Early life exposure to endocrine disrupting chemicals and child health

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General introduction to thesis

During the past decades the Western world has been rapidly developing. Major technical changes have enabled improvements in many areas such as food production and health care. Life expectancy has increased with every new generation and is currently still increasing (1). However, the abundance in food as well as changes in activity patterns due to a more sedentary lifestyle have resulted in increasing prevalences of obesity worldwide, to the proportions of an epidemic (2). Though at first, obesity seemed to occur predominantly among adults, it is clear now that increasing numbers of children have to deal with the health consequences and social stigma of being overweight (3). Obese children have a higher risk for bone and joint problems, and are more likely to have pre-diabetes and risk factors for cardiovascular disease (4). Furthermore they are more likely to be obese when they reach adulthood (5), which increases their risk for health problems associated with adult obesity. Even though the average life expectancy of new generations is still higher compared to previous generations, it is believed that the high prevalence of obesity decreases disability free life expectancy (6).

Besides the marked increase in the prevalence of childhood obesity, the number of children diagnosed with autism spectrum disorder (ASD) or attention deficit hyperactivity disorder (ADHD) has also been increasing. For ASD a 23% increase in estimated prevalence between 2006 and 2008 was measured, as reported by the Autism and Developmental Disablilties Monitoring (ADDM) Network (7), and according to the most recent figures from the Centers for Disease Control and Prevention (CDC), one in fifty children in the United States is diagnosed with ASD (8). The CDC reported furthermore an increase of 22% in children with parent reported ADHD diagnosis between 2003 and 2007 (9). Both ASD and ADHD may significantly decrease the quality of life of the individuals concerned and their environment. The majority of the children diagnosed with ASD require special education and are not able to have common social interactions with others (10). Children with ADHD may experience more difficulties at school, both regarding their education and social contacts (9, 11). Adults with ADHD report to have a worse general health (11) and are more often absent at work due to illness (12).

The increase in prevalence of children diagnosed with a neurobehavioral disorder such as ASD or ADHD is often attributed to changes in diagnostic criteria and instruments used, especially regarding ADHD. However, as was stated by Sciutto and Eisenberg, there is no sufficient evidence to state that ‘factors contributing to the misidentification of ADHD in children systematically favour false positives over false negatives’ or that ‘ADHD is systematically overdiagnosed’ (13). Also the argument that childhood obesity is caused by energy imbalance seems insufficient to explain individual differences in weight gain (14). Clearly, other factors should be considered.

In 2002 a review by Baillie-Hamilton was published which showed remarkable similarities between the obesity epidemic and the production of chemicals from 1930 and onwards (15). After the second World War the use of chemicals such as pesticides became popular and innovations in research made it possible to produce chemicals which were only available in limited amounts in nature or which did not occur in nature at all (16), resulting

in a increasing global chemical output ever since (figure 1). The innovations in the chemical industry have contributed substantially to the modernization of our world, and it could be argued that the increase in obesity prevalence and the similarities with chemical production are in fact similarities with changes in lifestyle resulting from modernization of the environment; e.g. a lack of physical activity and a surplus of food. However it is known that exposure to several chemicals may adversely affect health. The use of dichlorodiphenyltrichloroethane (DDT) as an insecticide was banned from agricultural usage in the United States in 1972, after concerns about possible carcinogenic effects in humans as well as the effects on wildlife, such as eggshell thinning (17). Diethylstilbestrol (DES), a synthetic, estrogenic drug given to women between 1940 and 1970 to prevent miscarriage, was withdrawn in 1971 because of carcinogenic effects as well as an increased risk for infertility in children who were in utero exposed (18).

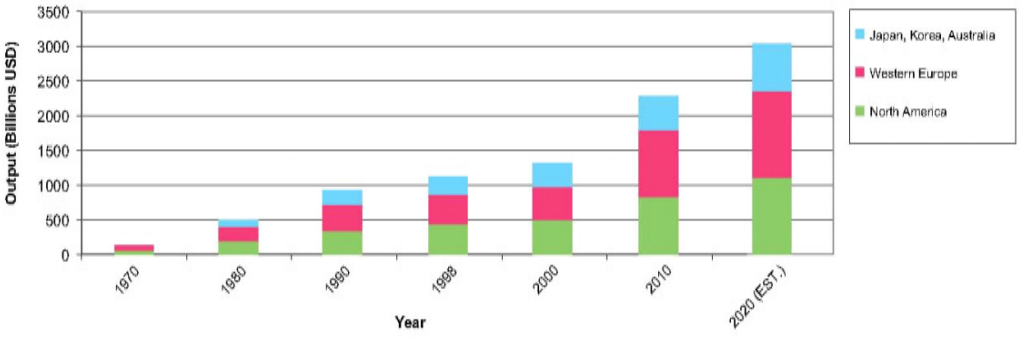

Figure 1.1. Chemical industry output, developed regions (19)

The example of diethylstilbestrol also illustrates another important concept, which is that in utero events may affect health later in life. This approach is referred to as the 'Developmental Origins of Health and Disease (DOHaD), and hypothesizes that many non-communicable diseases originate during development, as early as in utero and during childhood (20). This puts the health risks of chemical exposure in a new perspective, especially since research has shown that various chemicals may pass the placenta and reach the unborn child (21). The fetus is particularly vulnerable to the effects of environmental exposures as the prenatal period is characterized by periods of rapid cell devision and growth and as organs, including the brain, are developing (22).

The current hypothesis is that chemicals may interfere with hormonal functions in our body and that early life exposure in particular may have long-lasting health effects. These chemicals are therefore referred to as endocrine disrupting chemicals (EDCs), which are defined by the Inter-Organisation Programme for the Sound Management of Chemicals as 'an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations' (23). They may act on various parts related to the endocrine system, such as gene expression which may affect hormone production, and receptor binding which may

mimick or block hormonal activity. EDCs that 'inappropriately alter lipid homeostasis to promote adipogenesis and lipid accumulation, are also referred to as 'obesogens' (24).

A description of a subset of chemicals with endocrine disrupting properties will be given below. Furthermore the most commonly described pathways through which EDCs are suspected to promote childhood adiposity or to affect neurodevelopment will be given. These include peroxisome proliferator-activated receptor (PPAR), sex-steroid, thyroid hormone, and feeding circuit/leptin/insulin mediated effects. Also an outline of sources of EDC exposure will be given.

Sources and properties of EDCs

Several chemicals are currently known to have endocrine disrupting characteristics, and this list will be extending as research progresses. For the purpose of this thesis, sources and properties of five classes of chemicals will be described here.

Non-dioxin-like polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are generally divided in two groups, either dioxin-like PCBs, which have a dioxin-like mechanism of toxicity, or non-dioxin-like PCBs, including PCB-153 (figure 1). In total, 209 PCB congeners are possible, based on the number and position of chlorine atoms. Non dioxin-like PCBs were mostly applied as dielectric and coolant fluids, however due to health concerns, including potential carcinogenic effect, production of PCBs in Europe has been prohibited since 1985 (25). However, PCBs may still be released in the environment from old electrical appliances, and as they are furthermore known to be highly persistent in the environment, they may accumulate in fat tissue of animals and humans (26). The main source of exposure for people is through animal food (fish and meat), which is mostly contaminated with PCB-153 and PCB-138 (26). In Europe, dietary exposure to PCBs is monitored by the European Food Safety Authority (EFSA) (27).

Organochlorine pesticides – DDT and HCB

Dichloro-diphenyl-trichloroethane (DDT) and hexachlorobenzene (HCB) are part of group referred to as organochlorine pesticides. DDT was developed in the 1940s as an insecticide and was amongst others used to fight insect-borne diseases, such as malaria (28). Due to health concerns – DDT is carcinogenic – use of DDT was banned in the US in 1972 and in the Netherlands in 1973 (29). However, in developing countries DDT is still used for vector-control (malaria), which is currently also supported by the World Health Organization (WHO) (30). DDT is known to bioaccumulate and to be highly persistent in the environment. HCB is a byproduct of manufacturing processes of other chemicals, and has also been widely used as a pesticide. Due to probable carcinogenic effects, production

and use of HCB was prohibited by the Stockholm Convention (31). Like DDT, it accumulates in fat tissue and it persists in the environment.

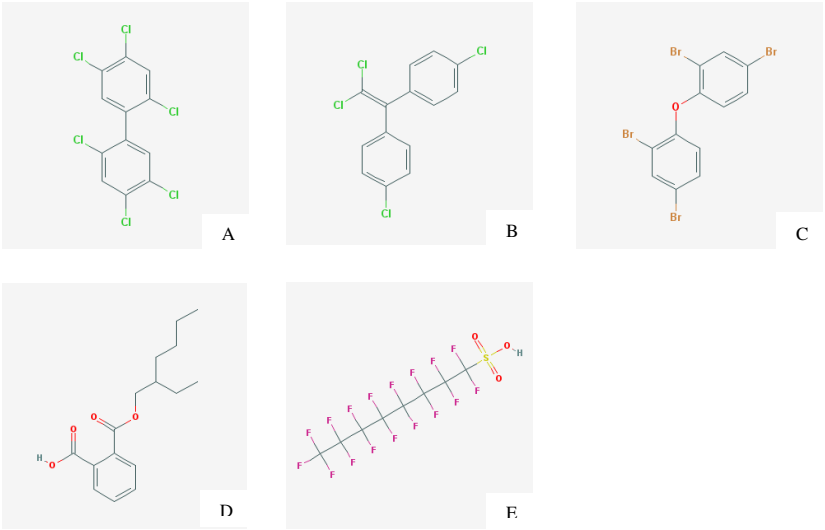

Figure 1.2 Chemical structures of A: PCB-153, B: DDE, C: PBDE-47, D: MEHP, E: PFOS

Brominated flame retardants

Brominated flame retardants (BFRs), including polybrominated diphenyl ethers (PBDEs) are chemicals used to prevent fires and slow down combustion time. They are for example applied in clothes, furniture, and electrical equipment, and are known to be persistent and to bioaccumulate (32). The Bromine Science and Environmental Forum (BSEF) estimated that in 2000 the annual production of BFRs was more than 200000 metric tons (33). Several BFRs, including BDE-47, BDE-99, and HBCD have PBT (persistent, bioaccumulative, and toxic) properties (34). BFRs are usually applied in mixtures and production of two common mixtures, pentaBDE (in which BDE-47 and BDE-99 are the most abundant) and octaBDE (including various BDE congeners which have on average 7.2 to 7.7 bromine atoms per molecule of diphenyl ether), has already been terminated in Europe (34). DecaBDE (BDE-209 as most prevalent congener) is also likely to be phased out soon.

Phthalates

Phthalates are used in plastics to increase flexibility, and are often referred to as plasticizers. They are also used as solvents and can be found in various products, ranging from vinyl on floors, to cosmetics and toys. Human exposure occurs mainly through diet

(35), as phthalates can be released from packaging material into the products. Phthalates are metabolized by the body and the metabolites usually pass the body through urine. They are not known to bioaccumulate (35). However, some studies to observe associations with health effects, and therefore also these chemicals are considered to be of concern.

Perfluorinated alkyl acids

Perfluorinated alkyl acids are fully fluorinated organofluorine compounds with a carboxylic acid or carboxylate functional group. They are used amongst others in surfactants, impregnation agents and water repellents, fabric protectors, and in fire-fighting foams. Two of these perfluors in particular, perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), are considered hazardous for health. PFOS was listed in annex B of the Stockholm convention, implying that its' use should be limited. In 2010 the use of PFOS was banned by the EU, with some exemptions of products such as hydraulic fluids for aviation (36). PFOA is currently considered by the EFSA 'a substance of very high concern because of its' CMR (carcinogenic, mutagenic, or toxic for reproduction) and PBT (persistent, bioaccumulative, and toxic) properties' (37). Various studies have determined the half-life of PFOA in humans, and periods from 2.3 to 8.5 years have been reported (37). As PFOA is not metabolized by the body and elimination rate is low, it accumulates in the body.

Mechanisms of action of EDCs

PPAR mediated effects

Peroxisome Proliferator-Activated Receptors, or PPARs, are a group of nuclear receptor proteins that are involved in control of cellular differentiation programs, but also in metabolism of lipids and carbohydrates by means of transcriptional control. Of the three varieties (α, β, and γ), PPARγ is considered most involved in regulating adipogenesis.

Agonists of PPARα promote peroxisome proliferation and fatty acid β-oxidation (38), therefore targeting of PPARα often has a hypolipodemic effect. Activation of PPARγ on the other hand, results in lipid biosynthesis and differentiation of preadipocytes into adipocytes (39). In vivo treatment with PPARγ antagonists prevented high fat diet induced weight gain in rodent models (40-43), and it is known that PPARγ agonist pharmaceuticals – used to improve insulin sensitivity in diabetes (44) – promote weight gain (45, 46).

There are several options through which endocrine disruptors may promote obesity through PPARγ. Usually a ligand is needed for PPARγ to bind co-activators, release co-repressors, decondensate the chromatin and activate transcription (47). However post-translational modifications, such as phosphorylation, may activate PPARγ in absence of ligand (48). EDCs may furthermore cause multipotent stromal cells (MSCs), cells which can differentiate into various tissues, to predominantly differentiate into adipose tissue (47, 49). This particular subset of MSCs expresses PPARγ (50). Trybutyltin, a known agonist of

PPARγ, has been shown to cause lipid accumulation in adipose tissues in prenatally exposed newborn mice (51). MSCs derived from the adipose tissue of these mice showed increased commitment to the adipocyte lineage compared to controls (52).

Besides from its' presence in adipose tissue, PPARγ is also expressed in the central nervous system, in particular in the area important for the central regulation of energy balance (53). Administration of the thiazolidinedione (TZD) Rosiglitazone (RSG) to rats resulted in higher food intake with a corresponding higher body weight change compared to controls (54). This effect was not observed after blocking of central nervous system CNS PPARγ receptors by an antagonist, indicating that activation of CNS PPARγ may result in higher food intake.

What also needs to be considered is that even though activation of PPARα stimulates lipid mobilization, it may also indirectly promote obesity. Prenatal activation may result in low birth weight, a known risk factor for obesity later in life. Furthermore, PPAR agonists and their metabolites may activate multiple PPAR isoforms. Bis(2-ethylhexyl)phthalate (DEHP), an EDC from the phthalate class, may activate PPARα, however its metabolite mono (2-ethylhexyl)phthalate (MEHP) may activate PPARγ (55, 56). And though lipid mobilization induced by PPARα activation requires continuous exposure, PPARγ may only need a single or episodic exposure to establish its effects in adipose tissues (43).

Recent research has also indicated potential for perfluorinated alkyl acids, such as perfluorooctane sulfonate (PFOS), to interfere with PPAR by inducing expression of PPARγ genes in mouse neonatal brain after prenatal exposure (57).

Estrogen receptor interference

Another potential route for endocrine disruptors to be involved in the aetiology of obesity is through estrogen receptors (ER). The main receptors, ERα and ERβ, are known for their function in reproduction, however they are also involved in brain development and several studies indicate their link to metabolism (58). For example, post-menopausal women often experience increase in white adipose tissue, an effect which can be reversed by estrogen supplementation (59). A similar observation was made in rodents with low oestrogen levels due to ovarectomy (58).

It is suggested that these effects are modulated mainly through ERα since in animal models in which ERα was knocked out (αERKO), no reversible effect of oestrogens on weight homeostasis was observed (60). Furthermore the balance of steroid hormones seems an important factor in weight homeostasis. Estrogen supplementation is an example of a correction of an imbalance occurring after menopause. Moreover in men who are treated for prostate cancer and in women who are treated for polycystic ovary syndrome with anti-androgenic therapy, weight gain is observed (43, 59).

Developmental exposure to estrogens exerts quite different effects than adult exposure, which was quite clearly demonstrated by Newbold et al. who exposed mice prenatally to diethylstilbestrol (DES) – a compound with estrogenic characteristics (61). At birth no effect on weight was seen in the low exposed group (1 µg DES/kg/day), but at later age the

exposed individuals gained significantly more weight than the unexposed. However it must be noted that in humans prenatal exposure to this compound does not seem to affect body weight in later life, as was observed in daughters of women who received prescriptions for DES to prevent miscarriage (62). On the other hand, Hatch et al. observed associations with preterm birth and higher risk for small for gestational age in a cohort prenatally exposed to DES (63), and being born small for gestational age (SGA) is a known risk factor for obesity later in life (64). Newbold et al. (61) also reported decreased birth weight in higher exposed groups of mice (10- 1000 µg/kg/day). In all groups weight remained decreased compared to controls throughout life, however in the highest exposed mice, low birth weight was followed by a period of catch-up growth during puberty, resulting in a significantly increased body weight compared to controls at two months of age.

Estrogens are also involved in brain development and estradiol in particular is important for dendritic growth, spine density, and synaptogenesis in the cerebellum (65, 66). This brain structure controls various behaviours, including attention, language, and motor control (67), which implies that estrogenic disruption in this area may affect neurodevelopment and therefore may be related to neurodevelopmental disorders. A recent study by Saili et al. showed that developmental exposure of zebrafish to either 17β-estradiol or GSK4716 (a synthetic estrogen which mainly targets ERγ), as well as BPA, resulted in hyperactivity in larvae and learning deficits in adult zebrafish (68). Even though evidence from experimental studies is scarce, Braun et al. also observed associations between gestational BPA exposure and externalizing scores in 2 year old girls (69), suggesting there is potential for exogenous estrogens to be involved in neurodevelopmental disorders.

Though steroid hormones such as estrogen are produced by the gonads, they can also be generated by the brain, including the cerebellum (70). One key enzyme in this process is cytochrome P450 aromatase (CYP19), which converts testosterone to 17β-estradiol (E2). Developmental exposure to estradiol has been found to decrease CYP19a1b expression in the brain of male rainbowfish (71), and aromatase activity was decreased in male rats prenatally exposed to a PCB-mixture (72). Other studies also suggest that aromatase activity may be affected by phthalates (73), DDE (74), and various pesticides (terbuthylazine, propiconazole and prothioconazole) (75), although findings need to be substantiated.

Thyroid hormone receptors

Also associated with metabolism and weight homeostasis, as well as brain development, are thyroid hormones, which at elevated levels accelerate metabolism, increase lipolysis and hepatic cholesterol biosynthesis and excretion, therefore stimulating weight loss (58). At lower levels the opposite effects are observed. They are furthermore essential for neurodevelopment as they regulate genes involved in myelination and neuronal cell differentiation (76)

EDCs have been shown to interfere with thyroid hormone (TH) receptors. Hydroxylated metabolites of PCBs were the first environmental chemicals discovered to bind to

transthyretin, a transport protein for T4, resulting in displacement of T4 (77). BPA has the ability to bind to TH receptor and has antagonistic properties, resulting in inhibition of transcriptional activity stimulated by triodothyronine (T3) (78). Furthermore BPA may enhance recruitment of corepressor N-CoR to the TH receptor, resulting in displacement of T3 from the TH receptor and gene suppression (79). Paradoxically, perinatal exposure to BPA may also result in increased levels of thyroxine (T4) (80). The latter effect was observed in offspring of dams exposed to BPA in drinking water during gestation and lactation (81). However, the mothers also experienced a decrease in free T4 compared to controls and therefore it was suggested that the increase in T4 observed in the offspring was a compensatory response.

Thyroid hormones may also be disrupted by brominated flame retardants, for example metabolites of polybrominated diphenyl ethers (PBDEs). Some isoforms have been shown to exert inhibiting effects for binding of T3 to the TH receptor (78). PBDE metabolites are also able to bind to transthyretin (82), which has also been observed for perfluorinated alkyl acids, such as PFOS and PFOA (83).

It should also be considered that thyroid hormones are important factors for brain development and that low T4 levels during development disturb normal maturation of the brain. Transthyretin is able to pass the placenta and delivers T4 across the blood-brain barrier. This implies that when binding PBDE metabolites or perfluorinated alkyl acids instead of T4, transthyretin may transport these compounds to the fetal brain compartment where they may accumulate (84). Even though discussion remains as to the importance of transthyretin compared to TBG for transport of thyroid hormones, developing foetuses and infants are especially sensitive to small changes in TH disruption (85, 86). In a cohort of 5-6 year old children, TSH correlated with worse neurophsychological functions, while T3 and T4 correlated with better outcome such as less ADHD and better behaviour (87). Furthermore, pentachlorophenol (PCP) was negatively related to T3, which would fit with the observed correlation between T3 and outcome. However, PBDE related positively to T3. Also in a study by Gascon et al., no significant associations between TSH, TT3, fT4 and postnatal levels of PBDE-47 were observed, nor did they significantly relate with neurodevelopmental outcomes (88).

Much remains to be clarified; however the role of thyroid hormones in metabolism and brain development and the potential disrupting abilities of some chemicals with regards to this pathway is significant, indicating that they may very well be involved in the aetiology of obesity as well as neurodevelopmental disorders.

Feeding circuits, lepin, and insulin

Regulation of energy balance is a basic concept in the aetiology of obesity. Several factors control appetite and energy expenditure, which are all integrated in the hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus is the centre for energy balance and appetite regulation. In adults, hormones such as leptin and insulin give signals to the arcuate nucleus of the hypothalamus (ARH), which in turn emits neuronal signals by means

of neurotransmitters such as pro-opiomelanocortin (POMC), cocaine and amphetamine-regulated transcript (CART), agouti-related peptide (AgRP), and neuropeptide Y (NPY). POMC and CART are known to decrease appetite, while NPY and AgRP are appetite stimulators. These peptidergic hormone factors are potential transcriptional targets for endocrine disrupting chemicals (43). It is for example known that estrogens may mimic actions of leptin on NPY (downregulation) and POMC neurons (upregulation), which would prohibit obesity development (89).

Not much is known on developmental exposure to EDCs in relation to later appetite regulation. The development of these feeding circuits starts prenatally. In rodents, generation of ARH neurons takes place between embryonic day 12 and 17. Development of projections of neurons to their targets on the other hand occurs only postnatally (90). Less is known about the development of hypothalamic feeding circuits in humans. Studies indicate that unlike rodents, in which development takes place partially postnatally, development in primates occurs completely prenatally (90) in the second and third trimesters (91).

Chemicals may also disrupt actions of leptin or insulin itself. It is clear that both leptin and insulin play important roles in development of feeding circuits which are comparable to sex steroid hormones with regard to the development of sexually dimorphic circuits (male, female) (92). Rats injected with insulin between embryonic day 15 and 20 (term is 22 days) were significantly more obese (93). Increases in leptin levels are observed during the first two weeks of life in rats (94). In this early period in life the need for energy is high and a leptin surge would be in contrast with this need. However, this surge coincides with a critical period in development of parts of the hypothalamus that control energy homeostasis, and it is therefore suggested that leptin is an important factor for brain development early in life rather than an anorexigenic factor (92, 94). In leptin-deficient mice also ARH circuit formation was affected (95). Leptin is also considered a regulator of fetal growth (96) and low leptin levels at birth have been associated with a higher risk for obesity and diabetes (97).

As a model for brain development, the pig and the sheep model are more comparable to human development than the rat model. In intra-uterine growth retarded (IUGR) newborn pigs lower leptin levels were observed in the circulation and in the placenta (90, 91). Furthermore changes in distribution of leptin receptors (OB-Rb) in the hypothalamus were observed (92). These receptors are usually located in the ARH, but in the IUGR newborn pigs expression was equal in the ARH and in the ventromedial premillary nuclei. This could cause lower sensitivity to leptin, resulting in altered food intake (92). As indicated before, EDCs can act on PPARγ. When PPARγ is activated by natural ligands, it inhibits OB-gene expression and leptin release in adipocytes (61, 93), resulting in decreased leptin levels (95). EDCs may exert similar effects via PPARγ and when this occurs during the fetal period, consequences for the hypothalamic development may be similar to what has been observed in IUGR pigs. This was confirmed by Boberg et al. who showed that prenatal

exposure to PPAR agonists diisobutylphthalate and rosiglitazone resulted in significantly lower leptin and insulin levels in fetal rats (98).

An overview of mechanisms of endocrine disrupting chemicals is given in table 1.1. These pathways will however not be investigated, as this is beyond the scope of this thesis.

Table 1.1 Mechanisms of endocrine disrupting chemicals

Target	Pathway	Effect	Chemical
PPARα	• Agonists stimulate lipid mobilization	• Prenatal activation may result in low birth weight	• DEHP
PPARγ	• Lipid biosynthesis, differentiation of preadipocytes in adipose tissue, inhibition of OB-gene expression • In CNS, PPAR is also found in areas important for regulation of energy balance	• Lower leptin levels prenatally, affecting distribution of leptin receptors in the hypothalamus • Activation of CNS PPARγ may result in higher food intake	• TBT • Pharmaceuticals • Phthalates • BPA • PFOS
ER	• Estradiol in cerebellum regulates dendritic growth, spine density, and synaptogenesis • Energy homeostasis	• Hyperactivity, learning deficits • Higher risk for preterm birth and SGA (human), low birth weight in mice followed by catch-up growth and weight gain	• DES
Thyroid hormones	• Binding to transport proteins (e.g. transthyretin) which may transport chemicals across the blood-brain barrier • Competitive binding to TH receptors with actual thyroid hormones	• Transport of chemicals across the blood-brain barrier • Displacement of actual TH from receptor •	• BPA • PBDE • PFOS/PFOA
Leptin		• IUGR associated with lower leptin levels • Low leptin levels at birth associated with higher risk for obesity	• Indirectly through PPARγ agonists

Outline of this thesis

It can be concluded that exposure to chemicals, particularly early in life, may be involved in the programming of childhood obesity and therefore also obesity later in life. Exposure may furthermore affect neurodevelopment and may therefore be related to behavioural disorders.

In this thesis the effects of early life exposure to five classes of endocrine disrupting chemicals on child health during the first 12 months of life are investigated. The focus will be predominantly on outcomes related to child growth, but also the potential for EDCs to be involved in the aetiology of neurodevelopmental disorders will be explored.

Firstly, an overview is given of current literature on early life exposure to EDCs and neurodevelopmental disorders such as attention deficit hyperactivity disorder and autism spectrum disorders (**chapter 2**), as well as childhood obesity (**chapter 3**). This is followed by a description of the LINC (LInking EDCs in maternal Nutrition to Child health) study, which was designed to study health effects of early life exposure to EDCs in a cohort of mother-child pairs in the Netherlands (**chapter 4**). The LINC study was started as part of the OBELIX project (OBesogenic Endocrine disrupting chemicals: LInking prenatal eXposure to the development of obesity later in life), which was an European Union funded research to study obesogenic effects of EDCs in both animal and human studies.

Chapters 5 and **6** describe the associations between early life EDC exposure and respectively weight and thyroid hormones at birth, as they were observed in the LINC study. Effects on growth during the first year after birth are discussed in **chapter 7**.

Finally, in the general discussion (**chapter 8**), results from chapters 4 to 7 are summarized, and a general reflection on the main outcome is given. Potential pathways and methodological challenges are addressed, and recommendations for future research are given.

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2

Does perinatal exposure to endocrine disruptors induce autism spectrum and attention deficit hyperactivity disorders? Review

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Abstract

Aim: To provide an overview of studies on perinatal exposure in humans to EDCs in relation to autism spectrum (ASD) and attention deficit hyperactivity (ADHD) disorders.

Methods: A review of the literature (PubMed) was performed. Exposure related keywords, including various chemicals, were matched with keywords describing outcome. Animal studies as well as publications not written in English were excluded. In total 834 titles were retrieved. The final selection included 21 publications.

Results: Positive associations were found for ASD in relation to exposure to all chemicals investigated, which included hazardous air pollutants, pesticides, and bisphenol A (BPA). Increased risks for ADHD or positive associations were found for exposure to polychlorinated biphenyls (PCBs), dialkyl phosphate (DAP), and chlorpyrifos. BPA, polybrominated diphenylethers (PBDEs), and low molecular weight (LMW) phthalates were positively associated with externalizing behaviour. Five out of seventeen studies did not find any association between exposure and ADHD.

Conclusion: Perinatal exposure to EDCs appears to be associated with the occurrence of ASD as well as ADHD. Disruption of thyroid hormone function and gamma-aminobutyric acid (GABA)ergic mechanisms may offer an explanation for the observed relations, though conclusive evidence in humans is limited.

Introduction

Toxic chemicals are produced in large quantities. They are incorporated in numerous products used in daily life, e.g. in plastics, food packaging material, furniture, and pesticides. Many of these chemicals make life more comfortable and, due to their frequent use, production of many of those continues to grow (1). Children today are at risk of exposure to 3000 synthetic chemicals produced in quantities of more than 1 million pounds per year (2). Along with the growth in production of toxic chemicals, an increase in prevalence of neurodevelopmental disorders such as Autism Spectrum Disorders (ASD) and Attention Deficit Hyperactivity Disorder (ADHD) has been observed over the past decades. Increased awareness and a shift in diagnosis may have contributed to the higher prevalences. However, they do not offer a sufficient explanation for these findings (3). It is hypothesized that exposure to chemicals with endocrine disrupting properties (endocrine disrupting chemicals – EDCs) (table 2.1) increases the risk for neurodevelopmental disorders such as ASD and ADHD.

Table 2.1 Classes of endocrine disrupting chemicals

Class of EDC	Chemical(s)
Perfluorinated alkyl acids	PFOS, PFOA
Organochlorine pesticides	HCB, DDE, DDT
Organophosphate pesticides	DAP, chlorpyrifos
Non-dioxin-like PCBs	PCB-153
Dioxin-like compounds	2,3,7,8-TCDD, PCB126
Brominated flame retardants	PBDE-47, PBDE-99, HBCD
Phthalates	DBP
Organohalogens (OHCs)	4OH-CB-146
Others	BPA, PCE, phytoestrogens

PFOS: perfluorooctane sulfonate; PFOA: perfluorooctanoic acid; HCB: hexachlorobenzene; DDE: dichlorodiphenyldichloroethylene; DDT: dichlorodiphenyltrichloroethane; DAP: dialkyl phosphate; PCB: polychlorinated biphenyl; TCDD: tetrachlorodibenzodioxin; PBDE: polybrominated diphenylether; HBCD: hexabromocyclododecane; DBP: dibutyl phthalate; OH-CB: hydroxylated polychlorinated biphenyls; BPA: bisphenol A; PCE: perchloroethylene or tetrachloroethylene.

Both ASD and ADHD typically are diagnosed during childhood, and even though their causal factors are to date not well understood (4), it is known that both genetic and environmental factors, as well as their interactions, are involved (5) (6). Detrimental effects of lead, methylmercury and polychlorinated biphenylethers (PCBs) have been reported previously (7, 8). More recently it has been observed that mice exposed to polybrominated diphenylethers (PBDEs) – a chemical used in flame retardants - had increased hyperactivity in adulthood compared to controls (9). Also alterations in spontaneous behaviour were observed (10). Furthermore, studies in rats have shown that exposure to phthalates causes hyperactivity reminding of the clinical picture of ADHD as observed in humans (11). Therefore endocrine disruption may be an interesting link between these exposures and the neurodevelopmental effects observed.

It is known that exposure to EDCs in adults may cause adverse health effects. Vietnam war veterans who have been exposed to 2,3,7,8-Tetrachlorodibenzodioxin (TCDD), more commonly known as agent orange, have an increased risk of diabetes and prostate cancer (12). Human exposure, however, may already start early in development, since the placenta does not completely block the transfer of EDCs from the maternal circulation to the foetus (13). The foetal period is the most important period in development, and hormones are key factors in many developmental events (14). Cell proliferation, differentiation, and apoptosis of many foetal tissues are regulated by hormones (14). Foetal growth rate is adjusted to foetal nutrient supply by hormones which signal the availability of nutrients and oxygen to foetal tissues (14). Also brain development is regulated and influenced by hormones, and especially thyroid hormones are known to be essential for normal embryonal and foetal neurogenesis (15). EDCs are known to affect thyroid hormonal function in particular. Therefore disruption of hormonal function during specific time periods important for brain development may have many consequences and may amongst others have adverse effects on neurodevelopment. Furthermore, Skinner et al. (16) showed that embryonic exposure to the endocrine disruptor vinclozolin resulted in changes in expression of various genes in both the hippocampus and amygdala, changes which were still observed in rats three generations removed from exposure . Increased anxiety-like behaviour was observed in the females of this generation, while young males showed hyperactivity. Exposure to EDCs may therefore also affect behaviour in multiple generations through epigenetic pathways. The objective of this review was to provide an overview of studies on perinatal exposure in humans to EDCs in relation to ASD and ADHD.

Methods

PubMed was searched for relevant publications, using terms relating to exposure in combination with outcome related key words. The following terms were used to describe exposure: environmental exposure, chemicals, EDC, endocrine disruption, pesticides, polychlorinated biphenyls (PCB), organochlorine, dichlorodiphenyldichloroethylene (DDE), dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), flame retardant, polybrominated diphenylether (PBDE), bisphenol A (BPA), phthalate, perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), and phytoestrogen. These terms were matched with each of the following key words describing ADHD and ASD related outcome: ADHD, hyperactivity, overactivity, inattentive, impulsive, ASD, autism, social behaviour, social interaction, communication, (child) behaviour.

Searches were – if necessary – refined by adding the key word ‘exposure’ to the search and by exclusion of animal studies and publications not written in English. In total 834 titles were retrieved. Publications were only included if exposure was assessed perinatally and if the outcome measured related to ASD or ADHD. Selection based on titles and abstracts resulted in the inclusion of 42 publications, reviews not included. To complete

information on exposure assessment and on outcome measures, the methodology section of articles were carefully searched. Reference lists of included publications were also searched for relevant studies, resulting in an additional two publications. The final selection included 21 publications.

Results

An overview of the occurrence of ASD and ADHD in relation to perinatal exposure to toxic chemicals in various cohorts is presented in table 2. ASD in relation to perinatal chemical exposure was assessed in four studies; all studies observed a positive association. Windham et al. (2006) (17) assessed exposure to hazardous air pollutants (HAPs) in both autism cases and controls and observed slightly – though not significantly – elevated adjusted odds ratios (AOR) for the fourth quartile of exposure to endocrine disruptors and developmental toxicants (table 2). In particular vinyl chloride and trichloroethylene were significantly elevated in the fourth quartile. Roberts et al. (2007) (18) observed that living near sites of pesticide application during gestation increased the likelihood for the occurrence of ASD in children by six times. Risk increased with poundage of pesticides used and as distance from field sites decreased. Similar to this result, Volk et al. (2011) found an increased AOR for children born to mothers living close to freeways at the time of birth (19). The most recent study on prenatal exposure to EDCs in relation to ASD measured BPA and phthalates in urine of women in their third trimester of pregnancy as a proxy for in utero exposure of the foetus (20). Prenatal exposure to BPA was not associated with ASD later in life. However, exposure to phthalates, and low molecular weight phthalates (LMW) in particular, was associated with greater social deficits, poorer cognition, communication, and social awareness.

Regarding ADHD, a positive association or an increased risk is found with increasing exposures to chemicals including DAP, PCBs, HCB, chlorpyrifos, and solvents (21-26). Prenatal exposure to DAP was reported on twice, both times from the same cohort. DAP-levels as measured in maternal urine during gestation were not associated with ADHD and attention scores on the CBCL when the children were 24 months of age (27). However these associations did become significant at 5 years of age at follow-up (21). Perinatal exposures to BPA, PCBs, LMW phthalates, and solvents were positively associated with externalizing scores (28-31). In a minority of the studies exposure was found to be related to either attention (31, 32) or activity only (33). In five out of seventeen studies no effect was observed of perinatal exposure on attention and/or activity (27, 34-37).

PCB exposure has been associated with ADHD in several cohorts, such as the one studied by Sagiv et al. which included children aged seven to eleven years (23). Mothers of these children were residing near a PCB-contaminated harbour and children in the highest quartile of exposure to a sum of four PCBs had a relative risk of 1.79 for ADHD compared to children in the lowest quartile. PCB exposure was also studied in Inuit children, which

are known for their high exposure to PCBs from seafood. Perinatal exposure was found to correlate with inattention and increased activity at eleven months of age when exposure was modelled with a validated physiologically based pharmacokinetic (PBPK)-model (38). Plusquellec et al (2010) however observed no associations for both attention and activity with increasing exposure to PCB-153 at five years of age in another Inuit cohort (35). Exposure was measured in cord serum only, not taking into account exposure through breastfeeding. Indeed, exposure through breastfeeding was also not taken into account in the children studied by Sagiv et al. (23) who also assessed PCB-exposure. Only 25% of the mothers breastfed their children longer than three months, while in the Inuit cohort of the group of Plusquellec the mean duration of breastfeeding was 57 weeks.

Janulewicz et al. (36) observed no effect of exposure. However, some modest associations were found in the lower exposed group. A model was used to calculate the cumulative mass of PCE entering homes through drinking water instead of assessment of direct human exposure. Due to likely misclassification of exposure because of the crude model used and inconsistency in dose-response relations observed, it was concluded that exposure was not associated with an increased risk of developing ADHD.

Table 2.2 ASD and ADHD in relation to perinatal exposure to EDCs in various cohorts. Sorted by outcome (ASD first) and year of publication

	Population	Exposure	Dose	Outcome	Result
Miodovnic 2011 (19)	n=137 7-9 years	Phthalates, BPA Urine, 3rd trimester	BPA: 1.3 (0.7–2.3). LMW phthalates: 430 (175–1090) Median (IQR) - µg/L	ASD SRS	LMW phthalates → greater social deficits (β=1.18, 95% CI 0.25 – 2.08) No effect for BPA.
Volk 2011 (19)	n=304 ASD n=259 controls	Traffic pollution birth residence	-	ASD	Shortest distance from freeway (10%) → AOR 1.86 (95% CI 1.03 – 3.45
Roberts 2007 (18)	n=465 ASD n=6975 controls	Pesticides Birth	-	ASD Registries	Living near sites of pesticide application → OR = 6.1 (95% CI 2.4 – 15.3)
Windham 2006 (17)	n=284 ASD n=657 controls	Air pollutants Birth residence	-	ASD Registries	Developmental toxicants, highest quartile: AOR 1.40 (95% CI 0.98 – 2.00)
Gascon 2011 (33)	n=332 4 years Menorca-INMA	PBDE-47 Cord blood (n=88)	2.10 (16.8) Median (max) - ng/g lipid <LOD = 48.9%	ADHD CP-SCS and ADHD DSM-IV	No effects of prenatal exposure.
Marks 2010 (20)	n=331 5 years CHAMACOS (California)	DAP Maternal urine (2x during pregnancy)	109.0 (99.4 – 119.6) Mean (95% CI) - nmol/L <LOD = LOD/√2	Attention, ADHD CBCL, K-CPT	Attention problems (CBCL) → β=0.7, 95% CI 0.2-1.2 ADHD (CBCL) → β=1.3, 95% CI 0.4-2.1 Composite score → OR=3.5, 95% CI 1.1-10.7
Verner 2010 (21)	n=168 11 months Inuit children	Simulated PCB-153 Perinatal	103 (15 – 706) Median (range) - ng/g lipid	Behaviour BSID-II	Prenatal PCB was associated with increased inattention, postnatal PCB predicted increased activity at 11 months.
Engel 2010 (30)	n=188 4-9 years New York	lmw phthalates 3rd trimester urine	2.24 (0.90 – 5.65) Median (IQR) - µM/L	Behaviour BASC-PRS	Conduct problems → β=2.40, 95% CI 1.34 – 3.46; Attention → β=1.29, 95% CI 0.16 – 2.41 Externalizing → β=1.75, 95% CI 0.61 – 2.88
Sagiv 2010 (22)	n=607 7-11 years Massachusetts	PCB (118, 138, 153, 180), p,p'-DDE Cord serum	∑PCB_4: 0.19 (0.01 – 4.41) p,p'-DDE: 0.31 (0.00–14.93) Median (range) - ng/g	ADHD CRS-T	∑PCB_4 , highest vs. lowest quartile: Conners' ADHD index → RR=1.76, 95% CI 1.06 – 2.92 DSM-IV total →RR=1.79, 95% CI 1.08 – 2.96 p,p'-DDE, highest vs. lowest quartile: Conners' ADHD index → RR=1.80, 95% CI 1.10 – 2.94 DSM-IV total →RR=1.69, 95% CI 1.01 - 2.83

	Population	Exposure	Dose	Outcome	Result
Plusquellec 2010 (34)	n=110, 5 years Inuit children	Pb, PCBs, Hg Cord serum	PCBs: 120.6 (21.6 – 407.4) Mean (range) - µg/kg	Behaviour IBRS, video	No associations found for attention and activity outcomes
Braun 2009 (27)	n=249 2 years Cincinnati, Ohio	BPA Urine, 16 & 26 weeks of gestation, birth	16 wks: 1.6 (<LOD–34.8) 26 wks: 2.0 (<LOD–583) Birth: 1.9 (<LOD–27.3) Median (range) - µg/L,	Behaviour BASC-2/BASC-PRS	Externalizing scores → β=6.0, 95% CI 0.1 – 12.0 (females) BPA at 16 wks was more strongly associated than at 26 wks
Roze 2009 (31)	n=62 5-6 years Compare (Groningen)	OHCs, PBDEs Maternal blood, week 35	BDE-47: 0.9 (<LOD-6.1) ng/g lipid BDE-99: 0.2 (<LOD-2.1) ng/g lipid PCB-153: 63.0 (34.0-162.2) pg/g 4OH-CB-146: 103.3 (36.3-290.1) pg/g *Median* (range)	Cognition, behaviour CBCL, teacher's report form and ADHD-questionnaire	Sustained attention: r = -0.264, p < 0.05 (BDE-47 and BDE-99) Total behavioural outcome: r = 0.276, p < 0.05 (BDE-99) Externalizing behaviour: CBCL → r = -0.278, p < 0.10 (OH-CB) Teacher → r = -0.328, p<0.05 (OH-CB) Teacher → r = -0.288, p<0.05 (PCB153)
Janulewicz 2008 (35)	n=2490 Cape Cod	PCE (drinking water) Model of exposure at birth	7.34 (4×10^{-5} – 1328) Median (range) – g Cumulative prenatal exposure	Attention, activity Parent report of diagnosis by doctor	Low prenatal exposure: ADD → OR=1.4, 95% CI 0.9 – 2.0 ADHD → OR=1.5, 95% CI 0.9 – 2.7 Low postnatal exposure: ADD → OR=1.3, 95% CI 0.9 – 1.9 ADHD → OR=1.4, 95% CI 0.8 – 2.5 High exposure: no sign. increased risk
Eskenazi 2007 (26)	n=356 – 396 24 months CHAMACOS (California)	DAP Maternal urine (gestation)	114.9 (105.7 – 125.0) Mean (95% CI) – nmol/L Average value gestation <LOD = LOD/√2	Neuro-development CBCL at 24 months	Total prenatal DAP: Attention → OR=0.77, 95% CI 0.27 – 2.24; ADHD → OR=1.34, 95% CI 0.50 – 3.59 PDD → OR=2.25 95% CI 0.99–5.16
Ribas-Fito 2007 (23)	n=475 4 years Menorca-INMA	HCB Cord serum	0.73 (0.14 – 9.82) Median (range) – ng/mL	ADHD CP-SCS and ADHD DSM-IV	HCB> 1.5 ng/mL: Poor social competence → RR=4.04, 95% CI 1.76 – 9.58 ADHD → RR=2.71, 95% CI 1.05 – 6.96

	Population	Exposure	Dose	Outcome	Result
Rauh 2006 (24)	n=254 12, 24, 36 months New York	Chlorpyrifos Cord plasma	<LOD – 63 Range – pg/g	Neuro-development CBCL (36 months)	High (>6.17 pg/g plasma) vs low exposed at 3 years of age: Attention → OR=11.26, 95% CI 1.79–70.99 ADHD → OR=6.50, 95% CI 1.09–38.69 PDD → OR=5.39, 95% CI 1.21–24.11
Laslo-Baker (2004) (25)	n=32 exposed n=32 controls 5-6 years Motherrisk	Solvents (occupation) Questionnaire	Exposure was at least 2 months during pregnancy and started in the first trimester	CBCL, CRS	Conner hyperactivity/impulsivity → β=0.27 (p=0.052) Conner DSM-IV hyperactivity → β=0.62 (p<0.001) Conner DSM-IV score → β=0.33 (p=0.02) CBCL externalizing not reported
Jacobson 2003 (36)	n=148 11 years Michigan	PCB Cord and maternal serum, milk	Cord: 2.7 (2.1) ng/mL Maternal: 5.9 (3.8) ng/mL Milk: 859.3 (388.2) ng/g fat Mean (SD), <LOD=7.4%	Sustained attention (*CPT*), focused attention	No effect of exposure on sustained attention.
Lai 2002 (28)	n=118 exposed n=118 controls 13-17 years	PCBs Yu-Cheng vs. controls	-	CBCL Rutter scale	Exposed vs. controls CBCL externalizing → β=2.55 (SE: 0.86)
Till 2001 (29)	n=33 exposed n=28 controls 3-5 years Motherrisk	Solvents Questionnaire Material safety data sheets	Exposure was at least 2 months during pregnancy, minimally 5 hours per week.	Attention, externalizing behaviour CBCL, CPT	Exposed: Externalizing → $\chi^2(1)=5.35$, p=0.02 CPT: exposure was not related to either errors of omission or commission
Chen 1994 (32)	n=118 exposed n=118 controls 3-12 years	PCBs Yu-Cheng vs. controls	-	Rutter scale	Cases scored 11% to 63% (mean = 28%) higher on activity than controls at each age (except for the 12-year-olds)

Discussion

The objective of this review was to present results of cohort studies on the relation between perinatal exposure to EDCs and occurence of ASD and/or ADHD in order to investigate current evidence for EDCs as a causal factor in the etiology of these neurodevelopmental disorders. Four studies assessed this relationship for ASD and positive associations were found for all chemicals investigated, which included hazardous air pollutants, pesticides and BPA. As for ADHD a larger pool of studies was available. Six out of seventeen studies observed positive associations or increased risks for a variety of chemicals including PCBs, DAP, and chlorpyrifos. Another five studies found positive associations with externalizing behaviour for chemicals such as BPA, PBDEs, and LMW phthalates. Five studies did not find any association between exposure and ADHD.

Dose-response relationships may not be straightforward, which may offer an explanation for the observations in the cohort of Janulewicz et al. (36) and may also be applicable to PBDE-47 exposure (32, 34). Gascon et al. (34) measured exposure in cord blood (median concentration: 2.10 ng/g lipid) and found no association with ADHD in four year old children. Roze et al. (32) measured exposure in maternal blood at 35 weeks of gestation. An association with decreased sustained attention was found for PBDE-47 in five and six year old children, even though concentrations were lower than in the study of Gascon et al. (median concentration: 0.9 ng/g lipid).

Perinatal exposure to chemicals was also found to be positively associated with externalizing behaviour. Though hyperactivity is only part of the construct of externalizing behaviour (39), ADHD is considered an externalizing disorder. Prenatal exposure to LMW phthalates, BPA, PCBs and solvents was observed to be associated with externalizing behaviour (28-32). High scores on externalizing or internalizing behaviour are reflected in the default network resting state functional connectivity (DN RSFC) of these children compared to normally developing children (40). Functional connectivity is a type of connectivity in the brain in which similarities of temporal characteristics of brain activity in multiple regions are recorded (41). Sex steroids such as testosterone may affect functional connectivity (42), and testosterone in particular is also potentially related to the development of ADHD (43). Several EDCs are known to disturb sex steroid levels and may therefore indirectly affect functional connectivity. Furthermore EDCs such as PBDEs are known to affect thyroid hormone (TH) levels which in turn are known to affect neuronal differentiation, migration, myelination, synaptogenesis and dendritic branching (44). They are therefore important factors for brain development and potentially also for development of the connectome. In mice with a heterozygous mutation of the TH receptor a1 a reduced density of GABAergic inhibitory interneurons in the hippocampus is observed, which was accompanied by more depressive and anxious behaviour (45). GABA is an inhibitory neurotransmitter in the mature brain. However in the embryonic and perinatal period, it triggers calcium influx and it is important for cell proliferation, migration, differentiation, synapse maturation and cell death (46). In children with autism, dysfunction of GABAergic

signalling is observed (46). Furthermore in mice it is demonstrated that BPA inhibits the GABAaR-mediated response and that BPA affects development of GABAergic and dopaminergic systems (47). Toxicology studies also have shown the potential for PCBs to affect the GABAa response (48, 49), indicating EDCs as risk factors for neurodevelopmental disorders through modification of GABAergic systems. Evidence from cohort studies thus far for these mechanisms is scarce. Only two of the included cohorts actually measured thyroid hormones (32, 34). Gascon et al. observed no significant associations between TSH, TT3, fT4 and postnatal levels of PBDE-47, nor did they significantly relate with neurodevelopmental outcomes. However, Roze et al. (32, 34) observed TSH to be correlated with worse neuropsychological functions, while T3 and T4 were found to be correlated with better outcome such as less ADHD and better behaviour. Pentachlorophenol (PCP) was negatively related to T3, which would fit with the observed correlation between T3 and outcome. However, PBDE related positively to T3.

Exposure to EDCs may also affect behaviour through pathways other than the endocrine pathway, e.g. epigenetic alterations resulting in reprogramming of the brain, as was demonstrated in an experimental study by the group of Skinner. They observed that the F3 generation of vinclozolin exposed rats had altered genes in the hippocampus and amygdala and altered behaviour (16), even though major effects on hormone levels were not observed (16, 50, 51). Behaviour may therefore also be potentially determined by exposures of previous generations. It should also be considered that enzymatic polymorphisms may result in variations in detoxification phenotypes, making some individuals more susceptible to environmental contaminants. An example of this is paraoxonase 1 (PON1), which is important in the detoxification of individual organophosphorous compounds (52). Furthermore, other potential risk factors for ASD and ADHD (53, 54), such as preterm birth, intrauterine growth restriction, and low birth weight, should also be considered, especially since some of them may also be linked to EDC exposure (55).

It can be concluded that especially regarding ASD insufficient data are available, but that studies so far seem to indicate an association between prenatal exposure to EDCs and occurrence of ASD. Moreover, observations indicate a relation between perinatal EDC exposure and prevalence of ADHD. A limitation of our study is however, that confounding factors have not been taken into account in the overview. Future research should include prospective, longitudinal cohort studies in which exposure to EDCs as well as hormone levels have to be assessed in relation to outcome in order to clarify mechanisms of action.

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3

Obesogenic effects of endocrine disruptors, what do we know from animal and human studies?

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Abstract

Background: Hormonal actions and activation of receptors involved in adipogenesis and brain development during the prenatal period may be affected by exposure to certain chemicals. Experimental studies have shown that amongst others polychlorinated biphenyl (PCB)-153 and dichlorodiphenyltrichloroethane (DDT) may have obesogenic effects in prenatally exposed mice.

Objective: To provide an overview of five classes of chemicals which have frequently been indicated as potential obesogens, and to discuss the evidence available regarding early life exposure to these compounds and overweight later in life.

Methods: Pubmed was systematically searched for publications which related early life exposure to endocrine disrupting chemicals (EDCs) to growth parameters later in life. We included 19 studies, which were published from 1995 and onwards.

Results: 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. Dose-response relations appear to be non-monotonic.

Conclusion: For certain EDCs, early life exposure may be associated with weight homeostasis later in life, however not necessarily in an obesogenic direction. More sensitive measures of adiposity as well as long-term follow-up is warranted for future studies.

Introduction

The prevalence of obesity continues to grow worldwide, presenting governments and health care organizations with a major challenge. Though at first obesity seemed to occur predominantly among adults, it is clear now that increasing numbers of children have to deal with the health consequences and social stigma of being overweight (1). In 2002 a review by Baillie-Hamilton was published which showed remarkable similarities between the obesity epidemic and the production of chemicals from 1930 and onwards (2). Also in experimental studies it was showed that various chemicals had obesogenic effects. Female progeny of rats exposed to polychlorinated biphenyl (PCB)-153 experienced accelerated growth compared to controls (3). PCB-153 furthermore stimulated adipogenesis in 3T3-L1 adipocytes (4). Body weight of mice was affected after in utero exposure to a mixture of chemicals, including the organochlorine pesticide dichlorodiphenyltrichloroethane (DDT), however dose-response was non-monotonic, with results for higher doses often being opposite from what was observed for lower doses (5). Moreover, exposure of mature adipocytes to dichlorodiphenyldichloroethylene (DDE), the metabolite of DDT, resulted in increased leptin release (6), a hormone which has been associated with fetal growth (7).

It has become clear that certain compounds may interfere with the function of hormones (endocrine disrupting chemicals, EDCs), including estrogen, testosterone, and thyroid hormones (reviewed by Bergman et al. (8)), which are involved in various processes in adults, but also in brain development early in life (9, 10). These hormones have also been associated with weight homeostasis, both early in development and later in life. Estrogens, for example, reverse weight gain often experienced by post-menopausal women (11). Moreover in men who are treated for prostate cancer and in women who are treated for polycystic ovary syndrome with anti-androgenic therapy, weight gain is observed (11, 12). EDCs may interact with estrogen receptors, but may also affect aromatase activity. Aromatase is an enzyme which converts testosterone to 17β-estradiol. Developmental exposure to estradiol EDCs has been found to decrease expression of the aromatase gene (CYP19a1b) in the brain of male rainbowfish (13). Decreased aromatase activity was however also observed in male rats prenatally exposed to a PCB-mixture (14). Other studies also suggest that aromatase activity may be affected by phthalates (15), DDE (16), and various pesticides (terbuthylazine, propiconazole and prothioconazole) (17), although findings need to be substantiated.

EDCs may also interfere with thyroid hormone (TH) receptors as well as transport proteins for TH. Some isoforms of polybrominated diphenylethers (PBDEs) have been shown to exert inhibiting effects for binding of triiodothyronine to the TH receptor (18). PBDE metabolites may furthermore bind to the transport protein transthyretin, resulting in displacement of thyroxine (19). This has also been observed for perfluorinated alkyl acids, such as PFOS and PFOA (20). This has been however predominantly shown in vitro.

Endocrine disruptors may furthermore promote obesity through peroxisome proliferator-activated receptor (PPAR) α and γ. Activation of PPARα stimulates lipid

mobilization, but may indirectly also be obesogenic as prenatal activation may result in low birth weight, a known risk factor for obesity later in life. There are several options through which EDCs may promote obesity through PPARγ. Usually a ligand is needed for PPARγ to bind co-activators, release co-repressors, decondensate the chromatin and activate transcription (21). However post-translational modifications, such as phosphorylation, may activate PPARγ in absence of a ligand (22). EDCs may furthermore cause multipotent stromal cells (MSCs), cells which can differentiate into various tissues, to predominantly differentiate into adipose tissue (21, 23). This particular subset of MSCs expresses PPARγ (24). This has in particular been found for organotins such as tributyltin (TBT).

What also needs to be considered is that PPAR agonists and their metabolites may activate multiple PPAR isoforms. Bis(2-ethylhexyl)phthalate (DEHP), an EDC from the phthalate class, may activate PPARα, however its metabolite mono (2-ethylhexyl)phthalate (MEHP) may activate PPARγ (25, 26). And though lipid mobilization induced by PPARα activation requires continuous exposure, PPARγ may only need a single or episodic exposure to establish its effects in adipose tissues (12). Recent research has also indicated potential for perfluorinated alkyl acids, such as perfluorooctane sulfonate (PFOS), to interfere with PPAR by inducing expression of PPARγ genes in mouse neonatal brain after prenatal exposure (27).

Regulation of energy balance is a basic concept in the aetiology of obesity. Several factors control appetite and energy expenditure, which are all integrated in the hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus, and in particular the arcuate nucleus of the hypothalamus (ARH), is the centre for energy balance and appetite regulation.

Not much is known on developmental exposure to EDCs in relation to later appetite regulation. Chemicals may disrupt actions of hormones related to energy balance, e.g. leptin and insulin. It is clear that both leptin and insulin play important roles in development of feeding circuits which are comparable to sex steroid hormones with regard to the development of sexually dimorphic circuits (28). Rats injected with insulin between embryonic day 15 and 20 (term is 22 days) were significantly more obese at 50 days of age (29). In leptin-deficient mice also ARH circuit formation was affected (30). Leptin is also considered a regulator of fetal growth (7) and low leptin levels at birth have been associated with a higher risk for obesity and diabetes (31).

Early life exposure to these toxicants may have different effects than exposure in adulthood, as perturbations during stages of developmental plasticity may give rise to more profound long-lasting effects (32). As endocrine disruption early in life seems to be a plausible mechanism which may predispose children to obesity, the aim of this study was to create an overview of six classes of chemicals which have frequently been indicated as potential obesogens in observational studies, and to discuss the evidence available regarding early life exposure (i.e. during the prenatal or early postnatal period) to these compounds and overweight later in life.

Methods

Articles were considered relevant when they determined effects of either dioxin-like compounds, non-dioxin like compounds, organochlorine pesticides, brominated flame retardants, phthalates or perfluorinated alkyl acids on growth and physical development in humans. PubMed was therefore systematically searched for publications by means of the following terms relating to exposure: chemical exposure, endocrine disruption (prenatal) environmental exposure, pesticides, bisphenol a (BPA), brominated flame retardant, DDE, DDT, hexachlorobenzene (HCB), organochlorines, organotin, perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), phthalates, polybrominated diphenyl ethers (PBDE), and PCB. Each of these terms was combined with the following terms relating to growth: obesity, overweight, fat, growth, anthropometry, cohort studies obesity, cohort studies overweight. In total 2832 publications were retrieved.
Articles were only considered when exposure was determined during pregnancy (or extrapolated to the period of pregnancy), or when it was measured in breast milk. Furthermore publications had to be written in English. Reference lists of articles included from the initial search were also searched for relevant publications. In total 19 publications of observational studies were included.

Results

An overview of observational studies included is given in table 3.1.

Organotins

Organotins are characterized by a tin atom which is bound to an organic chain. The most common varieties are tributyltin (TBT) and triphenyltin (TPT) which are generally found in wood preservatives and antifouling boat paints. Due to their organic chain, they are hydrophobic and they therefore bioaccumulate. Human exposure occurs mainly through diet, in particular through sea food (33).

Though observational studies are not available, obesogenic characteristics of TBT in particular have been well documented in experimental studies. Prenatal exposure to this compound in mice has been associated with adiposity at later age (34). Furthermore, MSCs derived from the adipose tissue of these mice showed increased commitment to the adipocyte lineage compared to controls (35, 36), at levels comparable to the tolerable daily intake of humans (37). Differentiation of pre-adipocytes into adipocytes was increased by TBT exposure in a dose and time dependent manner (38, 39). Moreover, these effects may be heritable as Chamorro-Garcia et al. observed these results in both the F2 and F3 generation of exposed mice (37). Gender-specific results have been reported, with effects on fat mass lasting longer in male mice than in female mice after prenatal exposure to TBT at human relevant levels (40).

Non-dioxin-like PCBs

Non-dioxin-like PCBs were mostly applied as dielectric and coolant fluids, however due to health concerns, including potential carcinogenic effect, production of PCBs in Europe has been prohibited since 1985 (41). However, PCBs may still be released in the environment from old electrical appliances, and as they are furthermore known to be highly persistent in the environment, they may accumulate in fat tissue of animals and humans (42). The main source of exposure for people is through animal food (fish and meat), which is mostly contaminated with PCB-153 and PCB-138 (42). In Europe, dietary exposure to PCBs is monitored by the European Food Safety Authority (EFSA) (43).

In vitro studies have shown that PCBs may enhance adipocyte proliferation and differentiation (4, 44). Futhermore, Sitarek and Gralewicz (2009) observed accelerated growth in female progeny of rats exposed to PCB-153 during pregnancy until 21 days after birth (3). Animal studies on obesogenic effects of NDL PCBs are however scarce.

Several observational studies report no association between PCB exposure and BMI, weight or height (45-48). Other studies did observe associations between perinatal PCB exposure and growth; however both positive and negative associations have been reported for weight as well as for height. BMI was positively associated with PCB exposure in both the study by Verhulst et al. (49) and the study by Valvi et al. (50). In both these studies, PCB exposure was assessed in cord blood. A positive association with BMI standard deviation scores (SDS) was observed in children 3 years of age (49) and an increased relative risk for overweight was found in children 6.5 years of age in the highest exposure tertile (50). However, no association between PCB exposure and BMI was seen in a group of 280 females aged 20-50 years for whom exposure was assessed in maternal serum (45). However, these samples were collected from the mothers at time of enrolment of the daughters and exposure levels were extrapolated back to the period of pregnancy. Also Blanck et al. (51) and Rylander et al. (52) measured PCBs in maternal serum at the time of enrolment of the children (aged 5 to 24 years and 4 to 7 years respectively) and converted exposure to the time of pregnancy. Both studies report negative associations between exposure levels and weight and no associations with height.

Organochlorine pesticides (DDT/DDE and HCB)

DDT and HCB are part of a group referred to as organochlorine pesticides. DDT was developed in the 1940s as an insecticide and was amongst others used to fight insect-borne diseases, such as malaria (53). Due to health concerns – DDT is genotoxic and a suspected carcinogen – use of DDT was banned in the United States in 1972 and in the Netherlands in 1973 (54). However, in developing countries DDT is still used for vector-control (malaria), which is currently also supported by the World Health Organization (55). DDT is known to bioaccumulate and to be highly persistent in the environment. HCB is a byproduct of manufacturing processes of other chemicals, and has also been widely used as a pesticide. Due to probable carcinogenic effects, production and use of HCB was prohibited by the

Stockholm Convention (56). Like DDT, it accumulates in fat tissue and it persists in the environment.

In vitro studies with preadipocytes have shown that DDE exposure at environmentally relevant levels resulted in increased proliferation (44) and differentiation (57). Howell and Mangum (2010) did not observe an effect on adipogenesis after exposure of NIH3T3-L1 cells to DDE, which may potentially be due to the lower concentration used (2-20 µM vs. 5-500 µM used by Chapados et al.) (6). They did however observe an increase in leptin release from mature adipocytes and an increase in basal fatty acid uptake, after exposure to DDE. Hojo et al. (2006) exposed rats to DDE through the diet and observed a decreased weight in F1 females nine weeks after birth, which was significant for the highest dose group (58). This dose may have been too high however, as they observed clear signs of systemic toxicity in this group (tremors, convulsions), and some females died after appearance of these symptoms.

Table 3.1 Observational studies on early life exposure to EDCs and effects on growth-related outcomes later in life (ordered by date of publication)

Author	Population	Exposure	Outcome	Results
Andersen et al. (2013)	n=811 7 years old	PFOS: 33.8 (6.4 – 106.7) PFOA: 5.25 (0.5 – 21.9) *Median (range) – ng/mL. MP, GW 8*	Weight, height	No significant associations between exposure and any of the selected outcomes
Maisonet et al. (2012)	n=447 20 months old	PFOS: 19.6 (3.8 – 112.0) PFOA: 3.7 (1.0 – 16.4) *Median (range) – ng/mL. MS, GW 15*	Weight, height (2, 9, and 20 months)	PFOS, 3rd tertile, 20 months of age • Weighed 580 g. more than girls in the lowest tertile No effect for PFOA
Halldorsson et al. (2012)	n=665 20 years old	PFOS: 21.5 (9.1) PFOA: 3.7 (2.0) *Median (IQR) – ng/mL. MS, GW 30*	BMI, WC, insulin, leptin, adiponectin	PFOA, highest (5.8 ng/mL) vs. lowest quartile (2.3 ng/mL): • Adjusted RR BMI>25 = 3.1 (95% CI 1.4, 6.9) • Adjusted RR WC>88 cm = 3.0 (95% CI 1.3, 6.8) • Only significant in females, other congeners not significant
Valvi et al. (2012)	n=344 6.5 years old	Total PCB (Σ of 7): 0.75 ± 1.70 DDE: 1.06 ± 2.45 DDT: 0.08 ± 3.81 *Mean ± SD – ng/mL. CB*	BMI	PCB, 3rd tertile, RR overweight = 1.70 (95% CI 1.09, 2.64) DDE, 2nd tertile, RR overweight = 1.67 (95% CI 1.10, 2.55) DDT: only associated with overweight in boys Girls: stronger associations
Garced et al. (2012)	n=253 1 year old	DDE 1st trimester: 6.3 ± 2.8 DDE 2nd trimester: 6.6 ± 2.9 DDE 3rd trimester: 7.6 ± 2.9 *Mean ± SD – ng/mL. MS*	Weight-for-age Length-for-age BMI-for-age HC-for-age	No significant associations between exposure and any of the selected outcomes
Andersen et al. (2010)	n=1010 5 and 12 months old	PFOS: 33.4 (6.4 – 106.7) PFOA: 5.21 (0.5 – 21.9) *Median (range) – ng/mL, MP, GW 8*	Weight, height, BMI	• PFOS, weight, 5 months: β = -0.8 g (95% CI: -4.2, 2.6) • PFOS, weight, 12 months: β = -5.8 g (95% CI:-10.4, -1.2) • PFOA, weight, 5 months: β = -9.4 g (95% CI: -28.6, 9.9) • PFOA, weight, 12 months: β = -19.0 g (95% CI: -44.9, 6.8) Similar pattern for BMI. No associations with height. After stratification effects more pronounced in boys, not significant in girls
Mendez et al. (2010)	n=657 14 months	DDE, Average growers: 125.40 (115.03–136.70) Rapid growers: 135.59 (119.48–153.87) *Mean (95% CI) – ng/g lipid. MS, 1st trim.*	Rapid growth BMI at 14 months	DDE > 1st quartile • Rapid growth, RR 2.42 (95% CI: 1.25, 4.67) • Elevated BMI at 14 months, RR 1.50 (95% CI: 1.11, 2.03) • Normal pre-pregnancy weight mothers. For other OCs no effect

Author	Population	Exposure	Outcome	Results
Pan et al. (2010)	n=210 breast fed >6 months, 12 months old	Total PCB (Σ of 18): 81 (12-708) DDE: 113 (15-2140) DDT: 5 (<LOD-36) *Median (range) – ng/g lipid. BM, 3 months*	Weight (record), height	No significant associations between exposure and any of the selected outcomes
Verhulst et al. (2009)	n=138 3 years old	PCB: 117 ± 76 DDE: 212 ± 243 *Mean ± SD – ng/g lipid. CB*	BMI SDS	DDE 450 vs. 63.7 ng/g lipid: • 0.13 difference in BMI SDS for non-smoking mothers • Smoking enhanced DDE effect PCB and BMI SDS: β = 0.003, p = 0.03
Karmaus et al. (2009)	n=280 females 20-50 years old	PCB, DDE, DDT *MS, at enrolment daughters. Converted to exposure at time of pregnancy*	BMI	DDE <1.503 µg/L vs. 1.503-2.9 µg/L → 1.65 increase in BMI DDE <1.503 µg/L vs. >2.09 µg/L → 2.88 increase in BMI No effect of PCB exposure
Smink et al. (2008)	n=405 6 years old	HCB: 0.68 (0.46 – 1.03) *Median (IQR) – ng/mL. CB*	BMI, obesity	HCB > 1.03 vs. < 0.46 ng/mL: BMI: β = 0.80, p = 0.03. RR obesity: 2.06 (95% CI: 1.06, 3.85)
Rylander et al. (2007)	n=55 low and n=119 normal birth weight 7 years old	PCB-153 *MB, at enrolment children. Converted to exposure during year of childbirth.*	Weight (4 and 7 years), height	Weight at 4 years: β = -0.4 kg (95% CI: 0.01, 0.70, p = 0.04) Weight at 7 years: β = -1.2 kg (95% CI 0.50, 1.90, p = 0.001) Effects on weight only found in normal birth weight children
Ribas-Fito et al. (2006)	n=1712 7 years old	*p,p'*-DDE: 24.4 (17.0–36.2) *Median (IQR) – µg/L* *MS, third trim.*	Weight, height (1, 4, and 7 years)	DDE: >/ 60 µg/L vs lowest <15 µg/L. • Height → 1 year: β = -0.72 (SE 0.37); 4 years: β = -1.14 (SE 0.56);7 years: β = -2.19 (SE 0.46) • In lower categories and for weight no association observed. • No effect of PCB exposures
Hertz-Picciotto et al. (2005)	n=399 5 years old	Total PCB: 696 (378 – 1115) Mean (5^{th} – 95^{th} percentile) – ng/g lipids *MS, 2^{nd} or 3^{rd} trim.*	Weight and height (birth, 5 yrs), HC, gestational age	Males, exposure was negatively associated with: • At birth: weight, weight-for-gestational age, HC • No association with growth at 5 years of age Females: At birth: smaller HC, shorter gestation • Height at 5 years: β = 4.5 (95% CI: 0.05, 9.00)
Gladen et al. (2004)	n=304 males 20 years old	DDE: 5.7 (1.0 – 25.1) *p,p'*-DDT: 1.9 (<LOD – 12.7) *o,p'*-DDT: 0.14 (<LOD – 1.33) *Median (range) – µg/g lipid. MS, 3^{rd} trim.*	BMI, triceps & subscapular skinfold, serum DHEAS, serum, testosterone	No significant associations between exposure and any of the selected outcomes

Author	Population	Exposure	Outcome	Results
Blanck et al. (2002)	n=308 females 5-24 years old	PCB: 5 (<LOD – 78) *Median (range) – ppb. MS, at enrolment, proxy for exposure during pregnancy*	Weight, height	PCB > 5 ppb: • Weight (adjusted for height): β = -11.76 (95% CI: -4.20, -19.30)
Gladen et al. (2000)	n=594 Follow up at puberty	Transplacental = average of all matrix types PCB: 1.7 (1.5-5.5) ppm DDE: 2.4 (1.3-23.8) ppm Lactational = levels in breast milk*duration PCB: 5.0 (0.2-23.1) mg DDE: 6.2 (0.2 – 96.3) mg *Median (range). MB, CB, BM, placenta*	Weight, height (all self-reported)	• PCB white girls, highest (≥3 ppm) vs. low (0-1 ppm) transplacental exposure: 5.4 kg heavier (p = 0.09) • DDE boys, highest vs. low transplacental exposure: 6.3cm taller and 6.9 kg heavier • PCB boys and DDE girls: no effect of lactational or transplacental exposure
Patandin et al. (1998)	n=105 breast-fed (BF) n=102 formula-fed (FF) 42 months old	PCB maternal: 2.04 (0.59 – 7.35) PCB cord: 0.40 (0.08 – 2.08) PCB milk: 391.5 (173.7 – 1226.4) μg/kg fat *Median (range) – μg/L. MP, last month gestation. CP. BM, 2nd week*	(Birth) weight, height, HC. 10 days, 3, 7, 18, 42 months	P90 (0.8 μg/L) vs. P10 (0.20 μg/L) • Weighed 165 g. less at birth Lower growth rate from birth to 3 months, not at later ages (only in FF group, not in BF group)
Guo et al. (1995) PCB	n=118 exposed to PCB in utero n=117 controls	PCB: 49.3; 26.8 *Mean; median - ppb* *MS, end of pregnancy*	Weight, height, total lean mass	Exposed children vs. controls: • Age range 6 months – 7 years: 7% lighter and 3% shorter • Age range 6 – 13 years: 2.3% shorter, weight similar • Lower total lean and soft tissue mass,15% lower birth weight

BF: breastfed; BM: breast milk; CB: cord blood; CP: cord plasma; FF: formula-fed; GW: gestational week; HC: head circumference; LOD: limit of detection; MB: maternal blood; MP: maternal plasma; MS: maternal serum; OC: organochlorine; trim: trimester; WC: waist circumference.

Several cohort studies (45, 49, 50, 59) have observed a positive association between prenatal DDE exposure and BMI at later age. Prenatal DDE exposure measured in cord blood was associated with a 0.13 difference in BMI SDS in high vs. low exposed 3-year olds (49), and a relative risk of 1.67 for overweight in 6.5-year olds (50). Also DDE exposure assessed in first trimester maternal serum was associated with a relative risk (RR) of 1.50 for elevated BMI in 14 months old children (59). Karmaus et al. determined exposure in maternal serum, which was collected at the time of enrolment of the daughters (20-50 years of age) and then converted to exposure during pregnancy (45). They observed a non-linear trend across quintiles of exposure, and found a 2.88 increase in BMI in the three upper quintiles of adult female offspring versus the lowest two quintiles. Pan et al. found no effect of DDE exposure on BMI in 12 months old children (47). Prenatal DDE exposure has furthermore been associated with increased weight in boys at puberty (60). However, in a later study by Gladen et al., which included only males, no effect of DDE exposure on weight was observed (61). Also in the cohort of Ribas-Fito et al. no associations for weight and DDE exposure were seen (48).

Smink et al. measured prenatal exposure to HCB in cord blood of 405 children (62). At 6 years of age, the high exposure group (>1.03 ng/mL) had an increased BMI compared to the low exposure group (<0.46 ng/mL) ($\beta = 0.80$, $p = 0.03$). The highly exposed group had a relative risk of 2.06 (95% CI: 1.06, 3.85) for obesity compared to the low exposed children. Although Mendez et al. and Verhulst et al. observed an association between DDE and BMI, no effects for HCB were observed (49, 59).

Brominated flame retardants

Brominated flame retardants (BFRs), including PBDEs are chemicals used to prevent fires and slow down combustion time. They are for example applied in clothes, furniture, and electrical equipment, and are known to be persistent and to bioaccumulate (63). The Bromine Science and Environmental Forum estimated that in 2000 the annual production of BFRs was more than 200000 metric tons (64). Several BFRs, including BDE-47, BDE-99, and hexabromocyclododecane (HBCD) have PBT (persistent, bioaccumulative, and toxic) properties (65). BFRs are usually applied in mixtures and production of two common mixtures, pentaBDE (in which BDE-47 and BDE-99 are the most abundant) and octaBDE (including various BDE congeners which have on average 7.2 to 7.7 bromine atoms per molecule of diphenyl ether), has already been terminated in Europe (65). DecaBDE (BDE-209 as most prevalent congener) is also likely to be phased out soon.

Exposure of mice to high concentrations (150 – 2500 mg/kg body weight) of decaBDE during gestation resulted in inhibited fetal growth and development, and disrupted lipid metabolism in the F0 mice (66). Bondy et al. (2013) exposed rats to BDE-71 before mating, during gestation, weaning, and continued exposure of the F1 generation until sacrifice (postnatal day 42) (67). They observed a non-dose dependent increase in bodyweight in the F1 rats. However also in this study concentrations exceeded human exposure levels. From a recent publication it appears that BDE-47 may enhance adipocyte differentiation in a dose-

dependent manner (2.5 – 25 μM) (38). However, experimental studies assessing effects of pre- or early postnatal exposure to BDE's on e.g. growth, weight, or adipogenesis, are scarce. Also observational studies on this topic are lacking. Some have determined the association between prenatal exposure and birth weight, predominantly observing negative associations (68-70), but none have reported on growth later in life.

Phthalates

Phthalates are used in plastics to increase flexibility, and are often referred to as plasticizers. They are also used as solvents and can be found in various products, ranging from vinyl on floors, to cosmetics and toys. Human exposure occurs mainly through diet (71), as phthalates can be released from packaging material into the products. Phthalates are metabolized by the body and the metabolites usually are excreted in urine. They are not known to bioaccumulate (71).

In utero DEHP exposed offspring of mice showed increased visceral fat at a dose of 0.05 mg/kg body weight per day, which is an environmentally relevant dose (72). Also body weight of female offspring at this dose was elevated. At a higher dose (5.0 mg/kg body weight per day), visceral fat weight was still increased in females, but decreased in males. Body weight in females, at this dose, was higher compared to controls, and also in males a significant increase in body weight compared to controls was observed. Hao et al. (2013) exposed both 3T3-L1 cells and pregnant mice to DEHP (73). No effect on adipocyte concentration was observed, however the in utero exposed mice showed increased body weight, adipose tissue deposition, serum lipids, and glucose levels at postnatal day 60 compared to controls. Exposure of 3T3-L1 cells to MEHP concentrations relatively high compared to human exposure levels, did however result in increased adipogenesis (4).

There are no studies available which have determined early life exposure to phthalates in association with child growth. Reports on associations between phthalate exposure and any growth related outcome, have often determined exposure in the population at the same time as when the outcome was determined. Wang et al. (2013) for example reported positive associations between levels of phthalates in urine of Chinese school children and BMI and waist circumference (74). Also in a cross-sectional study of the National Health And Nutrition Examination Survey (NHANES) data differences in BMI and waist circumference were observed across quartiles of exposure to various phthalates (75). Trends were different between genders and age-groups and mostly showed non-linear dose response relations.

Perfluorinated alkyl acids

Perfluorinated alkyl acids are fully fluorinated organofluorine compounds with a carboxylic acid or carboxylate functional group. They have various uses, including as surfactants, impregnation agents and water repellents, fabric protectors, and in fire-fighting foams. Two of these perfluors in particular, PFOS and PFOA, are considered hazardous for health. PFOS was listed in annex B of the Stockholm convention, implying that its' use should be

limited. In 2010 the use of PFOS was banned by the European Union, with some exemptions of products such as hydraulic fluids for aviation (76). PFOA is currently considered by the European Chemical Agencu (ECHA) 'a substance of very high concern because of its' CMR (carcinogenic, mutagenic, or toxic for reproduction) and PBT properties' (77). Various studies have determined the half-life of PFOA in humans, and periods from 2.3 to 8.5 years have been reported (77). As PFOA is not metabolized by the body and elimination rate is low, it accumulates in the body.

In vitro or in vivo studies looking at obesogenic effects of perfluorinated alkyl acids are scarce. A recent publication by Bastos-Sales et al. (2013) showed modest to no effects of PFOS and PFOA on adipocyte differentiation at a concentration of 10 μM (38). In utero exposure of mice to PFOA was however associated with higher leptin and insulin levels in females (78). Also observational studies are few. Halldorsson et al. (2012) determined exposure to PFOS and PFOA in cord blood and performed follow-up when offspring was 20 years of age (79). They observed an increased relative risk for a BMI higher than 25 and a waist circumference higher than 88 cm. in 20-year old females in the highest quartile of prenatal PFOA exposure (median: 5.8 ng/mL); range 4.8 – 19.8) compared to lower exposed females. The association between PFOA and both BMI and waist circumference was non-linear, with a significantly larger effect observed for Q4 than for the other quartiles. No association was observed for males and for PFOS exposure. A non-linear dose-response was also observed for PFOA exposure and weight in a cohort of British girls at the age of 20 months, though results were insignificant (80). Higher PFOS exposure was however associated with decreased birth weight and increased weight at 20 months. Andersen et al. (2010) on the other hand, reported an inverse association between prenatal PFOS and PFOA exposure and weight and BMI in childrens' first year of life, and after stratification, the effect was more pronounced in boys than in girls (81). This association was not apparent anymore when the children were seven years of age (82). However, they collected maternal blood early in pregnancy, while Maisonet et al. collected maternal blood during gestational week 30 and Halldorsson et al. assessed exposure in cord blood. These exposures reflect different time periods in fetal development.

A short summary of all studies according to their results, is given in table 3.2.

Table 3.2. Summarized overview of observational studies, according to their effect

Chemical	Primary outcome	Results		
		Negative	**No effect**	**Positive**
PCB	BMI	-	• Karmaus et al. (2009)	• Valvi et al. (2011) • Verhulst et al. (2009)
	Weight	• Rylander et al. (2007) • Blanck et al. (2002)	• Pan et al. (2010) • Ribas-Fito et al. (2006) • Hertz-Picciotto et al. (2005) (5 years of age, males) • Gladen et al. (2000) (boys) • Patandin et al. (1998)	• Gladen et al. (2000) (white girls)
	Height	• Guo et al. (1995)	• Pan et al. (2010) • Ribas-Fito et al. (2006) • Hertz-Picciotto et al. (2005) (5 years of age, males)	• Hertz-Picciotto et al. (2005) (5 years of age, females)
DDE	BMI		• Garced et al. (2012) • Gladen et al. (2004)	• Valvi et al. (2011) • Mendez et al. (2010) • Verhulst et al. (2009) • Karmaus et al. (2009)
	Weight		• Garced et al. (2012) • Pan et al. (2010) • Ribas-Fito et al. (2006) • Gladen et al. (2000) (girls)	• Gladen et al. (2000) (boys)
	Height	• Ribas-Fito et al. (2006)	• Garced et al. (2012) • Pan et al. (2010) • Gladen et al. (2000) (girls)	• Gladen et al. (2000) (boys)
	Rapid growth			• Mendez et al. (2010)
PFOS PFOA	BMI		• Andersen et al. (2013) (7 years old)	• Halldorsson et al. (2012)
	Weight	• Andersen et al. (2010) (5 and 12 months old)		• Maisonet et al. (2012) (PFOS, girls)
HCB	BMI			• Smink et al. (2008)

Discussion

The objective of this study was to present evidence available on the relation between early life exposure to EDCs and their propensity to promote weight gain and develop obesity, for six classes of chemicals. For each class there are indications that exposure in early life does affect adipogenesis or growth later in life, however results lack consistency. For some classes of chemicals, such as organotins, brominated flame retardants and phthalates, experimental and observational studies are scarce. Thus far, no clear dose-response relation can be observed, and results seem to differ between males and females.

Nonmonotonicity in dose-response relations between exposure to endocrine disruptors and health outcomes, has been reviewed by Vandenberg et al. (2012) (83). The main implication is that one cannot predict low dose effects from effects seen at high doses and vice versa. Various studies included in this overview did not show a linear relation between exposure and outcome. Whether these are truly nonmonotonic dose-response relations remains to be clarified. Effects shown in these studies at various doses may be incidental significant associations or chance findings. For a given effect to be truly acceptable as non-monotonic, it must be completely reproducible with the same dose-response relationships and the same significant effects. Current findings do not indicate this. This is also supported by a recent systematic review on obesogenic effects of phthalate exposure in humans, in which the authors concluded that this hypothesis could neither be confirmed nor rejected as results of included studies were far from consistent (84). Furthermore, comparison of results between studies is complicated as exposures are determined in various matrices, e.g. cord blood, which is assumed to reflect prenatal exposure, versus maternal blood, sampled each trimester, to assess exposure. The latter may also reflect prenatal exposure, however as the timing of sampling and the type of matrix are very different, a comparison should not be made unless a conversion factor is available. This has for example been done by Govarts et al. (2012), who did a meta-analysis of studies looking at early life exposure to PCB-153 and DDE, in relation with birth weight. The authors applied conversion factors to transform exposure levels in breast milk and maternal blood to cord blood values, and thus to create more uniformity in exposure levels across studies. A small, but significant negative effect for PCB-153 on birth weight was observed; no effect for DDE was reported.

Some studies reported gender specific effects (60, 79, 85), however most studies, when including a cohort with both males and females, only reported their results for the overall population. The chemicals reviewed in this paper act on the endocrine system and most of them are thought to have (anti-)estrogenic or (anti)androgenic properties. 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 (86). Male rats showed increased serum insulin levels, and both female and male rats had a significantly lower birth weight than controls. Placental exposure to organohalogenated xenoestrogens, including DDT, was associated with increased birth weight in 14 month old boys but not in

girls (87). As gender specific effects are thus not unexpected for EDCs, future studies should aim to stratify their results for males and females.

As indicated, some studies (51, 52, 88) find PCB exposure to be associated with lower weight; only one study associated prenatal exposure to PCBs with increased weight at puberty in girls (60), although this result was not significant. However, the inverse associations between exposure and weight as reported by Guo et al. (88) were only seen at young ages (6 months to 7 years of age) and did not persist during childhood. Furthermore Rylander et al. (52) measured exposure in blood samples of the mothers, which were collected at enrolment of the children (4 and 7 years of age), and converted this to exposure during pregnancy. This indirect method for calculation of exposure may not be as accurate as measurements performed at the time of pregnancy. Several studies (46-48, 85, 89, 90) find no effect of exposure (both DDE and PCBs) on weight as an individual parameter. Evidence in support of a significant association between prenatal exposure to PCBs or DDE and decreased or increased weight gain later in life, seems unconvincing.

Some studies observe exposure to DDE or PCBs to be associated with lower height (48, 88), but again several studies observe no effect of exposure on height or find a taller height with increasing exposure (Gladen et al. 2000, increased height in boys with increasing DDE exposure; Hertz-Picciotto et al. 2005, increased height in girls with increased PCB exposure). It could be that effects of prenatal exposure to EDCs are not substantial enough to be observed in either height or weight separately.

As for BMI, a parameter which includes both weight and height, exposure to EDCs was in none of the included studies related to a lower BMI and several studies observed a higher BMI with increasing exposure. For DDE in particular one study indicated that no association could be observed between prenatal exposure and BMI, however the outcome was assessed at twelve months of age, while in other cohorts in which DDE exposure was assessed, BMI was measured at later ages (89). Although BMI was not a direct metric determined in the cohort of Pan et al, they observed no effect of DDE exposure on either weight or height, also measured at twelve months of age, making potential obesogenic effects unlikely (47). However, in this cohort only breast-fed children were included. It could be suggested that these children are a positive selection of the general population and that they are therefore more likely to be healthier. This is supported by the observations in the cohort of Patandin et al. (90), looking at effects of PCB exposure on growth in both formula-fed and breast-fed children. A lower growth rate was observed until the age of three months. This effect was only significant in children who were formula-fed, not in children who were breast-fed. Furthermore, it seems that exposure to EDCs in breast milk was never associated with any growth outcome, also not when duration of breastfeeding was taken into account (47, 60). This finding may indicate the importance of prenatal exposure in relation to growth in later life. It could furthermore be argued that – with respect to growth in later life – beneficial effects of breast milk may still outweigh detrimental effects of EDCs present in the milk.

The majority of the observational studies did only include anthropometric measurements as a way of quantifying obesity or growth. In most studies this information was collected by trained researchers, and in some cases by self-report. Though BMI as a relative measure may give an indication of when a child is likely to have a high percentage of body fat, it is not a measure of adiposity (91). This is also true for weight as an individual measure. For this purpose it would be interesting for future studies to include a measure of fat mass, e.g. by using DEXA-scans. Moreover, information on hormone levels such as thyroid hormones and leptin, may give more insight into potential mechanisms through which these compounds may affect growth.

Diet as a confounding factor was not considered by most of the included studies, even though it is an important route of exposure to several compounds and it is intrinsically related to energy balance. Mothers consuming a high fat diet may very well have children who will consume a similar diet, and who are therefore higher exposed through the diet to both endocrine disruptors and fat. Another potential confounder not considered by these studies, is birth weight. Both high (92, 93) and low birth weight (94, 95) are associated with an increased risk of obesity later in life. In several studies exposure to EDCs has found to be inversely associated with birth weight. More specifically this result was found in case of exposure to PCBs (measured either in maternal blood or in maternal milk) (85, 88, 90, 96, 97), PFOS and/or PFOA (measured in maternal or cord blood) (98-101) and for phthalate exposure (102). However, birth weight may also be on the causal pathway between early life EDC exposure and long-term growth, and adjusting models for this factor may also result in overcorrection.

From the overview presented here it may be concluded that PCBs and DDE are the most frequently studied chemicals in long-term prospective studies assessing the effects of prenatal exposure to EDCs. These compounds have also been frequently assessed in older populations, in which associations were observed with BMI (103), characteristics of metabolic syndrome, including waist circumference and fasting glucose (104), as well as fat mass (105). Effects of early life exposure to HCB and perfluorinated alkyl acids, such as PFOS and PFOA, have also been determined, however studies focussing on these chemicals are scarce. Health effects of other chemicals such as brominated flame retardants and phthalates, have thus far only been determined in relation to exposure measured in older children or adults, and obesogenic effects of organotins have only been studied in vitro and in vivo.

Conclusions

It can be concluded that literature does suggest that early life exposure to certain EDCs is associated with weight homeostasis and growth later in life, however not necessarily in an obesogenic direction. Results lack consistency and observational studies for some groups of chemicals are not yet available. Future studies should aim to determine prenatal exposure in

particular and to include not only BMI as an outcome measure, but also more sensitive measures of adiposity, such as fat mass. As these chemicals are considered to be endocrine disruptors, stratifying results for gender is advised. Long-term follow-up is warranted, especially regarding exposure to chemicals other than PCBs and DDE, for which knowledge on long-term effects is still scarce.

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1

General introduction to thesis

During the past decades the Western world has been rapidly developing. Major technical changes have enabled improvements in many areas such as food production and health care. Life expectancy has increased with every new generation and is currently still increasing (1). However, the abundance in food as well as changes in activity patterns due to a more sedentary lifestyle have resulted in increasing prevalences of obesity worldwide, to the proportions of an epidemic (2). Though at first, obesity seemed to occur predominantly among adults, it is clear now that increasing numbers of children have to deal with the health consequences and social stigma of being overweight (3). Obese children have a higher risk for bone and joint problems, and are more likely to have pre-diabetes and risk factors for cardiovascular disease (4). Furthermore they are more likely to be obese when they reach adulthood (5), which increases their risk for health problems associated with adult obesity. Even though the average life expectancy of new generations is still higher compared to previous generations, it is believed that the high prevalence of obesity decreases disability free life expectancy (6).

Besides the marked increase in the prevalence of childhood obesity, the number of children diagnosed with autism spectrum disorder (ASD) or attention deficit hyperactivity disorder (ADHD) has also been increasing. For ASD a 23% increase in estimated prevalence between 2006 and 2008 was measured, as reported by the Autism and Developmental Disablilties Monitoring (ADDM) Network (7), and according to the most recent figures from the Centers for Disease Control and Prevention (CDC), one in fifty children in the United States is diagnosed with ASD (8). The CDC reported furthermore an increase of 22% in children with parent reported ADHD diagnosis between 2003 and 2007 (9). Both ASD and ADHD may significantly decrease the quality of life of the individuals concerned and their environment. The majority of the children diagnosed with ASD require special education and are not able to have common social interactions with others (10). Children with ADHD may experience more difficulties at school, both regarding their education and social contacts (9, 11). Adults with ADHD report to have a worse general health (11) and are more often absent at work due to illness (12).

The increase in prevalence of children diagnosed with a neurobehavioral disorder such as ASD or ADHD is often attributed to changes in diagnostic criteria and instruments used, especially regarding ADHD. However, as was stated by Sciutto and Eisenberg, there is no sufficient evidence to state that ‘factors contributing to the misidentification of ADHD in children systematically favour false positives over false negatives’ or that ‘ADHD is systematically overdiagnosed’ (13). Also the argument that childhood obesity is caused by energy imbalance seems insufficient to explain individual differences in weight gain (14). Clearly, other factors should be considered.

In 2002 a review by Baillie-Hamilton was published which showed remarkable similarities between the obesity epidemic and the production of chemicals from 1930 and onwards (15). After the second World War the use of chemicals such as pesticides became popular and innovations in research made it possible to produce chemicals which were only available in limited amounts in nature or which did not occur in nature at all (16), resulting

in a increasing global chemical output ever since (figure 1). The innovations in the chemical industry have contributed substantially to the modernization of our world, and it could be argued that the increase in obesity prevalence and the similarities with chemical production are in fact similarities with changes in lifestyle resulting from modernization of the environment; e.g. a lack of physical activity and a surplus of food. However it is known that exposure to several chemicals may adversely affect health. The use of dichlorodiphenyltrichloroethane (DDT) as an insecticide was banned from agricultural usage in the United States in 1972, after concerns about possible carcinogenic effects in humans as well as the effects on wildlife, such as eggshell thinning (17). Diethylstilbestrol (DES), a synthetic, estrogenic drug given to women between 1940 and 1970 to prevent miscarriage, was withdrawn in 1971 because of carcinogenic effects as well as an increased risk for infertility in children who were in utero exposed (18).

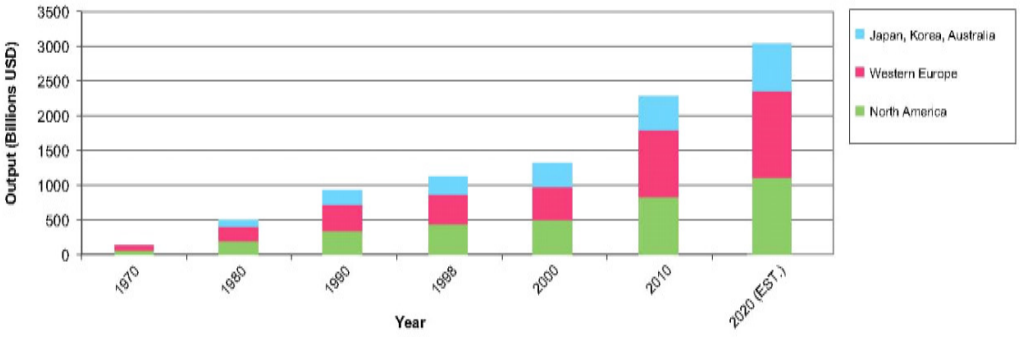

Figure 1.1. Chemical industry output, developed regions (19)

The example of diethylstilbestrol also illustrates another important concept, which is that in utero events may affect health later in life. This approach is referred to as the 'Developmental Origins of Health and Disease (DOHaD), and hypothesizes that many non-communicable diseases originate during development, as early as in utero and during childhood (20). This puts the health risks of chemical exposure in a new perspective, especially since research has shown that various chemicals may pass the placenta and reach the unborn child (21). The fetus is particularly vulnerable to the effects of environmental exposures as the prenatal period is characterized by periods of rapid cell devision and growth and as organs, including the brain, are developing (22).

The current hypothesis is that chemicals may interfere with hormonal functions in our body and that early life exposure in particular may have long-lasting health effects. These chemicals are therefore referred to as endocrine disrupting chemicals (EDCs), which are defined by the Inter-Organisation Programme for the Sound Management of Chemicals as 'an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations' (23). They may act on various parts related to the endocrine system, such as gene expression which may affect hormone production, and receptor binding which may

mimick or block hormonal activity. EDCs that 'inappropriately alter lipid homeostasis to promote adipogenesis and lipid accumulation, are also referred to as 'obesogens' (24).

A description of a subset of chemicals with endocrine disrupting properties will be given below. Furthermore the most commonly described pathways through which EDCs are suspected to promote childhood adiposity or to affect neurodevelopment will be given. These include peroxisome proliferator-activated receptor (PPAR), sex-steroid, thyroid hormone, and feeding circuit/leptin/insulin mediated effects. Also an outline of sources of EDC exposure will be given.

Sources and properties of EDCs

Several chemicals are currently known to have endocrine disrupting characteristics, and this list will be extending as research progresses. For the purpose of this thesis, sources and properties of five classes of chemicals will be described here.

Non-dioxin-like polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are generally divided in two groups, either dioxin-like PCBs, which have a dioxin-like mechanism of toxicity, or non-dioxin-like PCBs, including PCB-153 (figure 1). In total, 209 PCB congeners are possible, based on the number and position of chlorine atoms. Non dioxin-like PCBs were mostly applied as dielectric and coolant fluids, however due to health concerns, including potential carcinogenic effect, production of PCBs in Europe has been prohibited since 1985 (25). However, PCBs may still be released in the environment from old electrical appliances, and as they are furthermore known to be highly persistent in the environment, they may accumulate in fat tissue of animals and humans (26). The main source of exposure for people is through animal food (fish and meat), which is mostly contaminated with PCB-153 and PCB-138 (26). In Europe, dietary exposure to PCBs is monitored by the European Food Safety Authority (EFSA) (27).

Organochlorine pesticides – DDT and HCB

Dichloro-diphenyl-trichloroethane (DDT) and hexachlorobenzene (HCB) are part of group referred to as organochlorine pesticides. DDT was developed in the 1940s as an insecticide and was amongst others used to fight insect-borne diseases, such as malaria (28). Due to health concerns – DDT is carcinogenic – use of DDT was banned in the US in 1972 and in the Netherlands in 1973 (29). However, in developing countries DDT is still used for vector-control (malaria), which is currently also supported by the World Health Organization (WHO) (30). DDT is known to bioaccumulate and to be highly persistent in the environment. HCB is a byproduct of manufacturing processes of other chemicals, and has also been widely used as a pesticide. Due to probable carcinogenic effects, production

and use of HCB was prohibited by the Stockholm Convention (31). Like DDT, it accumulates in fat tissue and it persists in the environment.

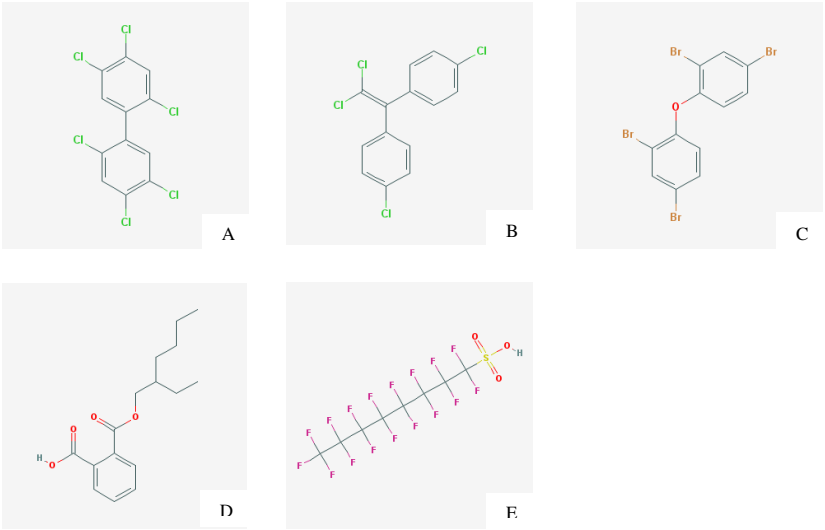

Figure 1.2 Chemical structures of A: PCB-153, B: DDE, C: PBDE-47, D: MEHP, E: PFOS

Brominated flame retardants

Brominated flame retardants (BFRs), including polybrominated diphenyl ethers (PBDEs) are chemicals used to prevent fires and slow down combustion time. They are for example applied in clothes, furniture, and electrical equipment, and are known to be persistent and to bioaccumulate (32). The Bromine Science and Environmental Forum (BSEF) estimated that in 2000 the annual production of BFRs was more than 200000 metric tons (33). Several BFRs, including BDE-47, BDE-99, and HBCD have PBT (persistent, bioaccumulative, and toxic) properties (34). BFRs are usually applied in mixtures and production of two common mixtures, pentaBDE (in which BDE-47 and BDE-99 are the most abundant) and octaBDE (including various BDE congeners which have on average 7.2 to 7.7 bromine atoms per molecule of diphenyl ether), has already been terminated in Europe (34). DecaBDE (BDE-209 as most prevalent congener) is also likely to be phased out soon.

Phthalates

Phthalates are used in plastics to increase flexibility, and are often referred to as plasticizers. They are also used as solvents and can be found in various products, ranging from vinyl on floors, to cosmetics and toys. Human exposure occurs mainly through diet

(35), as phthalates can be released from packaging material into the products. Phthalates are metabolized by the body and the metabolites usually pass the body through urine. They are not known to bioaccumulate (35). However, some studies to observe associations with health effects, and therefore also these chemicals are considered to be of concern.

Perfluorinated alkyl acids

Perfluorinated alkyl acids are fully fluorinated organofluorine compounds with a carboxylic acid or carboxylate functional group. They are used amongst others in surfactants, impregnation agents and water repellents, fabric protectors, and in fire-fighting foams. Two of these perfluors in particular, perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), are considered hazardous for health. PFOS was listed in annex B of the Stockholm convention, implying that its' use should be limited. In 2010 the use of PFOS was banned by the EU, with some exemptions of products such as hydraulic fluids for aviation (36). PFOA is currently considered by the EFSA 'a substance of very high concern because of its' CMR (carcinogenic, mutagenic, or toxic for reproduction) and PBT (persistent, bioaccumulative, and toxic) properties' (37). Various studies have determined the half-life of PFOA in humans, and periods from 2.3 to 8.5 years have been reported (37). As PFOA is not metabolized by the body and elimination rate is low, it accumulates in the body.

Mechanisms of action of EDCs

PPAR mediated effects

Peroxisome Proliferator-Activated Receptors, or PPARs, are a group of nuclear receptor proteins that are involved in control of cellular differentiation programs, but also in metabolism of lipids and carbohydrates by means of transcriptional control. Of the three varieties (α, β, and γ), PPARγ is considered most involved in regulating adipogenesis.

Agonists of PPARα promote peroxisome proliferation and fatty acid β-oxidation (38), therefore targeting of PPARα often has a hypolipodemic effect. Activation of PPARγ on the other hand, results in lipid biosynthesis and differentiation of preadipocytes into adipocytes (39). In vivo treatment with PPARγ antagonists prevented high fat diet induced weight gain in rodent models (40-43), and it is known that PPARγ agonist pharmaceuticals – used to improve insulin sensitivity in diabetes (44) – promote weight gain (45, 46).

There are several options through which endocrine disruptors may promote obesity through PPARγ. Usually a ligand is needed for PPARγ to bind co-activators, release co-repressors, decondensate the chromatin and activate transcription (47). However post-translational modifications, such as phosphorylation, may activate PPARγ in absence of ligand (48). EDCs may furthermore cause multipotent stromal cells (MSCs), cells which can differentiate into various tissues, to predominantly differentiate into adipose tissue (47, 49). This particular subset of MSCs expresses PPARγ (50). Trybutyltin, a known agonist of

PPARγ, has been shown to cause lipid accumulation in adipose tissues in prenatally exposed newborn mice (51). MSCs derived from the adipose tissue of these mice showed increased commitment to the adipocyte lineage compared to controls (52).

Besides from its' presence in adipose tissue, PPARγ is also expressed in the central nervous system, in particular in the area important for the central regulation of energy balance (53). Administration of the thiazolidinedione (TZD) Rosiglitazone (RSG) to rats resulted in higher food intake with a corresponding higher body weight change compared to controls (54). This effect was not observed after blocking of central nervous system CNS PPARγ receptors by an antagonist, indicating that activation of CNS PPARγ may result in higher food intake.

What also needs to be considered is that even though activation of PPARα stimulates lipid mobilization, it may also indirectly promote obesity. Prenatal activation may result in low birth weight, a known risk factor for obesity later in life. Furthermore, PPAR agonists and their metabolites may activate multiple PPAR isoforms. Bis(2-ethylhexyl)phthalate (DEHP), an EDC from the phthalate class, may activate PPARα, however its metabolite mono (2-ethylhexyl)phthalate (MEHP) may activate PPARγ (55, 56). And though lipid mobilization induced by PPARα activation requires continuous exposure, PPARγ may only need a single or episodic exposure to establish its effects in adipose tissues (43).

Recent research has also indicated potential for perfluorinated alkyl acids, such as perfluorooctane sulfonate (PFOS), to interfere with PPAR by inducing expression of PPARγ genes in mouse neonatal brain after prenatal exposure (57).

Estrogen receptor interference

Another potential route for endocrine disruptors to be involved in the aetiology of obesity is through estrogen receptors (ER). The main receptors, ERα and ERβ, are known for their function in reproduction, however they are also involved in brain development and several studies indicate their link to metabolism (58). For example, post-menopausal women often experience increase in white adipose tissue, an effect which can be reversed by estrogen supplementation (59). A similar observation was made in rodents with low oestrogen levels due to ovarectomy (58).

It is suggested that these effects are modulated mainly through ERα since in animal models in which ERα was knocked out (αERKO), no reversible effect of oestrogens on weight homeostasis was observed (60). Furthermore the balance of steroid hormones seems an important factor in weight homeostasis. Estrogen supplementation is an example of a correction of an imbalance occurring after menopause. Moreover in men who are treated for prostate cancer and in women who are treated for polycystic ovary syndrome with anti-androgenic therapy, weight gain is observed (43, 59).

Developmental exposure to estrogens exerts quite different effects than adult exposure, which was quite clearly demonstrated by Newbold et al. who exposed mice prenatally to diethylstilbestrol (DES) – a compound with estrogenic characteristics (61). At birth no effect on weight was seen in the low exposed group (1 µg DES/kg/day), but at later age the

exposed individuals gained significantly more weight than the unexposed. However it must be noted that in humans prenatal exposure to this compound does not seem to affect body weight in later life, as was observed in daughters of women who received prescriptions for DES to prevent miscarriage (62). On the other hand, Hatch et al. observed associations with preterm birth and higher risk for small for gestational age in a cohort prenatally exposed to DES (63), and being born small for gestational age (SGA) is a known risk factor for obesity later in life (64). Newbold et al. (61) also reported decreased birth weight in higher exposed groups of mice (10- 1000 µg/kg/day). In all groups weight remained decreased compared to controls throughout life, however in the highest exposed mice, low birth weight was followed by a period of catch-up growth during puberty, resulting in a significantly increased body weight compared to controls at two months of age.

Estrogens are also involved in brain development and estradiol in particular is important for dendritic growth, spine density, and synaptogenesis in the cerebellum (65, 66). This brain structure controls various behaviours, including attention, language, and motor control (67), which implies that estrogenic disruption in this area may affect neurodevelopment and therefore may be related to neurodevelopmental disorders. A recent study by Saili et al. showed that developmental exposure of zebrafish to either 17β-estradiol or GSK4716 (a synthetic estrogen which mainly targets ERγ), as well as BPA, resulted in hyperactivity in larvae and learning deficits in adult zebrafish (68). Even though evidence from experimental studies is scarce, Braun et al. also observed associations between gestational BPA exposure and externalizing scores in 2 year old girls (69), suggesting there is potential for exogenous estrogens to be involved in neurodevelopmental disorders.

Though steroid hormones such as estrogen are produced by the gonads, they can also be generated by the brain, including the cerebellum (70). One key enzyme in this process is cytochrome P450 aromatase (CYP19), which converts testosterone to 17β-estradiol (E2). Developmental exposure to estradiol has been found to decrease CYP19a1b expression in the brain of male rainbowfish (71), and aromatase activity was decreased in male rats prenatally exposed to a PCB-mixture (72). Other studies also suggest that aromatase activity may be affected by phthalates (73), DDE (74), and various pesticides (terbuthylazine, propiconazole and prothioconazole) (75), although findings need to be substantiated.

Thyroid hormone receptors

Also associated with metabolism and weight homeostasis, as well as brain development, are thyroid hormones, which at elevated levels accelerate metabolism, increase lipolysis and hepatic cholesterol biosynthesis and excretion, therefore stimulating weight loss (58). At lower levels the opposite effects are observed. They are furthermore essential for neurodevelopment as they regulate genes involved in myelination and neuronal cell differentiation (76)

EDCs have been shown to interfere with thyroid hormone (TH) receptors. Hydroxylated metabolites of PCBs were the first environmental chemicals discovered to bind to

transthyretin, a transport protein for T4, resulting in displacement of T4 (77). BPA has the ability to bind to TH receptor and has antagonistic properties, resulting in inhibition of transcriptional activity stimulated by triodothyronine (T3) (78). Furthermore BPA may enhance recruitment of corepressor N-CoR to the TH receptor, resulting in displacement of T3 from the TH receptor and gene suppression (79). Paradoxically, perinatal exposure to BPA may also result in increased levels of thyroxine (T4) (80). The latter effect was observed in offspring of dams exposed to BPA in drinking water during gestation and lactation (81). However, the mothers also experienced a decrease in free T4 compared to controls and therefore it was suggested that the increase in T4 observed in the offspring was a compensatory response.

Thyroid hormones may also be disrupted by brominated flame retardants, for example metabolites of polybrominated diphenyl ethers (PBDEs). Some isoforms have been shown to exert inhibiting effects for binding of T3 to the TH receptor (78). PBDE metabolites are also able to bind to transthyretin (82), which has also been observed for perfluorinated alkyl acids, such as PFOS and PFOA (83).

It should also be considered that thyroid hormones are important factors for brain development and that low T4 levels during development disturb normal maturation of the brain. Transthyretin is able to pass the placenta and delivers T4 across the blood-brain barrier. This implies that when binding PBDE metabolites or perfluorinated alkyl acids instead of T4, transthyretin may transport these compounds to the fetal brain compartment where they may accumulate (84). Even though discussion remains as to the importance of transthyretin compared to TBG for transport of thyroid hormones, developing foetuses and infants are especially sensitive to small changes in TH disruption (85, 86). In a cohort of 5-6 year old children, TSH correlated with worse neurophsychological functions, while T3 and T4 correlated with better outcome such as less ADHD and better behaviour (87). Furthermore, pentachlorophenol (PCP) was negatively related to T3, which would fit with the observed correlation between T3 and outcome. However, PBDE related positively to T3. Also in a study by Gascon et al., no significant associations between TSH, TT3, fT4 and postnatal levels of PBDE-47 were observed, nor did they significantly relate with neurodevelopmental outcomes (88).

Much remains to be clarified; however the role of thyroid hormones in metabolism and brain development and the potential disrupting abilities of some chemicals with regards to this pathway is significant, indicating that they may very well be involved in the aetiology of obesity as well as neurodevelopmental disorders.

Feeding circuits, lepin, and insulin

Regulation of energy balance is a basic concept in the aetiology of obesity. Several factors control appetite and energy expenditure, which are all integrated in the hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus is the centre for energy balance and appetite regulation. In adults, hormones such as leptin and insulin give signals to the arcuate nucleus of the hypothalamus (ARH), which in turn emits neuronal signals by means

of neurotransmitters such as pro-opiomelanocortin (POMC), cocaine and amphetamine-regulated transcript (CART), agouti-related peptide (AgRP), and neuropeptide Y (NPY). POMC and CART are known to decrease appetite, while NPY and AgRP are appetite stimulators. These peptidergic hormone factors are potential transcriptional targets for endocrine disrupting chemicals (43). It is for example known that estrogens may mimic actions of leptin on NPY (downregulation) and POMC neurons (upregulation), which would prohibit obesity development (89).

Not much is known on developmental exposure to EDCs in relation to later appetite regulation. The development of these feeding circuits starts prenatally. In rodents, generation of ARH neurons takes place between embryonic day 12 and 17. Development of projections of neurons to their targets on the other hand occurs only postnatally (90). Less is known about the development of hypothalamic feeding circuits in humans. Studies indicate that unlike rodents, in which development takes place partially postnatally, development in primates occurs completely prenatally (90) in the second and third trimesters (91).

Chemicals may also disrupt actions of leptin or insulin itself. It is clear that both leptin and insulin play important roles in development of feeding circuits which are comparable to sex steroid hormones with regard to the development of sexually dimorphic circuits (male, female) (92). Rats injected with insulin between embryonic day 15 and 20 (term is 22 days) were significantly more obese (93). Increases in leptin levels are observed during the first two weeks of life in rats (94). In this early period in life the need for energy is high and a leptin surge would be in contrast with this need. However, this surge coincides with a critical period in development of parts of the hypothalamus that control energy homeostasis, and it is therefore suggested that leptin is an important factor for brain development early in life rather than an anorexigenic factor (92, 94). In leptin-deficient mice also ARH circuit formation was affected (95). Leptin is also considered a regulator of fetal growth (96) and low leptin levels at birth have been associated with a higher risk for obesity and diabetes (97).

As a model for brain development, the pig and the sheep model are more comparable to human development than the rat model. In intra-uterine growth retarded (IUGR) newborn pigs lower leptin levels were observed in the circulation and in the placenta (90, 91). Furthermore changes in distribution of leptin receptors (OB-Rb) in the hypothalamus were observed (92). These receptors are usually located in the ARH, but in the IUGR newborn pigs expression was equal in the ARH and in the ventromedial premillary nuclei. This could cause lower sensitivity to leptin, resulting in altered food intake (92). As indicated before, EDCs can act on PPARγ. When PPARγ is activated by natural ligands, it inhibits OB-gene expression and leptin release in adipocytes (61, 93), resulting in decreased leptin levels (95). EDCs may exert similar effects via PPARγ and when this occurs during the fetal period, consequences for the hypothalamic development may be similar to what has been observed in IUGR pigs. This was confirmed by Boberg et al. who showed that prenatal

exposure to PPAR agonists diisobutylphthalate and rosiglitazone resulted in significantly lower leptin and insulin levels in fetal rats (98).

An overview of mechanisms of endocrine disrupting chemicals is given in table 1.1. These pathways will however not be investigated, as this is beyond the scope of this thesis.

Table 1.1 Mechanisms of endocrine disrupting chemicals

Target	Pathway	Effect	Chemical
PPARα	• Agonists stimulate lipid mobilization	• Prenatal activation may result in low birth weight	• DEHP
PPARγ	• Lipid biosynthesis, differentiation of preadipocytes in adipose tissue, inhibition of OB-gene expression • In CNS, PPAR is also found in areas important for regulation of energy balance	• Lower leptin levels prenatally, affecting distribution of leptin receptors in the hypothalamus • Activation of CNS PPARγ may result in higher food intake	• TBT • Pharmaceuticals • Phthalates • BPA • PFOS
ER	• Estradiol in cerebellum regulates dendritic growth, spine density, and synaptogenesis • Energy homeostasis	• Hyperactivity, learning deficits • Higher risk for preterm birth and SGA (human), low birth weight in mice followed by catch-up growth and weight gain	• DES
Thyroid hormones	• Binding to transport proteins (e.g. transthyretin) which may transport chemicals across the blood-brain barrier • Competitive binding to TH receptors with actual thyroid hormones	• Transport of chemicals across the blood-brain barrier • Displacement of actual TH from receptor •	• BPA • PBDE • PFOS/PFOA
Leptin		• IUGR associated with lower leptin levels • Low leptin levels at birth associated with higher risk for obesity	• Indirectly through PPARγ agonists

Outline of this thesis

It can be concluded that exposure to chemicals, particularly early in life, may be involved in the programming of childhood obesity and therefore also obesity later in life. Exposure may furthermore affect neurodevelopment and may therefore be related to behavioural disorders.

In this thesis the effects of early life exposure to five classes of endocrine disrupting chemicals on child health during the first 12 months of life are investigated. The focus will be predominantly on outcomes related to child growth, but also the potential for EDCs to be involved in the aetiology of neurodevelopmental disorders will be explored.

Firstly, an overview is given of current literature on early life exposure to EDCs and neurodevelopmental disorders such as attention deficit hyperactivity disorder and autism spectrum disorders (**chapter 2**), as well as childhood obesity (**chapter 3**). This is followed by a description of the LINC (LInking EDCs in maternal Nutrition to Child health) study, which was designed to study health effects of early life exposure to EDCs in a cohort of mother-child pairs in the Netherlands (**chapter 4**). The LINC study was started as part of the OBELIX project (OBesogenic Endocrine disrupting chemicals: LInking prenatal eXposure to the development of obesity later in life), which was an European Union funded research to study obesogenic effects of EDCs in both animal and human studies.

Chapters 5 and **6** describe the associations between early life EDC exposure and respectively weight and thyroid hormones at birth, as they were observed in the LINC study. Effects on growth during the first year after birth are discussed in **chapter 7**.

Finally, in the general discussion (**chapter 8**), results from chapters 4 to 7 are summarized, and a general reflection on the main outcome is given. Potential pathways and methodological challenges are addressed, and recommendations for future research are given.

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2

Does perinatal exposure to endocrine disruptors induce autism spectrum and attention deficit hyperactivity disorders? Review

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Abstract

Aim: To provide an overview of studies on perinatal exposure in humans to EDCs in relation to autism spectrum (ASD) and attention deficit hyperactivity (ADHD) disorders.

Methods: A review of the literature (PubMed) was performed. Exposure related keywords, including various chemicals, were matched with keywords describing outcome. Animal studies as well as publications not written in English were excluded. In total 834 titles were retrieved. The final selection included 21 publications.

Results: Positive associations were found for ASD in relation to exposure to all chemicals investigated, which included hazardous air pollutants, pesticides, and bisphenol A (BPA). Increased risks for ADHD or positive associations were found for exposure to polychlorinated biphenyls (PCBs), dialkyl phosphate (DAP), and chlorpyrifos. BPA, polybrominated diphenylethers (PBDEs), and low molecular weight (LMW) phthalates were positively associated with externalizing behaviour. Five out of seventeen studies did not find any association between exposure and ADHD.

Conclusion: Perinatal exposure to EDCs appears to be associated with the occurrence of ASD as well as ADHD. Disruption of thyroid hormone function and gamma-aminobutyric acid (GABA)ergic mechanisms may offer an explanation for the observed relations, though conclusive evidence in humans is limited.

Introduction

Toxic chemicals are produced in large quantities. They are incorporated in numerous products used in daily life, e.g. in plastics, food packaging material, furniture, and pesticides. Many of these chemicals make life more comfortable and, due to their frequent use, production of many of those continues to grow (1). Children today are at risk of exposure to 3000 synthetic chemicals produced in quantities of more than 1 million pounds per year (2). Along with the growth in production of toxic chemicals, an increase in prevalence of neurodevelopmental disorders such as Autism Spectrum Disorders (ASD) and Attention Deficit Hyperactivity Disorder (ADHD) has been observed over the past decades. Increased awareness and a shift in diagnosis may have contributed to the higher prevalences. However, they do not offer a sufficient explanation for these findings (3). It is hypothesized that exposure to chemicals with endocrine disrupting properties (endocrine disrupting chemicals – EDCs) (table 2.1) increases the risk for neurodevelopmental disorders such as ASD and ADHD.

Table 2.1 Classes of endocrine disrupting chemicals

Class of EDC	Chemical(s)
Perfluorinated alkyl acids	PFOS, PFOA
Organochlorine pesticides	HCB, DDE, DDT
Organophosphate pesticides	DAP, chlorpyrifos
Non-dioxin-like PCBs	PCB-153
Dioxin-like compounds	2,3,7,8-TCDD, PCB126
Brominated flame retardants	PBDE-47, PBDE-99, HBCD
Phthalates	DBP
Organohalogens (OHCs)	4OH-CB-146
Others	BPA, PCE, phytoestrogens

PFOS: perfluorooctane sulfonate; PFOA: perfluorooctanoic acid; HCB: hexachlorobenzene; DDE: dichlorodiphenyldichloroethylene; DDT: dichlorodiphenyltrichloroethane; DAP: dialkyl phosphate; PCB: polychlorinated biphenyl; TCDD: tetrachlorodibenzodioxin; PBDE: polybrominated diphenylether; HBCD: hexabromocyclododecane; DBP: dibutyl phthalate; OH-CB: hydroxylated polychlorinated biphenyls; BPA: bisphenol A; PCE: perchloroethylene or tetrachloroethylene.

Both ASD and ADHD typically are diagnosed during childhood, and even though their causal factors are to date not well understood (4), it is known that both genetic and environmental factors, as well as their interactions, are involved (5) (6). Detrimental effects of lead, methylmercury and polychlorinated biphenylethers (PCBs) have been reported previously (7, 8). More recently it has been observed that mice exposed to polybrominated diphenylethers (PBDEs) – a chemical used in flame retardants - had increased hyperactivity in adulthood compared to controls (9). Also alterations in spontaneous behaviour were observed (10). Furthermore, studies in rats have shown that exposure to phthalates causes hyperactivity reminding of the clinical picture of ADHD as observed in humans (11). Therefore endocrine disruption may be an interesting link between these exposures and the neurodevelopmental effects observed.

It is known that exposure to EDCs in adults may cause adverse health effects. Vietnam war veterans who have been exposed to 2,3,7,8-Tetrachlorodibenzodioxin (TCDD), more commonly known as agent orange, have an increased risk of diabetes and prostate cancer (12). Human exposure, however, may already start early in development, since the placenta does not completely block the transfer of EDCs from the maternal circulation to the foetus (13). The foetal period is the most important period in development, and hormones are key factors in many developmental events (14). Cell proliferation, differentiation, and apoptosis of many foetal tissues are regulated by hormones (14). Foetal growth rate is adjusted to foetal nutrient supply by hormones which signal the availability of nutrients and oxygen to foetal tissues (14). Also brain development is regulated and influenced by hormones, and especially thyroid hormones are known to be essential for normal embryonal and foetal neurogenesis (15). EDCs are known to affect thyroid hormonal function in particular. Therefore disruption of hormonal function during specific time periods important for brain development may have many consequences and may amongst others have adverse effects on neurodevelopment. Furthermore, Skinner et al. (16) showed that embryonic exposure to the endocrine disruptor vinclozolin resulted in changes in expression of various genes in both the hippocampus and amygdala, changes which were still observed in rats three generations removed from exposure . Increased anxiety-like behaviour was observed in the females of this generation, while young males showed hyperactivity. Exposure to EDCs may therefore also affect behaviour in multiple generations through epigenetic pathways. The objective of this review was to provide an overview of studies on perinatal exposure in humans to EDCs in relation to ASD and ADHD.

Methods

PubMed was searched for relevant publications, using terms relating to exposure in combination with outcome related key words. The following terms were used to describe exposure: environmental exposure, chemicals, EDC, endocrine disruption, pesticides, polychlorinated biphenyls (PCB), organochlorine, dichlorodiphenyldichloroethylene (DDE), dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), flame retardant, polybrominated diphenylether (PBDE), bisphenol A (BPA), phthalate, perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), and phytoestrogen. These terms were matched with each of the following key words describing ADHD and ASD related outcome: ADHD, hyperactivity, overactivity, inattentive, impulsive, ASD, autism, social behaviour, social interaction, communication, (child) behaviour.

Searches were – if necessary – refined by adding the key word ‘exposure’ to the search and by exclusion of animal studies and publications not written in English. In total 834 titles were retrieved. Publications were only included if exposure was assessed perinatally and if the outcome measured related to ASD or ADHD. Selection based on titles and abstracts resulted in the inclusion of 42 publications, reviews not included. To complete

information on exposure assessment and on outcome measures, the methodology section of articles were carefully searched. Reference lists of included publications were also searched for relevant studies, resulting in an additional two publications. The final selection included 21 publications.

Results

An overview of the occurrence of ASD and ADHD in relation to perinatal exposure to toxic chemicals in various cohorts is presented in table 2. ASD in relation to perinatal chemical exposure was assessed in four studies; all studies observed a positive association. Windham et al. (2006) (17) assessed exposure to hazardous air pollutants (HAPs) in both autism cases and controls and observed slightly – though not significantly – elevated adjusted odds ratios (AOR) for the fourth quartile of exposure to endocrine disruptors and developmental toxicants (table 2). In particular vinyl chloride and trichloroethylene were significantly elevated in the fourth quartile. Roberts et al. (2007) (18) observed that living near sites of pesticide application during gestation increased the likelihood for the occurrence of ASD in children by six times. Risk increased with poundage of pesticides used and as distance from field sites decreased. Similar to this result, Volk et al. (2011) found an increased AOR for children born to mothers living close to freeways at the time of birth (19). The most recent study on prenatal exposure to EDCs in relation to ASD measured BPA and phthalates in urine of women in their third trimester of pregnancy as a proxy for in utero exposure of the foetus (20). Prenatal exposure to BPA was not associated with ASD later in life. However, exposure to phthalates, and low molecular weight phthalates (LMW) in particular, was associated with greater social deficits, poorer cognition, communication, and social awareness.

Regarding ADHD, a positive association or an increased risk is found with increasing exposures to chemicals including DAP, PCBs, HCB, chlorpyrifos, and solvents (21-26). Prenatal exposure to DAP was reported on twice, both times from the same cohort. DAP-levels as measured in maternal urine during gestation were not associated with ADHD and attention scores on the CBCL when the children were 24 months of age (27). However these associations did become significant at 5 years of age at follow-up (21). Perinatal exposures to BPA, PCBs, LMW phthalates, and solvents were positively associated with externalizing scores (28-31). In a minority of the studies exposure was found to be related to either attention (31, 32) or activity only (33). In five out of seventeen studies no effect was observed of perinatal exposure on attention and/or activity (27, 34-37).

PCB exposure has been associated with ADHD in several cohorts, such as the one studied by Sagiv et al. which included children aged seven to eleven years (23). Mothers of these children were residing near a PCB-contaminated harbour and children in the highest quartile of exposure to a sum of four PCBs had a relative risk of 1.79 for ADHD compared to children in the lowest quartile. PCB exposure was also studied in Inuit children, which

are known for their high exposure to PCBs from seafood. Perinatal exposure was found to correlate with inattention and increased activity at eleven months of age when exposure was modelled with a validated physiologically based pharmacokinetic (PBPK)-model (38). Plusquellec et al (2010) however observed no associations for both attention and activity with increasing exposure to PCB-153 at five years of age in another Inuit cohort (35). Exposure was measured in cord serum only, not taking into account exposure through breastfeeding. Indeed, exposure through breastfeeding was also not taken into account in the children studied by Sagiv et al. (23) who also assessed PCB-exposure. Only 25% of the mothers breastfed their children longer than three months, while in the Inuit cohort of the group of Plusquellec the mean duration of breastfeeding was 57 weeks.

Janulewicz et al. (36) observed no effect of exposure. However, some modest associations were found in the lower exposed group. A model was used to calculate the cumulative mass of PCE entering homes through drinking water instead of assessment of direct human exposure. Due to likely misclassification of exposure because of the crude model used and inconsistency in dose-response relations observed, it was concluded that exposure was not associated with an increased risk of developing ADHD.

Table 2.2 ASD and ADHD in relation to perinatal exposure to EDCs in various cohorts. Sorted by outcome (ASD first) and year of publication

	Population	Exposure	Dose	Outcome	Result
Miodovnic 2011 (19)	n=137 7-9 years	Phthalates, BPA Urine, 3rd trimester	BPA: 1.3 (0.7–2.3). LMW phthalates: 430 (175–1090) Median (IQR) - µg/L	ASD SRS	LMW phthalates → greater social deficits (β=1.18, 95% CI 0.25 – 2.08) No effect for BPA.
Volk 2011 (19)	n=304 ASD n=259 controls	Traffic pollution birth residence	-	ASD	Shortest distance from freeway (10%) → AOR 1.86 (95% CI 1.03 – 3.45
Roberts 2007 (18)	n=465 ASD n=6975 controls	Pesticides Birth	-	ASD Registries	Living near sites of pesticide application → OR = 6.1 (95% CI 2.4 – 15.3)
Windham 2006 (17)	n=284 ASD n=657 controls	Air pollutants Birth residence	-	ASD Registries	Developmental toxicants, highest quartile: AOR 1.40 (95% CI 0.98 – 2.00)
Gascon 2011 (33)	n=332 4 years Menorca-INMA	PBDE-47 Cord blood (n=88)	2.10 (16.8) Median (max) - ng/g lipid <LOD = 48.9%	ADHD CP-SCS and ADHD DSM-IV	No effects of prenatal exposure.
Marks 2010 (20)	n=331 5 years CHAMACOS (California)	DAP Maternal urine (2x during pregnancy)	109.0 (99.4 – 119.6) Mean (95% CI) - nmol/L <LOD = LOD/√2	Attention, ADHD CBCL, K-CPT	Attention problems (CBCL) → β=0.7, 95% CI 0.2-1.2 ADHD (CBCL) → β=1.3, 95% CI 0.4-2.1 Composite score → OR=3.5, 95% CI 1.1-10.7
Verner 2010 (21)	n=168 11 months Inuit children	Simulated PCB-153 Perinatal	103 (15 – 706) Median (range) - ng/g lipid	Behaviour BSID-II	Prenatal PCB was associated with increased inattention, postnatal PCB predicted increased activity at 11 months.
Engel 2010 (30)	n=188 4-9 years New York	lmw phthalates 3rd trimester urine	2.24 (0.90 – 5.65) Median (IQR) - µM/L	Behaviour BASC-PRS	Conduct problems → β=2.40, 95% CI 1.34 – 3.46; Attention → β=1.29, 95% CI 0.16 – 2.41 Externalizing → β=1.75, 95% CI 0.61 – 2.88
Sagiv 2010 (22)	n=607 7-11 years Massachusetts	PCB (118, 138, 153, 180), p,p'-DDE Cord serum	∑PCB_4: 0.19 (0.01 – 4.41) p,p'-DDE: 0.31 (0.00–14.93) Median (range) - ng/g	ADHD CRS-T	∑PCB_4 , highest vs. lowest quartile: Conners' ADHD index → RR=1.76, 95% CI 1.06 – 2.92 DSM-IV total →RR=1.79, 95% CI 1.08 – 2.96 p,p'-DDE, highest vs. lowest quartile: Conners' ADHD index → RR=1.80, 95% CI 1.10 – 2.94 DSM-IV total →RR=1.69, 95% CI 1.01 - 2.83

	Population	Exposure	Dose	Outcome	Result
Plusquellec 2010 (34)	n=110, 5 years Inuit children	Pb, PCBs, Hg Cord serum	PCBs: 120.6 (21.6 – 407.4) Mean (range) - µg/kg	Behaviour IBRS, video	No associations found for attention and activity outcomes
Braun 2009 (27)	n=249 2 years Cincinnati, Ohio	BPA Urine, 16 & 26 weeks of gestation, birth	16 wks: 1.6 (<LOD–34.8) 26 wks: 2.0 (<LOD–583) Birth: 1.9 (<LOD–27.3) Median (range) - µg/L,	Behaviour BASC-2/BASC-PRS	Externalizing scores → β=6.0, 95% CI 0.1 – 12.0 (females) BPA at 16 wks was more strongly associated than at 26 wks
Roze 2009 (31)	n=62 5-6 years Compare (Groningen)	OHCs, PBDEs Maternal blood, week 35	BDE-47: 0.9 (<LOD-6.1) ng/g lipid BDE-99: 0.2 (<LOD-2.1) ng/g lipid PCB-153: 63.0 (34.0-162.2) pg/g 4OH-CB-146: 103.3 (36.3-290.1) pg/g *Median* (range)	Cognition, behaviour CBCL, teacher's report form and ADHD-questionnaire	Sustained attention: r = -0.264, p < 0.05 (BDE-47 and BDE-99) Total behavioural outcome: r = 0.276, p < 0.05 (BDE-99) Externalizing behaviour: CBCL → r = -0.278, p < 0.10 (OH-CB) Teacher → r = -0.328, p<0.05 (OH-CB) Teacher → r = -0.288, p<0.05 (PCB153)
Janulewicz 2008 (35)	n=2490 Cape Cod	PCE (drinking water) Model of exposure at birth	7.34 (4×10^{-5} – 1328) Median (range) – g Cumulative prenatal exposure	Attention, activity Parent report of diagnosis by doctor	Low prenatal exposure: ADD → OR=1.4, 95% CI 0.9 – 2.0 ADHD → OR=1.5, 95% CI 0.9 – 2.7 Low postnatal exposure: ADD → OR=1.3, 95% CI 0.9 – 1.9 ADHD → OR=1.4, 95% CI 0.8 – 2.5 High exposure: no sign. increased risk
Eskenazi 2007 (26)	n=356 – 396 24 months CHAMACOS (California)	DAP Maternal urine (gestation)	114.9 (105.7 – 125.0) Mean (95% CI) – nmol/L Average value gestation <LOD = LOD/√2	Neuro-development CBCL at 24 months	Total prenatal DAP: Attention → OR=0.77, 95% CI 0.27 – 2.24; ADHD → OR=1.34, 95% CI 0.50 – 3.59 PDD → OR=2.25 95% CI 0.99–5.16
Ribas-Fito 2007 (23)	n=475 4 years Menorca-INMA	HCB Cord serum	0.73 (0.14 – 9.82) Median (range) – ng/mL	ADHD CP-SCS and ADHD DSM-IV	HCB> 1.5 ng/mL: Poor social competence → RR=4.04, 95% CI 1.76 – 9.58 ADHD → RR=2.71, 95% CI 1.05 – 6.96

	Population	Exposure	Dose	Outcome	Result
Rauh 2006 (24)	n=254 12, 24, 36 months New York	Chlorpyrifos Cord plasma	<LOD – 63 Range – pg/g	Neuro-development CBCL (36 months)	High (>6.17 pg/g plasma) vs low exposed at 3 years of age: Attention → OR=11.26, 95% CI 1.79–70.99 ADHD → OR=6.50, 95% CI 1.09–38.69 PDD → OR=5.39, 95% CI 1.21–24.11
Laslo-Baker (2004) (25)	n=32 exposed n=32 controls 5-6 years Motherrisk	Solvents (occupation) Questionnaire	Exposure was at least 2 months during pregnancy and started in the first trimester	CBCL, CRS	Conner hyperactivity/impulsivity → β=0.27 (p=0.052) Conner DSM-IV hyperactivity → β=0.62 (p<0.001) Conner DSM-IV score → β=0.33 (p=0.02) CBCL externalizing not reported
Jacobson 2003 (36)	n=148 11 years Michigan	PCB Cord and maternal serum, milk	Cord: 2.7 (2.1) ng/mL Maternal: 5.9 (3.8) ng/mL Milk: 859.3 (388.2) ng/g fat Mean (SD), <LOD=7.4%	Sustained attention (*CPT*), focused attention	No effect of exposure on sustained attention.
Lai 2002 (28)	n=118 exposed n=118 controls 13-17 years	PCBs Yu-Cheng vs. controls	-	CBCL Rutter scale	Exposed vs. controls CBCL externalizing → β=2.55 (SE: 0.86)
Till 2001 (29)	n=33 exposed n=28 controls 3-5 years Motherrisk	Solvents Questionnaire Material safety data sheets	Exposure was at least 2 months during pregnancy, minimally 5 hours per week.	Attention, externalizing behaviour CBCL, CPT	Exposed: Externalizing → $\chi^2(1)=5.35$, p=0.02 CPT: exposure was not related to either errors of omission or commission
Chen 1994 (32)	n=118 exposed n=118 controls 3-12 years	PCBs Yu-Cheng vs. controls	-	Rutter scale	Cases scored 11% to 63% (mean = 28%) higher on activity than controls at each age (except for the 12-year-olds)

Discussion

The objective of this review was to present results of cohort studies on the relation between perinatal exposure to EDCs and occurence of ASD and/or ADHD in order to investigate current evidence for EDCs as a causal factor in the etiology of these neurodevelopmental disorders. Four studies assessed this relationship for ASD and positive associations were found for all chemicals investigated, which included hazardous air pollutants, pesticides and BPA. As for ADHD a larger pool of studies was available. Six out of seventeen studies observed positive associations or increased risks for a variety of chemicals including PCBs, DAP, and chlorpyrifos. Another five studies found positive associations with externalizing behaviour for chemicals such as BPA, PBDEs, and LMW phthalates. Five studies did not find any association between exposure and ADHD.

Dose-response relationships may not be straightforward, which may offer an explanation for the observations in the cohort of Janulewicz et al. (36) and may also be applicable to PBDE-47 exposure (32, 34). Gascon et al. (34) measured exposure in cord blood (median concentration: 2.10 ng/g lipid) and found no association with ADHD in four year old children. Roze et al. (32) measured exposure in maternal blood at 35 weeks of gestation. An association with decreased sustained attention was found for PBDE-47 in five and six year old children, even though concentrations were lower than in the study of Gascon et al. (median concentration: 0.9 ng/g lipid).

Perinatal exposure to chemicals was also found to be positively associated with externalizing behaviour. Though hyperactivity is only part of the construct of externalizing behaviour (39), ADHD is considered an externalizing disorder. Prenatal exposure to LMW phthalates, BPA, PCBs and solvents was observed to be associated with externalizing behaviour (28-32). High scores on externalizing or internalizing behaviour are reflected in the default network resting state functional connectivity (DN RSFC) of these children compared to normally developing children (40). Functional connectivity is a type of connectivity in the brain in which similarities of temporal characteristics of brain activity in multiple regions are recorded (41). Sex steroids such as testosterone may affect functional connectivity (42), and testosterone in particular is also potentially related to the development of ADHD (43). Several EDCs are known to disturb sex steroid levels and may therefore indirectly affect functional connectivity. Furthermore EDCs such as PBDEs are known to affect thyroid hormone (TH) levels which in turn are known to affect neuronal differentiation, migration, myelination, synaptogenesis and dendritic branching (44). They are therefore important factors for brain development and potentially also for development of the connectome. In mice with a heterozygous mutation of the TH receptor a1 a reduced density of GABAergic inhibitory interneurons in the hippocampus is observed, which was accompanied by more depressive and anxious behaviour (45). GABA is an inhibitory neurotransmitter in the mature brain. However in the embryonic and perinatal period, it triggers calcium influx and it is important for cell proliferation, migration, differentiation, synapse maturation and cell death (46). In children with autism, dysfunction of GABAergic

signalling is observed (46). Furthermore in mice it is demonstrated that BPA inhibits the GABAaR-mediated response and that BPA affects development of GABAergic and dopaminergic systems (47). Toxicology studies also have shown the potential for PCBs to affect the GABAa response (48, 49), indicating EDCs as risk factors for neurodevelopmental disorders through modification of GABAergic systems. Evidence from cohort studies thus far for these mechanisms is scarce. Only two of the included cohorts actually measured thyroid hormones (32, 34). Gascon et al. observed no significant associations between TSH, TT3, fT4 and postnatal levels of PBDE-47, nor did they significantly relate with neurodevelopmental outcomes. However, Roze et al. (32, 34) observed TSH to be correlated with worse neuropsychological functions, while T3 and T4 were found to be correlated with better outcome such as less ADHD and better behaviour. Pentachlorophenol (PCP) was negatively related to T3, which would fit with the observed correlation between T3 and outcome. However, PBDE related positively to T3.

Exposure to EDCs may also affect behaviour through pathways other than the endocrine pathway, e.g. epigenetic alterations resulting in reprogramming of the brain, as was demonstrated in an experimental study by the group of Skinner. They observed that the F3 generation of vinclozolin exposed rats had altered genes in the hippocampus and amygdala and altered behaviour (16), even though major effects on hormone levels were not observed (16, 50, 51). Behaviour may therefore also be potentially determined by exposures of previous generations. It should also be considered that enzymatic polymorphisms may result in variations in detoxification phenotypes, making some individuals more susceptible to environmental contaminants. An example of this is paraoxonase 1 (PON1), which is important in the detoxification of individual organophosphorous compounds (52). Furthermore, other potential risk factors for ASD and ADHD (53, 54), such as preterm birth, intrauterine growth restriction, and low birth weight, should also be considered, especially since some of them may also be linked to EDC exposure (55).

It can be concluded that especially regarding ASD insufficient data are available, but that studies so far seem to indicate an association between prenatal exposure to EDCs and occurrence of ASD. Moreover, observations indicate a relation between perinatal EDC exposure and prevalence of ADHD. A limitation of our study is however, that confounding factors have not been taken into account in the overview. Future research should include prospective, longitudinal cohort studies in which exposure to EDCs as well as hormone levels have to be assessed in relation to outcome in order to clarify mechanisms of action.

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3

Obesogenic effects of endocrine disruptors, what do we know from animal and human studies?

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Abstract

Background: Hormonal actions and activation of receptors involved in adipogenesis and brain development during the prenatal period may be affected by exposure to certain chemicals. Experimental studies have shown that amongst others polychlorinated biphenyl (PCB)-153 and dichlorodiphenyltrichloroethane (DDT) may have obesogenic effects in prenatally exposed mice.

Objective: To provide an overview of five classes of chemicals which have frequently been indicated as potential obesogens, and to discuss the evidence available regarding early life exposure to these compounds and overweight later in life.

Methods: Pubmed was systematically searched for publications which related early life exposure to endocrine disrupting chemicals (EDCs) to growth parameters later in life. We included 19 studies, which were published from 1995 and onwards.

Results: 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. Dose-response relations appear to be non-monotonic.

Conclusion: For certain EDCs, early life exposure may be associated with weight homeostasis later in life, however not necessarily in an obesogenic direction. More sensitive measures of adiposity as well as long-term follow-up is warranted for future studies.

Introduction

The prevalence of obesity continues to grow worldwide, presenting governments and health care organizations with a major challenge. Though at first obesity seemed to occur predominantly among adults, it is clear now that increasing numbers of children have to deal with the health consequences and social stigma of being overweight (1). In 2002 a review by Baillie-Hamilton was published which showed remarkable similarities between the obesity epidemic and the production of chemicals from 1930 and onwards (2). Also in experimental studies it was showed that various chemicals had obesogenic effects. Female progeny of rats exposed to polychlorinated biphenyl (PCB)-153 experienced accelerated growth compared to controls (3). PCB-153 furthermore stimulated adipogenesis in 3T3-L1 adipocytes (4). Body weight of mice was affected after in utero exposure to a mixture of chemicals, including the organochlorine pesticide dichlorodiphenyltrichloroethane (DDT), however dose-response was non-monotonic, with results for higher doses often being opposite from what was observed for lower doses (5). Moreover, exposure of mature adipocytes to dichlorodiphenyldichloroethylene (DDE), the metabolite of DDT, resulted in increased leptin release (6), a hormone which has been associated with fetal growth (7).

It has become clear that certain compounds may interfere with the function of hormones (endocrine disrupting chemicals, EDCs), including estrogen, testosterone, and thyroid hormones (reviewed by Bergman et al. (8)), which are involved in various processes in adults, but also in brain development early in life (9, 10). These hormones have also been associated with weight homeostasis, both early in development and later in life. Estrogens, for example, reverse weight gain often experienced by post-menopausal women (11). Moreover in men who are treated for prostate cancer and in women who are treated for polycystic ovary syndrome with anti-androgenic therapy, weight gain is observed (11, 12). EDCs may interact with estrogen receptors, but may also affect aromatase activity. Aromatase is an enzyme which converts testosterone to 17β-estradiol. Developmental exposure to estradiol EDCs has been found to decrease expression of the aromatase gene (CYP19a1b) in the brain of male rainbowfish (13). Decreased aromatase activity was however also observed in male rats prenatally exposed to a PCB-mixture (14). Other studies also suggest that aromatase activity may be affected by phthalates (15), DDE (16), and various pesticides (terbuthylazine, propiconazole and prothioconazole) (17), although findings need to be substantiated.

EDCs may also interfere with thyroid hormone (TH) receptors as well as transport proteins for TH. Some isoforms of polybrominated diphenylethers (PBDEs) have been shown to exert inhibiting effects for binding of triiodothyronine to the TH receptor (18). PBDE metabolites may furthermore bind to the transport protein transthyretin, resulting in displacement of thyroxine (19). This has also been observed for perfluorinated alkyl acids, such as PFOS and PFOA (20). This has been however predominantly shown in vitro.

Endocrine disruptors may furthermore promote obesity through peroxisome proliferator-activated receptor (PPAR) α and γ. Activation of PPARα stimulates lipid

mobilization, but may indirectly also be obesogenic as prenatal activation may result in low birth weight, a known risk factor for obesity later in life. There are several options through which EDCs may promote obesity through PPARγ. Usually a ligand is needed for PPARγ to bind co-activators, release co-repressors, decondensate the chromatin and activate transcription (21). However post-translational modifications, such as phosphorylation, may activate PPARγ in absence of a ligand (22). EDCs may furthermore cause multipotent stromal cells (MSCs), cells which can differentiate into various tissues, to predominantly differentiate into adipose tissue (21, 23). This particular subset of MSCs expresses PPARγ (24). This has in particular been found for organotins such as tributyltin (TBT).

What also needs to be considered is that PPAR agonists and their metabolites may activate multiple PPAR isoforms. Bis(2-ethylhexyl)phthalate (DEHP), an EDC from the phthalate class, may activate PPARα, however its metabolite mono (2-ethylhexyl)phthalate (MEHP) may activate PPARγ (25, 26). And though lipid mobilization induced by PPARα activation requires continuous exposure, PPARγ may only need a single or episodic exposure to establish its effects in adipose tissues (12). Recent research has also indicated potential for perfluorinated alkyl acids, such as perfluorooctane sulfonate (PFOS), to interfere with PPAR by inducing expression of PPARγ genes in mouse neonatal brain after prenatal exposure (27).

Regulation of energy balance is a basic concept in the aetiology of obesity. Several factors control appetite and energy expenditure, which are all integrated in the hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus, and in particular the arcuate nucleus of the hypothalamus (ARH), is the centre for energy balance and appetite regulation.

Not much is known on developmental exposure to EDCs in relation to later appetite regulation. Chemicals may disrupt actions of hormones related to energy balance, e.g. leptin and insulin. It is clear that both leptin and insulin play important roles in development of feeding circuits which are comparable to sex steroid hormones with regard to the development of sexually dimorphic circuits (28). Rats injected with insulin between embryonic day 15 and 20 (term is 22 days) were significantly more obese at 50 days of age (29). In leptin-deficient mice also ARH circuit formation was affected (30). Leptin is also considered a regulator of fetal growth (7) and low leptin levels at birth have been associated with a higher risk for obesity and diabetes (31).

Early life exposure to these toxicants may have different effects than exposure in adulthood, as perturbations during stages of developmental plasticity may give rise to more profound long-lasting effects (32). As endocrine disruption early in life seems to be a plausible mechanism which may predispose children to obesity, the aim of this study was to create an overview of six classes of chemicals which have frequently been indicated as potential obesogens in observational studies, and to discuss the evidence available regarding early life exposure (i.e. during the prenatal or early postnatal period) to these compounds and overweight later in life.

Methods

Articles were considered relevant when they determined effects of either dioxin-like compounds, non-dioxin like compounds, organochlorine pesticides, brominated flame retardants, phthalates or perfluorinated alkyl acids on growth and physical development in humans. PubMed was therefore systematically searched for publications by means of the following terms relating to exposure: chemical exposure, endocrine disruption (prenatal) environmental exposure, pesticides, bisphenol a (BPA), brominated flame retardant, DDE, DDT, hexachlorobenzene (HCB), organochlorines, organotin, perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), phthalates, polybrominated diphenyl ethers (PBDE), and PCB. Each of these terms was combined with the following terms relating to growth: obesity, overweight, fat, growth, anthropometry, cohort studies obesity, cohort studies overweight. In total 2832 publications were retrieved.
Articles were only considered when exposure was determined during pregnancy (or extrapolated to the period of pregnancy), or when it was measured in breast milk. Furthermore publications had to be written in English. Reference lists of articles included from the initial search were also searched for relevant publications. In total 19 publications of observational studies were included.

Results

An overview of observational studies included is given in table 3.1.

Organotins

Organotins are characterized by a tin atom which is bound to an organic chain. The most common varieties are tributyltin (TBT) and triphenyltin (TPT) which are generally found in wood preservatives and antifouling boat paints. Due to their organic chain, they are hydrophobic and they therefore bioaccumulate. Human exposure occurs mainly through diet, in particular through sea food (33).

Though observational studies are not available, obesogenic characteristics of TBT in particular have been well documented in experimental studies. Prenatal exposure to this compound in mice has been associated with adiposity at later age (34). Furthermore, MSCs derived from the adipose tissue of these mice showed increased commitment to the adipocyte lineage compared to controls (35, 36), at levels comparable to the tolerable daily intake of humans (37). Differentiation of pre-adipocytes into adipocytes was increased by TBT exposure in a dose and time dependent manner (38, 39). Moreover, these effects may be heritable as Chamorro-Garcia et al. observed these results in both the F2 and F3 generation of exposed mice (37). Gender-specific results have been reported, with effects on fat mass lasting longer in male mice than in female mice after prenatal exposure to TBT at human relevant levels (40).

Non-dioxin-like PCBs

Non-dioxin-like PCBs were mostly applied as dielectric and coolant fluids, however due to health concerns, including potential carcinogenic effect, production of PCBs in Europe has been prohibited since 1985 (41). However, PCBs may still be released in the environment from old electrical appliances, and as they are furthermore known to be highly persistent in the environment, they may accumulate in fat tissue of animals and humans (42). The main source of exposure for people is through animal food (fish and meat), which is mostly contaminated with PCB-153 and PCB-138 (42). In Europe, dietary exposure to PCBs is monitored by the European Food Safety Authority (EFSA) (43).

In vitro studies have shown that PCBs may enhance adipocyte proliferation and differentiation (4, 44). Futhermore, Sitarek and Gralewicz (2009) observed accelerated growth in female progeny of rats exposed to PCB-153 during pregnancy until 21 days after birth (3). Animal studies on obesogenic effects of NDL PCBs are however scarce.

Several observational studies report no association between PCB exposure and BMI, weight or height (45-48). Other studies did observe associations between perinatal PCB exposure and growth; however both positive and negative associations have been reported for weight as well as for height. BMI was positively associated with PCB exposure in both the study by Verhulst et al. (49) and the study by Valvi et al. (50). In both these studies, PCB exposure was assessed in cord blood. A positive association with BMI standard deviation scores (SDS) was observed in children 3 years of age (49) and an increased relative risk for overweight was found in children 6.5 years of age in the highest exposure tertile (50). However, no association between PCB exposure and BMI was seen in a group of 280 females aged 20-50 years for whom exposure was assessed in maternal serum (45). However, these samples were collected from the mothers at time of enrolment of the daughters and exposure levels were extrapolated back to the period of pregnancy. Also Blanck et al. (51) and Rylander et al. (52) measured PCBs in maternal serum at the time of enrolment of the children (aged 5 to 24 years and 4 to 7 years respectively) and converted exposure to the time of pregnancy. Both studies report negative associations between exposure levels and weight and no associations with height.

Organochlorine pesticides (DDT/DDE and HCB)

DDT and HCB are part of a group referred to as organochlorine pesticides. DDT was developed in the 1940s as an insecticide and was amongst others used to fight insect-borne diseases, such as malaria (53). Due to health concerns – DDT is genotoxic and a suspected carcinogen – use of DDT was banned in the United States in 1972 and in the Netherlands in 1973 (54). However, in developing countries DDT is still used for vector-control (malaria), which is currently also supported by the World Health Organization (55). DDT is known to bioaccumulate and to be highly persistent in the environment. HCB is a byproduct of manufacturing processes of other chemicals, and has also been widely used as a pesticide. Due to probable carcinogenic effects, production and use of HCB was prohibited by the

Stockholm Convention (56). Like DDT, it accumulates in fat tissue and it persists in the environment.

In vitro studies with preadipocytes have shown that DDE exposure at environmentally relevant levels resulted in increased proliferation (44) and differentiation (57). Howell and Mangum (2010) did not observe an effect on adipogenesis after exposure of NIH3T3-L1 cells to DDE, which may potentially be due to the lower concentration used (2-20 µM vs. 5-500 µM used by Chapados et al.) (6). They did however observe an increase in leptin release from mature adipocytes and an increase in basal fatty acid uptake, after exposure to DDE. Hojo et al. (2006) exposed rats to DDE through the diet and observed a decreased weight in F1 females nine weeks after birth, which was significant for the highest dose group (58). This dose may have been too high however, as they observed clear signs of systemic toxicity in this group (tremors, convulsions), and some females died after appearance of these symptoms.

Table 3.1 Observational studies on early life exposure to EDCs and effects on growth-related outcomes later in life (ordered by date of publication)

Author	Population	Exposure	Outcome	Results
Andersen et al. (2013)	n=811 7 years old	PFOS: 33.8 (6.4 – 106.7) PFOA: 5.25 (0.5 – 21.9) *Median (range) – ng/mL. MP, GW 8*	Weight, height	No significant associations between exposure and any of the selected outcomes
Maisonet et al. (2012)	n=447 20 months old	PFOS: 19.6 (3.8 – 112.0) PFOA: 3.7 (1.0 – 16.4) *Median (range) – ng/mL. MS, GW 15*	Weight, height (2, 9, and 20 months)	PFOS, 3rd tertile, 20 months of age • Weighed 580 g. more than girls in the lowest tertile No effect for PFOA
Halldorsson et al. (2012)	n=665 20 years old	PFOS: 21.5 (9.1) PFOA: 3.7 (2.0) *Median (IQR) – ng/mL. MS, GW 30*	BMI, WC, insulin, leptin, adiponectin	PFOA, highest (5.8 ng/mL) vs. lowest quartile (2.3 ng/mL): • Adjusted RR BMI>25 = 3.1 (95% CI 1.4, 6.9) • Adjusted RR WC>88 cm = 3.0 (95% CI 1.3, 6.8) • Only significant in females, other congeners not significant
Valvi et al. (2012)	n=344 6.5 years old	Total PCB (Σ of 7): 0.75 ± 1.70 DDE: 1.06 ± 2.45 DDT: 0.08 ± 3.81 *Mean ± SD – ng/mL. CB*	BMI	PCB, 3rd tertile, RR overweight = 1.70 (95% CI 1.09, 2.64) DDE, 2nd tertile, RR overweight = 1.67 (95% CI 1.10, 2.55) DDT: only associated with overweight in boys Girls: stronger associations
Garced et al. (2012)	n=253 1 year old	DDE 1st trimester: 6.3 ± 2.8 DDE 2nd trimester: 6.6 ± 2.9 DDE 3rd trimester: 7.6 ± 2.9 *Mean ± SD – ng/mL. MS*	Weight-for-age Length-for-age BMI-for-age HC-for-age	No significant associations between exposure and any of the selected outcomes
Andersen et al. (2010)	n=1010 5 and 12 months old	PFOS: 33.4 (6.4 – 106.7) PFOA: 5.21 (0.5 – 21.9) *Median (range) – ng/mL, MP, GW 8*	Weight, height, BMI	• PFOS, weight, 5 months: β = -0.8 g (95% CI: -4.2, 2.6) • PFOS, weight, 12 months: β = -5.8 g (95% CI:-10.4, -1.2) • PFOA, weight, 5 months: β = -9.4 g (95% CI: -28.6, 9.9) • PFOA, weight, 12 months: β = -19.0 g (95% CI: -44.9, 6.8) Similar pattern for BMI. No associations with height. After stratification effects more pronounced in boys, not significant in girls
Mendez et al. (2010)	n=657 14 months	DDE, Average growers: 125.40 (115.03–136.70) Rapid growers: 135.59 (119.48–153.87) *Mean (95% CI) – ng/g lipid. MS, 1st trim.*	Rapid growth BMI at 14 months	DDE > 1st quartile • Rapid growth, RR 2.42 (95% CI: 1.25, 4.67) • Elevated BMI at 14 months, RR 1.50 (95% CI: 1.11, 2.03) • Normal pre-pregnancy weight mothers. For other OCs no effect

Author	Population	Exposure	Outcome	Results
Pan et al. (2010)	n=210 breast fed >6 months, 12 months old	Total PCB (Σ of 18): 81 (12-708) DDE: 113 (15-2140) DDT: 5 (<LOD-36) *Median (range) – ng/g lipid. BM, 3 months*	Weight (record), height	No significant associations between exposure and any of the selected outcomes
Verhulst et al. (2009)	n=138 3 years old	PCB: 117 ± 76 DDE: 212 ± 243 *Mean ± SD – ng/g lipid. CB*	BMI SDS	DDE 450 vs. 63.7 ng/g lipid: • 0.13 difference in BMI SDS for non-smoking mothers • Smoking enhanced DDE effect PCB and BMI SDS: β = 0.003, p = 0.03
Karmaus et al. (2009)	n=280 females 20-50 years old	PCB, DDE, DDT *MS, at enrolment daughters. Converted to exposure at time of pregnancy*	BMI	DDE <1.503 µg/L vs. 1.503-2.9 µg/L → 1.65 increase in BMI DDE <1.503 µg/L vs. >2.09 µg/L → 2.88 increase in BMI No effect of PCB exposure
Smink et al. (2008)	n=405 6 years old	HCB: 0.68 (0.46 – 1.03) *Median (IQR) – ng/mL. CB*	BMI, obesity	HCB > 1.03 vs. < 0.46 ng/mL: BMI: β = 0.80, p = 0.03. RR obesity: 2.06 (95% CI: 1.06, 3.85)
Rylander et al. (2007)	n=55 low and n=119 normal birth weight 7 years old	PCB-153 *MB, at enrolment children. Converted to exposure during year of childbirth.*	Weight (4 and 7 years), height	Weight at 4 years: β = -0.4 kg (95% CI: 0.01, 0.70, p = 0.04) Weight at 7 years: β = -1.2 kg (95% CI 0.50, 1.90, p = 0.001) Effects on weight only found in normal birth weight children
Ribas-Fito et al. (2006)	n=1712 7 years old	*p,p'*-DDE: 24.4 (17.0–36.2) *Median (IQR) – µg/L* *MS, third trim.*	Weight, height (1, 4, and 7 years)	DDE: >/ 60 µg/L vs lowest <15 µg/L. • Height → 1 year: β = -0.72 (SE 0.37); 4 years: β = -1.14 (SE 0.56);7 years: β = -2.19 (SE 0.46) • In lower categories and for weight no association observed. • No effect of PCB exposures
Hertz-Picciotto et al. (2005)	n=399 5 years old	Total PCB: 696 (378 – 1115) Mean (5^{th} – 95^{th} percentile) – ng/g lipids *MS, 2^{nd} or 3^{rd} trim.*	Weight and height (birth, 5 yrs), HC, gestational age	Males, exposure was negatively associated with: • At birth: weight, weight-for-gestational age, HC • No association with growth at 5 years of age Females: At birth: smaller HC, shorter gestation • Height at 5 years: β = 4.5 (95% CI: 0.05, 9.00)
Gladen et al. (2004)	n=304 males 20 years old	DDE: 5.7 (1.0 – 25.1) *p,p'*-DDT: 1.9 (<LOD – 12.7) *o,p'*-DDT: 0.14 (<LOD – 1.33) *Median (range) – µg/g lipid. MS, 3^{rd} trim.*	BMI, triceps & subscapular skinfold, serum DHEAS, serum, testosterone	No significant associations between exposure and any of the selected outcomes

Author	Population	Exposure	Outcome	Results
Blanck et al. (2002)	n=308 females 5-24 years old	PCB: 5 (<LOD – 78) *Median (range) – ppb. MS, at enrolment, proxy for exposure during pregnancy*	Weight, height	PCB > 5 ppb: • Weight (adjusted for height): β = -11.76 (95% CI: -4.20, -19.30)
Gladen et al. (2000)	n=594 Follow up at puberty	Transplacental = average of all matrix types PCB: 1.7 (1.5-5.5) ppm DDE: 2.4 (1.3-23.8) ppm Lactational = levels in breast milk*duration PCB: 5.0 (0.2-23.1) mg DDE: 6.2 (0.2 – 96.3) mg *Median (range). MB, CB, BM, placenta*	Weight, height (all self-reported)	• PCB white girls, highest (≥3 ppm) vs. low (0-1 ppm) transplacental exposure: 5.4 kg heavier (p = 0.09) • DDE boys, highest vs. low transplacental exposure: 6.3cm taller and 6.9 kg heavier • PCB boys and DDE girls: no effect of lactational or transplacental exposure
Patandin et al. (1998)	n=105 breast-fed (BF) n=102 formula-fed (FF) 42 months old	PCB maternal: 2.04 (0.59 – 7.35) PCB cord: 0.40 (0.08 – 2.08) PCB milk: 391.5 (173.7 – 1226.4) μg/kg fat *Median (range) – μg/L. MP, last month gestation. CP. BM, 2nd week*	(Birth) weight, height, HC. 10 days, 3, 7, 18, 42 months	P90 (0.8 μg/L) vs. P10 (0.20 μg/L) • Weighed 165 g. less at birth Lower growth rate from birth to 3 months, not at later ages (only in FF group, not in BF group)
Guo et al. (1995) PCB	n=118 exposed to PCB in utero n=117 controls	PCB: 49.3; 26.8 *Mean; median - ppb* *MS, end of pregnancy*	Weight, height, total lean mass	Exposed children vs. controls: • Age range 6 months – 7 years: 7% lighter and 3% shorter • Age range 6 – 13 years: 2.3% shorter, weight similar • Lower total lean and soft tissue mass,15% lower birth weight

BF: breastfed; BM: breast milk; CB: cord blood; CP: cord plasma; FF: formula-fed; GW: gestational week; HC: head circumference; LOD: limit of detection; MB: maternal blood; MP: maternal plasma; MS: maternal serum; OC: organochlorine; trim: trimester; WC: waist circumference.

Several cohort studies (45, 49, 50, 59) have observed a positive association between prenatal DDE exposure and BMI at later age. Prenatal DDE exposure measured in cord blood was associated with a 0.13 difference in BMI SDS in high vs. low exposed 3-year olds (49), and a relative risk of 1.67 for overweight in 6.5-year olds (50). Also DDE exposure assessed in first trimester maternal serum was associated with a relative risk (RR) of 1.50 for elevated BMI in 14 months old children (59). Karmaus et al. determined exposure in maternal serum, which was collected at the time of enrolment of the daughters (20-50 years of age) and then converted to exposure during pregnancy (45). They observed a non-linear trend across quintiles of exposure, and found a 2.88 increase in BMI in the three upper quintiles of adult female offspring versus the lowest two quintiles. Pan et al. found no effect of DDE exposure on BMI in 12 months old children (47). Prenatal DDE exposure has furthermore been associated with increased weight in boys at puberty (60). However, in a later study by Gladen et al., which included only males, no effect of DDE exposure on weight was observed (61). Also in the cohort of Ribas-Fito et al. no associations for weight and DDE exposure were seen (48).

Smink et al. measured prenatal exposure to HCB in cord blood of 405 children (62). At 6 years of age, the high exposure group (>1.03 ng/mL) had an increased BMI compared to the low exposure group (<0.46 ng/mL) ($\beta = 0.80$, $p = 0.03$). The highly exposed group had a relative risk of 2.06 (95% CI: 1.06, 3.85) for obesity compared to the low exposed children. Although Mendez et al. and Verhulst et al. observed an association between DDE and BMI, no effects for HCB were observed (49, 59).

Brominated flame retardants

Brominated flame retardants (BFRs), including PBDEs are chemicals used to prevent fires and slow down combustion time. They are for example applied in clothes, furniture, and electrical equipment, and are known to be persistent and to bioaccumulate (63). The Bromine Science and Environmental Forum estimated that in 2000 the annual production of BFRs was more than 200000 metric tons (64). Several BFRs, including BDE-47, BDE-99, and hexabromocyclododecane (HBCD) have PBT (persistent, bioaccumulative, and toxic) properties (65). BFRs are usually applied in mixtures and production of two common mixtures, pentaBDE (in which BDE-47 and BDE-99 are the most abundant) and octaBDE (including various BDE congeners which have on average 7.2 to 7.7 bromine atoms per molecule of diphenyl ether), has already been terminated in Europe (65). DecaBDE (BDE-209 as most prevalent congener) is also likely to be phased out soon.

Exposure of mice to high concentrations (150 – 2500 mg/kg body weight) of decaBDE during gestation resulted in inhibited fetal growth and development, and disrupted lipid metabolism in the F0 mice (66). Bondy et al. (2013) exposed rats to BDE-71 before mating, during gestation, weaning, and continued exposure of the F1 generation until sacrifice (postnatal day 42) (67). They observed a non-dose dependent increase in bodyweight in the F1 rats. However also in this study concentrations exceeded human exposure levels. From a recent publication it appears that BDE-47 may enhance adipocyte differentiation in a dose-

dependent manner (2.5 – 25 μM) (38). However, experimental studies assessing effects of pre- or early postnatal exposure to BDE's on e.g. growth, weight, or adipogenesis, are scarce. Also observational studies on this topic are lacking. Some have determined the association between prenatal exposure and birth weight, predominantly observing negative associations (68-70), but none have reported on growth later in life.

Phthalates

Phthalates are used in plastics to increase flexibility, and are often referred to as plasticizers. They are also used as solvents and can be found in various products, ranging from vinyl on floors, to cosmetics and toys. Human exposure occurs mainly through diet (71), as phthalates can be released from packaging material into the products. Phthalates are metabolized by the body and the metabolites usually are excreted in urine. They are not known to bioaccumulate (71).

In utero DEHP exposed offspring of mice showed increased visceral fat at a dose of 0.05 mg/kg body weight per day, which is an environmentally relevant dose (72). Also body weight of female offspring at this dose was elevated. At a higher dose (5.0 mg/kg body weight per day), visceral fat weight was still increased in females, but decreased in males. Body weight in females, at this dose, was higher compared to controls, and also in males a significant increase in body weight compared to controls was observed. Hao et al. (2013) exposed both 3T3-L1 cells and pregnant mice to DEHP (73). No effect on adipocyte concentration was observed, however the in utero exposed mice showed increased body weight, adipose tissue deposition, serum lipids, and glucose levels at postnatal day 60 compared to controls. Exposure of 3T3-L1 cells to MEHP concentrations relatively high compared to human exposure levels, did however result in increased adipogenesis (4).

There are no studies available which have determined early life exposure to phthalates in association with child growth. Reports on associations between phthalate exposure and any growth related outcome, have often determined exposure in the population at the same time as when the outcome was determined. Wang et al. (2013) for example reported positive associations between levels of phthalates in urine of Chinese school children and BMI and waist circumference (74). Also in a cross-sectional study of the National Health And Nutrition Examination Survey (NHANES) data differences in BMI and waist circumference were observed across quartiles of exposure to various phthalates (75). Trends were different between genders and age-groups and mostly showed non-linear dose response relations.

Perfluorinated alkyl acids

Perfluorinated alkyl acids are fully fluorinated organofluorine compounds with a carboxylic acid or carboxylate functional group. They have various uses, including as surfactants, impregnation agents and water repellents, fabric protectors, and in fire-fighting foams. Two of these perfluors in particular, PFOS and PFOA, are considered hazardous for health. PFOS was listed in annex B of the Stockholm convention, implying that its' use should be

limited. In 2010 the use of PFOS was banned by the European Union, with some exemptions of products such as hydraulic fluids for aviation (76). PFOA is currently considered by the European Chemical Agencu (ECHA) 'a substance of very high concern because of its' CMR (carcinogenic, mutagenic, or toxic for reproduction) and PBT properties' (77). Various studies have determined the half-life of PFOA in humans, and periods from 2.3 to 8.5 years have been reported (77). As PFOA is not metabolized by the body and elimination rate is low, it accumulates in the body.

In vitro or in vivo studies looking at obesogenic effects of perfluorinated alkyl acids are scarce. A recent publication by Bastos-Sales et al. (2013) showed modest to no effects of PFOS and PFOA on adipocyte differentiation at a concentration of 10 μM (38). In utero exposure of mice to PFOA was however associated with higher leptin and insulin levels in females (78). Also observational studies are few. Halldorsson et al. (2012) determined exposure to PFOS and PFOA in cord blood and performed follow-up when offspring was 20 years of age (79). They observed an increased relative risk for a BMI higher than 25 and a waist circumference higher than 88 cm. in 20-year old females in the highest quartile of prenatal PFOA exposure (median: 5.8 ng/mL); range 4.8 – 19.8) compared to lower exposed females. The association between PFOA and both BMI and waist circumference was non-linear, with a significantly larger effect observed for Q4 than for the other quartiles. No association was observed for males and for PFOS exposure. A non-linear dose-response was also observed for PFOA exposure and weight in a cohort of British girls at the age of 20 months, though results were insignificant (80). Higher PFOS exposure was however associated with decreased birth weight and increased weight at 20 months. Andersen et al. (2010) on the other hand, reported an inverse association between prenatal PFOS and PFOA exposure and weight and BMI in childrens' first year of life, and after stratification, the effect was more pronounced in boys than in girls (81). This association was not apparent anymore when the children were seven years of age (82). However, they collected maternal blood early in pregnancy, while Maisonet et al. collected maternal blood during gestational week 30 and Halldorsson et al. assessed exposure in cord blood. These exposures reflect different time periods in fetal development.

A short summary of all studies according to their results, is given in table 3.2.

Table 3.2. Summarized overview of observational studies, according to their effect

Chemical	Primary outcome	Results		
		Negative	**No effect**	**Positive**
PCB	BMI	-	• Karmaus et al. (2009)	• Valvi et al. (2011) • Verhulst et al. (2009)
	Weight	• Rylander et al. (2007) • Blanck et al. (2002)	• Pan et al. (2010) • Ribas-Fito et al. (2006) • Hertz-Picciotto et al. (2005) (5 years of age, males) • Gladen et al. (2000) (boys) • Patandin et al. (1998)	• Gladen et al. (2000) (white girls)
	Height	• Guo et al. (1995)	• Pan et al. (2010) • Ribas-Fito et al. (2006) • Hertz-Picciotto et al. (2005) (5 years of age, males)	• Hertz-Picciotto et al. (2005) (5 years of age, females)
DDE	BMI		• Garced et al. (2012) • Gladen et al. (2004)	• Valvi et al. (2011) • Mendez et al. (2010) • Verhulst et al. (2009) • Karmaus et al. (2009)
	Weight		• Garced et al. (2012) • Pan et al. (2010) • Ribas-Fito et al. (2006) • Gladen et al. (2000) (girls)	• Gladen et al. (2000) (boys)
	Height	• Ribas-Fito et al. (2006)	• Garced et al. (2012) • Pan et al. (2010) • Gladen et al. (2000) (girls)	• Gladen et al. (2000) (boys)
	Rapid growth			• Mendez et al. (2010)
PFOS PFOA	BMI		• Andersen et al. (2013) (7 years old)	• Halldorsson et al. (2012)
	Weight	• Andersen et al. (2010) (5 and 12 months old)		• Maisonet et al. (2012) (PFOS, girls)
HCB	BMI			• Smink et al. (2008)

Discussion

The objective of this study was to present evidence available on the relation between early life exposure to EDCs and their propensity to promote weight gain and develop obesity, for six classes of chemicals. For each class there are indications that exposure in early life does affect adipogenesis or growth later in life, however results lack consistency. For some classes of chemicals, such as organotins, brominated flame retardants and phthalates, experimental and observational studies are scarce. Thus far, no clear dose-response relation can be observed, and results seem to differ between males and females.

Nonmonotonicity in dose-response relations between exposure to endocrine disruptors and health outcomes, has been reviewed by Vandenberg et al. (2012) (83). The main implication is that one cannot predict low dose effects from effects seen at high doses and vice versa. Various studies included in this overview did not show a linear relation between exposure and outcome. Whether these are truly nonmonotonic dose-response relations remains to be clarified. Effects shown in these studies at various doses may be incidental significant associations or chance findings. For a given effect to be truly acceptable as non-monotonic, it must be completely reproducible with the same dose-response relationships and the same significant effects. Current findings do not indicate this. This is also supported by a recent systematic review on obesogenic effects of phthalate exposure in humans, in which the authors concluded that this hypothesis could neither be confirmed nor rejected as results of included studies were far from consistent (84). Furthermore, comparison of results between studies is complicated as exposures are determined in various matrices, e.g. cord blood, which is assumed to reflect prenatal exposure, versus maternal blood, sampled each trimester, to assess exposure. The latter may also reflect prenatal exposure, however as the timing of sampling and the type of matrix are very different, a comparison should not be made unless a conversion factor is available. This has for example been done by Govarts et al. (2012), who did a meta-analysis of studies looking at early life exposure to PCB-153 and DDE, in relation with birth weight. The authors applied conversion factors to transform exposure levels in breast milk and maternal blood to cord blood values, and thus to create more uniformity in exposure levels across studies. A small, but significant negative effect for PCB-153 on birth weight was observed; no effect for DDE was reported.

Some studies reported gender specific effects (60, 79, 85), however most studies, when including a cohort with both males and females, only reported their results for the overall population. The chemicals reviewed in this paper act on the endocrine system and most of them are thought to have (anti-)estrogenic or (anti)androgenic properties. 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 (86). Male rats showed increased serum insulin levels, and both female and male rats had a significantly lower birth weight than controls. Placental exposure to organohalogenated xenoestrogens, including DDT, was associated with increased birth weight in 14 month old boys but not in

girls (87). As gender specific effects are thus not unexpected for EDCs, future studies should aim to stratify their results for males and females.

As indicated, some studies (51, 52, 88) find PCB exposure to be associated with lower weight; only one study associated prenatal exposure to PCBs with increased weight at puberty in girls (60), although this result was not significant. However, the inverse associations between exposure and weight as reported by Guo et al. (88) were only seen at young ages (6 months to 7 years of age) and did not persist during childhood. Furthermore Rylander et al. (52) measured exposure in blood samples of the mothers, which were collected at enrolment of the children (4 and 7 years of age), and converted this to exposure during pregnancy. This indirect method for calculation of exposure may not be as accurate as measurements performed at the time of pregnancy. Several studies (46-48, 85, 89, 90) find no effect of exposure (both DDE and PCBs) on weight as an individual parameter. Evidence in support of a significant association between prenatal exposure to PCBs or DDE and decreased or increased weight gain later in life, seems unconvincing.

Some studies observe exposure to DDE or PCBs to be associated with lower height (48, 88), but again several studies observe no effect of exposure on height or find a taller height with increasing exposure (Gladen et al. 2000, increased height in boys with increasing DDE exposure; Hertz-Picciotto et al. 2005, increased height in girls with increased PCB exposure). It could be that effects of prenatal exposure to EDCs are not substantial enough to be observed in either height or weight separately.

As for BMI, a parameter which includes both weight and height, exposure to EDCs was in none of the included studies related to a lower BMI and several studies observed a higher BMI with increasing exposure. For DDE in particular one study indicated that no association could be observed between prenatal exposure and BMI, however the outcome was assessed at twelve months of age, while in other cohorts in which DDE exposure was assessed, BMI was measured at later ages (89). Although BMI was not a direct metric determined in the cohort of Pan et al, they observed no effect of DDE exposure on either weight or height, also measured at twelve months of age, making potential obesogenic effects unlikely (47). However, in this cohort only breast-fed children were included. It could be suggested that these children are a positive selection of the general population and that they are therefore more likely to be healthier. This is supported by the observations in the cohort of Patandin et al. (90), looking at effects of PCB exposure on growth in both formula-fed and breast-fed children. A lower growth rate was observed until the age of three months. This effect was only significant in children who were formula-fed, not in children who were breast-fed. Furthermore, it seems that exposure to EDCs in breast milk was never associated with any growth outcome, also not when duration of breastfeeding was taken into account (47, 60). This finding may indicate the importance of prenatal exposure in relation to growth in later life. It could furthermore be argued that – with respect to growth in later life – beneficial effects of breast milk may still outweigh detrimental effects of EDCs present in the milk.

The majority of the observational studies did only include anthropometric measurements as a way of quantifying obesity or growth. In most studies this information was collected by trained researchers, and in some cases by self-report. Though BMI as a relative measure may give an indication of when a child is likely to have a high percentage of body fat, it is not a measure of adiposity (91). This is also true for weight as an individual measure. For this purpose it would be interesting for future studies to include a measure of fat mass, e.g. by using DEXA-scans. Moreover, information on hormone levels such as thyroid hormones and leptin, may give more insight into potential mechanisms through which these compounds may affect growth.

Diet as a confounding factor was not considered by most of the included studies, even though it is an important route of exposure to several compounds and it is intrinsically related to energy balance. Mothers consuming a high fat diet may very well have children who will consume a similar diet, and who are therefore higher exposed through the diet to both endocrine disruptors and fat. Another potential confounder not considered by these studies, is birth weight. Both high (92, 93) and low birth weight (94, 95) are associated with an increased risk of obesity later in life. In several studies exposure to EDCs has found to be inversely associated with birth weight. More specifically this result was found in case of exposure to PCBs (measured either in maternal blood or in maternal milk) (85, 88, 90, 96, 97), PFOS and/or PFOA (measured in maternal or cord blood) (98-101) and for phthalate exposure (102). However, birth weight may also be on the causal pathway between early life EDC exposure and long-term growth, and adjusting models for this factor may also result in overcorrection.

From the overview presented here it may be concluded that PCBs and DDE are the most frequently studied chemicals in long-term prospective studies assessing the effects of prenatal exposure to EDCs. These compounds have also been frequently assessed in older populations, in which associations were observed with BMI (103), characteristics of metabolic syndrome, including waist circumference and fasting glucose (104), as well as fat mass (105). Effects of early life exposure to HCB and perfluorinated alkyl acids, such as PFOS and PFOA, have also been determined, however studies focussing on these chemicals are scarce. Health effects of other chemicals such as brominated flame retardants and phthalates, have thus far only been determined in relation to exposure measured in older children or adults, and obesogenic effects of organotins have only been studied in vitro and in vivo.

Conclusions

It can be concluded that literature does suggest that early life exposure to certain EDCs is associated with weight homeostasis and growth later in life, however not necessarily in an obesogenic direction. Results lack consistency and observational studies for some groups of chemicals are not yet available. Future studies should aim to determine prenatal exposure in

particular and to include not only BMI as an outcome measure, but also more sensitive measures of adiposity, such as fat mass. As these chemicals are considered to be endocrine disruptors, stratifying results for gender is advised. Long-term follow-up is warranted, especially regarding exposure to chemicals other than PCBs and DDE, for which knowledge on long-term effects is still scarce.

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