Fetal programming in pregnancy-associated disorders
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General introduction
Fetal programming

Pregnancy is a dynamic process associated with physiological changes of the mother in order to nurture the developing fetus. The survival and development of the fetus is dependent on optimal maternal hemodynamic changes and the quality of intrauterine environment [1]. Adversity in the intrauterine environment can reset important physiological parameters in the fetus, a notion known as fetal programming [2]. The fetal programming ensures at least short-term benefits so that the offspring is well-adjusted to the adverse in utero environment. However, this might create a physiological conflict with the post-uterine environment, as most of the adverse stimuli are then abolished [3]. The discrepancy between the fetal adaptation and the later environment, therefore, can lead to aberrant mechanisms and increased susceptibility to diseases in later life [4–6].

The concept of fetal programming, also known as the Developmental Origins of Health and Disease (DOHaD) or Barker’s hypothesis, originates from epidemiological studies performed in the early ‘80s on the association between birth weight and morbidity and mortality in later life [7–9]. This initiated a worldwide interest and research in the area of fetal and developmental programming for clues and consequences of pregnancy complications and their effect in later life.

However, the first experimental studies showing evidence that early life stressors can lead to later adverse adjustment have been already done by Widdowson and McCance in the early ‘60s of the past century [10]. They showed that restricted nutrient availability during weaning leads to a decline in the growth rate of rat pups which is not restored after exposure to normal food intake. Later on, many other experimental studies have shown that undernutrition in utero can result in hypertension, diabetes, and neurocognitive disorders in later life [11–15]. These experimental models were almost always associated with a decreased birth weight, suggesting that fetal growth restriction is the most evident primary event of fetal programming towards disease.

Critical windows of development

During an organism’s development, there are periods when the phenotype is more responsive to intrinsic or extrinsic factors, also known as critical windows of development. These critical windows are typically defined by distinct starting and end time points and the presence of a stressor before or after those time points has little or no phenotypic effects [16]. However, during fetal development, these critical windows are likely to be with a variable time of duration and dose-dependent on the stressor [17,18]. This is in part due to an increased growth through the processes of cellular hyperplasia and hypertrophy that are abundant at different times of the development and may overlap at several time points [19,20]. Moreover, different tissues mature at a
different rate and some of them are composed of long-lived cells making them susceptible to intrauterine stimuli at distinct periods of development [21–23].

Fetal growth is a dynamic process and is primarily dependent on the placental nutrient delivery system [24]. The total protein deposition in human fetuses is rapidly increasing until 26th weeks of gestation and plateaus after 35th weeks of gestation [25]. On the other hand, fetal lipid accumulation is constant until the second half of pregnancy when the fat synthesis and deposition is increasing, leading to increased fetal weight gain [26]. The fast-growing tissues are at a high demand for nutrients and oxygen and exposure to shortage or environmental stressors will result in minor or major fetal growth abnormalities. It is easy to speculate that, in case the insult occurs early in development, it will result in a symmetrical reduction of the organ size and probably will affect the cell number leading to symmetrically growth-restricted fetus. In contrast, if the insult happens later in the gestation, the organ size will be differentially affected (probably without changes in the cell number per organ), therefore resulting in asymmetrical growth restriction. This responsiveness of the fetus to insults is also known as developmental plasticity.

Pregnancy-associated disorders

Pregnancy-associated disorders represent a heterogeneous group of diseases mainly arising from inadequate placentation or metabolic maladaptation. These are associated with significant maternal and fetal mortality and morbidity including decreased fetal weight. As decreased fetal weight is one of the indicators for fetal programming [27,28], here we explore the clinical features, pathophysiology and the programming effect of these disorders.

Preeclampsia

Preeclampsia is a heterogeneous, multisystemic pregnancy-associated disorder. Although the etiology is complex, it is still diagnosed with the clinical onset of hypertension (>140/90 mmHg) and proteinuria (>0.3 g in 24 hours urine) after the 20th week of gestation [29]. Risk factors for preeclampsia are multiple and include advanced maternal age, primiparity, new sexual partner, chronic hypertension, renal disease, previous preeclampsia, collagen vascular disorder, obesity and diabetes mellitus [30]. Furthermore, preeclampsia can be substratified as early and late-onset preeclampsia, depending on the occurrence of the symptoms before or after the 34th week of gestation [31,32]. In 10% of the preeclamptic cases, preeclampsia can be superimposed into HELLP syndrome, characterized by hemolysis, elevated liver enzymes, and low platelet count. It can also be complicated by eclampsia with further implications of the brain and development of seizures [33]. Women exposed to preeclampsia have at least 2- fold increased risk of developing cardiovascular pathologies in later life [34].
Preeclampsia is associated with increased risk for maternal and fetal morbidity and mortality [35]. Approximately 2/3 of the total preeclamptic pregnancies are complicated with fetal growth restriction and increased susceptibility to chronic diseases in later life (extensively overviewed in chapter 2 of this thesis).

**Intrauterine growth restriction**

Intrauterine growth restriction (IUGR) is characterized by failure of the fetus to reach their predetermined growth potential [36]. It affects 10-15% of the pregnancies and it is associated with increased mortality and morbidity in the offspring [37]. The term of fetal growth restriction (FGR) is much appropriate to be used when the diagnosis is based only on the weight of the fetus.

Risk factors for developing IUGR are advanced maternal age (>35 years), multiparity, hypertensive disorders of pregnancy including preeclampsia, pre-existing diabetes, alcohol, smoking and toxic substances abuse, maternal undernutrition fetal infections, genetic and congenital malformations and placental abnormalities [37]. These offspring are at increased risk for immediate or long-term consequences such as minor neurocognitive deficits, cardiovascular diseases, dyslipidemia, diabetes, obesity and metabolic syndrome in later life [38,39].

**Diabetes during pregnancy**

Diabetes in pregnancy occurs in around 14% of pregnancies and can be separated into three different categories: diabetes mellitus type 1, diabetes mellitus type 2 and gestational diabetes [40]. Diabetes mellitus type 1 is characterized by diminished structure and functionality of beta cells due to autoreactive CD4+ T cells attack and it is manifested with hyperglycemia and hypoinsulinemia [41]. Diabetes mellitus type 2 has unknown etiology yet, and it is characterized by global insulin resistance, hyperinsulinemia, and hyperglycemia [42]. Gestational diabetes (GDM) is a de novo onset of diabetes occurring during the second or third trimester of pregnancy, manifested with the same characteristics as type 2 diabetes [43,44]. Women diagnosed with GDM in the first trimester of pregnancy are considered as patients with pre-existing diabetes mellitus [45].

Pregnancies complicated with diabetes are at increased risk of developing miscarriages, pre-term delivery, preeclampsia, congenital malformations, macrosomia or even microsomia [46–48]. Although the glycemic disturbances are much more severe in pregnant women with type 1 diabetes, the fetal outcomes are equally or even much more severe if the pregnancies are complicated with type 2 diabetes in comparison to type 1 diabetes controls [49,50]. However, it was shown that children born to either gestational diabetes or pre-existing type 1 or type 2 diabetes are all equally exposed to hyperglycemic conditions [51] and that long-term outcomes in these offspring are not per se dependent on the type of maternal diabetes [52,53].
Animal models of pregnancy-associated disorders

Animal models are indispensable in the fetal programming research, primarily due to the possibility to test and combine different programming factors and check their long-term effects. As human pregnancy-associated disorders involve a meshwork of diverse mechanisms involving inflammation, immunity and angiogenesis, a comprehensive and translatable disease model is of great importance for better understanding of the mechanistic aspects and the programming consequences of such diseases.

Figure 1. Gross histological morphology of human (A) and mouse placenta (B) (reproduced with permission of [61], Dove Medical Press Ltd.).

Animal models of pregnancy-associated disorders can be developed in various species, however, the most often used ones are rodent animal models, e.g., mice and rats [54–56]. There is a spectrum of advantages using these models, namely short length of
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pregnancy (19-22 days) and multiparity, which enables studies on multiple fetuses at the same time [57]. Another important feature of the rodent placenta is that it has a similar gross morphology as the human placenta. Placentae of rodents and humans are of the same hemochorial type and both have a discoid shape [58]. The hemochorial placenta has, as a special feature, a direct contact of the trophoblast layer with the maternal circulation without being separated by endothelium. However, the human hemochorial interface is monochorionic, composed of only a syncytiotrophoblast layer and a discontinuous cytotrophoblast villous, while in mice the interface is trichorionic composed of three trophoblast layers including two syncytial layers and one cytotrophoblast layer [59,60]. The term placenta in rodents and humans can be roughly divided into three segments as represented in Figure 1 [61].

Firstly we recognize a basal plate that directly interacts with the maternal decidual layers. Secondly, the middle part of the placenta is recognized as a terminal villous unit in human and as labyrinth zone in the mouse placenta [62]. This part enables most of the nutrient and gas exchange. Lastly, on the fetal surface, the placenta is presented with a chorionic plate. Among other differences between these two types of placenta is the endocrine functionality. The human placenta produces placental estrogen and chorionic gonadotropin, whereas the rodent placenta does not. However, the junctional zone in mice is comprised of glycogen cells and spongiotrophoblasts that may contribute to the endocrine function of the placenta [62].

**Offspring response**

In the offspring, we can discern a broad spectrum of responses to a hostile intrauterine environment. In this thesis, we concentrate on several ones, which are individually described below (Figure 2).

**Body and organ size adaptations**

One of the most important and easily approachable phenotypic characteristic of the newborn is the body weight [28,63]. In the human population, it is already well known that small babies are at increased risk for a range of immediate neonatal morbidities. However, in the past decades, there is increasing evidence that size at birth is also associated with later life outcomes [63]. Therefore, a tight control of organ- and body weight is necessary to ensure optimal size, based on its genetic potential and functionality demands. Dysregulation of such processes can result in multiple phenotypic differences including growth restriction or an increased fetal weight and organ hypo- or hyperplasia [64]. Size control is dependent on major signaling pathways such as insulin/IGF1, mTOR,
JAK, that are involved in growth rate, cell division and growth coordination. During the cell cycle, the primary step is cell division, which is heavily dependent on environmental factors that in turn can modify the cellular homeostasis and enable cell size and number adaptation to the newly encountered environment [65].

**Figure 2.** Potential factors associated with fetal programming due to pregnancy-associated disorders.

Previously, it was shown that protein deprivation during rodent pregnancy leads to significantly smaller pups [66]. Intrauterine growth restriction can decrease the nephron number [67], reduce the beta cell mass in the pancreas [68] and alters the cardiac morphology [69–71]. Besides body weight, another often used parameter is the organ-to-body weight ratio which represents the relative organ weight. Usually, the growth restricted fetuses are associated with a brain sparing effect, whereby brain weight is relatively conserved compared to liver weight, resulting in an increased brain-to-liver ratio. These different growth patterns during organ development between growth restricted and control offspring favor the fetal physiology in such a way that gestational age at birth is increased together with the immediate chance of survival, however with a possible functional discrepancy of the neonatal physiology in later life.
Metabolic patterns

Metabolites are intermediate products of metabolic reactions with low molecular weight (equal or smaller than 1500 Da) [72]. During pregnancy, metabolic adaptations are essential to ensure adequate growth and development of the fetus. Principal changes occur during pregnancy in carbohydrate and lipid metabolism [73,74]. There is an increase in plasma glucose and free fatty acids, which enables a sufficient substrate availability to the fetus [75]. However, there is still not much known about the distinct metabolic patterns during pregnancy-associated disorders. This is of extreme importance, especially because maternal metabolism can influence fetal metabolism either directly via the placenta or indirectly via hormonal mediators or changes in placental metabolism.

Extensive analysis of metabolic patterns is pivotal in the comprehension of the clinical phenotype of a certain disease. Such analysis are possible with large-scale studies of metabolomics, based on a systematic detection, identification, and quantification of metabolites in biological tissues and/or fluids [76]. For instance, metabolomics can capture exposures that are extremely difficult to be quantified and can shed light on aberrant physiological processes that ultimately can point to a later-life disease [77].

Epigenetics

Epigenetics is the study of heritable (mitotically or meiotically) changes in gene expression without alterations in the DNA sequence [78]. The epigenetic field comprises several areas, including changes in DNA methylation, chromatin modifications and alterations in non-coding RNA molecules [79]. They are responsible for a flexible relationship between the genotype and the phenotype. Disruptions in the epigenetic marks can lead to an altered gene function which in turn is an underlying process for several chronic disorders [80]. Epigenetic changes are reversible, but nonetheless once established are relatively stable [81] and can be even transmitted to the next generation leading to transgenerational epigenetic inheritance [82].

During critical windows of fetal development, epigenetic marks are partially cleared and then re-established, in order to restore the developmental potency. This was exemplified in the mouse, where the epigenome is reprogrammed during conception at gestational day (GD) 3.5 and later when the primordial germ cells are formed (GD 13.5) [83]. In humans, similar reprogramming dynamics are present. DNA methylation drops between 5-7 weeks of gestation, resulting in hypomethylated germ cells of both sexes until 19 weeks of gestation [84,85]. This makes the embryo and the fetus extremely
vulnerable to hostile environmental stimuli especially during the periods of reprogramming. It was shown that exposure to maternal nutrient deprivation in the first trimester of human pregnancy is associated with increased risk for cardiovascular and neurocognitive disorders in later life. Exposure in the second trimester is associated with increased risk for kidney and lung disorders. Finally, exposure in the last trimester of pregnancy is associated with impaired glucose tolerance [86]. In addition, in utero exposure to the Dutch Famine at the end of the World War II was associated with aberrant methylation of several genes (in whole blood samples) involved in cardiovascular and metabolic diseases. Interestingly, some of these methylation changes in relation to the in utero nutritional deprivation were reported to be sex-specific [87,88].

**Aim and outline of the thesis**

The aim of this thesis was to develop new and more specific models of human pregnancy-associated diseases that would be suitable to study the underlying mechanisms of fetal programming. The studies are designed to deliver a better understanding of the complex pathophysiological effects of pregnancy-associated disorders on the fetal outcome. In future, this should contribute to the design of preventive and therapeutic strategies. Hence, our ultimate goal is to provide the phenotypic marks associated with fetal programming.

Preeclampsia is associated with increased risk for cardiovascular and metabolic disorders in later life of the offspring. In chapter 2 we summarized the available human and animal studies on preeclampsia with respect to the cardiometabolic outcome of the offspring. Furthermore, novel insights into the mechanisms of fetal programming obtained from these studies, are described.

Angiogenic dysbalance has been promoted as a successful model of translational research, elucidating possible mechanism in the genesis of preeclampsia [89]. Therefore, in chapter 3 we determined the effects of the antiangiogenic factor sFlt-1 (soluble fms like tyrosine kinase 1) on pregnancy and fetal outcomes. We demonstrate that antiangiogenic dysbalance solely does not explain the pathophysiology of preeclampsia entirely. However, we show that it can have a direct effect on the liver molecular phenotype, including modulation in the Ppara promoter methylation levels.

Preeclampsia is a multifactorial disorder and possibly two different pathophysiological mechanisms might be intertwined in its genesis. Frequently, preeclampsia is associated with inflammation and increased concentrations of
antiangiogenic factors. In chapter 4 we characterize a novel “double hit” preeclampsia model by induction of both an angiogenic dysbalance and a low-grade inflammation. We demonstrate that this was accompanied by changes in the metabolic footprint of the mother and the fetuses as well, showing sex-specific differences in the outcome.

Intrauterine growth restriction is a common complication of preeclampsia, but as well it can be registered as an isolated disorder without manifestation of maternal symptoms [90]. The hypothesis that growth restriction is associated with obesity and insulin resistance in later life was tested in chapter 5. First, we characterized a novel IUGR model with conditional deletion of transcriptional factor Tfap2c in the junctional zone of the placenta. Next, in the adulthood of the offspring, we assessed the metabolic parameters. We demonstrate sex-specific differences in the molecular parameters with predominant affection on the female offspring.

Gestational diabetes is another frequent pregnancy-associated disorder that shows an increased risk for complications in the offspring [91]. However, an ideal animal model for type 2 diabetes in pregnancy is still a challenge. In chapter 6 we explored a novel model of generalized insulin resistance (by conditional global deletion of the insulin receptor (IR)) in pregnancy. We determined the maternal and fetal characteristics and we show that fetuses exposed to hyperinsulinemia and hyperglycemia have altered expression and methylation levels of the sterol regulatory binding factor 2 gene (Srebf2) in fetal liver and brain. This factor is already known to be adjusted in diabetic patients, but this is the first study showing that it can also serve as a phenotypic mark of fetal programming due to the diabetic intrauterine environment.

Finally, in chapter 7 we give an overview of the most relevant findings described in the previous chapters and provides suggestions for further research in the field of fetal programming.
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