Chapter 6

General discussion
The global rise in prevalence of obesity is not fully explained by genetics or lifestyle factors. The developmental origins of health and disease (DOHaD) paradigm proposes that environmental factors during early life could play a role. In this perspective, perinatal exposure to endocrine disrupting compounds (EDCs) has been hypothesized as a programming factor for obesity and related metabolic disorders later in life. Programming comprises the ability of a factor to alter the developmental trajectory of an organism during early life, resulting in a phenotype prone for diseases later in life. The goal of this thesis was to study in a mouse model whether perinatal exposure to EDCs programs obesity and metabolic alterations later in life, and to determine if epigenetics, specifically DNA methylation, is an underlying mechanism. We have studied four EDCs with different endocrine disrupting mechanisms: bisphenol A (BPA), perfluorooctanoic acid (PFOA), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyl 153 (PCB 153). C57BL/6JxFVB hybrid mice were exposed during gestation and lactation via maternal feed to a wide subtoxic dose range of each EDC. Generally, the top dose was at or below the reproductive toxic dose calculated by regulatory agencies such as EFSA, and human exposure levels were approached or included in the dose ranges. After weaning, offspring were followed into adulthood without further exposure. Metabolic parameters such as body weight, organ and fat pad weights, glucose and insulin tolerance, and endocrine and lipid profile of serum, but also neurobehavioral and immunological parameters were determined.

Do EDCs program obesity and metabolic alterations?

Our animal studies showed that exposure to different EDCs early in life results in compound- and sex-specific programming of metabolic alterations, but not obesity, later in life. We tested programming by exposing dams during the complete perinatal period and ceasing exposure at weaning age. We had a long follow-up into adulthood and studied a wide variety of metabolic-related parameters. An overview of the results is presented in Table 1. In the BPA study, especially females showed an altered metabolic profile (e.g. decreases in body weight, white and brown fat pad weights, changes in serum lipid and endocrine profile), but males showed some alterations as well (e.g. body and liver weight increase and serum glucagon decrease) (Chapter 2). In the PFOA study, both sexes showed increased liver weights, hepatic foci of cellular alterations and nuclear dysmorphology, with, similar as for BPA, females showing
more alterations than males (Chapter 4). F1 females had permanent changes in body weight (decrease) and effects on growth, serum lipids, organ and fat pad weights and tibia composition/function. In F1 males the decrease in body weight did not persist until the end of the study. In adult males, only effects on growth and cortical density and length of the tibia were observed. Perinatal TCDD exposure led to programming of both metabolic and immune homeostasis in adult offspring, again with different effects between sexes (Chapter 5). F1 females had an increase in white fat tissue and in IFN\(\gamma\) production of the splenic immune cells, while F1 males had a decrease in white fat tissue and in IL-4 production. Finally, PCB 153 exposure also resulted in sex-specific metabolic programming, though limited (Chapter 5). Offspring showed alterations in glucose homeostasis; a decrease in glucagon and pancreas weight in F1 females, and an increase in glucose in F1 males.

Animal studies in literature have shown adult health effects of early life exposure to EDCs, with research focus on the brain, reproduction and cancer (Haugen et al., 2014). Programmed effects can be expressed earlier or later in life, and particularly delayed effects, i.e. effects that remain hidden until triggered by an additional factor, such as ageing, are of interest for the scope of this thesis. Studies focusing on metabolic programming that apply a similar design as ours are limited in literature (Haugen et al., 2014). Of the four EDCs studied in this thesis, programmed effects of TCDD and PCB 153 have hardly been studied by others. Remarkably, for comparable studies that are available, effects observed in offspring show much variation, especially for BPA, and experimental conditions seem to act as confounding factors (e.g. species, strain, sex, diet, exposure route and window, age of testing and microbiome). For example, the standard NIH-07 diet in our mouse studies was specifically chosen because it contains low levels of natural phytoestrogens, which have been shown to promote normal physiology in mice, in contrast to phytoestrogen free diets (Ruhlen et al., 2008). Importantly, in line with the DOHaD paradigm, phytoestrogens can have similar endocrine disruptive activities as EDCs (Jefferson and Newbold, 2000; Newbold et al., 2005). Therefore, interference with effects of EDCs by phytoestrogens can be expected and can explain different outcomes among studies.
Table 1. Overview of results in F1 mice after early life exposure to EDCs

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>BPA F</th>
<th>PFOA F</th>
<th>TCDD F</th>
<th>PCB 153 F</th>
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<tbody>
<tr>
<td>Body weight</td>
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<tr>
<td>Growth with standard diet</td>
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<tr>
<td>Growth with high fat diet</td>
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<td>↑↑↑↑</td>
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<tr>
<td>Body size</td>
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<td>↓</td>
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<tr>
<td>Food consumption</td>
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<tr>
<td>Organ weights</td>
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<td>↑</td>
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<tr>
<td>Fat pad weights</td>
<td>–</td>
<td>↓↓↓</td>
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<td>Adipocyte size (WAT)</td>
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<tr>
<td>Lipid accumulation (BAT)</td>
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<td>↓</td>
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<tr>
<td>Ucp1 expression</td>
<td>–</td>
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<tr>
<td>Serum lipid profile</td>
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<td>↓↓</td>
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<tr>
<td>Serum endocrine profile</td>
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<tr>
<td>Glucose homeostasis</td>
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<td>Neurobehavior</td>
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<tr>
<td>Immune system</td>
<td>nd</td>
<td>nd</td>
<td>↑</td>
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</tbody>
</table>

↑, increase; ↓, decrease; ↓↓ both increase and decrease observed depending on parameter measured. Number of arrows indicates the weight of the effect depending on the number of affected parameters (see definitions below) and/or duration of the effect (body weight, growth). * 1 or more parameters allowed for the calculation of an informative BMDL (lowest 5% lower confidence bound of the benchmark dose at a critical effect size of 5%).

Definitions of categories listed are (according to availability of data):
- Body weight: weekly body weight data; Growth with standard diet: weekly ratios of body weight / body weight of week 5 in animals fed the standard NIH-07 diet; in the BPA study the denominator was the first week that an effect of body weight appeared; Growth with high fat diet: weekly ratios of body weight / body weight of week 5 in animals fed the high fat diet; in the BPA study the denominator was the body weight at the start of the high fat diet period; Body size: body length, femur length, both absolute and relative to body weight; in the PFOA study tibia length was also included; Food consumption: average food consumption measured over three weeks relative to average body weight; Organ weights: both absolute and relative to body weight measurements of adrenal glands, brain, femur, liver, quadriceps femoris muscle, pancreas; in the TCDD and PCB 153 study, spleen, testes and thymus were also included; Fat pad weights: interscapular, perirenal and perigonadal fat pad weights, both absolute and relative to body weight; in the BPA study mesenterial, subcutaneous mammary caudal/rostral and the sum of fat pads were also included; Glucose homeostasis: glucose and insulin tolerance tests and blood levels of glucose and HbA1c; in the PCB 153 study, an extra glucose tolerance test in animals fed the high fat diet was included; Neurobehavior: physical activity, object recognition and sweet preference tests; Immune system: splenocyte responsiveness tests (adherent and nonadherent) and serum C-reactive protein levels.

In the studies described here, body weight was measured weekly throughout life and the observed dose-responses have indicated that the effect on body weight varied over time for every compound and programming of overt obesity has not been shown. In general, maximum effect sizes of body weights were only in the range of 5–10%.
even after a high fat diet at the end of the study. These effects are mild compared to body weight increases observed in ob/ob mice, a genetic mouse model for obesity (Giesbertz et al., 2015). In rats, prolonged feeding with high fat diets induces an increase in body weight in susceptible strains of 10–20% compared to standard diet fed controls (Buettner et al., 2007). In contrast to the hypothesis of this thesis, effects of perinatal exposure comprised mainly decreases of body weight, with a permanent negative effect on body weight in BPA and PFOA females (Chapters 2 and 4), and a transient effect in PFOA males (Chapter 4) and both PCB 153 males and females (Chapter 5). Only males exposed perinatally to BPA showed a permanent increase in body weight (Chapter 2). In addition, both sexes in the TCDD study showed transient increases during juvenility in males and during adulthood with a high fat diet in females (Chapter 5). In contrast, body weight of TCDD males and females was decreased during lactation. In line with these observations, other animal studies (summarized in Table 4 for BPA (Chapter 2) and Table 4 for PFOA (Chapter 4)) also show positive, negative or no associations between perinatal EDC exposure and body weight. Body weight is an apical endpoint that reflects energy homeostasis. Energy homeostasis is a complex process that is regulated by a multitude of checks and balances. Other parameters, that are further upstream in the pathway towards body weight effects, may be more sensitive and should therefore form an integrated part of the assessment of effects on energy homeostasis. For instance, alterations in white fat mass have been observed in TCDD offspring without changes in body weight (Chapter 5). Regrettably, researchers that review EDCs as obesogens hardly address the possibility of observing changes in energy homeostasis without a change in body weight (Janesick et al., 2014; Newbold, 2010). Although this shift in energy homeostasis is not reflected in body weight changes and thus an obvious symptom of obesity is lacking, the effects can be considered adverse. Homeostasis is permanently disturbed and this altered phenotype can be more sensitive to additional adverse triggers later in life. Moreover, in the mouse model an obesogenic phenotype is absent, but in humans, changes in homeostasis could have more dramatic implications for health.

To unveil whether the hypothesized disruption in programming by early life exposure to EDCs could increase the sensitivity to an obesogenic stimulus later in life, we triggered the offspring with a high fat diet, containing 45 kcal% fat of animal origin (lard), as recommended (Buettner et al., 2007), during the final weeks of the study. In the PFOA study, the high fat diet enhanced the observed positive effect on growth of
males and the negative effect on growth of females, as induced by perinatal exposure to PFOA (Table 1; Chapter 4). In perinatally exposed TCDD males, decreases in growth were only observed during weeks 20–29 with the standard diet and continuously with the high fat diet (weeks 43–52; Table 1; Chapter 5). These findings suggest an interaction of perinatal exposure to either PFOA or TCDD, and the response to a high fat diet later in life. The high fat diet did not show any effects in the BPA and PCB 153 studies (Table 1). Similar to our TCDD males, a study in mice has also shown an interaction of perinatal TCDD exposure with high fat diet in later life (Sugai et al., 2014) as discussed in Chapter 5. In contrast to our BPA study, other studies have indicated the interaction of exposure to BPA early in life and a diet high in saturated fats later in life (Somm et al., 2009; Strakovsky et al., 2015; Wei et al., 2011). These studies in rats showed that the high fat diet exacerbated the effects of perinatal BPA exposure, however, the effects were most pronounced in males which were not challenged with a high fat diet in our BPA study (Chapter 2). For a better comparison between exposure to an EDC only and the interaction with a high fat diet, it would have been useful to have included mice on a standard diet throughout the complete study. Possibly extending the period of high fat diet and/or starting the high fat diet earlier may have been advisable, as obesity induction in rodents is most effective when the high fat diet is started at a young age (Buettner et al., 2007).

Finally, as most epidemiological studies on the association between EDC exposure and obesity are cross-sectional, few epidemiological studies that indicate metabolic programming effects of EDCs exist (de Cock and van de Bor, 2014). Especially some classes of EDCs, such as perfluoroalkylated substances (PFASs), are hardly studied in humans. These epidemiological studies need to measure EDC levels in cord blood or maternal serum/milk and, after a long follow-up, measure metabolic parameters more sensitive than body mass index (BMI) in (young) adults. However, current indications from epidemiology for programming by EDCs together with the programming effects in energy (and immune) homeostasis shown in the animal studies in this thesis, may be considered as an alert and evoke the need for further study. Thus both well-designed animal and epidemiological studies on metabolic programming are warranted.
Does altered DNA methylation underlie the effects observed?

Epigenetic changes have been proposed as the leading mechanism underlying programming effects induced by environmental factors (Gluckman et al., 2009), including EDCs (Vaiserman, 2014). Studies have focused on DNA methylation, since new DNA methylation patterns are established during embryogenesis and considered to be more stable throughout life than histone modifications (Gluckman et al., 2009). In our BPA study, the programmed metabolic phenotype observed in female offspring was not associated with changes in DNA methylation in the liver (Chapter 3). Possibly, epigenetic changes did occur, but were missed due to tissue-specificity and limitations of the DNA methylation assay applied. However, it is extremely difficult to choose the most appropriate assay, taking into account technical issues such as the choice of epigenomic regulatory mechanism (e.g. DNA methylation or histone modifications), target tissue, and a genome wide or targeted approach (Greally and Jacobs, 2013). Following Waterland (2014) and Greally and Jacobs (2013) who discuss several possibilities to study endocrine disruptors and epigenetics, our study design is one of the most favorable, because we used an inbred mouse strain, we analyzed liver, which is an important organ in energy metabolism, and we tested DNA methylation both globally and at specific CpG sites with a genome-wide methylation assay followed by validation of candidate markers with pyrosequencing. In contrast to our findings, in a perinatal BPA rat study, F1 males at PND90 did show an affected phenotype (an increased fat/lean mass ratio) and altered DNA methylation and histone marks together with changes in hepatic expression that were measured at PND1 (Strakovsky et al., 2015). In the agouti mouse model, Anderson et al. (2012) showed a decrease in body weight after perinatal BPA exposure (2 weeks premating – PND22) via the diet to 0.01 µg/kg bw/d at PND22 together with a global hypomethylation of tail tissue for all doses (0.01–10–10,000 µg/kg bw/d) and a decrease of DNA methylation in the promoter of the *Agouti* gene for the top dose. Effects did not differ between sexes and data were combined. Importantly, these studies had continuous exposure to BPA up to the age of testing, in contrast to our BPA study which included a recovery phase. In addition, we did not measure DNA methylation at weaning age in our BPA females, making comparison between the studies difficult. Importantly, the potential reversibility of the observed epigenetic effects in the studies of Strakovsky et al. (2015) and Anderson et al. (2012) cannot be excluded and definitive epigenetic programming has not yet been shown.
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Animal studies such as the BPA study described in this thesis (Chapters 2 and 3) are essential to understand possible epigenetic mechanisms of programming by EDCs, while human epigenetic studies for metabolic diseases have limitations. The genetic variation in humans is a major determinant of interindividual epigenetic variation (Rakyan et al., 2011). Therefore, the results of human studies have genetic confounding as a disadvantage. Furthermore, the only easily accessible tissue in humans is peripheral blood. However, peripheral blood cells are not important regulators in energy metabolism (Waterland, 2014). In contrast, energy-related tissues, such as hypothalamus, white adipose tissue and liver, the primarily studied tissues in animal models, are hard to harvest from humans. Moreover, epigenetic measurements in adults do not prove causality of metabolic diseases since they do not preclude the possibility that epigenetic changes, which can be reversible, occurred as a consequence of metabolic disorders (Waterland, 2014).

Many models within the DOHaD-field, both in vitro and in vivo, are used in the study of epigenetics, and each of them has advantages and disadvantages (Greally and Jacobs, 2013; Rasoulpour et al., 2011). The complexity of our hypothesis does not allow an in vitro model to provide an answer. To study programming, apical endpoints in multiple physiological systems that can indicate a permanent change in phenotype need to be analyzed, which is not possible with, for example, human stem cells. Importantly, the benchmark approach used in this study allowed for a minimal amount of animals relative to the amount of information gained, taking into account Reduction in the 3Rs philosophy (Slob, 2014a; Slob, 2014b). Nonetheless, in vitro studies can be used as supplementary tools to provide mechanistic information. In contrast to the animal organs, single cells will not have the variation caused by the mixture of cells present in intact organs. Therefore, small changes in gene expression and DNA methylation can be identified more easily (Legler et al., 2011). In one OBELIX study, EDC exposure resulted in metabolic and inflammatory gene expression changes in human peripheral blood mononuclear cells (Wens et al., 2013). Other OBELIX studies showed that EDCs can influence murine adipocyte differentiation, and concomitantly have effects on global and locus-specific methylation (Bastos Sales et al., 2013; Kamstra et al., 2014).

Several theories explain how EDCs exert these permanent epigenetic changes (Rissman and Adli, 2014). EDCs can directly act on the epigenome by changing the composition or the activity of epigenetic chromatin regulator complexes, such as
DNA methyltransferases (DNMTs) and histone methyltransferases. Otherwise, they can act indirectly, by binding to nuclear receptors (Rissman and Adli, 2014). These receptors subsequently become activated and function as transcription factors that change the expression of these chromatin regulators (e.g. DNMTs) and hence, the state of the epigenome. Furthermore, it is postulated that early in life, during the first contact of endogenous hormones with their receptors, receptors are lifelong primed on how to respond (e.g. sensitivity) to the hormones later in life (Csaba, 2011). Epigenetics is recognized as a mechanism involved in this hormonal imprinting. The EDCs that mimic endogenous hormones could interfere with this process (Vaiserman, 2014). Therefore, possibly, the receptors that are activated by the EDCs tested in this thesis, such as ERs (BPA), PPARs (PFOA), AhR (TCDD), CAR/PXR (PCB 153), may have been mediators in the observed metabolic programming. For example, AhR target genes of TCDD-exposed zebrafish embryos showed alterations in DNA methylation of promoter regions (Aluru et al., 2015). In addition, a perinatal mouse study with BPA displayed changes in the expression of ERs and DNMTs in the brain, associated with changes in DNA methylation of ERα (Kundakovic et al., 2013).

Alternative candidate mechanisms that underlie programming have also been proposed (Waterland and Garza, 1999). For example, EDCs could affect organ structure (gross morphology, above cellular level) or cell number. Early in life, adipocyte cell number is established (Lee et al., 2014) and hormonal signals control cellular proliferation. Thus, obesogenic EDCs may act by increasing this number (Janesick et al., 2014). Consequently, the metabolic activity of the adipose tissue can be altered (Waterland 1999). These different mechanisms that potentially underlie programming could act in combination with epigenetics (Waterland, 2014). In addition, they can be induced by epigenetics or permit epigenetic changes. Therefore, the interdependency between epigenetics and alternative candidate mechanisms means that they must be studied in an integrated approach.

**Sexual dimorphism**

All four EDCs tested in this thesis have shown alterations of the adult phenotype in a sex-specific manner. Other animal studies have also shown sex-specific effects of EDCs, in particular neurobehavioral effects after BPA exposure (Adriani et al., 2003; Sobolewski et al., 2014), as well as PFOA, TCDD (Sobolewski et al., 2014),
and PCB 153 (Holene et al., 1999). Sex-dependent metabolic alterations in adult F1 mice have been observed after lifelong exposure to a mixture of BPA, TCDD, PCB 153 and di-(2-ethylhexyl) phthalate (DEHP) (Naville et al., 2013). Epidemiological studies have also shown different associations for early life exposure to EDCs regarding risk of overweight in later life between males and females (Halldorsson et al., 2012; Tang-Peronard et al., 2014). Sex hormones are the most obvious regulators for different responses of the sexes to environmental factors. Sex-linked genes are already differentially expressed in the early embryo in humans, even before the actual production of sex hormones (Ober et al., 2008). These genes are present on the X- and Y-chromosomes, but also on the autosomes, and can be responsible for the observed sex-differences in alterations of the adult phenotypes. Differential expression of sex-linked genes between sexes is observed in all organs, albeit in varying degrees (Gabory et al., 2009). The liver is the most well-studied organ, showing a high percentage (70%) of sexual dimorphism in gene expression (Gabory et al., 2009). Due to a sex-dependent expression of cytochrome P450 enzymes, liver metabolism of xenobiotics, including EDCs, differs between males and females (Hochberg 2011). Although liver metabolism is still developing in the fetus, this could have played a role in the observed sex-specific effects. More importantly, male and female placentas respond differently to environmental factors resulting in different adverse health outcomes in adult life (Gabory et al., 2013). For example, sex-dependent changes in placental gene expression, including changes in expression of epigenetic chromatin regulators (e.g. histone methyltransferases), were observed in mice after exposure to a high fat diet (Gabory et al., 2012). In addition, mRNA expression of nuclear receptors in placentas of BPA-exposed mice was sex-specifically altered (Imanishi et al., 2003). The placenta is the interface between mother and fetus, plays a pivotal role in fetal growth and development, and is derived from both maternal and fetal contributions with the majority of the tissue of fetal origin, which explains the sex-specificity (Rossant and Cross, 2001). The placenta has multiple functions, such as metabolism of xenobiotics (e.g. EDCs) and the production of hormones that have important effects on both maternal and fetal physiology. Therefore, it is a key regulator in the response to EDCs in the fetal body. Regardless of the underlying mechanism, the variation of effects between sexes underlines the importance of assessing both males and females to determine the most sensitive sex.
Risk assessment

Currently, formal criteria to regulate EDCs do not exist, and the best approach to identify EDCs that are of concern for human health is under extensive debate (Autrup et al., 2015; Bergman et al., 2013; Dietrich et al., 2013; Gore et al., 2013; Holmes, 2013; Rhomberg et al., 2012; Vandenberg et al., 2013). Regulation of EDCs is of importance in various legislation, e.g. under REACH (Registration, Evaluation and Authorization of CHemicals), for plant protection products, biocides, medical devices, etc. The Organization for Economic Co-operation and Development (OECD) is continuously in the process of developing guidelines and has developed a conceptual framework for testing endocrine active substances including EDCs. The need for the incorporation of OECD-validated testing guidelines that include permanent effects emerging later in life after early exposure, has been emphasized (EFSA, 2013; Felter et al., 2015). Commonly, risk assessment consists of four steps: hazard identification, dose-response assessment, exposure assessment and risk characterization (Felter et al., 2015). In assessing the risk of chemicals, fetuses, children and the elderly are considered to be more sensitive than adults. Sensitivity of fetuses and children to EDCs can be different since toxicokinetics (absorption, distribution, metabolism and excretion), toxicodynamics, and exposure, differ compared to adults. The capacity for metabolism of xenobiotics, including EDCs, is still highly under development in fetuses and also growth of the fetus and organs (i.e. blood flow to the liver and kidney) plays a role. Moreover, the interaction of EDCs with hormones, which have age-dependent circulating concentrations, is of importance. In this respect, little is known about differences in toxicodynamics between fetuses/newborns and adults, but they can be expected to exist (Felter et al., 2015). In addition to a different sensitivity, susceptibility of fetuses and newborns can be different due to different exposure levels compared to children and adults. This depends on many factors, e.g. fetuses and newborns are exposed via the mother. Indeed, in our study, we found for example higher BPA serum concentrations in pups than in dams (Chapter 2).

The experimental model used in this thesis to study programming has a number of strengths that make it appropriate for risk assessment purposes. First of all, we used rodents as a model, also the main model used in risk assessment in general, and in the DOHaD/EDC field specifically (Haugen et al., 2014). Therefore, differences between rodents and humans are well characterized. For instance, of all available nonprimate animal models, the rodent placenta is known to be most similar to the organization of
the human placenta (Myllynen et al., 2005). Furthermore, the mouse model provides access to necessary informative tissues, inaccessible in humans. Moreover, the use of two inbred strains avoided genetic confounding, as is present in human observational studies. Secondly, the study design and experimental conditions in our studies were structured to mimic the human situation as closely as possible. Exposure via the diet mirrored the main exposure route for humans, since route of exposure can influence toxicokinetics. Dams were exposed to the EDCs before mating to have steady state levels in their circulation. A wide range of doses was tested, with lower doses reflecting human exposure. Doses were maximally at or below reproductive toxic dose to avoid toxicity so we could distinguish the observed effects from reproductive and teratogenic effects (Schug et al., 2011). This is especially important since sensitivity can differ depending on dose, due to the influence of saturation of metabolic pathways for high doses (Felter et al., 2015). Exposure was ceased at weaning age to have a latent phase during follow-up in order to investigate permanent effects and test the programming concept. Developmental windows of organs differ between mice and humans, but these are well-described. Maternal exposure of offspring throughout the complete perinatal period covered the sensitive developmental phase since functional maturation of organs in mice is reached at 3 weeks of age (Coogan, 2013). Third, the long follow-up of six months (BPA, PFOA) or one year (TCDD, PCB 153) mimics the occurrence of overweight and obesity in young and middle-aged adults. Finally, the study design is amenable to risk assessment, i.e. it was comprised of one unexposed and seven exposed groups, allowing for the application of the benchmark dose (BMD) approach. BMDLs are more precise points of departure (PODs) than no-observed-adverse-effect-levels (NOAELs) (EFSA, 2009; Slob, 2014b).

The adversity of metabolic changes observed after perinatal exposure to EDCs in our studies, e.g. serum triglycerides, glucagon and adipocytokine levels, can be debated. Defined scientific criteria for assessment of adversity do not exist. EFSA (2013) reported: ‘In general, but not always, transient, inconsistent and minor fluctuations at the biochemical and molecular level may be considered adaptive, i.e. non-adverse. Changes at the cell-, organ-, organism-, or (sub)population-level resulting in pathology or functional impairment in vivo, as well as altered timing of development, may be considered adverse.’ The fluctuations at the biochemical and molecular level observed throughout our studies, however, are not a result of direct interaction with the EDC, but are permanent changes indicating a differently programmed homeostasis. In principle, the biochemical effects in our studies could be
considered as a non-adaptive situation, because there was no exposure to the EDCs at the time of evaluation. However, full appraisal of adversity requires further evaluation of the animal’s ability to resist additional physiological challenges with this altered homeostasis. The interaction of high fat diet later in life with perinatal exposure to PFOA and TCDD, as discussed above, indicates that the perinatally exposed animals in these studies reacted differently to a physiological challenge, suggesting adversity.

Currently, however, programming of metabolic phenotype is not included in risk assessment of chemicals and the inclusion of endpoints such as increase or decrease in body weight or adipose weight in risk assessment is under much debate. Our studies also included the analysis of specific target organs that already receive more critical attention in risk assessment: skeletal, endocrine, neurological and immune systems (Felter et al., 2015). The results of the animal studies presented in this thesis can be used to determine if current standards set for toxicants, such as EDCs, in food are sufficiently protective of long-term effects of perinatal exposure. For BPA, we here consider EFSA’s most recent method for tolerable daily intake (TDI) calculation (EFSA, 2015), which differs from the 2014 method as applied in our previously published study (Chapter 2). Calculating with our BMDL of 233 µg/kg bw/d for interscapular fat pad weight decrease (Chapter 2), would produce a TDI which is 38 times lower than EFSA’s TDI based on the BMDL10 of 8960 µg/kg bw/d for mean relative kidney weight. This TDI would be below the current dietary intake estimate of BPA for infants and toddlers, which is up to 0.875 µg/kg bw/d as well as the aggregated exposure for adolescents estimated at 1.449 µg/kg bw/d. It should be noted, however, that the adversity of this particular effect and the validity of comparing exposures at different time windows (early development in our study versus infancy/adolescence in humans) needs to be established. The study with PFOA showed a BMDL of 46 µg/kg bw/d for perirenal fat pad weight which is more than 10 times lower than the most sensitive developmental BMDL of 616 µg/kg bw/d established by a developmental study of Lau et al. (2006) and 6.5 times lower than the BMDL10 of 300 µg/kg bw/d used for setting the TDI (EFSA, 2008). Applying our developmental BMDL of 46 µg/kg bw/d would lower the TDI to 230 ng/kg bw/d. Still, this level is a factor 15 higher than the human dietary exposure range of 0.16–15 ng/kg bw/d in infants (EFSA, 2012), suggesting a sufficient margin of safety (Chapter 4). The situation is different for the other two tested compounds. The effects found in the TCDD and PCB 153 studies indicate that current TDIs are sufficiently protective for potential metabolic programming effects of these chemicals (Chapter 5).
Chapter 6

Future perspectives

Mechanistic studies
The in vivo studies in this thesis are not mechanistic but descriptive since we studied apical endpoints (e.g., body weight, organ and fat pad weights) and endpoints that can only point in the direction of a perturbed pathway (e.g., histopathology, serum lipid and endocrine profile). We did not explore the involvement of the known endocrine mechanisms of action of the EDCs and due to time and financial constraints, only F1 females perinatally exposed to BPA were studied for DNA methylation as an underlying mechanism (Chapter 2). It would be intriguing to unravel the mechanisms underlying the compound- and sex-specific programming effects of BPA, PFOA, TCDD and PCB 153, for example by performing whole genome methylation and gene expression studies. In this respect, discovering the adverse outcome pathways (AOPs) of EDCs is of interest. An AOP is ‘a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event (e.g., a molecular interaction between a xenobiotic and a specific biomolecule) and an adverse outcome at a biological level of organization relevant to risk assessment’ (Ankley et al., 2010). In our case, this would imply the link between the altered programmed metabolic phenotype in adult mice induced by perinatal exposure to EDCs and the hypothesized epigenetic changes, and the links in-between, are laid down. The OECD (2013) already has a guidance document to provide insights into the identification and documentation of AOPs, and their use in integrated testing strategies and risk assessment is under development (Patlewicz et al., 2015). So revealing the AOPs of programming by EDCs would provide a mode (and/or mechanism) of action and, therefore, would increase the weight-of-evidence in risk assessment (Dekant and Colnot, 2013). In addition, untangling the AOPs could provide clues in the predictions of effects of EDCs and less (animal) studies will be needed to assess the programmed health risks of EDCs with similar AOPs.

Mixture effects
In our animal studies, programming of individual compounds was evaluated. Studying one compound provides information on the specific effects the compound can induce and the possibility to gain more knowledge on the underlying mechanism. To better mimic the realistic situation, studies with mixtures should be performed. EDCs activate receptors and among these receptors crosstalk occurs (Pascussi et al., 2008). Therefore, safe doses of individual EDCs in a mixture can result in effects due to additive or
synergistic actions. Perinatal animal studies have already shown the importance of low dose mixture effects of EDCs (Axelstad et al., 2014; Manikkam et al., 2013; Sobolewski et al., 2014). Interestingly, in one study, mice were exposed lifelong to a mixture of BPA, TCDD, PCB 153 and DEHP, in low doses close to the TDI (Naville et al., 2013). Offspring showed sex-dependent metabolic alterations without general toxicity or weight gain. In addition, comparing single EDC exposures to mixtures is relatively new in human prospective studies. With this approach a recent study found an association for prenatal exposure to organochlorine compounds with overweight in 7-year old children (Agay-Shay et al., 2015). Risk assessment can be improved by taking into account mixture effects (Hass et al., 2012; Kortenkamp, 2008). However, developing a framework that can model mixtures of EDCs will be a challenge.

**Holistic approach**
Combining the divergent metabolic effects observed for each EDC, it is hard to point towards one pathway that is altered. In general, a clear cohesive pattern of all the observed changes was not revealed. These results are in line with other studies, which also show varying metabolic alterations, for which BPA is a typical example (Table 4 in Chapter 2). Moreover, the additional analysis of neurological and immune parameters in the final two studies suggests that multiple physiological systems were affected, as in the TCDD study, programming of immune homeostasis was shown (Chapter 5). More evidence from animal and epidemiological studies exists that developmental exposure to EDCs can result in adult immune dysfunction (Dietert, 2012; Dietert and Piepenbrink, 2006). Although neurological alterations were not revealed in the TCDD and PCB 153 studies (Chapter 5), several reviews describe animal and epidemiological studies that support neurological programming of EDCs (Berghuis et al., 2015; León-Olea et al., 2014; Rosenfeld, 2015; Sioen et al., 2013). Since the effects of exposure to EDCs in our and other programming studies are induced during development, it is indeed plausible multiple physiological systems are affected. Moreover, functions of these systems are intertwined and alterations in one system can affect functioning of another. For example, the immune system has a critical role in brain development (Bilbo and Schwarz, 2012). Furthermore, perinatal neuro-immune interactions have been suggested capable of programming offspring obesity (Jasoni et al., 2015). Cytokines produced after aberrant immune activation can affect brain development via, for example, changes in the development of hypothalamic feeding centers. Disruption of centers that regulate body weight can result in an elevated risk for obesity later in life.
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Thus, effects of EDCs on multiple disease or organ endpoints, like brain, cancer, immune system, lung, obesity and reproduction have been found (Haugen et al., 2014). In addition, different endpoints can be affected by one EDC and yet similar effects are induced by multiple EDCs with different endocrine disrupting activities. Even more, completely different environmental factors, such as stress and nutrition, share a large overlap with early EDC-induced adult life effects (Prescott, 2014). To explain these observations it has been proposed that all environmental factors, e.g. EDCs, nutrition, stress etc., interact with each other and the fetus during development, likely via epigenetic mechanisms. Eventually, the final sum of these factors results in a specific programmed adult phenotype that holds an increased risk for an altered homeostasis in one or more systems or even an increased risk for one or more disease endpoints. One school of thought advocates research using this holistic approach instead of a focused approach of one environmental factor and one (disease) endpoint to finally see the big(ger) picture (Hamlin, 2012). For instance, both Prescott and Dietert, independently of each other, hypothesize inflammation, which can be active in many organ systems, as the key player in this early life programming of dysregulation later in life (Dietert and Piepenbrink, 2006; Prescott, 2013; Prescott, 2014).

**Intervention versus prevention**

Understanding the big(ger) picture could result in the establishment of effective intervention and prevention strategies. Current interventions in children and adults are not sufficient since the health burden of overweight, obesity and metabolic disorders is still escalating (Atkinson et al., 2012; Gluckman et al., 2011). To achieve the target of the World Health Organization (WHO) to halt the rise in obesity and diabetes by 2025, new and better approaches are needed (WHO, 2013). The evidence of a substantial contribution of environmental factors early in life to these diseases is compelling (Schug et al., 2013) and the importance of interventions in early life, starting already before conception, to increase human health later in life is increasingly recognized (Doyle et al., 2009). However, direct application of environmental interventions in clinical practice is currently still a challenge (Painter, 2015). This is mainly due to the complexity of factors involved and the current gaps in knowledge (Lumeng et al., 2015). For example, the reciprocity of the different environmental factors with each other and the fetus is a point of interest. If the relative contributions of all environmental factors could be determined, the most cost effective health improving recommendations and interventions can be developed and applied.
Although diet and physical activity are more important contributors to the societal costs of obesity (Cecchini and Sassi, 2015), calculations for EDC-attributable costs have been made and are substantial (Trasande et al., 2015). In the European Union, a conservative estimate of minimal 18 billion Euros for EDC-attributable costs of obesity and diabetes has been calculated (Legler et al., 2015). Calculations for separate EDC classes were performed. For example, prenatal BPA exposure was identified with a 20% to 69% probability to increase the prevalence of childhood obesity with 0.89% to 2.90%, causing 42,400 cases of childhood obesity, which is associated with 1.54 billion Euros of societal costs per year (Legler et al., 2015). Keeping in mind that EDCs also contribute to other types of health effects, such as cognitive deficits, neurodevelopmental disorders and male reproductive health, calculated by the same method (Bellanger et al., 2015; Hauser et al., 2015) or other methods with similar outcomes (Olsson, 2014; Rijk and van den Berg, 2015), the burden of societal costs attributable to EDCs is even higher. Trasande et al. (2015) have reported the burden of diseases attributable to EDCs to be in the hundreds of billions of Euros per year. It should be noted that these high health care costs are calculated with a probability of causation evaluated based on existing data of epidemiological and animal studies, which is high for some EDCs (e.g. DDE), and lower for others (e.g. BPA), but still support the importance of reducing and/or preventing early life exposure to EDCs.

Targets for intervention and prevention strategies already exist. In the case of epigenetics as the underlying mechanism, the acquired epigenetic profile as a newborn could be a ‘fingerprint’ for the programmed adult phenotype (Haugen et al., 2014). Examples of future useable tissues for acquiring the epigenetic ‘fingerprint’ are cord (blood) or the placenta (Gabory et al., 2013). Inflammatory deviations as potential biomarkers have also been suggested (Dietert, 2012; Prescott, 2014). The potential for reversibility of the early epigenetic profile and/or programmed adult phenotype has been shown in animal intervention studies comprised of, for example, neonatal leptin treatment (Vickers et al., 2005) or maternal dietary supplementation with the phytoestrogen genistein or folic acid (Dolinoy et al., 2007) after perinatal undernutrition and BPA exposure respectively. Therefore, offering lifestyle recommendations to (future) parents or interventions on a nutritional or perhaps even on a pharmacological level, based on the epigenetic ‘fingerprint’ or other biomarkers early in life, has the prospect to function as a prevention strategy. In addition, other sensitive time windows in life, such as puberty, are also important. Long-lasting health can best be achieved by complementing early life interventions with interventions throughout the life course.
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(Wachs et al., 2014). These interventions need to take into account all complex factors involved in obesity and their interactions with each other and the individual (Kleinert and Horton, 2015). For example, as discussed above, the in vivo studies of this thesis already showed the interaction of perinatal exposure to PFOA or TCDD and a high fat diet later in life. Not only biological, but also psychological, social, and economic factors play a role in an individual’s vulnerability towards the development of obesity (Roberto et al., 2015). The efficiency of these intervention and prevention strategies in halting or even lower the prevalence of childhood and adult obesity and associated metabolic disorders will depend on this integrated approach.
Conclusions

Overall, the studies in this thesis did not show programming of obesity in our mouse model by early life exposure to EDCs. However, our studies showed compound- and sex-specific effects for metabolic alterations, such as changes in fat pad weights and histology and serum levels of lipids and hormones, that persisted into adulthood supporting the perinatal programming concept (Chapters 2, 4, and 5). The variation of results within our studies and literature suggests that the relation between early life exposure and apical outcomes at adulthood is not straightforward and may be influenced by secondary factors, such as diet, and microbiome. This complexity indicates translation of the observed effects in mice to humans is also not straightforward.

Programmed effects as studied in this thesis are not considered in current risk assessment. If this would be the case, our lowest observed BMDL for BPA (Chapter 2) would lead to a TDI below estimated human intakes. The lowest BMDL in the PFOA study would lower the TDI, but compared to current exposure levels, the margin of safety would still be sufficient (Chapter 4), whereas the derived TDIs for TCDD and PCB 153 for potential metabolic programming effects would be within limits of current TDIs (Chapter 5).

Our BPA epigenetic study results did not support the hypothesis that DNA methylation is a mechanism by which perinatal exposure to EDCs programs metabolic alterations later in life (Chapter 2). Although many suggestions for epigenetic changes as the underlying mechanism exist in literature, substantiated evidence that precludes other mechanisms is still lacking. Multiple factors, including the complexity of causality in humans, the latent time period between early life exposure and adult outcomes, and the potential for reversibility of epigenetic marks, make it particularly challenging to unravel whether programming is mediated via epigenetic changes.

In addition to unraveling the involvement of epigenetics, we need to gain more knowledge on the development of obesity, the role of chemical exposure in obesity, and finally acquire complete AOPs. To grasp the big(ger) picture, future research needs to integrate the different environmental factors during development that may lead to adult health effects. In the end, understanding of the big(ger) picture could result in the set-up of effective intervention and prevention strategies.
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References


General discussion


Chapter 6


General discussion


Rijk I., van den Berg M. (2015) Putting a price on your exposed brain. A case-study towards prenatal exposure to polybrominated diphenyl ethers (PBDEs), organophosphate pesticides (OPs) and associated socioeconomic cost of IQ loss in the Netherlands, IRAS.


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