Early life exposure to endocrine disrupting chemicals and child health

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2014

document version
Publisher's PDF, also known as Version of record

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Prenatal exposure to endocrine disrupting chemicals and birth weight – a prospective cohort study

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Submitted to Chemosphere

Abstract

Background: Prenatal exposure to endocrine disrupting chemicals may affect fetal development through disruption of hormonal actions and epigenetic modifications, potentially predisposing individuals to later on-set health risks, such as obesity.

Objective: The objective of this study was to determine associations between biological exposure markers of various endocrine disrupting chemicals and birth weight in a newly established, prospective mother-child cohort in the Netherlands.

Methods: Birth weight was obtained from birth records, and exposure to dichlorodiphenyldichloroethylene (DDE), three di-2-ethylhexyl phthalate (DEHP) metabolites, polychlorinated biphenyl-153, perfluorooctanesulfonic acid (PFOS), and perfluorooctanoic acid was determined in cord plasma or breast milk. Linear regression analysis was used to determine associations between compounds and birth weight, which were stratified for gender and adjusted for a priori defined covariates.

Results: Increased exposure to DDE was to some extent associated with higher birth weight in girls (≥107.50 ng/L, +424.3 grams, 95% CI 24.29 to 824.40). For PFOS a higher birth weight was observed in girls in the highest exposure quartile. For most of the DEHP metabolites, lower birth weights were observed in girls in higher exposure quartiles, though mostly not significant.

Conclusion: It can be concluded that prenatal exposure to DDE, PFOS, and mono(2-ethyl-5-oxohexyl)phthalate was associated with changes in birth weight in this population. Associations were gender specific, and appeared to be non-monotonic. Since the population was relatively small, results should be interpreted with caution.

Introduction

Childhood obesity is a major worldwide problem. An imbalance between energy intake and expenditure is generally regarded to underpin the problem of weight gain, but fails to explain individual susceptibility to obesity (1). Every human is a unique product of genes and environment, resulting also in variation in physical processes involved in weight gain (2). The foetus may be especially vulnerable to environmental factors, and therefore the prenatal period in particular may determine health later in life. This is illustrated by the consequences of prenatal tobacco or alcohol exposure. Maternal smoking may result in low birth weight and increased risk for obesity and metabolic syndrome later in life, while prenatal alcohol exposure may cause growth restriction, central nervous system impairment, and distinct dysmorphic facial features (3-5).

Next to prenatal tobacco and alcohol exposure, exposure to Endocrine Disrupting Chemicals (EDCs) may also affect fetal development through interference with hormonal balance and epigenetic modifications. Birth weight is an interesting outcome to consider in this context as both high and low birth weights have been associated with increased body mass index (BMI) in children (6, 7). Prenatal polychlorinated biphenyl (PCB) exposure has been associated with reduced birth weight, as demonstrated in a recent meta-analysis of multiple European birth cohorts (8, 9). For other EDCs such as dichlorodiphenyldichloroethylene (DDE) and hexachlorobenzene (HCB), reported associations with birth weight are less consistent than for PCBs. Some studies find an effect of DDE or HCB exposure on birth weight (10-12), while several other studies show no effect (8, 9, 13, 14).

A mother-child cohort was recruited in the Netherlands, as part of the OBELIX project (OBesogenic Endocrine disrupting chemicals: Linking prenatal eXposure to the development of obesity later in life) (15). The aim of the present study was to determine associations between biological markers of prenatal EDC exposure and birth weight. A broad range of EDCs were selected, including non-dioxin-like PCB-153 and DDE, as well as perfluorinated alkyl acids (PFAAs), and several di-2-ethylhexyl phthalate (DEHP) metabolites, which have been identified as potential health threats as well, but have not yet been investigated as thoroughly in prospective studies.

Methods

Subjects

Recruitment started in January 2011 in the area of Zwolle in the Netherlands, and was finished in January 2013. Six midwifery clinics participated in the recruitment of pregnant women, who were invited to participate during the first antenatal visit to the midwife. Women were considered eligible for participation if they were able to fill out Dutch questionnaires. Twin pregnancies and major congenital anomalies were reasons for

exclusion, but no subject was excluded because of these criteria. Signed informed consent was obtained from every participant. The study was approved by the medical ethics committee of the VU University Medical Centre.

Birth weight

Birth weight was measured by a midwife or a nurse and was obtained from registries of the midwives. Newborns were put on the weighing scale without a diaper and weight was determined when the infant was in a calm state. Weighing scales were provided by the midwives and were calibrated daily.

Chemical exposure

Umbilical cord blood was collected immediately after birth when the health of mother and child was ascertained. The blood was delivered to the lab within twelve hours by a courier in case of home delivery or by hospital staff in case of delivery at the hospital. At the lab, cord blood was centrifuged for 10 minutes at 2000g. after which the plasma layer was transferred to plasma tubes. Plasma was stored at -80°C.

Breast milk was collected in polypropylene bottles during the second month after birth (mean [SD] weeks after birth: 6.3 [2.5]). Materials were checked for contamination problems by means of blanks. In total a minimum of 100 mL. was collected, spread over five to ten days to minimize the burden to the mothers in case of low milk flow. Mothers were instructed to note the dates on which they collected a sample and to store the milk in the freezer in between sampling days. They were allowed to use a breast pump for collection.

Compounds were analysed in cord plasma. For DDE we also analysed breast milk samples from mothers for whom an insufficient amount of cord blood was available. perfluorooctanesulfonic acid (PFOS), and perfluorooctanoic acid (PFOA) were analysed by applying isotope dilution and large volume injection using an on-line trapping column coupled to liquid chromatography and triple quadropole mass spectrometry. The breast milk samples were extracted with solid phase extraction using Oasis WAX cartridges. For the cord plasma samples, the proteins were precipitated by adding methanol and centrifuging the mixture prior to injection onto the analytical system. After drying both the cord plasma and the breast milk samples with Kieselguhr, the organochlorine pesticides HCB and p,p'-DDE, and PCB-153 were extracted with a mixture of dichloromethane and hexane. Cleanup of the extracts was done using sulphuric acid silica columns and for the analysis gas chromatography with mass spectrometric detection in negative chemical ionization mode was used. For the analyses of the DEHP metabolites, a deconjugation step using deconjugation enzymes was carried out. After addition the internal standards used for isotope dilution, the breast milk samples were extracted using Oasis MAX cartridges, while for the cord plasma samples a simple protein precipitation step using formic acid was applied. The extracts were analyzed after large volume injection using an on-line trapping column coupled to liquid chromatography and triple quadropole mass spectrometry.

Problems due to the occurrence of contamination of the breast milk samples are to be expected for mono(2-ethylhexyl)phthalate (MEHP), the hydrolytic monoester of DEHP, that has shown to also be formed in the matrix due to the residual activity of enzymes (lipases, esterases), even after prolonged storage periods at -20°C. Therefore, MEHP is a very unreliable parameter for the assessment of DEHP exposure and should not be used for breast milk. In contrast, mono(2-ethyl-5-carboxypentyl)phthalate (MECPP), mono(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP), and mono(2-ethyl-5-oxohexyl)phthalate (MEOHP) are not susceptible to contamination as they are major metabolites of DEHP created by the liver. They are only formed in vivo and serve as a reliable parameter for DEHP exposure.

The coefficient of variation for the chemicals measured, was 16 – 17%. More information on chemical analysis, including limits of quantification and quality control parameters, is given in the Supplemental Material.

Covariates

Covariates were selected based on literature (16-20). Weight and length of both parents were measured by the midwife at inclusion, approximately 10-12 weeks in pregnancy. Measurement of maternal weight was repeated at 36 weeks of pregnancy to determine gestational weight gain (GWG). Midwives received a measuring tape as well as strict instructions on how to perform these measurements. Weighing scales were provided by the midwife and were calibrated daily. Gestational age was determined by means of ultrasound.

Questionnaires were administered at inclusion and the start of the second trimester to collect information on education (having a bachelor or master degree, yes or no), birth date of the mother, parity, and maternal smoking (yes or no) and alcohol intake during the first trimester (drinks per week). At inclusion, fish intake (grams per day) was determined by food frequency questionnaire which included both frequencies and portion sizes (21). One month after birth another questionnaire was administered to inquire on folic acid intake during pregnancy (yes or no).

Data-analysis

Data analysis was performed using SPSS version 20 (22). For each compound separate linear regression models were composed which were stratified for gender. Exposure values below the limit of quantification (LOQ) were replaced by LOQ/√2 (9). As DDE accumulates in lipid, a conversion factor based on what has been published for other European cohorts was used to transform levels in breast milk to levels in cord blood (9).

[DDE, cord plasma (ng/L)] = 1.20 X [DDE, breast milk (ng/g lipid)]

For PCB-153 in cord plasma, over 50% of the samples was <LOQ. As PCB-153 was determined predominantly in cord blood, it was included as a dichotomous variable (>LOQ vs. <LOQ). None of the compounds had a linear association with birth weight, therefore each exposure (other than PCB-153) was split up in quartiles and included as dummy

variables in the model. Models were adjusted for various a priori defined covariates, which initially included gestational age, maternal BMI, height, and age at birth, GWG, and parity. The fully adjusted models also included smoking, alcohol intake, paternal BMI and height, fish and folic acid intake. Each of these covariates was checked for linearity with birth weight and was included in the models regardless of the degree of confounding. As GWG is mostly seen as a confounder for lipophilic compounds, we also performed sensitivity analysis for this variable (23). For each model the residuals were plotted and checked for normal distribution. Effect modification by smoking was taken into consideration and was included if the interaction term was significant ($p<0.05$).

Results

In total 148 women participated in this study. Fourteen subjects dropped-out before delivery due to lack of time, and for 43 subjects no exposure data was available due to insufficient volumes of sample, resulting in the inclusion of 91 mother-child pairs for analysis (table 5.1). Overall, 64% of the infants were male and the mean birth weight was 3607 grams.

Table 5.1 Population characteristics (n=91)

	Overall	**Boys**	**Girls**
Gender (boy, %)	64	-	-
Birth weight (g)	3607.4 (455.9)	3639.8 (495.2)	3584.5 (401.7)
Gestational age (weeks)	39.8 (1.3)	39.7 (1.5)	40.1 (1.0)
Parity (nulliparous, %)	39	43	32
Caesarean (yes, %)	3	4	3
BMI mother (start pregnancy, kg/m^2)	23.3 (3.7)	23.6 (3.4)	23.2 (4.2)
BMI father (kg/m^2)	24.4 (2.9)	24.0 (3.0)	24.9 (2.8)
Gestational weight gain (kg.)	12.4 (4.5)	12.7 (4.2)	11.9 (5.3)
Age mother (years)	30.9 (4.5)	30.3 (3.7)	32.0 (3.5)
Age father (years)	33.2 (5.3)	32.4 (5.6)	34.4 (4.3)
Education mother (bachelor/master, %)	68	72	53
Smoking (first trimester, yes, %)	4	4	7
Alcohol (first trimester, yes, %)	7	6	7
Folic acid use (yes, %)	94	92	97
Fish intake (g/day)	21.6 (19.0)	23.6 (17.4)	25.6 (19.6)

Values are mean (SD) unless stated otherwise.

For DDE, and HCB, average wet weight levels in breast milk were significantly higher than in cord plasma (table 5.2). However, lipid adjusted levels were similar, reflecting the high fat content of the breast milk. For these compounds the percentage of samples with exposure levels >LOQ was also higher in breast milk than in cord blood. HCB concentrations in cord blood were <LOQ in more than 98% of the samples, and were therefore not included in analysis. All DEHP metabolites as well as the PFAAs had

relatively high quantification rates. In contrast to DDE, and HCB, levels of PFOS and PFOA were higher in cord plasma compared to breast milk.

Table 5.2 Exposure levels in cord plasma and/or breast milk

Compound		n	Mean	Median	Range	LOQ	<LOQ (%)
PCB-153							
Cord plasma	- ng/L	54	35.81	29.14	22.63 – 96.00	21 – 43	56
	- ng/g lipid	54	36.16	30.17	17.95 – 88.89	14 – 53	56
DDE							
Cord plasma	- ng/L	55	113.86	87.00	28.28 – 470.00	33 – 73	24
	- ng/g lipid	55	114.71	83.33	28.83 – 580.25	23 – 86	24
Breast milk	- ng/L	26	2381.15	1950.00	400 – 11390.00	9.20 – 13.00	0
	- ng/g lipid	26	59.68	43.98	12.11 – 277.80	0.13 – 0.53	0
Total [a]	- ng/L	81	100.30	75.00	14.53 – 470.00		16
HCB							
Cord plasma	- ng/L	54	44.03	44.55	28.28 – 78.00	40 – 79	98
	- ng/g lipid	54	45.96	46.13	17.68 – 82.11	25 – 96	98
Breast milk	- ng/L	26	611.54	605.00	300.00 – 1060.00	9.20 – 13.00	0
	- ng/g lipid	26	16.08	14.95	10.36 – 26.88	0.16 – 0.68	0
MECPP							
Cord plasma	- ng/mL	67	0.31	0.26	0.11 – 1.00	0.13 – 0.28	8
MEHHP							
Cord plasma	- ng/mL	67	0.33	0.27	0.10 – 1.00	0.14 – 0.27	9
MEOHP							
Cord plasma	- ng/mL	67	0.29	0.23	0.12 – 0.87	0.17 – 0.33	22
PFOA							
Cord plasma	- ng/L	67	934.03	870.00	200 – 2700	50 – 140	0
PFOS							
Cord plasma	- ng/L	67	1601.2	1600.0	570 – 3200	44 – 140	0

[a] For total DDE, cord plasma exposure data were merged with breast milk exposure levels converted to cord plasma levels.

Results for PCB-153 are presented in table 5.3. For the other compounds associations with birth weight are given in table 5.4, and 5.5, for respectively boys and girls (for results of the overall group, see Supplemental Material, Table S5.2). Effects were predominantly observed in girls. A lower birth weight was observed for girls with PCB-153 levels >LOQ when models were partially adjusted (-479.0 grams, 95% CI: -854.31 to -103.59). For DDE exposure on the other hand, a higher birth weight was observed in those highest exposed to DDE when models were unadjusted (+548.6 grams, 95% CI: 0.70 to 1096.44) and partially adjusted (+424.3 grams, 95% CI: 24.29 to 824.40). No differences were observed for the other quartiles.

For MECPP, a lower birth weight was observed in Q2 (-267.3 grams, 95% CI -534.58 to -18.03) and Q4 (-373.8 grams, 95% CI -677.23 to -70.42), in the partially adjusted model for the overall group. After further adjustment, these differences were attenuated and no longer statistically significant. When stratified for gender, no effects were observed for MECPP. MEOHP exposure in the overall group and in girls, differences between Q3 and

Q1 were statistically significant in the partially adjusted model (overall: -361.0 grams, 95% CI: -673.63 to -48.28; girls: -721.9 grams, 95% CI: -1204.17 to -239.70), but not in the fully adjusted model. In boys no difference in birth weight across the quartiles of MEOHP exposure was observed. For MEHHP exposure no effect on birth weight was observed in either groups.

For PFOS a higher birth weight was observed for girls in the highest exposure quartile (+595.9 grams, 95% CI 88.77 to 1103.04) in the fully adjusted model. Results were similar for girls (Q4: +557.9 grams, 95% CI 56.65 to 1059.09). Boys in the second quartile of PFOA exposure had a lower birth weight (-602.7 grams, 95% CI: -995.63 to -209.71), however this was attenuated and insignificant after adjustment for covariates.

Removing GWG from the models mostly affected results for boys (results not shown). For PFOA, Q2 also showed a lower birth weight with the partially adjusted model (-768.9 grams, 95% CI -1348.32 to -189.48). Also results for DDE were affected, however in an incosistent manner, with a lower birth weight for Q4 when the model was partially adjusted, and a higher birth weight for those in Q2 when the model was fully adjusted. In the overall population, and in girls, removing GWG from the models for phthalates attenuated results, which became insignificant.

Table 5.3 Regression coefficients for PCB-153 (<LOQ vs. >LOQ) and birth weight (grams), stratified for gender

		n	<LOQ	>LOQ		
				β (95% CI)		p-value
Overall	Crude	55	Ref.	1.3	(-253.38 – 255.88)	0.992
	Model A[1]	42	Ref.	-113.2	(-373.25 – 146.88)	0.381
	Model B[2]	37	Ref.	-102.6	(-484.09 – 278.88)	0.575
Male	Crude	52	Ref.	105.8	(-218.17, 429.84)	0.514
	Model A[1]	42	Ref.	99.5	(-196.23, 395.17)	0.496
	Model B[2]	37	Ref.	74.1	(-340.94, 489.14)	0.706
Female	Crude	52	Ref.	-323.5	(-790.97, 143.97)	0.171
	Model A[1]	42	Ref.	-479.0	(-854.31, -103.59)	0.014
	Model B[2]	37	Ref.	-472.2	(-1158.96, 214.51)	0.161

[1] Adjusted for gestational age, maternal BMI, maternal height, maternal age at birth, gestational weight gain, and parity.
[2] Model A + additionally adjusted for smoking, alcohol intake, paternal BMI, paternal height, fish intake, and folic acid intake.

Table 5.3 Regression coefficients for various exposures (ng/L) in boys (in quartiles) and birth weight (grams)

Compound	n	Q1 (ng/L)	Q2 (ng/L) β (95% CI)	p-value	Q3 (ng/L) β (95% CI)	p-value	Q4 (ng/L) β (95% CI)	p-value
DDE		**<41.80**	**41.80 – 74.50**		**74.51 – 107.50**		**≥107.51**	
Crude	70	Ref	90.0 (-309.76, 489.76)	0.655	130.0 (-246.99, 506.99)	0.494	-240.7 (-594.36, 112.94)	0.179
Model A[1]	65	Ref	92.7 (-256.10, 441.59)	0.595	116.2 (-184.79, 417.19)	0.441	-190.6 (-482.54, 101.36)	0.195
Model B[2]	61	Ref	379.3 (-83.98, 842.65)	0.105	206.8 (-158.47, 572.15)	0.255	-196.9 (-569.73, 175.95)	0.288
MECPP		**<0.22**	**0.22 – 0.27**		**0.28 – 0.38**		**≥0.39**	
Crude	63	Ref	-1.5 (-405.48, 402.39)	0.994	76.6 (-327.3, 480.57)	0.705	-152.0 (-565.44, 261.44)	0.465
Model A[1]	57	Ref	-170.1 (-522.56, 182.41)	0.333	45.5 (-334.28, 425.27)	0.809	-330.2 (-738.31, 77.87)	0.109
Model B[2]	52	Ref	-175.4 (-700.98, 350.26)	0.492	127.2 (-353.85, 608.17)	0.585	-189.1 (-784.02, 405.75)	0.513
MEHHP		**<0.18**	**0.18 – 0.27**		**0.28 – 0.39**		**≥0.40**	
Crude	63	Ref	-105.8 (-522.47, 310.97)	0.613	-89.3 (-551.82, 373.21)	0.700	-110.8 (-562.25, 340.75)	0.625
Model A[1]	57	Ref	159.6 (-246.38, 565.57)	0.430	124.9 (-367.05, 616.92)	0.609	102.6 (-313.96, 519.16)	0.620
Model B[2]	52	Ref	244.3 (-294.08, 782.59)	0.353	419.8 (-299.95, 1139.52)	0.236	224.3 (-294.48, 743.09)	0.376
MEOHP		**<0.17**	**0.17 – 0.23**		**0.24 – 0.41**		**≥0.42**	
Crude	63	Ref	168.8 (-246.52, 584.02)	0.419	258.8 (-156.52, 674.02)	0.217	50.8 (-380.81, 482.31)	0.815
Model A[1]	57	Ref	-34.5 (-422.56, 353.52)	0.857	-93.9 (-510.19, 322.43)	0.649	-33.7 (-434.72, 367.30)	0.865
Model B[2]	52	Ref	-27.0 (-592.50, 538.46)	0.921	-104.5 (-712.36, 503.34)	0.722	74.0 (-412.14, 560.13)	0.753
PFOS		**<996**	**996 – 1600**		**1601 – 2000**		**≥2001**	
Crude	62	Ref	-200.2 (-649.36, 248.88)	0.376	173.3 (-286.88, 633.55)	0.454	67.3 (-407.97, 542.64)	0.778
Model A[1]	57	Ref	-140.5 (-616.45, 335.37)	0.552	157.4 (-363.06, 677.92)	0.543	-67.7 (-595.16, 459.73)	0.796
Model B[2]	52	Ref	246.6 (-394.52, 887.64)	0.430	401.9 (-205.90, 1009.66)	0.182	382.8 (-254.19, 1019.84)	0.223
PFOA		**<591**	**591 – 870**		**871 – 1150**		**≥1511**	
Crude	62	Ref	-602.7 (-995.63, -209.71)	0.003	-103.3 (-476.96, 270.41)	0.582	-207.7 (-573.86, 158.53)	0.261
Model A[1]	57	Ref	-115.1 (-713.98, 483.82)	0.698	107.1 (-330.01, 544.29)	0.621	122.5 (-328.19, 573.11)	0.584
Model B[2]	52	Ref	-282.4 (-1132.49, 567.63)	0.494	-24.8 (-668.78, 619.22)	0.936	-34.6 (-681.17, 612.05)	0.912

[1] Adjusted for gestational age, maternal BMI, maternal height, maternal age at birth, GWG, and parity.

[2] Model A + additionally adjusted for smoking, alcohol intake, paternal BMI, paternal height, education, fish intake, and folic acid intake.

* $p < 0.050$

Table 5.4 Regression coefficients for various exposures (ng/L) in girls (in quartiles) and birth weight (grams)

Compound	n	Q1 (ng/L)	Q2 (ng/L) β (95% CI)	p-value	Q3 (ng/L) β (95% CI)	p-value	Q4 (ng/L) β (95% CI)	p-value
DDE		**<41.80**	**41.80 – 74.50**		**74.51 – 107.50**		**≥107.51**	
Crude	70	Ref	156.7 (-327.56, 640.95)	0.521	308.6 (-191.56, 808.71)	0.222	548.6 (0.70, 1096.44)	0.050
Model A[1]	65	Ref	-28.3 (-391.62, 335.07)	0.876	157.1 (-171.02, 485.28)	0.340	424.3 (24.29, 824.40)	0.038
Model B[2]	61	Ref	22.3 (-380.06, 729.08)	0.910	271.1 (-186.86, 572.15)	0.235	352.3 (-158.62, 863.13)	0.169
MECPP		**<0.22**	**0.22 – 0.27**		**0.28 – 0.38**		**≥0.39**	
Crude	63	Ref	-359.0 (-886.03, 168.03)	0.178	-23.0 (-607.69, 561.69)	0.937	-319.0 (-939.16, 301.16)	0.307
Model A[1]	57	Ref	-387.2 (-808.87, 34.39)	0.071	-334.1 (-807.69, 139.57)	0.161	-396.4 (-856.67, 63.96)	0.089
Model B[2]	52	Ref	-251.8 (-777.02, 273.44)	0.327	-349.4 (-910.28, 211.40)	0.207	-741.1 (-1569.71, 87.58)	0.077
MEHHP		**<0.18**	**0.18 – 0.27**		**0.28 – 0.39**		**≥0.40**	
Crude	63	Ref	-22.5 (-636.92, 591.92)	0.942	-166.3 (-680.31, 347.81)	0.520	-83.8 (-698.17, 530.67)	0.786
Model A[1]	57	Ref	-5.5 (-490.29, 479.20)	0.982	-347.6 (-721.21, 25.95)	0.067	-122.1 (-627.17, 382.99)	0.626
Model B[2]	52	Ref	67.4 (-704.41, 839.24)	0.856	6.6 (-560.08, 573.21)	0.981	225.8 (-454.75, 906.39)	0.495
MEOHP		**<0.17**	**0.17 – 0.23**		**0.24 – 0.41**		**≥0.42**	
Crude	63	Ref	-374.0 (-906.73, 158.73)	0.165	-181.3 (-751.51, 389.01)	0.527	-483.3 (-989.51, 22.84)	0.061
Model A[1]	57	Ref	-237.3 (-640.96, 166.31)	0.240	-721.9 (-1204.17, -239.7)	0.005	-373.3 (-761.70, 15.06)	0.059
Model B[2]	52	Ref	37.2 (-632.47, 706.92)	0.908	-712.1 (-1557.12, 132.85)	0.094	-181.7 (-761.33, 397.99)	0.519
PFOS		**<996**	**996 – 1600**		**1601 – 2000**		**≥2001**	
Crude	62	Ref	-20.4 (-545.1, 504.35)	0.938	-54.4 (-677.51, 568.76)	0.862	65.6 (-431.46, 562.71)	0.792
Model A[1]	57	Ref	132.7 (-319.69, 585.08)	0.555	218.0 (-332.15, 768.13)	0.426	224.3 (-193.07, 641.68)	0.282
Model B[2]	52	Ref	377.7 (-134.73, 890.04)	0.139	487.6 (-157.17, 1132.33)	0.130	595.9 (88.77, 1103.04)	0.024
PFOA		**<591**	**591 – 870**		**871 – 1150**		**≥1511**	
Crude	62	Ref	-337.1 (-779.69, 105.58)	0.133	24.4 (-651.76, 700.51)	0.943	-353.6 (-841.19, 133.94)	0.152
Model A[1]	57	Ref	-147.4 (-551.16, 256.37)	0.463	-233.2 (-842.90, 376.56)	0.442	-11.3 (-495.07, 472.55)	0.963
Model B[2]	52	Ref	170.6 (-406.08, 747.23)	0.542	192.5 (-634.15, 1019.15)	0.631	71.7 (-609.58, 753.06)	0.827

[1] Adjusted for gestational age, maternal BMI, maternal height, maternal age at birth, GWG, and parity.

[2] Model A + additionally adjusted for smoking, alcohol intake, paternal BMI, paternal height, education, fish intake, and folic acid intake.

* $p < 0.050$

Discussion

The objective of the current study was to determine associations between exposure markers of various EDCs and birth weight. Even though our sample size was relatively small, we observed potentially relevant associations. Higher exposure to DDE to a certain extent was associated with higher birth weight in girls. Also for PFOS a higher birth weight was observed in girls in the highest exposure quartile. These results differ from what has been reported in other studies. In a meta-analysis of 12 European cohorts, no overall effect of DDE on birth weight was observed (9), and other cohorts outside Europe mostly show no effect or an inverse association (24, 25). However, levels of DDE in this study are lower than in other European cohorts (e.g. median DDE exposure in a Flemish cohort: 220.0 ng/L vs. median DDE current study: 100.3 ng/L). Also for PFOS most studies report an inverse association, but each of these studies also reported higher exposure levels (26, 27).

The possibility that dose-response between endocrine disruptors and various health outcomes may be nonmonotonic has been extensively reviewed by Vandenberg et al. (2012) (28). The main implication is that one cannot predict low dose effects from effects seen at high doses and vice versa. Another important point made in this publication is the case of low dose effects. As stated by Vandenberg: 'the endocrine system evolved to function when unbound physiologically active ligands (hormones) are present at extremely low doses' , and 'EDCs that mimic natural hormones have been proposed to follow the same rules and therefore have biological effects at low doses'. Our results for DDE and PFOS may be low dose results, as exposure levels in this cohort are lower than what has been reported in other studies. It remains to be clarified however whether the observed effects are truly nonmonotonic dose-response relations as our results may be incidental significant associations or chance findings.

In this study, secondary phthalate metabolites were measured in cord blood and milk, novel matrices that are challenging to analyse. These compounds are usually measured in urine, making comparison across studies difficult. A few reports of the primary DEHP metabolite mono(2-ethylhexyl)phthalate (MEHP) exist in cord blood, including one Chinese study of low-birth weight infants which observed a mean MEHP level of 9.94 mg/L in cord blood (29), which is 1000 times higher than the level measured in our cohort (data not shown). These results should be interpreted with caution, however, as analysis of MEHP in blood and milk is hampered by problems of contamination during storage and analysis (30). Several studies have related phthalate exposure to birth weight. Some observed no effect of early life phthalate exposure on birth outcomes, including birth weight (31, 32). However, prenatal di-n-butyl phthalate exposure was associated with a higher risk for low birth weight in newborns from Shanghai (33). and inverse associations with birth weight have been observed for prenatal 2,4-dichlorophenol and 2,5-dichlorophenol exposure (34, 35). Furthermore in another Dutch cohort maternal occupational exposure to phthalates (yes or no) was associated with impaired fetal growth, including fetal weight, during pregnancy (36). This is in line with our observations for

MEOHP in girls, in whom higher exposure was associated with lower birth weight. Though each of the phthalate metabolites showed significant inter-correlation, we cannot explain why no effect was observed for MECPP and MEHHP on birth weight in girls. This might be due to the small sample size and therefore a lack of power.

Regarding PCB-153 exposure, a decreased birth weight was observed for girls with quantifiable levels in cord blood, however when we made additional corrections for certain lifestyle characteristics, this effect was no longer significant. In the meta-analysis of Govarts et al. (2012) an overall negative effect was reported, however for cohorts with comparable concentrations of PCB-153, no significant effects were observed when considering cohort-specific results (9). It may be that PCB-153 affects birth weight at higher levels than the levels in the current cohort. Furthermore the chosen cut-off level (<LOQ vs. >LOQ) might not have been a clinically relevant one. However, as there is no agreed upon clinically relevant cut-off level and as we had a very high level of non-detects, we chose to dichotomize PCB-153 at the LOQ. As for PFOA, levels in the current study are similar to the study of Apelberg et al. (2007) who observed a negative association between PFOA exposure and birth weight (26). This is similar to our results in males. In females we also observe a somewhat lower birth weight for higher exposed subjects, though not significant, which may be due to the low sample size as the sample in the study of Apelberg et al. was approximately twice as large.

Women in the cohort were on average more highly educated than the general Dutch population (68.2% vs. 28.2%) (37). Furthermore the male/female ratio of offspring was 1.7, which is higher than average. We have no explanation for this, as the cohort was recruited prospectively. In a recent review by Nieminen et al. (2013) no indication was observed that developmental exposure to PCBs altered male/female ratio (38), and as is suggested by van Larebeke et al. (2008) one would expect environmental exposures to have a negative impact on sex ratio, if any at all, as sex ratios worldwide seem to decline and use of chemicals is increasing (39),

A limitation of this study is the small sample size. Power was furthermore reduced by dividing participants in quartiles according to exposure levels, and by stratification of results for gender. However, as associations between outcome and exposure were non-linear, using quartiles was considered necessary. The cut points for these quartiles were not based on clinical relevance, which might have evoked non-relevant effects, or obscured real effects. We considered it appropriate to present results stratified for gender, as growth is a gender-specific process, and as the chemicals included may affect the endocrine system The confounders added to the models did not affect the model standard errors, and they were therefore included to reduce bias by confounding as much as possible. Based on the large number of statistical tests we performed, we cannot rule out that our statistically significant findings were (in part) chance findings. We have not used corrections such as a Bonferroni correction, because this would further reduce the power of our statistical tests. However, this does imply that our results may very well be false positive findings, and that they need confirmation in larger study populations.

Data within this cohort was collected prospectively, and though small, this population was very homogenous. Associations are therefore less likely to be confounded by demographic or socio-economic factors. Adjusting our models for GWG might be considered inappropriate as maternal weight gain during pregnancy is partly a result of fetal growth (40). However in a recent publication by Verner et al. (2013) it is stated that GWG may strongly confound the association between lipophilic exposures and birth weight (23). Sensitivity analysis excluding GWG from the models affected results for lipophilic compounds, but also for phthalates, which are not lipophilic. We have no explanation for these findings.

This study clearly indicated gender dimorphic associations, even though levels of exposures did not differ significantly between boys and girls. Gender specific effects of EDCs may be related to their (anti-)estrogenic or (anti-)androgenic properties. Developmental exposure of rats to suspected androgen DEHP resulted in impaired glucose tolerance and insulin secretion in females (41). Males showed increased insulin levels, and both females and males had a lower birth weight than controls, similar to what we observe for some DEHP metabolites. Placental exposure to organohalogenated xenoestrogens, including DDT, was associated with increased birth weight in 14 month old boys but not in girls (42), which is similar to our results for DDE.

Conclusion

We conclude that in our study population prenatal exposure to DDE, PFOS, and to a lesser extent PFOA, PCB-153, and MEOHP, are associated with birth weight. The associations seem to have a non-monotonic dose-response relationship and differ between boys and girls. For MECPP, and MEHHP associations with birth weight were observed. Results from our study should be interpreted with caution due to the limited sample size, and in particular regarding phthalates and PFAAs, results should be regarded as exploratory. Larger studies are warranted to confirm these results.

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Supplemental Material - Prenatal exposure to endocrine disrupting chemicals and birth weight – a prospective cohort study

Analysis of organochlorine pesticides and PCB 153

For the determination of the organochlorine pesticides (HCB and p,p'-DDE) and PCBs (PCB153), 3 ml of cord plasma or 12 ml breast milk were dried with Kieselguhr after addition of the internal standard ($^{13}C_6$-HCB, $^{13}C_{10}$-PCB153 (from Cambridge Isotope Laboratories) and BDE58). The dried samples were extracted with 30 ml hexane/dichloromethane (7:3 v/v) for 30 minutes by sonication. The extracts were purified over sulphuric acid silica columns (5 g, 40% sulphuric acid w/w) that were eluted with 30 ml hexane/dichloromethane (7:3 v/v). The eluates were evaporated under a gentle stream of nitrogen. Finally, the extracts were quantitatively transferred to GC-vials, with a final volume of 100 µl iso-octane.

The samples were analyzed using an Agilent 6890 GC with a 5975 Mass Spectrometric Detector in negative chemical ionization mode. The GC was equipped with a CPSil-8 CB column (Varian, 50 m x 0.25 mm ID x 0.25 µm film thickness). The samples were injected in pulsed splitless mode with the injector at 275°C. The oven was programmed as follows: initial temperature: 90°C for 3 min; then to 210°C at 30°C/min and held for 20 min; finally to 290 °C at 5°C/min and held for 3 minutes (total run time 45 minutes). Carrier gas was helium at a flow rate of 2.7 ml/min. The compounds of interest were quantified by using m/z 283.8 for HCB; m/z 359.8 for PCB 153 and m/z 35 for p,p'-DDE (compound confirmation by GC-MS/EI measurement of m/z 246).

Analysis of DEHP metabolites

To assess the exposure to DEHP, the secondary metabolites MEOHP (mono(2-ethyl-5-oxohexyl) phthalate), MEHHP (mono (2-ethyl-5-hydroxyhexyl) phthalate) and MECPP (mono(2-ethyl-5-carboxypentyl) phthalate) were quantitatively determined. The remaining enzymatic activity in the sample material was quenched by adding 0.02 ml 1 M phosphorous acid to 0.3 ml cord plasma or 0.04 ml 1 M phosphorous acid to 0.5 ml breast milk. The mixtures were then sonicated for 5 minutes. To adjust the pH of the plasma and milk samples to 6.2, 0.04 ml and 0.06 ml 1M NaOH was added to the samples, respectively. After the addition of the internal standard ($^{13}C_4$ – MEOHP, $^{13}C_4$ – MEHHP, $^{13}C_4$ – MECPP and MEHP- d_4, all from Cambridge Isotope Laboratories), 5 µl β–glucuronidase from E. Coli K12 (from Roche) in 0.2 ml 2.5 M ammonium acetate buffer (pH 6.2) was added. The samples were incubated for 90 minutes at 37°C. The completeness of the deconjugation step was checked by adding 4-methylumbelliferone-glucuronide to each sample. The β–glucuronidase activity was stopped by adding 0.06 ml formic acid to the plasma samples and 0.06 ml ammonium hydroxide to the milk samples followed by sonication for 15 minutes and overnight storage at -20°C.

The deconjugated plasma samples were thawed and centrifuged for 15 minutes at 17000 rpm. The supernatants are transferred to a vial to which 0.2 ml water is added.
The milk samples were also centrifuged for 15 minutes (17000 rpm) to remove the lipids. The remaining part was extracted by solid phase extraction (SPE). The Oasis MAX 3cc 60 mg cartridges were conditioned with 3 ml methanol and 3 ml milliQ. After the samples were loaded at 1 ml/min, the cartridges were washed with 1 ml 5% ammonium hydroxide and 1 ml 75% tetrahydrofuran in methanol. The metabolites were eluted from the cartridges with 5 ml 5% formic acid in methanol. To the eluates 0.2ml milliQ was added before evaporation to a volume of 0.2 ml. Finally, 0.2 ml 4% ammonium hydroxide and 0.2ml milliQ were added to the extracts.
The obtained extracts were injected onto a RAM (restricted access material) phase cartridge (LiChrospher RP-8 ADS, 25 µm, 25 x 4 mm). After trapping and cleanup, the analytes were eluted in backflush mode and transferred to the analytical column (Luna Phenyl-hexyl 75 x 4.6 mm) using a gradient of 0.1 % acetic acid and acetonitrile with a flow rate of 0.25 ml/min. The LC system was an Agilent 1200 Series (Palo Alto, CA, USA) coupled with an Agilent 6410 electro spray interface (ESI) operated in the negative ion mode prior to triple-quadrupole mass spectrometric detection. For MECPP, the ion transition used for quantification was m/z 307.1 – m/z 159.1, for MEHHP the ion transition m/z 293.1 – m/z 145.1, for MEOHP the ion transition m/z 291.1 – m/z 143.1, and for MEHP the ion transition m/z 277.1 – m/z 134.1 were used.

Analysis of PFOS and PFOA

For the determination of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), the breast milk samples were thawed and homogenized after stabilizing them at a temperature of 38°C. Subsequently, aliquots of 0.5ml of each sample were taken for analysis. The sample was sonicated for 30 min after addition of the internal standards ($^{13}C_4$-PFOA and $^{13}C_4$PFOS, from Wellington Laboratories) and 0.5ml 1M formic acid. Solid phase extraction was carried out using 1cc, 30 mg Oasis Wax cartridges. The cartridges were conditioned with 1 ml methanol and 1 ml MilliQ. The samples were loaded at a flow rate of 1ml/min along with the rinse volume of the sample tube, i.e. 1 ml 25 mM ammonium acetate pH4. The cartridges were washed with 1ml 25 mM ammonium acetate pH4 and 0.5 ml 25% tetrahydrofuran in methanol. The PFASs were eluted from the cartridge with 0.4 ml 1% NH_4OH in methanol and 0.4 ml 0.1M formic acid was added to the eluate.
For the analysis of the cord plasma samples 0.2 ml cord plasma was mixed with 0.2 ml methanol. After addition of the internal standard the mixture was homogenized and centrifuged for 15 minutes at 17000 rpm. The obtained supernatants were diluted and mixed with 0.5 ml 0.1 M formic acid.
The total volume of the extracts was injected and the PFASs are trapped on a C8-column (Xterra MS C_8, 10 mm x 4.6 mm, particle size 5 µm) in an on-line system with the analytical column (Betasil C8, 50 mm x 2.1 mm, particle size 3 µm). Subsequently the

PFASs are eluted from the trapping column and separated on the analytical column using gradient elution at a flow rate of 0.3 ml/min. For the gradient 20 mM NH_4AC pH4 and acetonitrile were used. The LC system was an Agilent 1200 Series (Palo Alto, CA, USA) coupled with an Agilent 6410 electro spray interface (ESI) operated in the negative ion mode prior to triple-quadrupole mass spectrometric detection. For PFOA, the ion transition used for quantification was m/z 413 – m/z 369, and for PFOS the ion transition m/z 499 – m/z 80 was used.

Determination of lipid content of breast milk and cord plasma samples

In the breast milk samples, the lipid content was determined using a method adapted from Manirakiza et al. (1). To 6 ml of sample, 13 ml of isoproanol and 15 ml of cyclohexane were added. The mixture was shaken vigorously for 5 minutes. Subsequently, 10 ml water was added. The cyclohexane phase containing the lipids was separated from the mixture. This procedure was repeated with a mixture of 15 ml isopropanol and cyclohexane (13:87 v/v). After combining the two cyclohexane fractions, the solvent was evaporated by a gentle nitrogen stream till dryness. The remaining lipids were gravimetrically determined after drying for 1 hr at 105°C.

In cord plasma, the lipid content was determined using standard protocols by the measurement of the triglycerides and cholesterol at the clinical laboratory of the academic hospital (ISO 15189 accredited) of the VU University, VUmc (Amsterdam, NL).

QA/QC procedures

For all the analyses described, no Certified Reference Materials (CRMs) were available. Therefore, in every measurement series (< 16 samples) a procedure blank, an enriched sample (similar/same matrix) and a sample from a previous series were included. The analytical values obtained for the enriched sample should fall within 20% of the known level. The re-analysis of a sample from a previous series, should give a result with z-values < |2|.

In case the procedure blank revealed that the compound(s) to be analyzed were present above the limit of detection (LOD), the series was repeated. All results were corrected using the long term average blank value. The obtained blank data were used for the determination of the LOD (as 3* standard deviation in the blank) and the limit of quantitation (LOQ, defined as 3*LOD).

The performance characteristics of all methods – LOD, recovery and repeatability for breast milk and cord plasma - are given in table S5.1.

Table S5.1 Performance characteristics of all the methods used for the assessment of exposure markers in breast milk and cord plasma

	Milk			Plasma		
	LOD (pg/ml)	Recovery (%)	Repeatability (%)	LOD (pg/ml)	Recovery (%)	Repeatability (%)
PCB153	10	96 (89-105)	7	13	105 (97-113)	5
4,4'-DDE	17	98 (91-109)	9	13	96 (82-119)	13
PFOS	1.4	105 (89–105)	9	5	86 (81-90)	3
PFOA	0.4	102 (98–104)	8	7	84 (75-90)	6
MECPP	20	94 (84-104)	5	40	100 (96-106)	3
MEHHP	30	95 (78-107)	11	10	92 (81-89)	4
MEOHP	30	91 (67-113)	16	20	92 (82-98)	6
MEHP	60	96 (65-120)	16	30	75 (70-83)	5

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Table S5.2 Regression coefficients for various exposures (ng/L), in quartiles, and birth weight (grams)

Compound	n	Q1	Q2 β (95% CI)		p-value	Q3 β (95% CI)		p-value	Q4 β (95% CI)		p-value
DDE		**<41.79**		**41.80 – 74.50**			**74.51 – 107.50**			**≥107.51**	
Crude	81	Ref	68.8	(-228.69 – 366.33)	0.65	134.3	(-155.69 – 424.26)	0.36	-22.8	(-316.39 – 270.77)	0.88
Model A[a]	62	Ref	-33.0	(-304.76 – 238.76)	0.81	99.5	(-140.33 – 339.25)	0.41	-3.6	(-272.84 – 265.63)	0.98
Model B[b]	55	Ref	86.5	(-244.23 – 417.28)	0.60	181.4	(-111.77 – 474.61)	0.22	-45.8	(-399.39 – 307.88)	0.79
MECPP		**<0.22**		**0.22 – 0.27**			**0.28 – 0.38**			**≥0.39**	
Crude	67	Ref	-129.7	(-423.06 – 163.65)	0.38	68.1	(-241.64 – 377.85)	0.66	-181.8	(-502.79 – 139.09)	0.26
Model A[a]	52	Ref	-276.3*	(-534.58 – -18.03)	0.04	-102.4	(-393.92 – 189.05)	0.48	-373.8*	(-677.23 – -70.42)	0.02
Model B[b]	46	Ref	-243.7	(-587.19 – 99.74)	0.16	-83.7	(-423.23 – 255.88)	0.61	-345.4	(-760.40 – 69.63)	0.10
MEHHP		**<0.18**		**0.18 – 0.27**			**0.28 – 0.39**			**≥0.40**	
Crude	67	Ref	-30.9	(-339.65 – 277.87)	0.84	-69.2	(-386.11 – 247.80)	0.66	-45.0	(-372.00 – 282.08)	0.78
Model A[a]	52	Ref	15.9	(-283.55 – 315.44)	0.92	-175.2	(-466.04 – 115.64)	0.23	-48.9	(-360.08 – 262.39)	0.75
Model B[b]	46	Ref	101.6	(-281.55 – 484.73)	0.59	139.7	(-332.33 – 611.80)	0.55	146.78	(-242.72 – 536.24)	0.44
MEOHP		**<0.17**		**0.17 – 0.23**			**0.24 – 0.41**			**≥0.42**	
Crude	67	Ref	-8.2	(-315.00 – 298.52)	0.96	114.5	(-197.05 – 425.98)	0.47	-127.9	(-434.70 – 178.82)	0.41
Model A[a]	52	Ref	-165.5	(-449.63 – 118.70)	0.25	-361.0*	(-673.63 – -48.28)	0.03	-241.8	(-512.00 – 28.39)	0.08
Model B[b]	46	Ref	-43.4	(-460.94 – 374.21)	0.83	-242.5	(-736.89 – 251.93)	0.32	-7.7	(-367.83 – 352.48)	0.97
PFOS		**<1001**		**1001 – 1700**			**1701 – 2154**			**≥2155**	
Crude	67	Ref	-97.3	(-393.89 – 199.27)	0.51	176.4	(-141.36 – 494.23)	0.27	114.1	(-198.56 – 426.69)	0.47
Model A[a]	52	Ref	19.6	(-285.95 – 325.22)	0.90	241.6	(-117.32 – 599.44)	0.18	118.4	(-202.77 – 439.59)	0.46
Model B[b]	46	Ref	345.2	(-12.15 – 702.59)	0.06	510.3	(97.43 – 923.14)	0.02	520.0	(120.93 – 919.15)	0.01
PFOA		**<591**		**591 – 910**			**911 – 1200**			**≥1201**	
Crude	67	Ref	-486.3	(-773.17 – -199.33)	0.001	-145.3	(-432.23 – 141.61)	0.32	-246.0	(-528.45 – 36.39)	0.09
Model A[a]	52	Ref	-122.5	(-441.41 – 196.40)	0.44	2.6	(-313.76 – 319.00)	0.99	41.5	(-260.34 – 343.24)	0.78
Model B[b]	46	Ref	-51.3	(-446.11 – 343.50)	0.79	100.8	(-339.70 – 541.34)	0.64	121.1	(-279.02 – 521.26)	0.54

[1] Adjusted for gestational age, maternal BMI, maternal height, maternal age at birth, GWG, and parity.

[2] Model A + additionally adjusted for smoking, alcohol intake, paternal BMI, paternal height, education, fish intake, and folic acid intake.

* $p < 0.050$