: adjusting for preterm in the main analysis or restricting to term babies did not alter our results. In addition, we did not collect information on some potentially important confounders such as maternal intelligence and home environment (Bradley et al., 2001; Deater-Deckard et al., 2009; Price et al., 2013), although inclusion of psychosocial covariates such as mother's age, education and mental health partly controls for this.

6. Conclusion

In this prospective cohort study, we analyzed the association between a large group of POPs and behavioral problems during the first two years of life using statistical methods novel in this setting, such as PCA, ENET and BMA. Within a mixture of toxicants, the variable selection methods used in this paper consistently selected *p,p′*-DDT as the single toxicant most related to behavioral problems at 12 months. There were no consistent relations between toxicants and behavioral problems at 24 months. More effort to understand the complex relations between multiple pollutants and neuropsychological development is warranted.

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Competing financial interests

The authors declare they have no actual or potential competing financial interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2016.07.014.

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