Introduction

Prevalence of overweight (body mass index (BMI) of ≥ 25 < 30 kg/m²) and obesity (BMI of ≥ 30 kg/m²) has reached pandemic proportions and is increasing rapidly in children. Obesity is a health concern since it is a major risk factor for noncommunicable diseases, including diabetes, cardiovascular disease and certain cancers. The global rise in prevalence of obesity is not fully explained by genetics or lifestyle factors, such as the consumption of energy-dense foods and insufficient physical activity. The developmental origins of health and disease (DOHaD) paradigm proposes that environmental factors during early life could play a role. In this perspective, early life exposure to endocrine disrupting compounds (EDCs) has been hypothesized as a programming factor for obesity and related metabolic disorders later in life. Programming refers to the process through which the translation from genotype to phenotype is tuned towards optimal adaptation to the environment. Disrupted programming, for example by early life exposure to EDCs, may lead to a maladapted or adverse phenotype with an increased risk for diseases later in life. Daily exposure of humans to man-made EDCs occurs through food contamination and compound release from various consumer products (e.g. packaging materials, cookware, cosmetics). The EU-funded project ‘OBELIX’ (2009–2013) aimed to comprehensively study the hypothesis that EDCs, in particular when exposed early in development, may act as ‘obesogens’, chemicals that stimulate fat cell differentiation and/or predispose or sensitize individuals to obesity. OBELIX is an abbreviation for OBesogenic Endocrine disrupting chemicals: LInking prenatal eXposure to the development of obesity later in life. This project combined in vitro, animal and epidemiological studies, of which the animal studies are described in this thesis. Our specific objectives were 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 (Chapter 1).

We have studied four EDCs representing different endocrine modes of action: bisphenol A (BPA), perfluorooctanoic acid (PFOA), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyl 153 (PCB 153) in an experimental design that is relevant for human exposure. C57BL/6JxFVB hybrid mice were exposed during gestation and lactation via maternal feed to a wide range of subtoxic doses of each EDC. In every study, one unexposed and seven exposed groups were included. Generally, the top dose was at or below the reproductive toxic dose calculated by
regulatory agencies such as EFSA. The benchmark dose (BMD) approach was applied to analyze the full dose range data. Subsequently, the calculated lowest derived lower bound of 90% confidence interval of the benchmark dose (BMDL) can be used in risk assessment. After weaning, offspring were followed into adulthood (half a year or one year) without further exposure. Through termination of the exposure after weaning, we could test our hypothesis that observed adult effects are not caused by direct exposure, but caused by permanent effects induced during exposure early in life. Follow-up included measurement of a broad range of metabolic parameters. Immune and neurobehavioral parameters were also examined, since the involvement of both immune and neurobehavioral functions have been implicated in the development of obesity. *In vivo* endpoints were: body weight, growth, glucose and insulin tolerance, food consumption and neurobehavior (physical activity, object recognition, sucrose preference). During the final weeks of each study, offspring were challenged with a high fat diet to test a possible change in response to energy-dense food. *Ex vivo* analyses included: testing function of adherent and nonadherent immune cells in the spleen (lymphoproliferative response), weighing and histopathology of organs and fat pads, and clinical chemistry parameters, like endocrine and lipid profile in blood. In addition, external and internal dose measurements were performed to confirm exposure.

**BPA**

BPA, the first EDC studied (Chapter 2), is a compound released from plastics and other consumer products used in everyday life. Obesogenic effects of BPA were already extensively studied and programming studies have shown a wide variety of results. Perinatal exposure to BPA (3–3000 µg/kg body weight/day (µg/kg bw/d)) resulted in dose-dependent increases of body and liver weights, no effects on fat pad weights, slightly increased lipid accumulation in brown adipose tissue (BAT), a dose-dependent decrease in circulating glucagon and a decrease in physical activity in adult male offspring (23 weeks of age). Female offspring showed an altered metabolic profile with a dose-dependent decrease in body weight, liver, muscle and fat pad weights, adipocyte size, serum lipids, serum leptin and serum adiponectin. In addition, lipid depletion in BAT was observed together with a dose-dependent decrease in *Ucp1* expression and a suggested increase in physical activity. The effects in females were more reliable and robust than in males due to wide confidence intervals and potential confounding by
litter size for male data. The most sensitive effect was the decrease of interscapular fat pad weight in females, with a BDML of 233 µg/kg bw/d.

We concluded that BPA can program for an altered metabolic phenotype, but in view of the sexual dimorphism of effects and diversity of outcomes among studies similar in design as ours, BPA could not be marked as a specific obesogen. For comparison to current standards applied in risk assessment, the method of EFSA (2014) was used in Chapter 2. However, recently EFSA (2015) used a different calculation method to set a tolerable daily intake (TDI) for BPA, and applying this latest EFSA method (EFSA, 2015) to our most sensitive BMDL would result in a TDI below the current dietary exposure estimates. It should be noted, however, that the adversity of the 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.

Epigenetics

Epigenetics is the molecular mechanism through which one genotype can give rise to variant phenotypes without changes in DNA sequence. An epigenetic profile is established early in life and EDCs are hypothesized to interact with this establishment. Alterations in the epigenetic profile by EDCs is a proposed mechanism underlying programming of obesity later in life. Epigenetic changes can occur on three different layers of which DNA methylation is mostly studied. Therefore, adult liver, as a key organ in energy homeostasis, of females exposed to BPA early in life were investigated for DNA methylation changes (Chapter 3). Females were chosen because they showed the most prominent metabolic alterations compared to males. Both global DNA methylation and genome-wide DNA methylation analysis at specific CpG sites with the digital restriction enzyme analysis of methylation (DREAM) assay were assessed to identify associated epigenetic alterations. Measurement of global DNA methylation did not show any changes. DREAM analysis in control and 3000 µg BPA/kg bw/d females revealed potential differences, that could not be confirmed by bisulfite pyrosequencing.

Overall, we demonstrated that the observed altered metabolic phenotype in female offspring after maternal exposure to BPA was not detectably associated with liver DNA methylation changes. This observation, however, does not exclude epigenetic
effects in other tissues with a regulating role in energy homeostasis, such as the hypothalamus, adipose tissue or the pancreas islets.

**PFOA**

PFOA, the second EDC studied (Chapter 4), is a man-made compound that is able to repel both water and oils. PFOA has many applications such as coatings for fabrics, carpets and food packaging. The study was comprised of perinatal exposure to PFOA in a dose range of 3–3000 µg/kg bw/d, and after a latency period of 23–25 weeks, the adult metabolic phenotype of the offspring was analyzed. Both male and female offspring showed a dose-dependent decrease in body weight from postnatal day 4 to adulthood. Growth under high fat diet in the last 4–6 weeks of follow-up was decreased in female and increased in male offspring. Subsequently, the decrease in body weight in males did not persist until the end of the study. Both sexes showed increased liver weights, hepatic foci of cellular alterations and nuclear dysmorphology. In females, which showed more alterations than males, reductions in perigonadal and perirenal fat pad weights, serum triglycerides and cholesterol were also observed. Endocrine parameters, such as glucose tolerance, serum insulin and leptin, were not affected.

We concluded that our study with early life exposure to PFOA resulted in metabolic effects in adult offspring which were more pronounced in females (Chapter 4). Most likely the effects induced by PFOA are a result of maladapted programming. However, the assayed endpoints did not provide a mechanistic explanation. Finally, the lowest BMDL of effects (46 µg/kg bw/d; decreased perirenal fat pad weight in females) in our study is below the known BMDL for developmental toxicity and also below the lowest BMDL used as the basis for the current TDI established by EFSA, but still higher than the estimated human dietary exposure range.

**TCDD and PCB 153**

TCDD and PCB 153 were the last two EDCs studied (Chapter 5). TCDD is the most potent aryl hydrocarbon receptor (AhR) agonist, while in contrast, PCB 153 is a potent constitutive androstane receptor/pregnane X receptor (CAR/PXR) agonist.
AhR, CAR and PXR are suggested to be involved in energy homeostasis and immune responses. CAR and PXR share a large overlap in their regulated genes, while AhR controls an almost completely different set of genes. We hypothesized that TCDD and PCB 153 represent two ligands which may lead to similar metabolic effects, though through different pathways. TCDD is mainly a side-product of several organic synthesis processes and incineration. PCBs were used in cooling and insulating fluids for electric equipment. They are currently banned, but production as a side-product still occurs. Early life exposure included a low dose range of 10–10,000 pg/kg bw/d for TCDD and 0.09–1406 µg/kg bw/d for PCB 153. Results showed that body weight was transiently affected by both compounds throughout the follow-up (up to 1 year of age), mainly during the midlife stage. TCDD-exposed males showed a decrease in white fat pad and spleen weights, and an increase in IL-4 production of ex vivo splenic immune cells. In contrast, females showed an increase in fat pad weights and in IFNγ production. PCB 153-exposed males showed an increase in glucose, whereas females showed an increase in glucagon together with a decrease in pancreas weight and an increase in thymus weight.

We concluded that the data suggest that early life exposure to TCDD can affect the programming of energy and immune homeostasis in offspring. Effects of perinatal PCB 153 on programming were mainly on glucose homeostasis. Both compounds acted differently between sexes. The lowest derived BMDLs in this study for both TCDD (156 pg/kg bw/d; decreased perigonadal fat pad weight in males) and PCB 153 (86 µg/kg bw/d; serum increased glucagon in females) are not lower than current TDIs.
Conclusions

Overall, the studies described in this thesis did not show programming of obesity in our mouse model by early life exposure to EDCs (Chapter 6). However, the studies showed compound- and sex-specific metabolic alterations supporting the perinatal programming hypothesis, e.g. changes in fat pad weights and serum levels of lipids and hormones, that persisted into adulthood. The variation of results within our studies and in literature suggests that the relation between early life exposure and apical outcomes at adulthood is not linear and may be influenced by secondary factors, such as diet and microbiome. The translation of the observed effects in mice to humans may therefore be equally complex. Future research needs to integrate the different environmental factors during development that in combination with exposure to EDCs may lead to adult health effects.

Our BPA epigenetic study was not conclusive regarding the hypothesis that DNA methylation is a mechanism by which perinatal exposure to EDCs programs metabolic alterations later in life. Further research is needed to unravel the involvement of epigenetics in perinatal programming of metabolic alterations.

Programmed effects as studied in this thesis are not considered in current risk assessment. If this would be the case, our observations might drive the reconsideration of TDIs for BPA and PFOA, whereas current human health standards would be sufficient for TCDD and PCB 153.