Early life exposure to endocrine disrupting chemicals and child health

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First year growth in relation to prenatal exposure to endocrine disruptors – A Dutch prospective cohort study

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Abstract

Background: Growth in the first year of life may already be predictive of obesity later in childhood. The objective was to assess the association between prenatal exposure to various EDCs and child growth during the first year.

Methods: Dichlorodiphenyldichloroethylene (DDE), mono(2-ethyl-5-carboxypentyl)phthalate (MECPP), mono(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP), mono(2-ethyl-5-oxohexyl)phthalate (MEOHP), polychlorinated biphenyl-153 , perfluorooctanesulfonic acid, and perfluorooctanoic acid were measured in cord plasma or breast milk. Data on weight, length, and head circumference (HC) until 11 months after birth was obtained from 89 mother-child pairs. Mixed models were composed for each health outcome and exposure in quartiles.

Results: For MEOHP, boys in quartile 1 had a higher BMI than higher exposed boys ($p = 0.029$). High DDE exposure was associated with low BMI over time in boys (0.8 kg/m2 difference at 11 m). Boys with high MECPP exposure had a greater HC (1.0 cm difference at 11 m) than other boys ($p = 0.047$), as did girls in the second quartile of MEHHP ($p = 0.018$) and DDE ($p < 0.001$) exposure.

Conclusion: In conclusion, exposure to phthalates and DDE was associated with BMI as well as with HC during the first year after birth. These results should be interpreted with caution though, due to the limited sample size.

Introduction

Optimal development and health early in life are key factors for health and wellbeing during childhood and adulthood. It has been hypothesized that adult health and disease have their origin in the prenatal and early postnatal environment, a concept referred to as the Developmental Origins of Health and Disease (1). This is illustrated by the Dutch Hunger Winter Study, in which exposure to famine during gestation was associated with an increased risk for amongst others coronary heart disease and obesity (2). These associations were furthermore dependent on the timing of the famine during gestation, as they were only observed for those exposed early in pregnancy .

There are various parameters early in life which are indicators for development later in life. Birth weight, for example, is inversely associated with hypertension (3) and type 2 diabetes (4) in adulthood, and both high (5) and low (6) birth weight are associated with obesity. Rapid growth in infancy, in terms of both weight and height, is considered an independent risk factor for childhood obesity (7, 8). A recent study by Gittner et al. showed that children who were obese at the age of five years already had distinct body mass index (BMI) patterns before 12 months of age. Children who had a normal BMI at age five always had a lower BMI from age 6 months and onwards compared to children who were obese at age five years (9). They furthermore showed that BMI patterns over time differed between male and female children, with female children who were overweight at the age of five gaining weight faster in the first two years of life compared to male overweight children (9).

Childhood obesity has also been related to exposure to endocrine disrupting chemicals (EDCs) early in life. Several studies have observed positive associations with BMI and early childhood growth for chemicals such as organochlorine pesticides (e.g. dichlorodiphenyltrichloroethane [DDT], hexachlorobenzene [HCB]) (10-13) and perfluorinated alkyl acids (PFAAs) (14). Several studies on the other hand have reported no effect (15-18), as we have described in a recently published review (19). Furthermore associations seem to differ between males and females, and dose-response relations are not clear. Exposure to these chemicals is widespread. The organochlorine pesticides, such as DDT, of which dichlorodiphenyldichloroethylene (DDE) is the major metabolite, and HCB, have been banned from production (20, 21), but remain in the food chain due to their lipophilic and bioaccumulating properties. DDT is furthermore still in use in developing countries for vector control (malaria) (22). Polychlorinated biphenyls (PCBs), which were mainly applied as dielectric and coolant fluids and which were banned due to their carcinogenic characteristics (23), have also bioaccumulated in the food chain (24). Exposure to these chemicals is slowly decreasing over time, however it is not certain if low dose exposure implies safe exposure (25). Not all EDCs are persistent, though exposure to non-persistent EDCs may occur on a continuous basis. Examples of these are phthalates which are used to soften plastics, and perfluorinated alkyl acids (PFAAs), surfactants used in various consumer products. Furthermore, the presence of many chemicals, such as

PFAAs, PCBs, organochlorine pesticides, and phthalates, can be detected in 99-100% of pregnant women (26). As several of these chemicals have also been detected in e.g. cord blood (12, 27) and amniotic fluid (28, 29), it is clear that the placenta does not protect the fetus from these exposures (30, 31). EDCs are suspected – as implied by their name – to disrupt hormonal action, and exposure early in life may have long lasting effects (32). Experimental studies have furthermore shown that they may cause epigenetic changes (33), which implies that effects of these chemicals may potentially be trans-generational.

In light of prevention, it is essential to determine to which extent EDCs affect child growth as early as in the first 12 months after birth. As infants in the Netherlands are eligible for free health monitoring by youth health care organizations, each child is measured and weighed regularly, especially in the first twelve months. The objective of this study was to determine possible associations between markers of prenatal exposure to various EDCs and child growth in the first year of life. The compounds included were DDE, PCB-153, three metabolites of di(2-ethylhexyl) phthalate (DEHP), including mono(2-ethyl-5-carboxypentyl)phthalate (MECPP), mono(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP), and mono(2-ethyl-5-oxohexyl)phthalate (MEOHP), as well as the PFAAs perfluorooctanesulfonic acid (PFOS), and perfluorooctanoic acid (PFOA).

Methods

Study procedures and subjects

Six midwifery clinics in the area of Zwolle, the Netherlands, participated in the recruitment of pregnant women, which started in January 2011 and finished in January 2013. The community Zwolle is located in the 'Salland' area, which is characterized by a relatively low level of urbanization. Women were invited to participate during the first antenatal visit (between 10 and 12 weeks of pregnancy) to the midwife and were considered eligible for participation if they were able to fill out Dutch questionnaires. In total 148 mother-child pairs were included. Twin pregnancies and major congenital anomalies were reasons for exclusion, however no participant was excluded because of these criteria. Signed informed consent was obtained from every participant. Cord blood and breast milk were collected for determination of markers of early life exposure to several EDCs. Youth health care organizations in the area of Zwolle were approached for data on growth during the first year which they collect during scheduled follow-up visits of each child in the Netherlands. About 99.5% of parents in the Netherlands visit youth health care centres with their child during the first year (34). On average each child is seen six times during this period. Information on parental anthropometry was obtained from the midwives, and questionnaires were administered during pregnancy to collect information on parental health and lifestyle, and previous pregnancies. Fourteen subjects dropped-out before delivery due to lack of time of the mother to participate, and for 45 subjects no exposure

data was available, resulting in the inclusion of 89 mother-child pairs for analysis. The study was approved by the medical ethics committee of the VU University medical centre.

First year growth

Data on weight, height (supine length), and head circumference were obtained from youth health care organizations. Parents were contacted by youth health care for follow-up visits at 1, 2, 4, 6, 9, and 11 months after birth. Infant BMI was calculated from weight and height (BMI = weight [kg] / height^2 [m^2]).

Chemical exposure

Umbilical cord blood was collected immediately after birth when the health of mother and child was ascertained. Midwives and nurses were instructed to collect as much blood as possible and to transfer it to EDTA tubes. 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 the second month after birth (mean [SD]: 6.3 [2.5] weeks). 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. PFOA and PFOS were analysed by applying isotope dilution and large volume injection using an on-line trapping column coupled to liquid chromatography and triple quadrupole mass spectrometry. The isotope labelled standards $^{13}C_4$-PFOA and $^{13}C_4$PFOS were obtained from Wellington Laboratories (Guelph, Canada). The breast milk samples were extracted with solid phase extraction using Oasis WAX cartridges (Waters, Milford, MA, USA). 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 (Supelco, Sigma-Aldrich, St. Louis, MO, USA), the organochlorine pesticide p,p'-DDE, and PCB153 were extracted with a mixture of dichloromethane and hexane. As internal standard, $^{13}C_{12}$-PCB153, obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA), was used. 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, enzymatic deconjugation was carried out. After addition of the internal standards used for isotope dilution, the breast milk samples were extracted using Oasis MAX (Milford, MA, USA) cartridges, while for the cord plasma samples a simple protein precipitation step using formic acid was applied. The

isotope labelled standards $^{13}C_4$ – MEOHP, $^{13}C_4$ – MEHHP, $^{13}C_4$ – MECPP and MEHP- d_4 were all obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). The extracts were analysed after large volume injection using an on-line trapping column coupled to liquid chromatography and triple quadrupole 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, MECPP, MEHHP, and 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.

Data-analysis

Data-analysis was performed using IBM SPSS version 20, Armonk, NY, United States (35). For each compound, exposure values below the limit of quantification (LOQ) were replaced by LOQ/√2 (36). For DDE a conversion factor, based on publications of other European cohorts, was used to transform levels in breast milk to levels in cord blood, as it accumulates in lipid (36). The following conversion factor was used:

[DDE, cord plasma (ng/L)] = 1.20 X [DDE, breast milk (ng/g lipid)] (36)

For PCB-153, over 50% of the cord plasma samples had an exposure level that could not be quantified. As PCB-153 was determined predominantly in cord blood it was decided to only include cord blood levels of PCB-153 and to dichotomize at LOQ (>LOQ vs. <LOQ). For the other compounds no conversion factor was applied and only cord blood levels were used.

Mixed models were used for the main analysis. For each compound a separate model was composed for weight, height, BMI, and head circumference. Linearity with outcome was checked for each compound, and as none of the compounds showed a linear association, each exposure was split up in quartiles. Exposure quartiles, timing of anthropometric measurement, sex and their interactions were added to the models as fixed effects and a random effect was added for subject. Time was included as a categorical variable. Covariates were selected based on literature and included maternal/paternal BMI and height, birth weight, gestational age, parity, alcohol, smoking, education, and breast feeding duration. As sample size was relatively small, covariates were only added to the models if they were deemed relevant (change in β-coefficient of exposures > 10%). Using this cut-off, birth weight, gestational age, and maternal height influenced the estimates in

the majority of analyses and were therefore selected for all models. Sex and time specific marginal means were calculated from the models and were used to plot the development over time in growth parameters for the exposure quartiles and for boys and girls separately. Interactions were considered significant at a p-value of 0.10, considering the limited power of this study.

Covariates

Birth weight was measured after birth 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 birth weight was determined when the infant was in a calm state. Weighing scales were provided by the midwives and were calibrated daily. Gestational age was determined by midwives by means of early ultrasound.

Maternal height was measured by the midwife at inclusion, approximately 10-12 weeks in pregnancy. Midwives received a measuring tape which was attached to a wall in the midwife's office, as well as instructions on how to perform this measurement, including how the participants should be positioned and the precision required for the measurement.

Results

Out of the 89 children included in the analysis, 56 were boys (table 7.1). Compared to girls, boys had a higher weight gain between 1 and 11 months of age (5.3 versus 4.5 kg.), as well as a larger increase in height (21.5 versus 19.7 cm.) and head circumference (9.0 versus 8.1 cm.). The majority of the children were breastfed for a period longer than three months (boys: 69.2%, girls: 64.3%). An overview of exposure levels of the various compounds is given in table 7.2. Information on exposure quartiles is given in table 7.3. Due to the high fat content of breast milk samples, wet weight levels of the lipophilic compound DDE were lower than lipid corrected levels. There was no difference in exposures between boys and girls (not shown).

BMI

Sex specific BMI curves for DDE, MECPP, MEHHP, and MEOHP are given in figures 7.1A-D. For the other EDCs they can be found in Supplemental Material p. 5-6. For all compounds, significant main effects were observed for both time and sex, but not for compound (table 7.4). We did find a relevant main effect for MECCP which was 1.5 and 2.2 at 11 months for boys and girls respectively (p=0.051). Interaction effects were observed for total DDE and MEOHP.

Total DDE showed an interaction with time, independent of sex, meaning that BMI differed between exposure quartiles over time (p = 0.078). Boys in the highest DDE exposure quartile had a BMI which was lower than boys with a lower DDE exposure at all ages (figure 1A). At the age of 11 months, the difference with the nearest quartile reached

0.8 kg/m^2 (mean BMI Q4: 16.8 kg/m^2, 95% CI 16.09 to 17.49; mean BMI Q3: 17.6 kg/m^2, 95% CI 16.93 to 18.35). A similar result was observed for girls in the third exposure quartile.

Table 7.1 Population characteristics of the study population

	Boys (n=56)	Girls (n=33)
Weight, age = 1 month (kg.)	4.68 (3.00 – 5.87)	4.25 (3.21 – 5.38)
Weight, age = 11 months (kg.)	10.13 (8.49 – 12.85)	8.92 (6.88 – 10.55)
Weight gain, first year (kg.)	5.31 (4.16 – 7.59)	4.46 (3.41 – 5.60)
Height, age = 1 month (cm.)	54.8 (51.0 – 59.0)	53.9 (51.0 – 59.9)
Height, age = 11 months (cm.)	76.6 (71.0 – 81.4)	74.6 (71.0 – 77.0)
Growth, first year (cm.)	21.5 (18.0 – 25.0)	19.7 (16.0 – 23.3)
BMI, age = 1 month (kg/m^2)	15.5 (12.8 – 18.7)	14.8 (12.0 – 17.0)
BMI, age = 11 months (kg/m^2)	17.1 (15.6 – 21.0)	16.2 (13.3 – 18.5)
BMI change, first year (kg/m2)	1.8 (-1.6 – 5.2)	1.6 (-0.8 – 3.2)
Head circumference, age = 1 month (cm.)	38.0 (34.2 – 40.6)	37.3 (35.1 – 40.1)
Head circumference, age = 11 months (cm.)	46.8 (43.4 – 49.5)	45.2 (42.6 – 48.0)
Head circumference change, first year (cm.)	9.0 (7.9 – 11.2)	8.1 (6.7 – 10.4)
Birth weight (g.)	3624.5 (491.7)	3564.2 (397.5)
Gestational age (weeks)	39.7 (1.5)	40.1 (1.0)
Parity (nulliparous, %)	23 (42.6 %)	10 (30.3 %)
Height mother (cm.)	171.4 (5.7)	171.9 (4.6)
Height father (cm.)	185.2 (6.8)	183.6 (6.2)
BMI mother (start pregnancy, kg/m^2)	23.6 (3.3)	23.0 (4.2)
BMI father (kg/m^2)	24.0 (3.0)	25.0 (2.8)
Age mother (years)	30.3 (3.8)	31.9 (3.5)
Age father (years)	32.4 (5.6)	34.2 (4.2)
Breastfeeding		
No breastfeeding	4 (7.7 %)	3 (10.7 %)
0-3 months breastfeeding	12 (23.1 %)	7 (25.0 %)
>3 months breastfeeding	36 (69.2 %)	18 (64.3 %)
Education mother (bachelor/master, %)	41 (75.9 %)	19 (59.4 %)
Smoking (first trimester, yes, %)	2 (3.7 %)	2 (6.1 %)
Alcohol (first trimester, yes, %)	3 (2.7 %)	2 (6.6 %)

Unless stated otherwise, values are median (range) or mean ± SD.

For MEOHP a significant interaction with time and sex on BMI was observed (p = 0.029), indicating that BMI differed between exposure quartiles during the first year, and that these patterns were different for boys and girls (figure 1D). In boys, weight at one month of age was similar for all quartiles. However, from 3 months onwards, boys in the lowest exposure quartile showed a very deviant development in their BMI from all other boys and girls in the sample. On average their BMI was much higher.

Weight and height

Sex specific weight and height curves for each compound can be found in Supplemental Material p. 7-14. Main effects of time and sex were significant for all compounds for both

weight and height (table 7.4). No main effects of exposure were observed, except for PFOA and height.

For MEOHP results regarding weight and height were similar to BMI, showing a significant interaction with both time and sex in a similar direction.

Head circumference

Sex specific head circumference curves for DDE, MECPP, MEHHP, and MEOHP are given in figures 7.2A-D. For the other EDCs they can be found in Supplemental Material p. 15-16. Similar to BMI, main effects for both time and sex on head circumference were significant for all compounds, whereas no main effects for exposure were observed (table 7.4). Interaction effects were observed for total DDE, PFOA, MECPP, and MEHHP.

For total DDE a significant interaction with time and sex on head circumference was observed ($p < 0.001$), indicating that head circumference differed between exposure quartiles during the first year, and that these patterns were different for boys and girls. Girls in the second quartile of exposure had a greater head circumference from six months onwards. Boys in the highest exposure quartile showed a similar pattern.

Boys in the highest MECPP exposure quartile had a consistently greater circumference over time than the other boys (figure 2B). Those in the lowest quartile on the other hand had a consistently smaller head circumference than the other quartiles. There was also a significant interaction for MEHHP exposure with time and sex ($p = 0.018$). In girls, the second exposure quartile in particular showed a consistently greater head circumference over time compared to the other quartiles (figure 2C). At six months of age the difference compared to the two quartiles showing the smallest head circumference was 2.0 cm., which was not statistically significant (head circumference Q2: 44.9 cm., 95% CI 43.2 to 46.7; head circumference Q3: 42.9 cm., 95% CI 42.2 to 43.5).

Table 7.2 Exposure profile of the study cohort

Compound		n	Mean	Median	Range	LOQ	<LOQ (%)
PCB-153							
Cord plasma	ng/L	52	35.94	29.14	22.63 – 96.00	21 – 43	55.8
	ng/g lipid	52	36.31	31.04	17.95 – 88.89	14 – 53	55.8
DDE							
Cord plasma	ng/L	52	115.61	83.00	28.28 – 470.00	33 – 73	23.1
	ng/g lipid	52	116.16	82.06	28.83 – 580.25	23 – 86	23.1
Breast milk	ng/L	24	2527.92	1950.00	400.00 – 11390.00	9.20 – 13.00	0
	ng/g lipid	24	62.58	45.33	12.11 – 277.80	0.13 – 0.53	0
Total [a]	ng/L	76	102.82	74.50	14.53 – 470.00		15.8
HCB							
Cord plasma	ng/L	52	43.70	43.14	28.28 – 78.00	40 – 79	98.1
	ng/g lipid	51	45.80	45.00	17.68 – 82.11	25 – 96	98.1
Breast milk	ng/L	24	630.42	655.00	300.00 – 1060.00	9.20 – 13.00	0
	ng/g lipid	24	16.27	4.25	10.36 – 26.88	0.16 – 0.68	0
MECPP							
Cord plasma	ng/mL	61	0.31	0.27	0.11 – 1.00	0.13 – 0.28	6.6
MEHHP							
Cord plasma	ng/mL	61	0.32	0.26	0.10 – 1.00	0.14 – 0.27	11.5
MEOHP							
Cord plasma	ng/mL	61	0.29	0.22	0.12 – 0.87	0.17 – 0.33	26.2
PFOA							
Cord plasma	ng/L	61	940.2	870.0	300 – 2700	50 – 140	0
PFOS							
Cord plasma	ng/L	61	1611.0	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.

Table 7.3 Exposure profile of the study cohort

Compound	Q1	n	Q2	n	Q3	n	Q4	n
DDE (Total) (ng/L)	<44.74	19	44.74-74.50	19	74.51-113.11	19	>113.11	19
MECPP (ng/mL)	<0.16	15	016-0.24	15	0.25-0.34	15	>0.34	15
MEHHP (ng/mL)	<0.20	16	0.20-0.23	17	0.24-0.37	11	>0.37	17
MEOHP (ng/mL)	<0.13	17	0.13-0.20	17	0.21-0.34	13	>0.34	14
PFOA (ng/L)	<591	17	591-870	14	871-1100	15	>1100	15
PFOS (ng/L)	<1001	18	1001-1600	15	1601-2000	14	>2000	14

PCB-153 was included as a dichotomous variable (<LOQ vs. >LOQ)

Table 7.4 P-values of tests of main effects and interactions of compound with time, sex, or time and sex, for each health outcome.

EDC	Effect	BMI P	Weight P	Height P	Head circumference P
DDE	Time	<0.001*	<0.001*	<0.001*	<0.001*
	DDE	0.293	0.647	0.910	0.778
	Gender	<0.001*	<0.001*	0.024*	<0.001*
	Time x DDE	0.078*	0.84	0.490	0.721
	DDE x gender	0.420	0.205	0.484	0.905
	Time x DDE x gender	0.298	0.695	0.134	<0.001*
PFOS	Time	<0.001*	<0.001*	<0.001*	<0.001*
	PFOS	0.586	0.802	0.975	0.649
	Gender	<0.001*	<0.001*	0.040*	<0.001*
	Time x PFOS	0.878	0.910	0.485	0.800
	PFOS x gender	0.655	0.425	0.749	0.485
	Time x PFOS x gender	0.679	0.617	0.321	0.742
PFOA	Time	<0.001*	<0.001*	<0.001*	<0.001*
	PFOA	0.813	0.350	0.045*	0.774
	Gender	<0.001*	<0.001*	0.019*	<0.001*
	Time x PFOA	0.389	0.126	<0.001*	0.001*
	PFOA x gender	0.242	0.169	0.165	0.467
	Time x PFOA x gender	0.348	0.812	0.152	0.387
MECPP	Time	<0.001*	<0.001*	<0.001*	<0.001*
	MECPP	0.051	0.265	0.726	0.314
	Gender	0.004*	<0.001*	0.002*	0.003*
	Time x MECPP	0.117	0.090*	0.944	0.604
	MECPP x gender	0.496	0.277	0.185	0.047*
	Time x MECPP x gender	0.204	0.929	0.978	0.886
MEHHP	Time	<0.001*	<0.001	<0.001*	<0.001*
	MEHHP	0.279	0.204	0.133	0.144
	Gender	0.005*	0.002	0.163	0.012*
	Time x MEHHP	0.593	0.962	0.294	0.100
	MEHHP x gender	0.383	0.326	0.073*	0.197
	Time x MEHHP x gender	0.127	0.485	0.011*	0.018*
MEOHP	Time	<0.001*	<0.001*	<0.001*	<0.001*
	MEOHP	0.315	0.151	0.112	0.119
	Gender	<0.001*	0.001*	0.390	<0.001*
	Time x MEOHP	0.152	0.948	0.084*	0.335
	MEOHP x gender	0.104	0.831	0.207	0.253
	Time x MEOHP x gender	0.029*	0.037*	<0.001*	0.240
PCB-153 (>LOQ vs. <LOQ)	Time	<0.001*	<0.001*	<0.001*	<0.001*
	PCB	0.203	0.544	0.598	0.729
	Gender	<0.001*	<0.001*	0.001*	<0.001*
	Time x PCB	0.491	0.518	0.946	0.861
	PCB x gender	0.338	0.738	0.115	0.586
	Time x PCB x gender	0.808	0.775	0.086*	0.102

* Main effects considered significant at $p \leq 0.05$, interaction effects at $p \leq 0.10$.

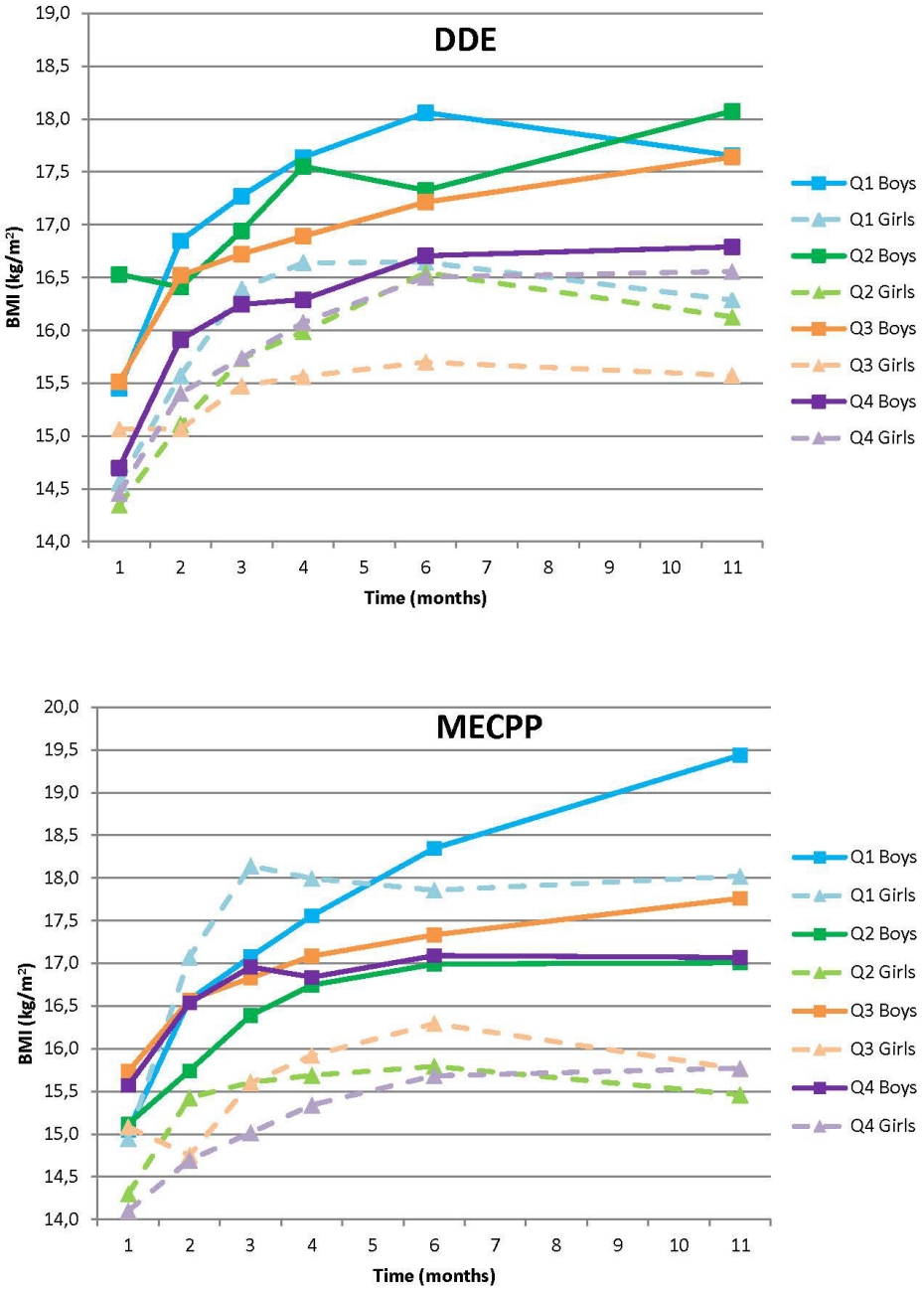

Figure 7.1 A-B. Sex specific BMI curves for early life DDE and MECPP exposure

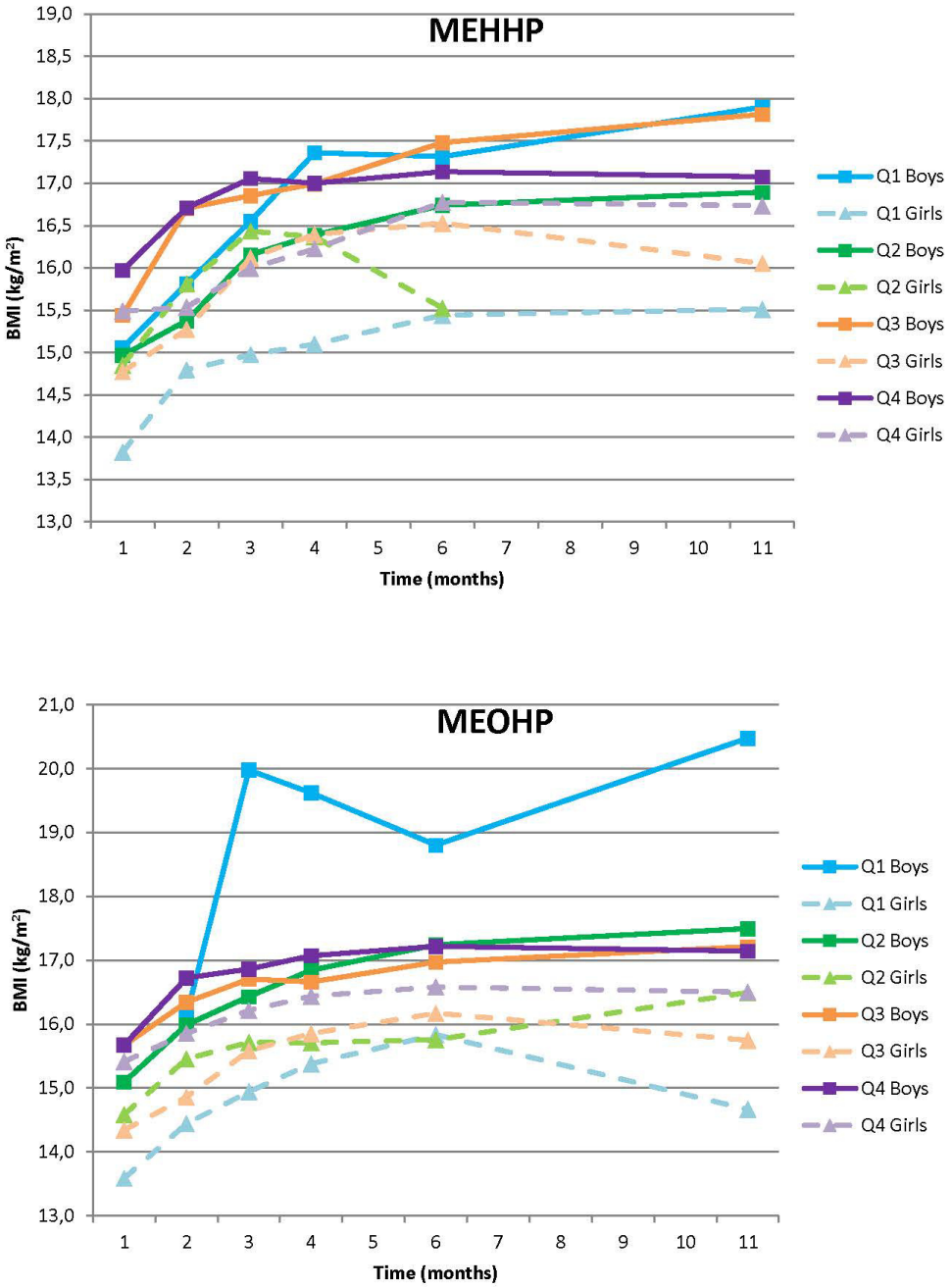

Figure 7.1 C-D. Sex specific BMI curves for early life MEHHP and MEOHP exposure

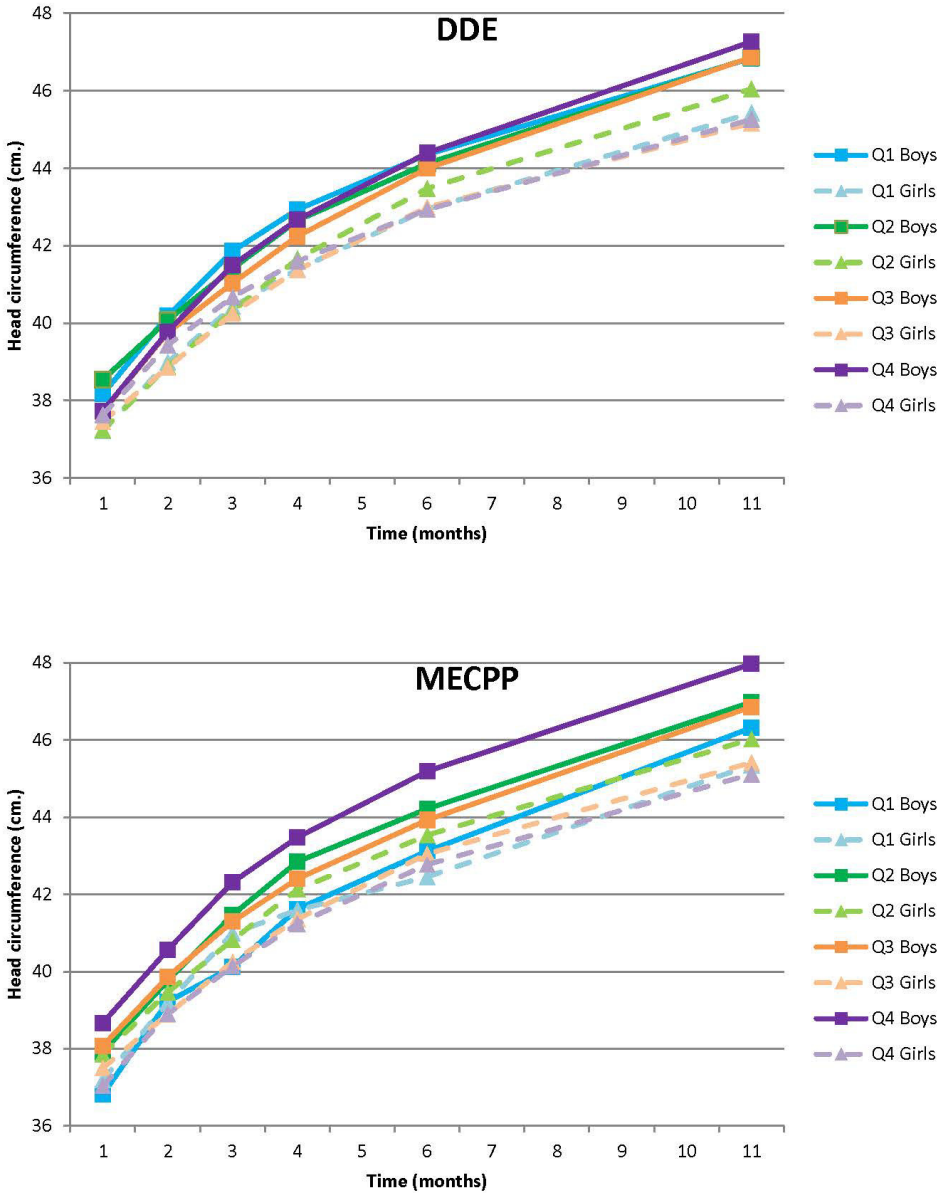

Figure 7.2 A-B. Sex specific head circumference curves for early life DDE and MECPP exposure

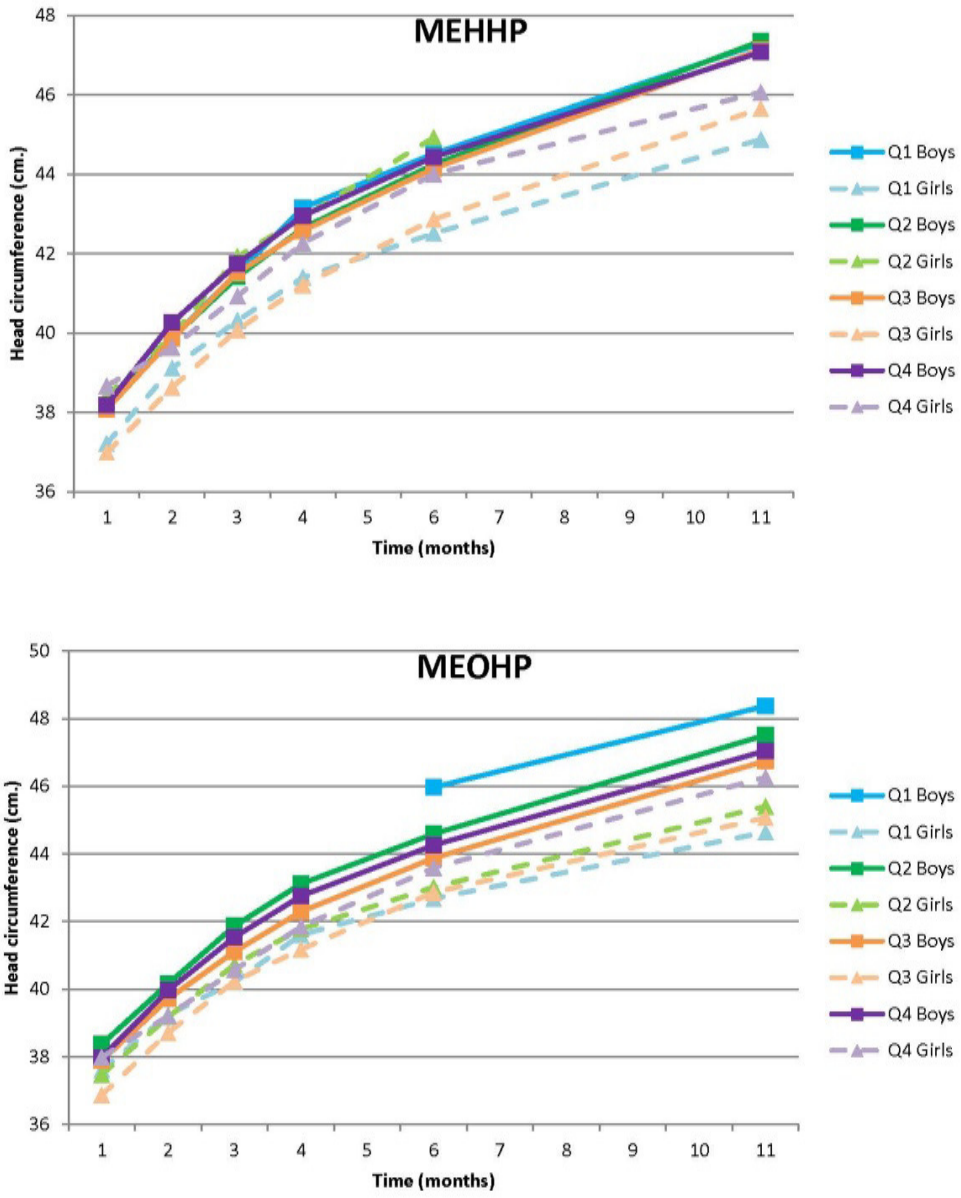

Figure 7.2 C-D. Sex specific head circumference curves for early life MEHHP and MEOHP exposure

Discussion

The objective of this study was to determine the possible associations between early life exposure to various EDCs and child growth in the first year of life. This is, to our knowledge, the first study to examine prenatal phthalate exposure in relation to growth in early childhood. Importantly, in our study we have measured phthalate metabolites in cord blood, whereas most studies use maternal urine as a proxy for prenatal exposure. Although our sample was relatively small, our analyses revealed some potentially relevant findings. Phthalate metabolites were associated with both BMI and head circumference, with low exposure levels associated with higher BMI values, and high exposure levels associated with a greater head circumference. Also DDE exposure was associated with BMI and head circumference, however patterns were less consistent. None of the compounds showed a linear dose-response relation with the outcomes included.

For all growth parameters effects of DEHP exposure were observed. Low exposure levels in particular were associated with higher BMI values. We furthermore observed continuous increases in BMI between 6 and 11 months of age for boys in the first and third exposure quartiles of all DEHP metabolites. Similar early life growth patterns have been observed for children who were overweight or obese when five years old, as observed in the study by Gittner et al. (2013) (9). Reference BMI curves of the World Health Organization generally show a decline in BMI in the second half of the first year (37). In this cohort, girls did show a stabilization of or a decline in BMI during the last six months. These results suggest that prenatal DEHP exposure may possibly predispose children, and perhaps boys in particular, to a more obese growth pattern. However, these results do need to be replicated in larger studies with more longitudinal follow-up as numbers in the current study are very low. Experimental studies have also indicated that maternal DEHP exposure in rodents leads to increases in body weight in offspring (38). The biological mechanism underlying DEHP effects on obesity has been proposed to be through its activation of peroxisome proliferator-activated receptors (PPAR), master regulatory genes in lipid metabolism and adipocyte differentiation (38, 39).

Early life exposure to phthalates was also related to changes in head circumference during the first year. In particular in boys and girls with the highest exposure to respectively MECPP and MEHHP exposure a higher head circumference was observed during the first year. We could not find any other studies which investigated this, except for a few papers which related prenatal exposure to head circumference at birth, but none of those showed any significant effect on head circumference at birth for MECPP, MEHHP, or MEOHP (40-43). For MEHHP exposure, we observed a range of 2 cm. across exposure quartiles at 6 months of age, and even though this difference was not significant, the absolute difference in itself is quite large as the range between P10 and P90 at this age in girls is 3.4 cm. according to the WHO reference tables (44). Follow-up of these children is essential to see if these variations persist throughout childhood and to clarify whether these results are relevant regarding neurodevelopment.

For DDE, a difference in BMI between exposure quartiles over time was observed. Boys highest exposed to DDE had a consistently lower BMI compared to their peers in the other quartiles, and for girls the third exposure quartile in particular showed less increase in BMI over time compared to the other girls. Several studies have reported no effect of prenatal DDE exposure on growth, including a cohort of Mexican boys with a median age of 18 months at follow-up (45), as well as another Mexican cohort with follow-up until 12 months of age (15). DDE exposure in both these cohorts was however much higher than in our sample. Interestingly, however, our study indicated that lower exposures to DDE were associated with an increase in BMI between 6 and 11 months of age. Similarly, in the Spanish INMA cohort, with DDE levels similar to this cohort, prenatal exposure was associated with rapid weight gain in the first six months, and a higher BMI at the age of 14 months (10). In Belgian children, prenatal DDE exposure was associated positively with BMI in 3-year-olds (27). Animal studies have indicated that long term, multigenerational exposure to DDT results in weight gain in offspring, though biological mechanisms have not been elucidated (46).

Effects for PCB-153, PFOS, and PFOA were less evident. For PCB-153 changes in height were significant over time and sex. These differences between groups were however only visible during the first three months, after which results became similar for all groups. For the other growth parameters no effect was observed. For head circumference a deviation for PCB-153 curves was observed compared to curves for other compounds, which showed a continuous increase in head circumference over time, while a decrease was observed for PCB-153 between four and six months of age. This is probably due to variations in the number of participants, which differs across quartiles as well as between compounds. The decrease was however insignificant.

For PFOS, no significant interaction effects were shown, and even though for PFOA exposure quartiles developed differently over time regarding height and head circumference, variation between quartiles was relatively small. Studies regarding prenatal PFAA exposure and childhood anthropometry are scarce. Halldorsson et al. showed a positive association with BMI for 20-year-old women who were prenatally exposed to PFOA (14). In the Danish National Birth Cohort inverse associations with BMI were seen for both PFOS and PFOA when the children were twelve months of age (47), however at seven years of age these associations were no longer apparent (18). More research is required to elucidate whether prenatal PFAA exposure affects growth in children.

Results of the current study showed some sex specific effects, which have also been observed in some previous studies. In a cohort of 7-year-old children from the Faroe Islands, associations between prenatal PCB and DDE exposure and BMI were only observed in girls, and in particular in girls who had mothers with a high pre-pregnancy BMI (48). In the INMA cohort, higher exposures to DDE were related to an increased risk for overweight, and this result was strongest in girls (12). Higher DDT exposure on the other hand, was associated with an increased risk for being overweight in boys only. As mentioned before, studies on prenatal phthalate exposure and obesity/overweight are not

available, however in a cross-sectional study, Hatch et al. reported that exposure to various phthalates, as determined in urine, was positively associated to BMI in males aged 20-59 years, and in females aged 12-19 years (49). Not all studies report results stratified for sex, and as these compounds are suspected to disrupt the endocrine system – which is specific for males and females – future studies should aim to include sex specificity in their results. Further experimental research would also be important to understand mechanisms underlying the sex specific effects of EDCs.

Various mechanisms are possible through which early life exposure to EDCs may affect anthropometry in a sex-specific fashion. Most EDCs are thought to have (anti-)estrogenic or (anti)androgenic properties. Studies in sheep have shown that prenatal exposure to testosterone is associated with lower birth weight, developmental changes in insulin-like growth factor (IGF)/IGF binding protein system, and insulin resistance (reviewed in (50)). In a recent study in which rats were developmentally exposed to the suspected androgen DEHP, female rats were observed to have impaired glucose tolerance and insulin secretion (51). Male rats showed increased serum insulin levels, and both female and male rats had a significantly lower birth weight than controls. Environmental exposure to chemicals may also affect anthropometry through interference with glucocorticoids. Increased maternal glucocorticoid levels have been associated with lower birth weight and altered set-point of the hypothalamic-pituitary-adrenal (HPA) axis (reviewed in (52)). Hydroxy-PCBs have been shown to have anti-glucocorticoid properties in human placenta (53), however in adipocytes BPA and dicyclohexyl phthalate (DCHP) activated the glucocorticoid receptor and increased lipid accumulation (54).

None of the compounds included showed a linear dose-response relation with any of the growth outcomes. This has however also been reported by other studies on early life EDC exposure and growth (14, 16, 55). Nonmonotonic dose-response relations have been reviewed by VandenBerg et al. (2012), who indicate that the endocrine system is designed to work at low concentrations and that low dose effects cannot be predicted from effects observed at higher doses (25). Whether the associations we observe are truly nonmonotonic remains to be clarified by larger studies.

Models were adjusted for birth weight, as birth weight in itself may predispose individuals to a more obese growth pattern. However, several studies have shown that prenatal exposure to certain EDCs is associated with birth weight (see meta-analysis by Govarts et al. (36)) and therefore birth weight may also be in the causal pathway between exposure and childhood obesity. Including this factor in the model would then lead to overcorrection. We therefore performed a sensitivity analysis for BMI without birth weight in the models (not shown), which did not affect any of the interaction effects, except between DDE and sex, which became significant, but no changes were observed in terms of relevance. For boys in the first quartile of PFOA exposure, and the third quartile of both PFOS and MEHHP exposure, a higher BMI was observed compared to the other groups if birth weight was not included in the models. These quartiles where however also relatively high when birth weight was included in the models.

A limitation of this study is the small sample size. As associations between outcomes and exposures were not linear, participants were divided in quartiles based on exposure levels. This also reduced the power. In addition, cut points were therefore not based on clinical relevance which might have obscured real effects or evoked non-relevant effects. However, no accepted cut-off level exists to our knowledge. Moreover we stratified results for sex, which reduced power of the tests. However, as growth differs between boys and girls and the chemicals in question may affect the endocrine system, stratification was necessary. Finally, our inclusion of covariates in the model could have potentially further reduced power. However standard errors were not affected by including them into the models. They were therefore included in order to reduce bias by confounding as much as possible. Another limitation results from the fact that we performed multiple tests which has inflated the risk of type I errors. We have chosen not to adjust our alpha for multiple testing, as this would further reduce the power of our statistical tests, but acknowledge that some of our significant findings may very well be false positive findings.
The strengths of the study are the prospective data collection, the large range of compounds included, and the fact that the cohort was very homogenous. Associations are therefore less likely to be confounded by demographic or socio-economic factors. The BMI of children, when four weeks old, was slightly higher compared to the general Dutch, Caucasian population. According to the fifth National Growth Study performed in 2009, median BMI at this age was 14.75 for boys, and 14.55 for girls, compared to 15.53, and 14.83 for boys and girls respectively in the current study (56). In one-year-olds, median BMI was 17.18 in boys, and 16.76 in girls in the general population, compared to respectively 17.14, and 16.21 in our cohort, indicating that girls had a slightly lower BMI at this age than would be expected. Furthermore, mothers in this cohort have more often received higher education compared to the general Dutch population (57), and the sex-ratio (1.7) of the offspring is higher than in general. 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. There is no indication that early life exposure to EDCs alter male/female ratio (58). Considering these factors, extrapolation of results to other populations should be performed with caution.

Conclusions

In conclusion, based on our study on first year growth, low exposure to phthalates may be associated with a higher BMI over time, which was reflected in both higher weight and height. Furthermore there may be an inverse association between DDE exposure and BMI. Results from our study should be interpreted with caution due to the limited sample size. Therefore confirmation of our results in larger studies is warranted. In addition there is a need for a longer follow-up to examine whether these associations persist into later childhood.

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Supplemental Material - First year growth in relation to prenatal exposure to endocrine disruptors – A Dutch prospective cohort study

Analysis of organochlorine pesticides and PCB 153

For the determination of the organochlorine pesticide 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 ($^{13}C_{10}$-PCB153 (from Cambridge Isotope Laboratories) 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 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 S7.1.

Table S7.1. 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

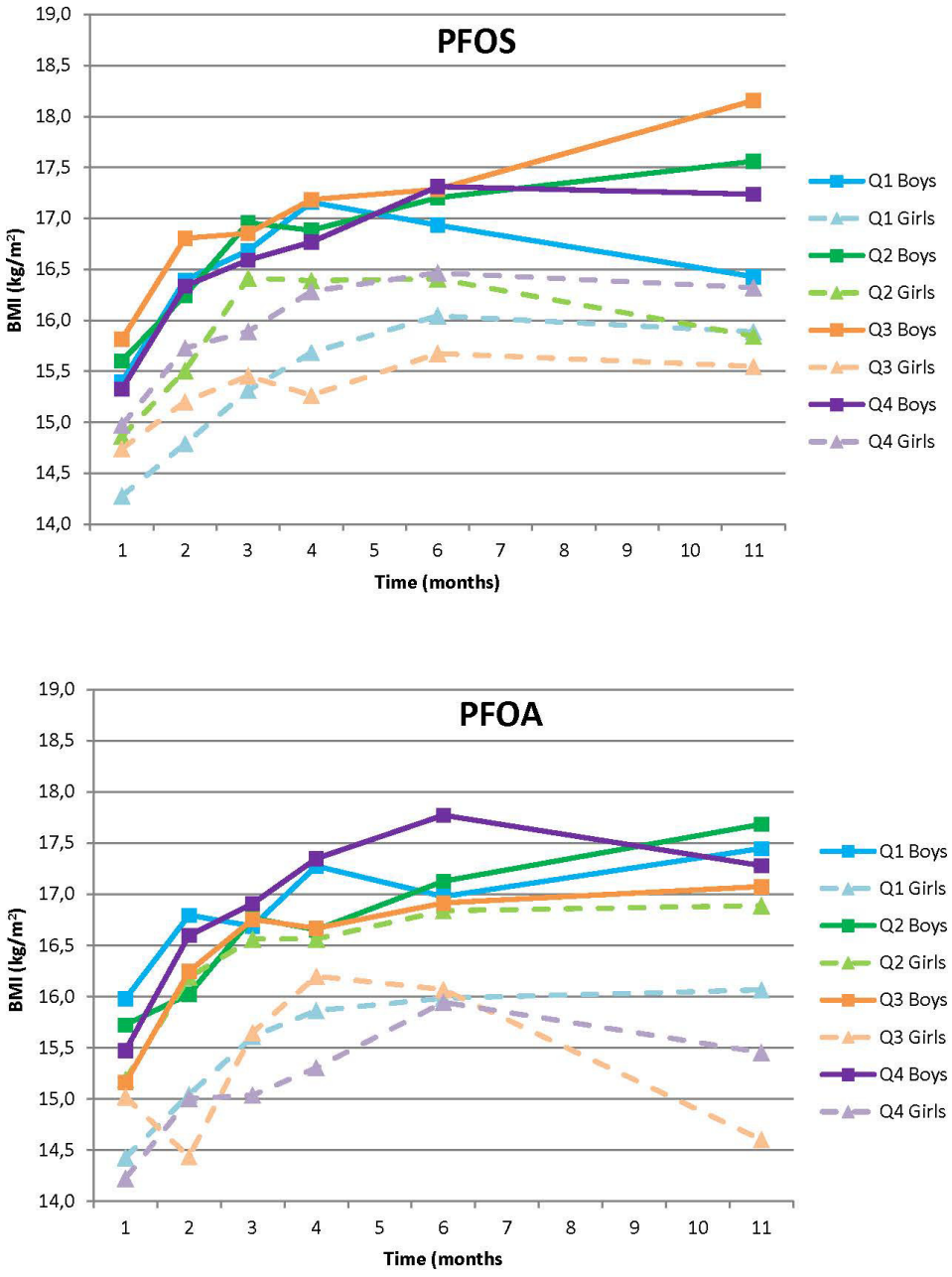


Figure S7.1 A-B Sex specific BMI curves for early life PFOS and PFOA exposure

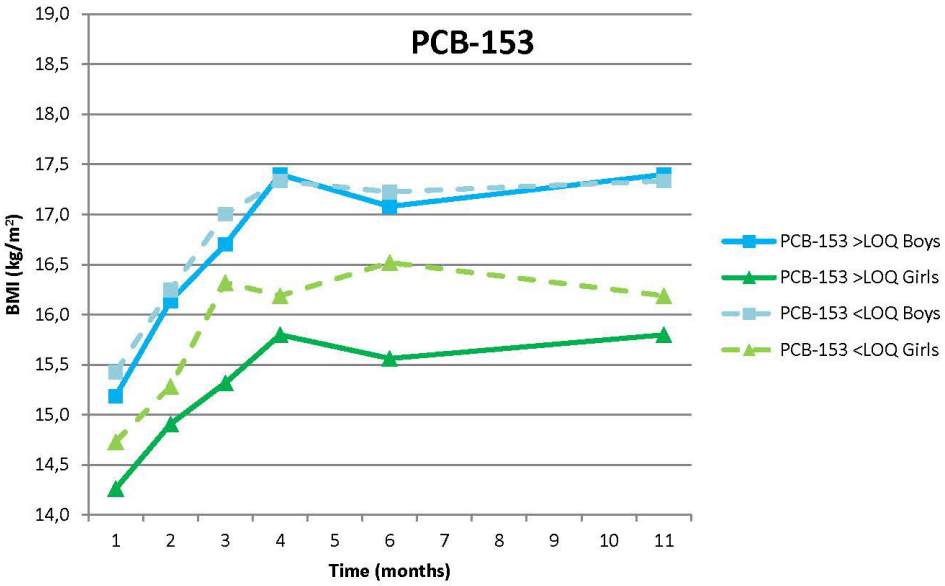


Figure S7.1 C Sex specific BMI curve for early life PCB-153 exposure

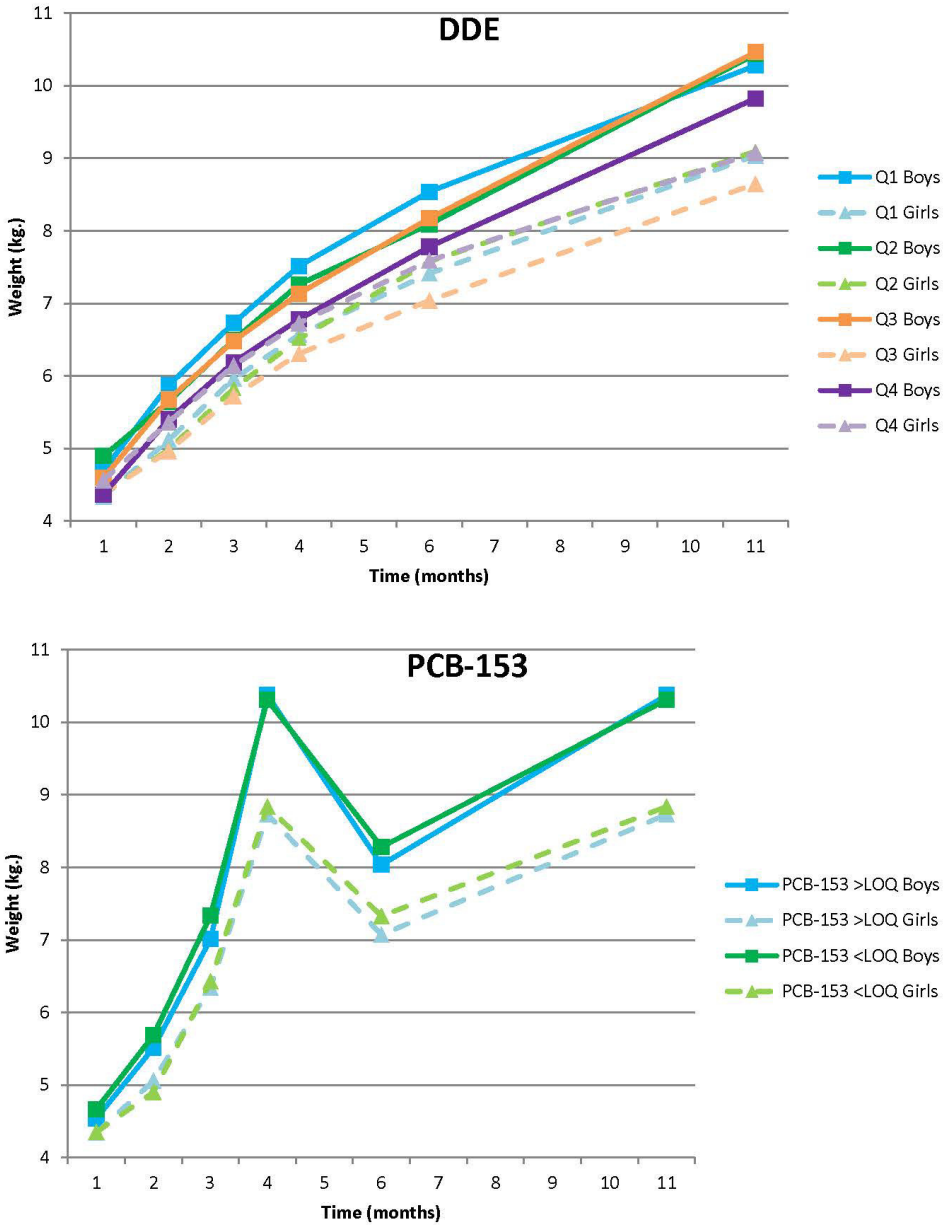


Figure S7.2 A-B Sex specific weight curves for early life DDE and PCB-153 exposure

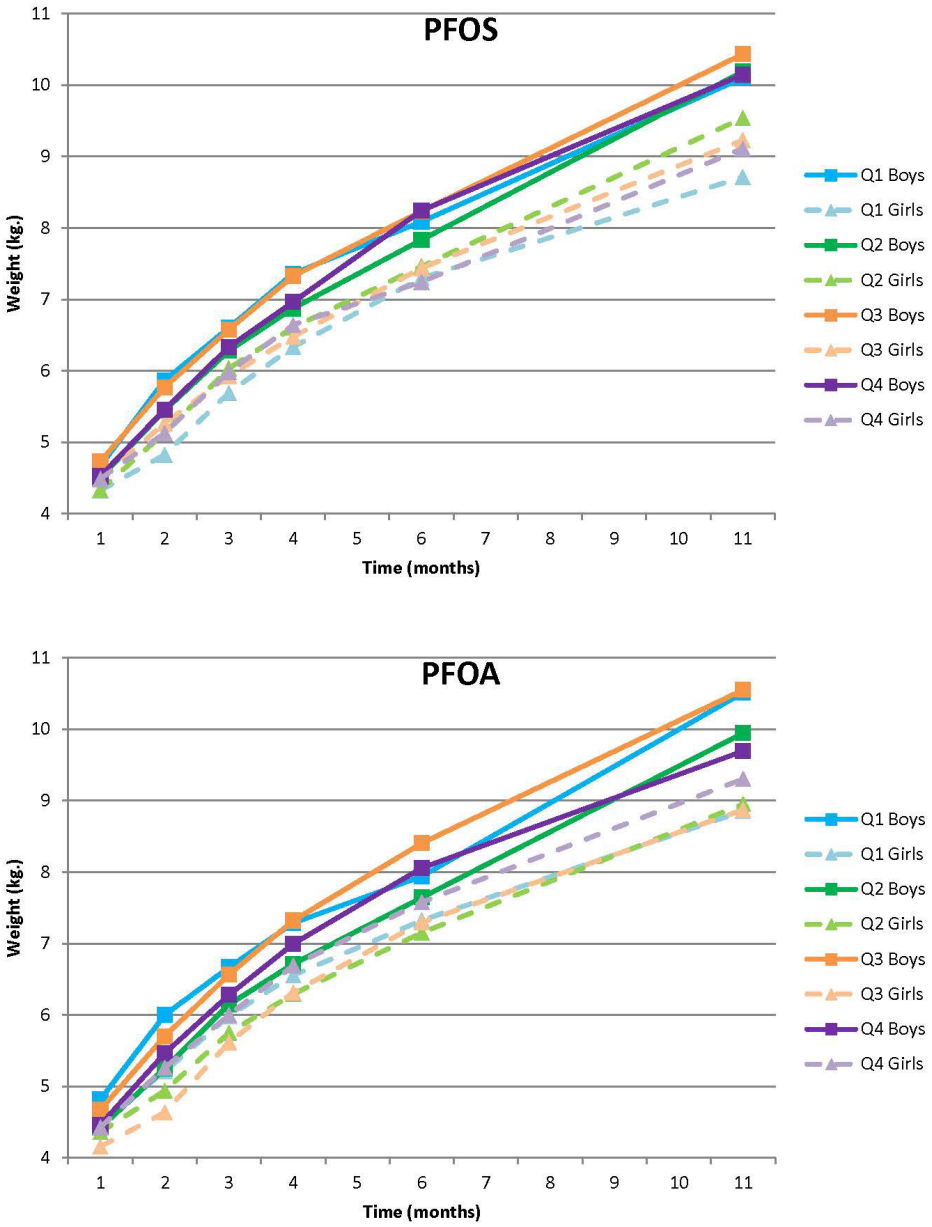


Figure S7.2 C-D Sex specific weight curves for early life PFOS and PFOA exposure

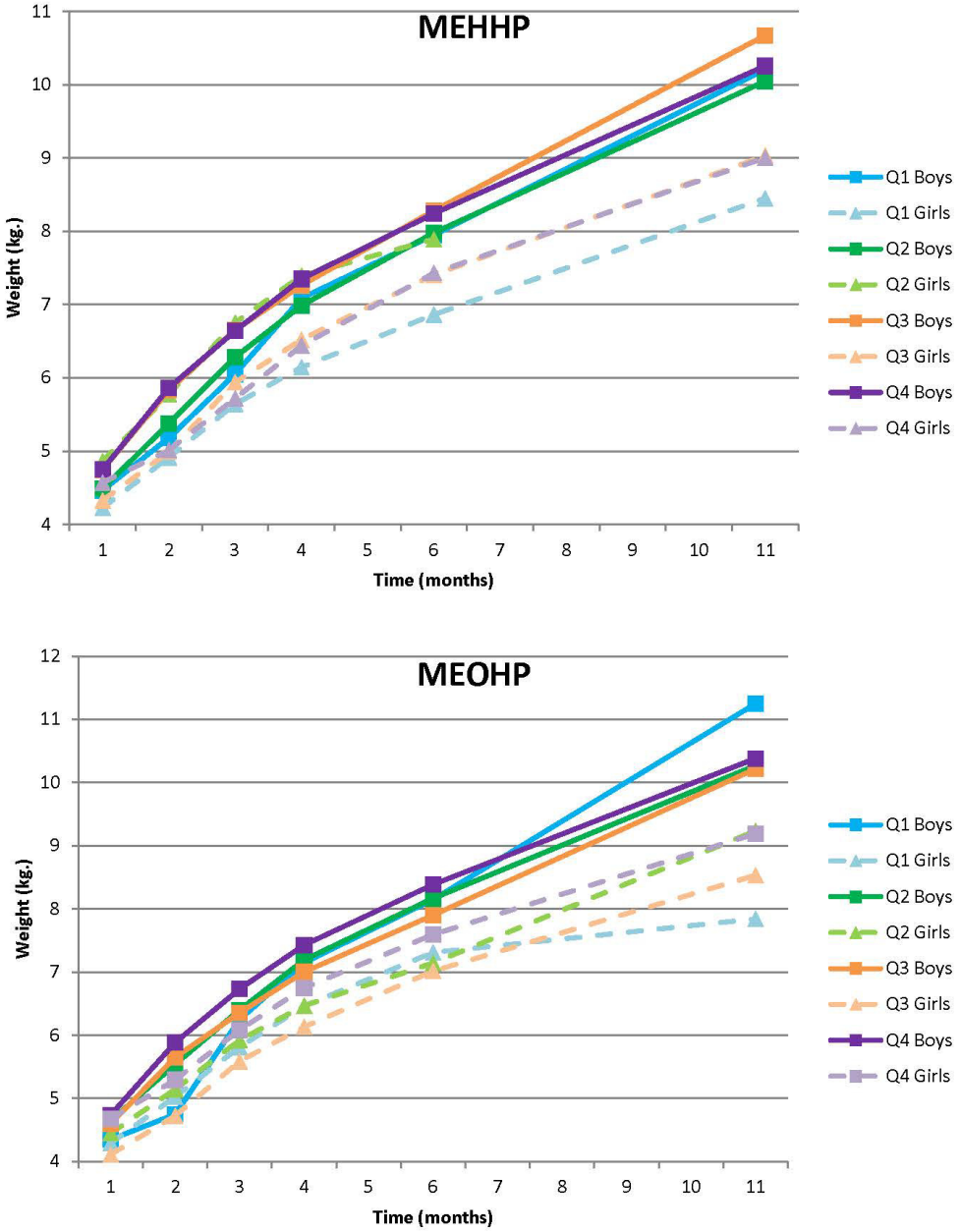


Figure S7.2 E-F Sex specific weight curves for early life MEHHP and MEOHP exposure

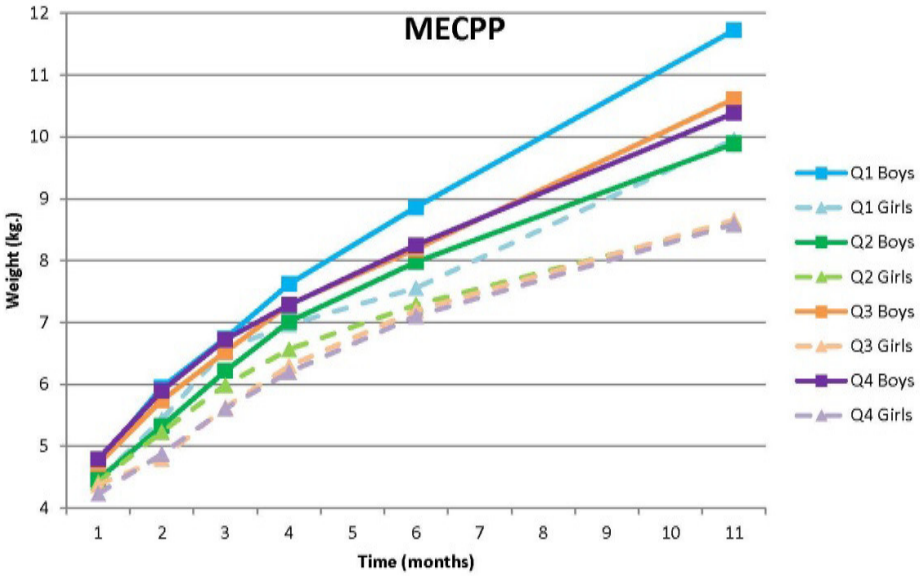
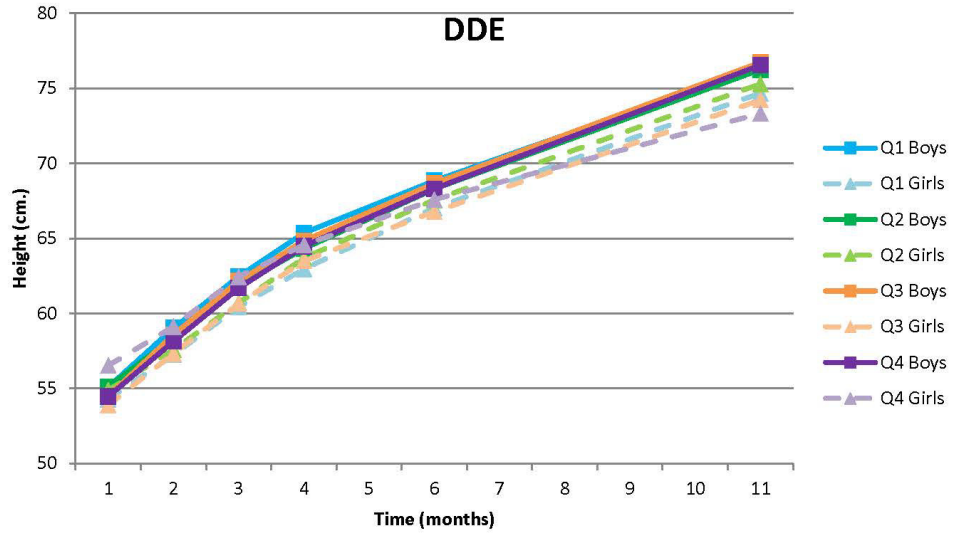


Figure S7.2 G Sex specific weight curve for early life MECPP exposure



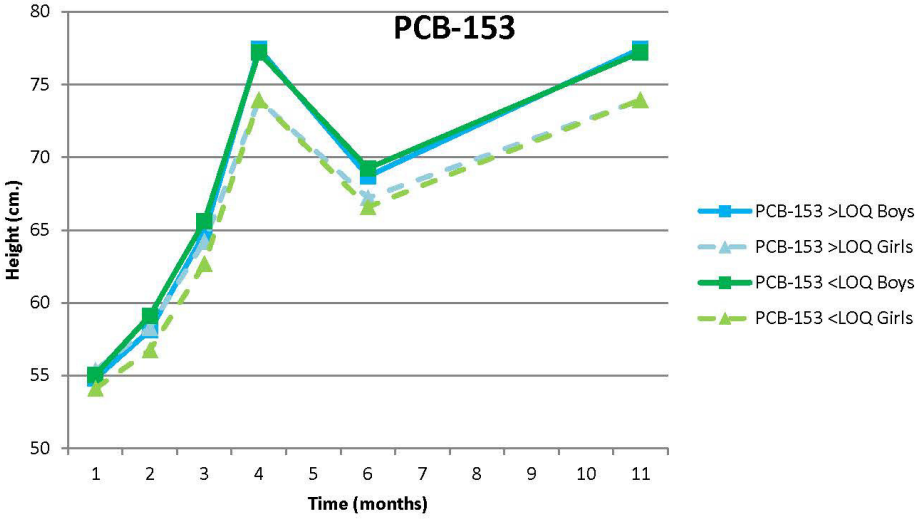


Figure S7.3 A-B Sex specific height curves for early life DDE and PCB-153 exposure

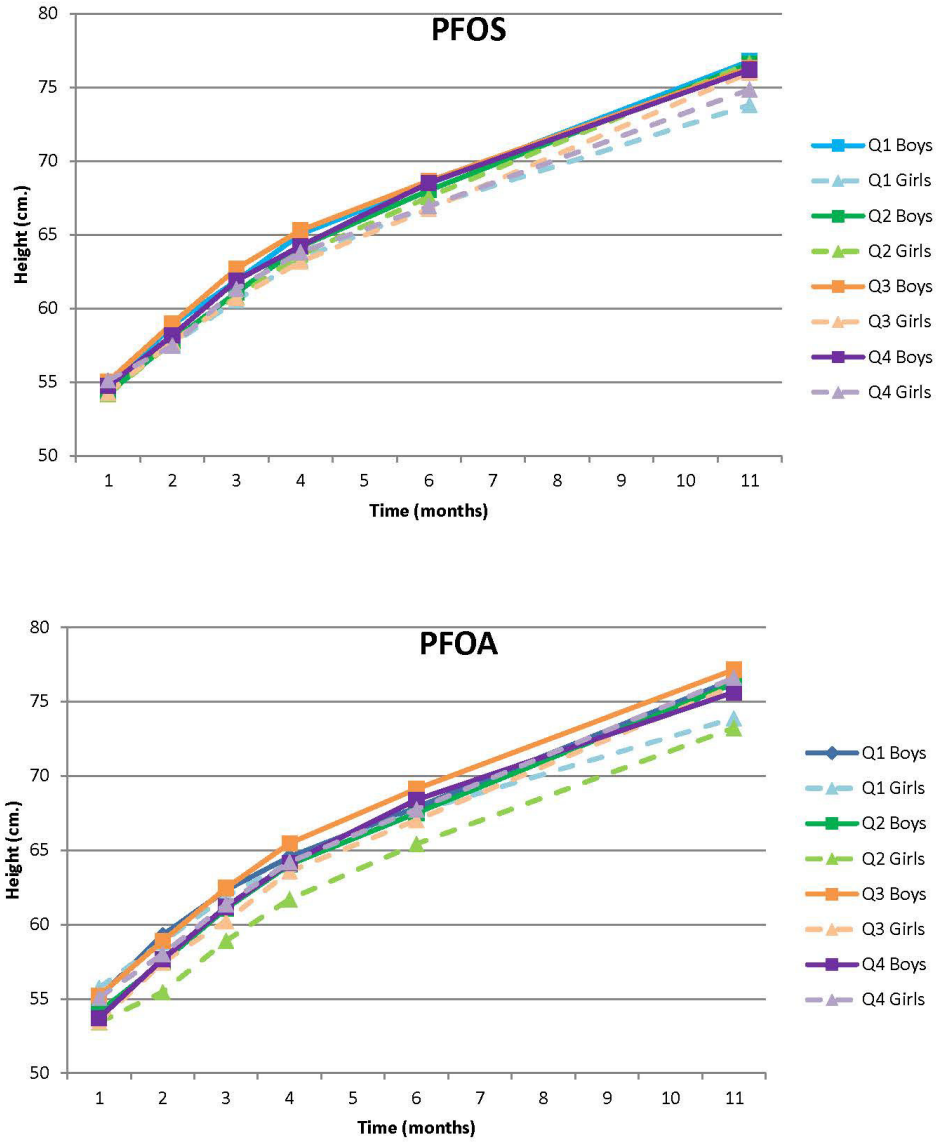


Figure S7.3 C-D Sex specific height curves for early life PFOS and PFOA exposure

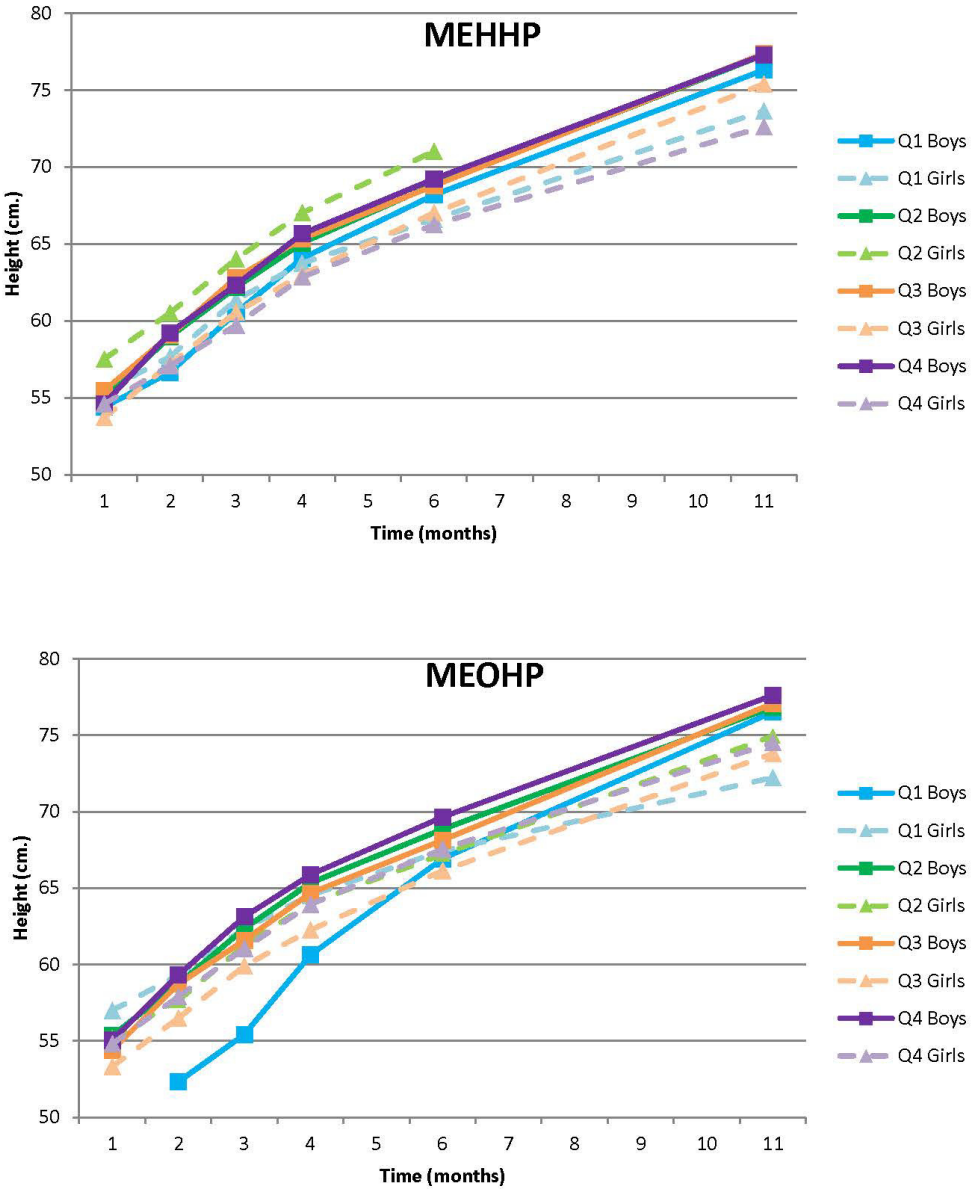


Figure S7.3 E-F Sex specific height curves for early life MEHHP and MEOHP exposure

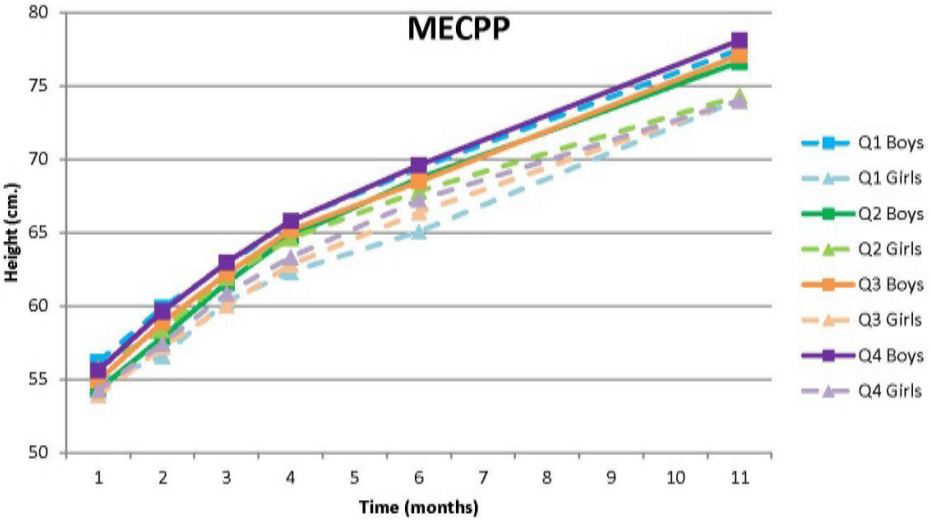


Figure S7.3 G Sex specific height curve for early life MECPP exposure

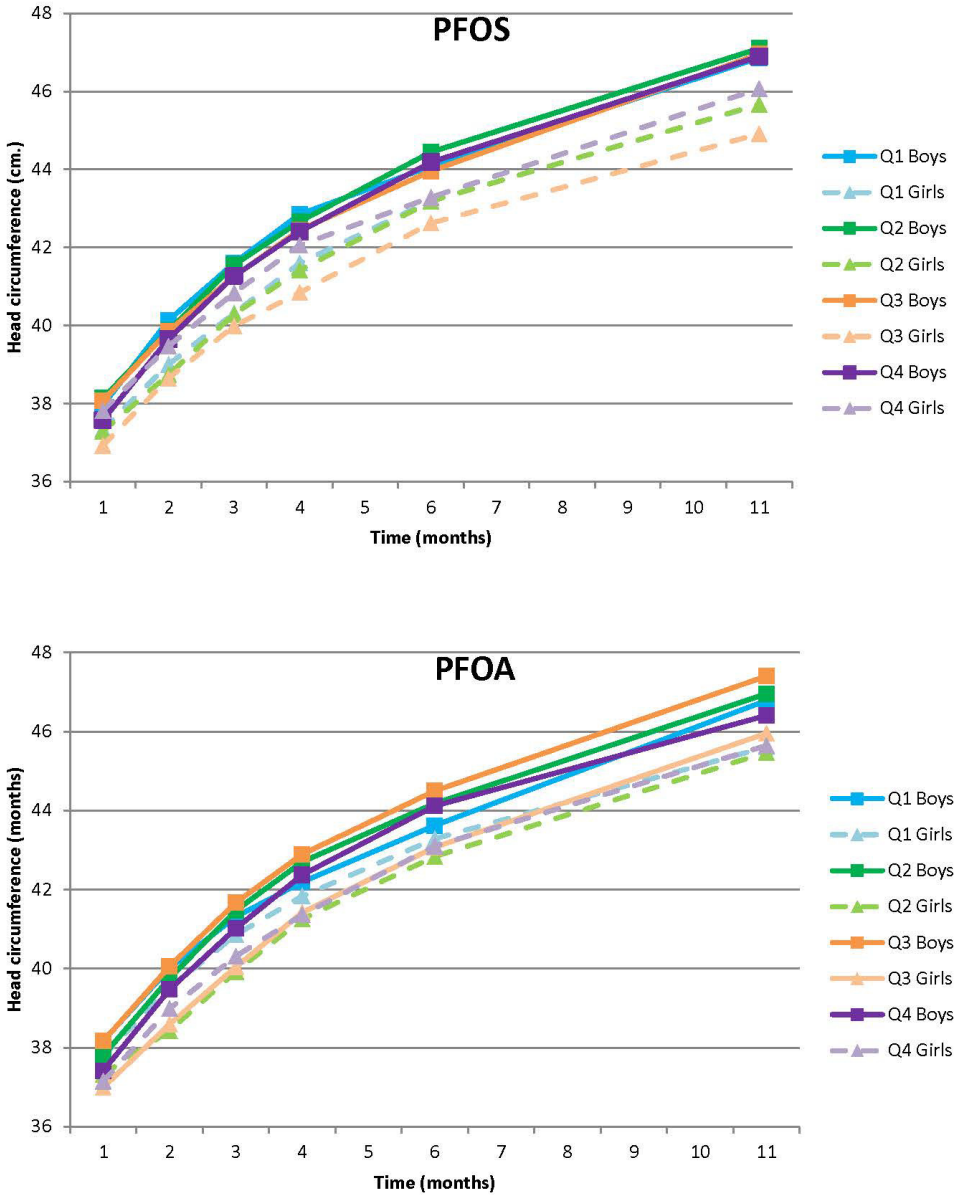


Figure S7.4 A-B Sex specific head circumference curves for early life PFOS and PFOA exposure

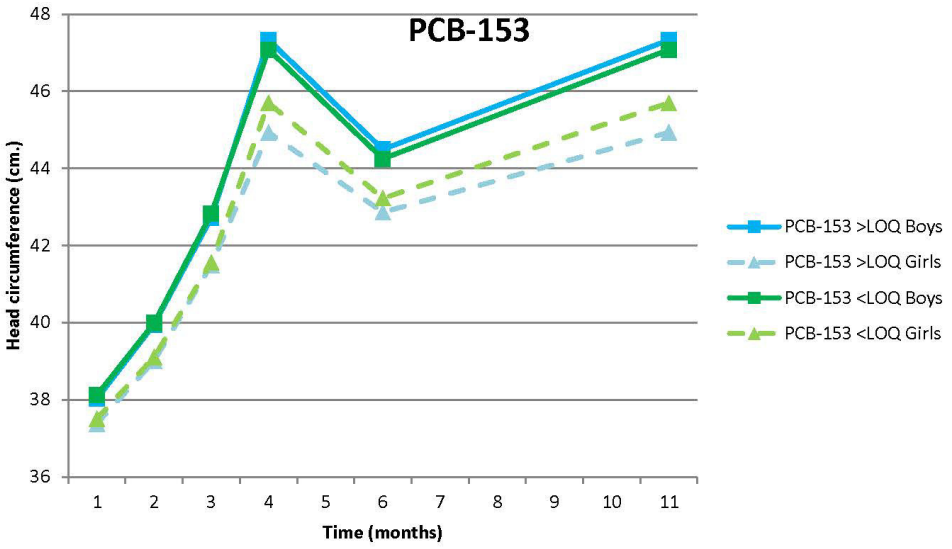


Figure S7.4 C Sex specific head circumference curve for early life PCB-153 exposure

References

1. Manirakiza P, Covaci A, Schepens P 2001 Comparative Study on Total Lipid Determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Bligh & Dyer Extraction Methods. Journal of Food Composition and Analysis **14**:93-100.

8

General discussion

The overall aims of this thesis were to relate exposure markers of endocrine disrupting chemicals (EDCs) to thyroid function and weight at birth as well as growth during the first year of life, in a newly recruited prospective cohort in the Netherlands. Girls with a relatively high exposure to dichlorodiphenyldichloroethylene (DDE) or perfluorooctanoic acid (PFOA) had higher thyroxine (T4) levels shortly after birth than those with low exposure levels. This was also observed for boys in the second quartile of perfluorooctanesulfonic acid (PFOS) exposure, however only in the partially adjusted model. No differences were observed for the other compounds. For girls with high PFOS exposure, and to some extent also for high DDE exposure, a higher birth weight was observed. MEOHP exposure on the other hand was associated with a lower birth weight in girls in the third quartile of exposure. Boys and girls with low MECPP exposure had a relatively high body mass index (BMI) during the first year after birth compared to their peers. This was also observed for boys with low exposure to MEOHP. Boys with a high MECPP exposure on the other hand had a higher head circumference during the first year than other boys, which was also observed for girls in the second quartile of MEHHP and DDE exposure.

Integration of results and implications for follow-up research

Results regarding birth weight, thyroxine levels, and first year growth were integrated in an overview (table 8.1), and show that quartiles in which participants had the highest birth weight according to our models, are often also the quartiles in which they had the highest BMI at 11 months of age. Accordingly, quartiles in which participants had the lowest birth weight, also tended to have the lowest BMI at 11 months of age. This was seen for DDE, MECPP, MEHHP, PFOS, and PFOA.

For DDE, the highest exposed group of girls had the highest birth weight, but also had the highest BMI at 11 months of age. A similar pattern was seen in boys in the second exposure quartile. Furthermore, these boys showed an increase in BMI between 6 and 11 months of age. This increase was also observed in boys in the third exposure quartile, who also had a relatively high birth weight compared to their lowest exposed peers. Moreover, we also observed that boys with the highest exposure had the lowest birth weights, and also the lowest BMI at 11 months of age, which was exactly opposite from our observations in girls in this quartile. This was also seen in the lowest exposure quartile in girls.

Girls with the lowest exposure to MECPP had the highest birth weight and also the highest BMI during the first year and at 11 months of age, which was increased compared to 6 months of age. The opposite was observed for girls with the higher MECPP exposure. For mono(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP) exposure in girls, results were opposite to those for MECPP exposure: the lowest exposure group had the lowest birth weight and the lowest BMI during the first year and at 11 months of age, while the highest exposure group had the highest birth weight and BMI at 11 months of age.

Table 8.1 Integrated overview of results within this thesis, showing highest (+) and lowest (-) levels of outcomes, as well as significance of results (colors)

Compound	Outcome	Boys				Girls			
		Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
DDE	Birth weight		+		-	-			+
	BMI during first year				-			-	
	BMI at 11 months		+		-			-	+
	Increase in BMI 6-11 months		+	+					
	Head circumference						+		
	T4	-	+			-			+
MECPP	Birth weight			+	-	+			-
	BMI during first year	+				+			
	BMI at 11 months	+	-		-	+	-		
	Increase in BMI 6-11 months	+		+					
	Head circumference	-			+		+		
	T4	+	-					-	+
MEHHP	Birth weight	-		+		-		-	+
	BMI during first year					-			
	BMI at 11 months	+		+		-			+
	Increase in BMI 6-11 months	+		+					
	Head circumference						+		+
	T4		-		+	+		-	
MEOHP	Birth weight			-	+		+	-	
	BMI during first year	+				-			
	BMI at 11 months	+				-	+		+
	Increase in BMI 6-11 months	+					+		
	Head circumference								
	T4		+	-			-	+	
PFOS	Birth weight	-		+		-			+
	BMI during first year								
	BMI at 11 months	-		+				-	+
	Increase in BMI 6-11 months		+	+					
	Head circumference								
	T4	+		-			-		+
PFOA	Birth weight	+	-			-		+	
	BMI during first year						+	-	
	BMI at 11 months		+				+	-	
	Increase in BMI 6-11 months	+	+						
	Head circumference								
	T4		+	-			-		+

\+ Highest birth weight/T4/BMI at 11 months of age. Consistently higher BMI/head circ. during first year

\- Lowest birth weight/T4/BMI at 11 months of age. Consistently lower BMI/head circ. during first year

Higher birth weight/T4 than Q1, fully adjusted model ($p<0.05$). Interaction BMI during first year ($p<0.10$)

Lower birth weight/T4 than Q1, partially adjusted model ($p<0.05$)

For mono(2-ethyl-5-oxohexyl)phthalate (MEOHP) the lower exposed girls had the highest weight at birth, as well as the highest BMI at 11 months of age. For boys, patterns are less clear. High MECPP exposure was related to a lower birth weight, and to a

relatively low BMI at 11 months of age. The exposure range in which the highest birth weight was found, did not show any extremes at later age, however for these boys an increase in BMI between 6 and 11 months of age was observed. For MEHHP in boys, exposure groups with the highest as well as the lowest birth weights had a relatively high BMI at 11 months of age, which furthermore also showed an increase between 6 and 11 months of age. For MEOHP in boys, no matches between highest or lowest birth weight and BMI during first year were observed.

Regarding the perfluorinated compounds, both boys and girls in respectively the third and the fourth quartile of PFOS exposure showed a high birth weight, as well as a high BMI at 11 months of age. For boys there was an increase in BMI between 6 and 11 months of age, which was not shown in girls. Furthermore, the lowest exposed boys had the lowest birth weight, and the lowest BMI at 11 months of age. For PFOA on the other hand, we only found a match between the exposure group with the lowest birth weight in boys, which had the highest BMI at 11 months of age, and girls with the highest birth weight, who had the lowest BMI at 11 months of age.

It is not unexpected to see these matches at both ends of our observations. Both high and low birth weight are risk factors for overweight later in life (1, 2), indicating that those with a high birth weight have a higher risk to stay in the upper percentiles of the growth curves, but that also those with a lower birth weight have a higher risk to end up in these percentiles. None of our cases were low birth weight babies, however it is likely that, rather than applying a cut-off, this type of development happens on a continuous scale.

There seemed to be no clear association between results for head circumference and other growth parameters. For two of the phthalates, MECPP and MEHHP, a higher head circumference during the first year was observed in respectively boys and girls, which coincided with a higher T4 level at birth. This finding was however not consistent for the other compounds.

Whereas there seems to be a quite clear pattern in matches between the ‘extremes’ in birth weight and the ‘extremes’ in first year BMI, the role of T4 in this pattern is less clear. For girls, the highest T4 levels were consistently observed in the highest exposure quartiles of each compound, with exception of MEOHP, where, according to the model applied, the highest T4 level was found in the third exposure quartile. Only for DDE the difference with the lowest exposure quartile was significant however. In boys we observed only for MECPP and MEHHP that the highest T4 levels are found in the highest exposed subjects. For the other compounds there seems to be no clear pattern.

Is T4 a potential mediator between early life exposure to EDCs and child growth? Observational studies which have both assessed both these parameters are scarce and often they determined T4 in cord blood. This measurement is less reliable than heel prick blood spots as cord blood levels may have been confounded by stress of the delivery. Our results do suggest that T4 may be a potential pathway for EDCs to affect growth, especially for DDE, and potentially also for MEHHP. Exposure quartiles which showed both a relatively high birth weight and BMI later in life, also had a relatively high T4 level. For DDE this

was independent of gender. Also for both of the perfluorinated alkyl acids, T4 may be involved, especially in boys, although for this compound high T4 levels at birth were matched with a relatively low weight at birth, while low T4 levels lined up with relatively high birth weights. Thyroid hormones are associated with metabolism and stimulate lipolysis and hepatic cholesterol biosynthesis and excretion, therefore stimulating weight loss (3). It may be that the perfluorinated alkyl acids at a certain dose enhance the effect of T4, therefore resulting in babies with lower birth weights, while DDE on the other hand may inhibit T4 activity, resulting also in high T4 levels but also in babies with a relatively high birth weight. However, this is mere speculation which requires substantiation from experimental studies.

As previously indicated, our models showed increases in BMI between 6 and 11 months of age for several compounds. This was in particular observed for boys regarding the DEHP metabolites. According to growth charts issued by the World Health Organization, usually a decrease in BMI is observed in this age period, and previous research has shown that this increase may be a risk factor for childhood obesity (4, 5). It is therefore essential to perform follow-up in these children at later age to see how growth patterns develop, and to determine the long-term consequences of prenatal exposure to DEHP.

Even though all children in the current study had T4 levels within the normal range, previous research has shown that even variations within the normal range were associated with outcomes such as attention deficit hyperactivity disorder (6, 7). Therefore it would most certainly also be interesting to follow these children regarding their behavioural development and to see whether early life exposure to these compounds affects behaviour, and if so, if this may be mediated even by relatively small changes in thyroid hormone levels.

Gender specificity

Gender specific effects have been observed for all health outcomes. This is not unexpected, even though levels of exposure to the selected compounds did not differ between boys and girls. The chemicals included in this study are suspected to disrupt the endocrine system, which is already gender specific in itself. The levels in which certain hormones are present in the body differ per sex, and chromatin structure and epigenetic marks, which both are important for gene expression, also differ between brain samples of males and females (8, 9).

Most endocrine disruptors are thought to have (anti-)estrogenic or (anti)androgenic properties. Studies in sheep have shown that prenatal exposure to testosterone is associated with lower birth weight, developmental changes in insulin-like growth factor (IGF)/IGF binding protein system, and insulin resistance (reviewed in (10)). Estrogen treatment in rats resulted in overexpression of the IGF-2 gene in the hypothalamus (11). IFG-I and II, and insulin are the main hormones responsible for growth during the fetal period (12), and

chemicals with estrogenic or androgenic properties may very well have similar effects. In a recent study in which rats were developmentally exposed to the suspected androgen DEHP, female rats were observed to have impaired glucose tolerance and insulin secretion (13). Male rats showed increased serum insulin levels, and both female and male rats had a significantly lower birth weight than controls, similar to what we observe for DEHP metabolites in our study. Placental exposure to organohalogenated xenoestrogens, including dichlorodiphenyltrichloroethane (DDT), was associated with increased birth weight in 14 month old boys but not in girls (14), which is similar to the results we observed for DDE.

Endocrine disrupting chemicals may also interact with various other factors in the body which may have gender dimorphic results. A key player in sexual differentiation of the brain is the enzyme aromatase (CYP 19), which converts testosterone to 17β-estradiol (E2). It is therefore mostly associated with the 'male direction' of brain development (15). Hany et al. (1999) observed that in male rats prenatally exposed to a polychlorinated biphenyl (PCB)-mixture, aromatase activity (AA) was reduced in the hypothalamus/preoptic area of the brain (15). This was also reflected in their behaviour, as treated males displayed higher preference for sweetness than controls, which is characteristic of female behaviour. AA was not determined in female offspring, however uterine wet weights were elevated, which is a marker for estrogenic activity. In a study by Shanthanagouda et al. (2013), developmental exposure to E2 was associated with decreased CYP19a1b expression in the brain of male rainbowfish, which would be in line with the observations from Hany et al. In the female brain however, CYP19a1b expression was increased. This may result in a more masculine development of the female brain. Not much is known about aromatase activity in humans and anthropometry, however in a case description of aromatase deficiency in an Indian girl it was reported that she amongst others presented with obesity (16). Obesity is also one of the characteristics of polycystic ovary syndrome (PCOS), in which women have an excess of androgens and dysfunctioning ovaria (17). Research has now indicated a possible link between various polymorphisms in the aromatase gene and the risk for PCOS, where some polymorphisms are associated with AA (18, 19), and aromatase inhibitors are currently under study as a treatment option for PCOS patients (20, 21). Experimental studies have observed that phthalates may also interfere with AA. Male rats which were exposed to DEHP in utero and during lactation, showed inhibition of AA at low doses, but increased AA at higher doses. In females increased AA was observed across all doses, and while AA in males seemed to be more affected at postnatal day (PND) 1 than in females, this was the opposite at weaning (PND 22) (22). Furthermore, in female rats prenatally exposed to MEHP altered mRNA expression of the aromatase gene was observed (23).

There are no indications we know of for gender-specific effects regarding disruption of thyroid hormones (24), however another potential factor which could be involved in gender-specific endocrine disruption affecting metabolism and weight homeostasis, is a group of recently discovered peptides referred to as kisspeptins (25). Although knowledge is still limited on the precise functions and pathways in which these peptides are involved, it is known that they are involved in the control of the hypothalamic-pituitary-gonadal

(HPG) axis (26) and therefore reproductive function. Kisspeptin is amongst others present in large numbers in the arcuate nucleus (ARC) of the hypothalamus (reviewed in (27)), and both in sheep (28) and humans (29) females are observed to have higher numbers of kisspeptin neurons in the ARC than males. This gender difference is however not observed in rodents (30). Estrogens are potent modulators of kisspeptin system (31), indicating a potential pathway for chemicals with estrogenic properties. Patisaul et al. (2009) report lower KISS fiber density in the ARC of female rats neonatally exposed to bishpenol A (BPA) (32). Prenatal PCB exposure on the other hand resulted in higher kisspeptin fiber density in the anteroventral periventricular nucleus (33) and increased kisspeptin receptor (GPR54) expression in the preoptic area (POA) of female rats (34). In male rats however a decrease in expression of GPR54 in the POA was observed (34). Furthermore kisspeptin is also expressed in adipose tissue. Administration of kisspeptin to adult male rhesus monkeys increased plasma adiponectin levels (35). Therefore kisspeptin may also be involved in adipocyte function. Most evidence regarding kisspeptin originates from experimental studies. However, in women with PCOS, levels of kisspeptin are higher than in controls, and in obese patients kisspeptin levels correlated with the free androgen index (36). Kisspeptin may therefore be an important factor in the metabolic problems related to PCOS, however it may also be that conditions of PCOS, such as excess androgens, affect the kisspeptin system.

Environmental exposure to chemicals may also affect anthropometry through interference with glucocorticoids. Increased maternal glucocorticoid levels have been associated with lower birth weight and altered set-point of the hypothalamic-pituitary-adrenal (HPA) axis (reviewed in (37)). Hydroxy-PCBs have been shown to have anti-glucocorticoid properties in human placenta (38), however in adipocytes BPA and dicyclohexyl phthalate (DCHP) activated the glucocorticoid receptor and increased lipid accumulation (39). Another regulator of growth is leptin (40), a hormone secreted by adipose tissue. Low leptin levels at birth have been associated with a higher risk for obesity and diabetes (41) , and there are indications for gender differences in leptin levels, with females having higher leptin levels than males (40). Though research on environmental exposures and leptin is scarce, it has been reported that prenatal exposure to diisobutyl phthalate reduced plasma leptin levels in male and female rats (42), and that DDE exposure increased leptin release from mature adipocytes (43). Sexually dimorphic effects have been reported for prenatal BPA exposure in mice, where no effect on leptin levels was observed in males but levels were increased in females (44).

We may conclude from the above paragraphs that due to the endocrine disrupting nature of certain chemicals, many potential pathways are possible through which exposure may result in gender-specific affects. It is therefore important that future studies, both observational and experimental, consider and incorporate this aspect in their designs.

Mixtures

We measured a variety of compounds with endocrine disrupting properties and assessed for each chemical individually the association with several health outcomes. The reality is however that humans are exposed to a mixture of chemicals on a daily basis and that peak exposure to a single compound is more likely to occur by exception. The compound by compound approach is not an accurate representation of our body burden and fails to take into account that one may have a relatively low exposure to compound A, but a relatively high exposure to compound B and C (45). Moreover, the mixture of environmental chemicals is a complex mixture (46), as we do not know exactly what chemicals we are exposed to.

Another reason why the compound by compound approach is not sufficient, is that mathematically adding effects of individual compounds will not always give a correct estimation of the actual physical effect. According to Hernandez et al. (2013), combining two or more compounds may result in either the compounds working independently from each other, or an additive effect, or an interaction effect (i.e. the combined effect is larger or smaller than the addition of individual effects) (47). This is illustrated by a study in which various mixture ratios of 30 androgen receptor (AR) antagonists were tested by means of a gene reporter assay (48). Each of the individual compounds was added in a concentration that would not have induced effects by itself. The mixture was however observed to illicit AR antagonistic effects, indicating why it is important to step beyond the compound by compound approach.

Thus far several studies have tested mixtures of EDCs, where the exact composition of the mixture was known. Some studies select chemicals in these mixtures based on their biological effect, such as the AR antagonists selected by Orton et al. (2013) (48). Flippin et al. (2009) exposed female rats for four days to a mixture of thyroid hormone synthesis inhibitors and stimulators of thyroxine clearance in the liver (49). The highest dose of the mixture resulted in a 45% decrease in serum thyroxine, and similar to the study by Orton et al., effect addition was the least accurate predictive model, resulting in overestimation of the observed effects..

The environmental chemical mixture is however likely to consist of chemicals with varying mechanisms, and combining chemicals with opposite effects may not always result in ‘no effect’ (50). It should furthermore be considered that combined effects of chemicals may also depend on the doses of the respective components. Hu et al. (2012) exposed Sertoli cells to various combinations of nonylphenol and monobutyl phthalate (51). At higher concentrations, a significant antagonistic effect on cell viability was observed, however at lower concentrations a synergistic effect occurred. These results complicate the testing of mixtures even further, as this would imply that the number of potential mixtures possible, as well as their effects, is limitless.

Several mathematical models have been designed to calculate mixture effects. Johnson et al. (2013) for example tested a model, aRP, which may be used for compounds which act

upon the same molecular target (52). They observed that for estrogenic or androgenic activity, the aRP model was more accurate that the toxicity equivalent factor (TEF) approach, though both methods correlated well. Furthermore, in 75% of the mixtures, modelled estimates were statistically comparable to actual measured responses. Also models based on dose addition, effect addition, or integrated addition are mostly used for compounds with a similar molecular target or mode of action (49, 53). This also presents a limitation to the use of models, however, predictive modelling may serve as a useful contribution to assessing toxicity of mixtures.

As the task of disentangling mixture effects may seem quite overwhelming, it may be more efficient to focus on effect biomarkers instead. These biomarkers should be able to reflect complex exposures as well as the accumulation of exposures over time (46). They are furthermore able to relate cause and (health) effect. Acetylcholinesterase inhibition by mixtures of organophosphate pesticides is an example of such a biomarker of effect, as it is a common mechanism for organophosphates (54). Gene expression profiles may also be of interest. As we suspect many chemicals to be endocrine disruptors, hormone levels may be valuable effect biomarkers as well. Identification of reliable and accessible biomarkers may be complicated, but it may also be a relatively fast approach contrary to, for example, case-control studies. This latter study-design would however also be an interesting option in approaching mixtures, as one can select subjects with a certain health outcome (cases) and compare their 'exposure profile' to subjects without this health outcome (controls). These types of studies are however time-consuming, especially for long-term effects of early life exposures, and may not be as informative regarding causality.

Strengths and limitations

Mixture effects are a complex problem and present a challenge to the field of EDCs which should not be underestimated. The current study analysed health effects in a compound-by-compound approach, as mixture effects were beyond the scope of this thesis. However, this approach certainly presents one of the limitations of the research presented here, and for follow-up of the LINC-cohort, the question of mixtures should also be addressed. Potential options may be found in using a – preferably validated - model to calculate mixture effects, and/or to include a larger variety of hormones in the measurements as biomarkers of effect.

Another limitation of this study was the low participation rate. Recruiting women to participate has proven to be a major challenge, as many find the study duration to be too long or consider the combination of this study with pregnancy (sometimes already having children) as well as labour and raising a child too much of a burden. Collection of cord blood from the women who did participate was also challenging as women in the Netherlands are offered the possibility of home delivery. Collection of samples in the home situation turned out to be quite a success, however in case of transfer of the mother to the hospital during delivery due to the clinical condition of mother and foetus, collection of the

samples often failed. This may have possibly been due to stress, or due to transfer of responsibility to an obstetrician. However, this does also imply that there was less risk of acute DEHP exposure, which is present in many medical devices, including intravenous tubing (55).

Furthermore we determined exposure for some participants in breast milk as cord blood was not available for all subjects. For DDE we decided on application of a conversion factor described in a publication of Govarts et al. (2012) (56), who established this factor based on data from a large number of European cohorts. This paper also provided a conversion factor for PCB-153, but due to the large number on non-detects, we decided to only include cord blood samples and to dichotomize this variable at the LOD. We considered breast milk mainly as an important matrix for compounds which accumulate, including PFOS and PFOA. Information on conversion factors was available for these perfluorinated alkyl acids, however as it was based on a small number of studies, we decided to perform sensitivity analysis including the highest, the lowest, as well as the median factor available from these studies. Applying either a higher or lower factor did significantly affect results however, and it was therefore decided to include only data from cord samples as there was too much uncertainty regarding which conversion factor to apply.

As mentioned before, participation rate was less than anticipated. The majority of the women who did participate had received higher education (bachelor or master degree), which is not uncommon for research populations. Furthermore, they were predominantly from Dutch or otherwise European origin, and they mostly had relatively healthy lifestyles (only a minority smoked or consumed alcohol during pregnancy). As a result the population was very homogenous, and results were therefore less likely to be confounded by demographic or socio-economic factors, which was one of the main strengths of this study. Moreover, we were able to determine early life exposure for most participants in cord blood. This matrix is most preferred for prenatal exposure assessment as it is material from the foetus or newborn as opposed to maternal blood or maternal urine during gestation. Furthermore collection of cord blood is non-invasive for both mother and child.

Using state of the art methodology, we were able to assess a large variety of endocrine disrupting chemicals, including PCB-153, DDE, and HCB, which have already been assessed quite frequently in other studies. This facilitated comparison of our population to other cohorts. A previous study by Patandin et al. (57) also measured PCB in Dutch children who were born between 1990 and 1992. They reported a median (range) sum PCB level of 0.40 µg/L (0.08 – 2.08), which included PCB's 118, 138, 153, and 180. This level is about 13 times higher than the level observed within the LINC-study, and even though we only included PCB-153, this may suggest that PCB levels are decreasing in the Netherlands. Furthermore the levels of PCB-153 and DDE observed in the LINC-study are also relatively low when compared to other European cohorts (56), even compared to Belgium and Germany.

Moreover we also determined exposure to three brominated flame retardants, however these compounds could not be detected in any of the cord blood samples and only in a few of the breast milk samples. This is similar to what has been observed in cohorts in Spain, Belgium, and France (58), which also had high percentages (86 – 99%) of samples below the limit of quantification for BFRs in cord blood. However, in the U.S., where PBDEs are still in use, levels are much higher (59). We also included phthalates and perfluorinated alkyl acids as thus far only few observational studies have included these chemicals. However, comparison is complicated as most studies have predominantly measured phthalate exposure in urine. One Chinese study of low birth weight infants observed a mean MEHP level of 9.94 mg/L in cord blood (60), which is about 300 times higher than the mean MEHP level reported here. However, measurements of MEHP may be hampered by contamination problems, and are therefore less reliable. Regarding the perfluorinated compounds, levels are lower but in a similar range compared to what has been reported in the large meta-analysis of European cohorts (58).

Another strength of this thesis is that we analysed the exposures in a similar, consistent manner for all health outcomes. This enables comparison and integration of the results as shown in table 1. However, power was limited due to the small sample size, and was further reduced by analysing participants in quartiles based on exposure levels, and by stratification of results for gender. It must be noted though that this approach was considered necessary as for none of the compounds a linear dose-response was observed, and sex-specific results were to be expected due to the nature of the endocrine system.

Future of the LINC study and societal impact

There are many challenges in the field of endocrine disruption, and the purpose of the LINC study is to address these challenges and to expand knowledge on the compatibility of chemicals and our health. Recruitment is currently still ongoing and two new locations, Purmerend and Den Helder, have been added. The purpose of these extra locations is to increase recruitment rate, as numbers are vital for the power of the study, as well as to increase heterogeneity. Though homogeneity was one of the strengths in this thesis, heterogeneity in demographics as well as geographical areas may also create more variety in exposure levels and will create the possibility to extrapolate results to other populations. The LINC study should furthermore aim to follow-up participants for a longer period of time, and to include more biological sampling to keep track of participants' body burden of certain chemicals. Moreover, to increase knowledge on mixture effects, a larger variety of hormones should be determined to serve as effect biomarkers.

The LINC study also has an import role to play for society, as there is currently still a lack of awareness about EDCs among the public and specific target groups, such as pregnant women and mothers of young children. Results of this study will therefore be communicated to various stakeholders and the public.

Evidence for certain health effects caused by specific chemicals is not conclusive. However, the European Union stands by the so called 'precautionary principle'. As stated on the website of the EU: 'The precautionary principle enables rapid response in the face of a possible danger to human, animal or plant health, or to protect the environment (61). In particular, where scientific data do not permit a complete evaluation of the risk, recourse to this principle may, for example, be used to stop distribution or order withdrawal from the market of products likely to be hazardous.' This implies that when there is reasonable evidence to assume certain chemicals are hazardous for human or animal health, this evidence should be enough to convince the EU to stop distribution or production of these particular chemicals. Indisputable human evidence will take cohort studies with several decades of follow-up, while time is precious, especially when it comes to our own health and that of our children. For many compounds, such as PFOA and DEHP, evidence is already compelling, and national governments as well as the EU have the duty to act on this evidence.

It should furthermore be acknowledged that use of chemicals in the world of today in inevitable. Industry has the responsibility to prove that chemicals are safe, however criteria for determining which chemicals are endocrine disruptors, do not exist. The industry should be provided with strict – though helpful - rules and guidelines regarding the testing of new chemicals. We currently state that for science the compound-by-compound approach is no longer adequate; however, in the chemical industry safety of chemicals is tested compound-by-compound. Science and industry should cooperate in improving techniques for testing of safety of chemicals. This may hopefully create more environmental as well as human friendly products.

Conclusions

The results of this thesis showed that children in the Netherlands are exposed to chemicals as early as before birth, and that these exposures are associated with various parameters of child growth at birth as well as growth during the first year of life, including head circumference. Results furthermore indicated that exposure to endocrine disrupting chemicals was associated with thyroxine levels at birth, a hormone which is essential for brain development. An important finding was that associations between exposures and health outcomes had a non-monotonic dose-response, and that results were gender specific.

The current challenge for research is to step beyond the compound specific approach, as we are exposed to mixtures of chemicals instead of single compounds. Though the precise mechanism needs further clarification, evidence that these chemicals affect our health is increasing. Awareness needs to be created among the general public as well as policy makers, in order for more strict safety regulations to arise. Furthermore scientists should cooperate with industry to improve the safety testing of chemicals.

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Summary

Rationale

Children are exposed to chemicals on a daily basis, mostly through everyday products and practices. Exposure starts, however, already as early as before birth, as chemicals present in the body of the mother may pass the placenta to reach the unborn child. Certain chemicals may disrupt the endocrine system (endocrine disrupting chemicals, EDCs), and early life exposure to EDCs may be particularly detrimental due to the vulnerability of the fetus. Hormones are involved in many processes during development, and endocrine disruption during this stage may have long-lasting health effects.

In this thesis an overview is given of literature on early life exposure to EDCs and effects on child growth as well as behavior. Furthermore research is presented on several environmental chemicals determined in a population of newborns in the area of Zwolle. In this population we examined if there were associations between levels of chemicals and growth in early childhood (birth weight, and growth during the first year after birth), as well as with thyroxine levels at birth. The latter was tested to examine the hypothesis that EDCs may affect growth through disruption of thyroid hormones.

What do we know from previous research?

Several studies have looked at the relation between prenatal or early postnatal exposure to EDCs and the prevalence of attention deficit hyperactivity disorder (ADHD) in children. Increased risks for ADHD or positive associations were found for amongst others polychlorinated biphenyls (PCBs), bisphenol A, and polybrominated diphenylethers (PBDEs). Moreover, low molecular weight phthalates were positively associated with externalizing behavior, which is also related to ADHD. Studies looking at autism are scarce, but those that have done so indicate positive associations.

It is also clear that for certain EDCs early life exposure may be associated with weight homeostasis later in life, however not necessarily in an obesogenic direction. Both positive and negative associations are observed between early life exposure and weight or height at various ages, including as early as 14 months, as well as until 20 years of age. In none of the included studies negative associations between perinatal exposure to EDCs and body mass index (BMI) were found and in several studies a positive association was observed.

Previous research also shows that dose-response relations appear to be nonmonotonic, meaning that an increase or decrease in exposure does not necessarily have to be accompanied with a similar in- or decrease in response.

Exposure to chemicals in children of the LINC study

Pregnant women from the area of Zwolle in the Netherlands were asked to participate in a study designed to determine early life exposure to EDCs and associations with child health. Levels of chemicals to which the child was exposed during pregnancy were determined in cord blood and breast milk. Information on lifestyle and various other factors was collected via questionnaires administered during pregnancy and during the first year after the child was born. Data on growth of the child was collected through youth health care centers which were visited by the parents seven times in the first year after birth.

The levels of PCB-153 and dichlorodiphenyldichloroethylene (DDE) observed in the LINC-study are relatively low when compared to other European cohorts, even compared to Belgium and Germany. A previous study also measured PCB in Dutch children who were born between 1990 and 1992. They reported a sum PCB level of about 13 times higher than the level observed within the LINC-study, and even though we only included PCB-153. This may suggest that PCB levels are decreasing in the Netherlands.

All three of the selected brominated flame retardants could not be detected in cord blood, however they could be measured in some of the breast milk samples. This is similar to what has been observed in cohorts in Spain, Belgium, and France, which also had high percentages (86 – 99%) of samples below the limit of quantification for BFRs in cord blood. Regarding the perfluorinated alkyl acids, levels are lower but in a similar range compared to what has been reported in the large meta-analysis of European cohorts.

EDCs and growth in children

When examining early life EDC exposure and growth in the sample of Dutch participants, effects were observed for weight at birth as well as growth during the first year after birth. High perfluorooctane sulfonate (PFOS) and high DDE exposure were associated with higher birth weight, although this was only observed in female children. Low exposure to mono(2-ethyl-5-carboxypentyl)phthalate (MECPP) was associated with a higher BMI during the first year, both in boys and in girls. Similar effects were observed for low mono(2-ethyl-5-oxohexyl)phthalate (MEOHP) exposure in boys. Moreover, for most compounds boys showed an increase in BMI between six and eleven months of age.

Exposure to DDE, MECPP, and mono(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP) was associated with head circumference during the first year after birth. High exposure to DDE was associated with a greater head circumference in boys, while the opposite was observed for girls. Also high MECPP exposure was related to a greater head circumference in boys. MEHHP exposure on the other hand seemed to mainly affect girls, in particular those with a relatively low exposure, who showed a greater head circumference than others.

Endocrine disruption - thyroxine

As experimental studies have shown that these compounds are endocrine disruptors, we tested whether levels of EDCs were associated with thyroid hormone levels at birth. Thyroid hormones are involved in various processes in the body, including metabolism. At birth, heel prick blood samples are collected from each child in the Netherlands to check for certain congenital disorders. This includes measurement of thyroxine, or T4. Positive associations with T4 were observed for DDE and perfluorooctanoic acid (PFOA) in girls, while for boys PFOS appeared to be of influence. When considering the results for T4 in light of findings for growth, it seems that results for DDE are most consistent. Exposure levels of DDE which were associated with both a relatively high birth weight and BMI later in life, also showed a relatively high T4 level.

Conclusions and future studies

We can conclude that children today are exposed to various chemicals as early as before birth. Even certain pesticides, which have been banned for several decades, can still be detected, although levels do appear to be decreasing. Associations between exposure levels and weight at birth as well as growth during the first year were observed. Moreover, association were observed with thyroxine, a hormone which is essential for metabolism as well as brain development. Results were sex-specific, and most associations between exposure levels and health outcomes had a non-monotonic dose-response.

Follow-up of these children is essential to see if effects observed during the first year, persist in later childhood. Boys, for example, showed an increase in BMI between six and eleven months of age for exposure to most compounds. According to growth standards of the WHO, generally a decrease in BMI is observed in this age period, and other studies have furthermore shown that this may be a risk factor for obesity in later childhood. Also regarding behavioral development, follow-up would most certainly be interesting, as previous research has shown that even variations in thyroxine levels within the normal range were associated with outcomes such as ADHD.

Future research should aim to disentangle mixture effects. Currently, most studies report effects compound by compound, which is not an accurate representation of reality. Furthermore awareness needs to be created among the general public as well as policy makers, in order for more strict safety regulations to arise. Scientists should cooperate with industry to improve the safety testing of chemicals.

Samenvatting

Achtergrond

Kinderen worden dagelijks blootgesteld aan chemische stoffen uit onze omgeving. Maar deze blootstelling begint eigenlijk al voor de geboorte, via stoffen die aanwezig zijn in het lichaam van de moeder en via de placenta bij het ongeboren kind kunnen komen. Bepaalde chemicaliën kunnen het hormonale systeem verstoren (hormoonverstorende chemicaliën, ofwel endocrine disrupting chemicals – EDCs), en vanwege de kwetsbaarheid van de foetus, vormt juist deze vroege blootstelling een risico. Hormonen maken deel uit van vele processen gedurende de ontwikkeling en hormoonverstoring in deze periode kan mogelijk langdurige gevolgen hebben voor de gezondheid.

In dit proefschrift wordt een overzicht gegeven van de literatuur over prenatale blootstelling aan EDCs en de effecten daarvan op groei en gedrag van kinderen. Daarnaast worden de resultaten gegeven van onderzoek naar EDCs dat is uitgevoerd bij pasgeboren kinderen uit de regio Zwolle. Bij deze kinderen is onderzocht of er verband was tussen blootstelling aan EDCs en groei in het eerste levensjaar en thyroxine (T4) gehaltes bij de geboorte. Dit laatste is bekeken om de hypothese te testen dat EDCs van invloed zijn op groei door verstoring van schildklierhormonen.

Wat weten we van voorgaand onderzoek?

Het verband tussen blootstelling aan EDCs rond de geboorte en de prevalentie bij attention deficit hyperactivity disorder (ADHD) is meerdere malen onderzocht. Verhoogde risico's of positieve verbanden zijn onder andere gevonden voor polychloorbifenyls (PCBs), bisfenol A, en gebromeerde vlamvertragers (PBDEs). Daarnaast werden ook positieve verbanden gevonden voor ftalaten met een laag molecuul gewicht en externaliserend gedrag, wat wordt gerelateerd aan ADHD. Tot nu toe is er nog weinig onderzoek gedaan naar verbanden met autisme, maar de huidige bevindingen wijzen in de richting van een positief verband.

Ook blijkt uit onderzoek dat voor bepaalde EDCs effecten op het gewicht worden gevonden, hoewel de resultaten niet altijd wijzen op een obesogeen effect. Zowel positieve als negatieve associaties met gewicht of lengte worden gevonden, op vroege leeftijd (14 maanden) en op volwassen leeftijd (20 jaar). Geen enkele studie heeft negatieve verbanden tussen blootstelling en BMI gerapporteerd en in meerdere studies is sprake van een positief verband.

Daarnaast blijken verbanden tussen blootstelling aan EDCs en de effecten op de gezondheid vaak nonmonotoon. Dit wil zeggen dat een toe- of afname in blootstelling niet noodzakelijk samen hoeft te gaan met een vergelijkbare toe- of afname van gezondheidseffecten.

Blootstelling aan EDCs bij kinderen van de LINC-studie

Zwangere vrouwen uit de regio Zwolle, Nederland, werden gevraagd om mee te werken aan een onderzoek over prenatale blootstelling aan EDCs en de effecten daarvan op de gezondheid van kinderen. De hoeveelheid chemische stoffen waaraan een kind was blootgesteld tijdens de zwangerschap werden bepaald in navelstrengbloed en moedermelk. Informatie over leefstijl en andere factoren werden verzameld via vragenlijsten tijdens de zwangerschap en gedurende het eerste levensjaar van het kind. Gegevens over groei van het kind werden verzameld via consultatiebureaus waar ouders zeven keer komen in het eerste jaar na de geboorte.

Gehaltes van PCB-153 en dichlorodiphenyldichloroethyleen (DDE) in de LINC-studie zijn relatief laag in vergelijking met andere Europese cohorten, zelfs in vergelijking met België en Duitsland. In een eerder onderzoek werden ook gehaltes gemeten van PCBs in Nederlandse kinderen geboren tussen 1990 en 1992. De som van PCBs was toen 13 keer hoger dan wat gemeten is in de LINC-studie, en hoewel wij alleen PCB-153 gemeten hebben, zou dit kunnen suggereren dat gehaltes van PCBs aan het dalen zijn in Nederland.

De drie geselecteerde gebromeerde vlamvertragers konden niet gemeten worden in navelstrengbloed, maar konden wel bepaald worden in sommige moedermelkmonsters. Dit is vergelijkbaar met wat wordt gerapporteerd voor cohorten uit Spanje, Belgie en Frankrijk, waar ook een hoog percentage van de monsters onder de kwantificatielimiet was. Gehaltes van perfluoralkylzuren waren lager maar vergelijkbaar met andere Europese cohorten.

EDCs en groei bij kinderen

Met betrekking tot het verband tussen blootstelling aan EDCs en groei, werden er verbanden gezien met zowel geboortegewicht en groei gedurende het eerste levensjaar. Hoge blootstelling aan DDE en perfluoroctaansulfanaat (PFOS) was geassocieerd met een hoger geboortegewicht bij meisjes. Lage blootstelling aan mono(2-ethyl-5-carboxypentyl)ftalaat (MECPP) was gerelateerd aan een hogere BMI gedurende het eerste jaar bij zowel jongens als meisjes. Vergelijkbare resultaten werden gevonden voor lage blootstelling aan mono(2-ethyl-5-oxohexyl)ftalaat (MEOHP) bij jongens. Daarnaast was er bij jongens voor de meeste stoffen een toename in BMI tussen 6 en 11 maanden zichtbaar.

Blootstelling aan DDE, MECPP, en mono(2-ethyl-5-hydroxyhexyl)ftalaat (MEHHP) was geassocieerd met hoofdomtrek in het eerste levensjaar. Hoge blootstelling aan DDE was gerelateerd aan een grotere hoofdomtrek bij jongens, terwijl het tegenovergestelde werd waargenomen bij meisjes. Ook werd een hogere blootstelling aan MECPP gerelateerd aan een grotere hoofdomtrek bij jongens. Lage blootstelling aan MEHHP daarentegen was juist bij meisjes aan een grotere hoofdomtrek gerelateerd.

Hormoonverstoring - thyroxine

Uit experimenteel onderzoek is gebleken dat de stoffen die onderzocht zijn in de LINC-studie een hormoonverstorende werking hebben. Daarom hebben we gekeken of ze geassocieerd zijn met schildklierhormoon bij de geboorde. Schildklierhormonen zijn betrokken bij allerlei processen in het lichaam, waaronder het metabolisme. Bij ieder kind in Nederland wordt na de geboorte via de hielprik gekeken naar aangeboren afwijkingen. Hierbij wordt ook thyroxine (T4) bepaald. In de LINC-studie werden positieve verbanden gevonden tussen T4 en DDE en perfluoroctaanzuur (PFOA) bij meisjes, terwijl voor jongens PFOS meer van invloed leek te zijn. Gekeken naar de resultaten van zowel groei als T4, lijken de bevinden voor DDE het meest consistent. Gehaltes van DDE die geassocieerd waren met zowel een hoog geboortegewicht als een hoog BMI in het eerste levensjaar, waren ook geassocieerd met een hogere T4 waarde rond de geboorte.

Conclusies en toekomstig onderzoek

We kunnen concluderen dat kinderen tegenwoorden worden blootgesteld aan meerdere chemicaliën en dat dit al zo vroeg als tijdens de zwangerschap gebeurt. Zelfs pesticiden die al tientallen jaren niet meer worden gebruikt, kunnen nog steeds worden gemeten. Blootstelling aan EDCs kon gerelateerd worden aan zowel geboortegewicht als groei gedurende het eerste levensjaar. Daarnaast werden ook effecten gezien op T4 gehaltes bij de geboorte, een hormoon dat betrokken is bij metabolisme en de ontwikkeling van de hersenen. Resultaten waren geslachts-specifiek en de meeste verbanden toonden een nonmonotone relatie.

Het is belangrijk dat deze kinderen gevolgd blijven worden om te zien of de huidige resultaten ook op de lange termijn zichtbaar blijven. Bij jongens zagen we bijvoorbeeld voor de meeste stoffen een toename in BMI tussen de leeftijd van zes en elf maanden. Volgens de WHO wordt in deze periode over het algemeen een daling in BMI gezien, en onderzoek heeft aangetoond dat deze toename mogelijk een risicofactor is voor obesitas bij kinderen. Ook wat betreft gedrag is het interessant om de kinderen te blijven volgen, omdat zelfs kleine variaties in T4 gerelateerd is aan uitkomsten zoals ADHD.

Een belangrijk doel van toekomstig onderzoek is het oplossen van ‘cocktail’ effecten. Momenteel rapporteren de meeste studies gezondheidseffecten per stof, wat geen correcte representatie is van de daadwerkelijke body burden. Daarnaast moet er meer bekendheid komen onder zowel burgers als beleidsmakers over dit onderwerp, zodat er striktere regelgeving kan komen. Wetenschappers moeten samenwerken met de industrie om de veiligheid van chemische stoffen te verbeteren.

Dankwoord

Dit proefschrift had niet tot stand kunnen komen zonder de hulp van velen.

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About the author

Marijke de Cock was born on May 20, 1985, in Hulst, the Netherlands,. After completing secondary school at het Reynaertcollege in Hulst, she studied Nutrition and Health at Wageningen University and Research Center. She received her Bachelor of Science in 2006 and completed the Master's degree in 2008. Part of her Master included an internship at Sara Lee International, and a research project on nutritional diversity in Kenya. After being employed as a junior researcher at Wageningen University, Marijke was accepted as a PhD-student on the OBELIX project at VU University in Amsterdam. She presented her findings at various international conferences, and analyzed data of multiple European cohorts. Furthermore, she supervised several students on a regular basis. Currently she is employed as a post-doc researcher for the DENAMIC project.