CHAPTER 1

GENERAL INTRODUCTION & SCOPE

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GENERAL INTRODUCTION

Obesity is one of the hallmarks of the metabolic syndrome that further comprises glucose intolerance, fatty liver and a disturbed lipid profile. People with the metabolic syndrome have an increased risk to develop type 2 diabetes and cardiovascular disease. Now, numbers on obesity and obesity-related diseases are still rising, in spite all effort to promote weight loss and healthier lifestyle. More disturbingly, there is a significant increase in the proportion of obese children and adolescents. In general, body weight, genetic makeup, aging and a sedentary lifestyle are considered key players in the pathophysiology of the metabolic syndrome. A yet underestimated factor in the etiology of the metabolic syndrome and related diseases may be the early developmental environment that is known to exert long-lasting influences by so called ‘metabolic programming’.

The phenomenon “Metabolic Programming”

The term programming was first defined by Lucas, who defined programming as; “a stimulus or insult operating at a critical or sensitive period of growth and development that results in long term consequences for the structure or function of the organism” [1]. Shortly thereafter, Hales and Barker described “the thrifty phenotype” hypothesis which suggests that fetal adaptations in answer to a nutritionally environment may permanently reset or program fetal metabolism in order to optimize its chances of survival [2]. This response is advantageous for the developing organism when this poor environment is met, but becomes detrimental in a nutritional rich postnatal environment.

A large number of epidemiological studies have shown the association between impaired fetal nutrition and development of obesity, hypertension, diabetes and cardiovascular disease [3-7].

The role of fetal programming in the development of Non-Alcoholic Fatty Liver Disease in epidemiological studies

Several epidemiological studies implicate an intrauterine contribution to adult liver disease. Although there is little literature about the specific effects of maternal nutrition on birth size in humans, there are many papers highlighting these effects under extreme conditions, like severe starvation (exemplified by the Dutch hunger winter, see [8]). It should be noted, however, that intrauterine growth restriction may have a plethora of other (often unknown) causes. Human infants born small-for-gestational-age (SGA) were found to have reduced liver dimensions, as measured
by ultrasonography at birth [9]. In addition, low birth size in men, but not women, is associated with increased total cholesterol in blood, which is at least partially regulated by the liver [10]. A large Danish prospective record linkage study showed the strongest correlation between measures of birth size and cause-specific mortality for deaths attributed to liver cirrhosis [11]. Fraser and colleagues examined the association of birth weight with adult markers of liver damage and function in a random sample of 2101 British women. They found a small but consistent inverse linear association between birth weight and adult age-adjusted levels of alanine aminotransferase (ALT) and gamma glutamyltransferase (GTT) [12]. ALT and GGT are liver-specific markers and are considered biomarkers of non-alcoholic fatty liver disease (NAFLD). The inverse association of birth weight with ALT and GGT supports the hypothesis that intrauterine exposure may contribute to the onset of NAFLD.

Evidence of fetal programming in the development of non-alcoholic fatty liver disease (NAFLD) from animal models

While the relationship between early developmental events and adult disease has become evident, the biological mechanisms behind these programming effects have remained largely unclear, although epigenetic modifications and differences in cell type composition may be involved [13,14]. The importance of fetal and early postnatal life is currently extensively studied in animal models to clarify the physiological and molecular links between events during this developmental period and long-term health.

The first evidence for programming of NAFLD comes from models using nutritionally-restricted diets, although the greatest impact is seen in over-nourished models. Mouse models of maternal overweight or over-nutrition have shown convincing evidence of fetal programming of NAFLD in offspring [15-19].

Hepatic hypertrophy - Structural changes have been noted in the liver of offspring from malnourished mothers. Hepatic lobules of protein-restricted rat offspring were described as having double the mean volume of lobules from control livers, without changes in relative liver weight [20]. Moreover, several mouse models of maternal malnutrition show hepatic hypertrophy in the offspring [21,22]. While not in every study hepatic hypertrophy reported, increases in liver derived enzymes (ALT and AST) are sometimes observed [17,23,24], a change that often accompanies hepatic hypertrophy and may appear in plasma after liver enlargement. Taken together, these results indicate that hepatic hypertrophy is a frequently-noted phenomenon accompanying gestational malnutrition.
Hepatic hypertrophy can be induced by several factors including altered oxidative status, fatty acid metabolism, energy production and utilization, cell turnover and altered hepatocellular cytoplasmic, and nuclear morphology [25]. Several of these factors have been reported in fetal programming of offspring health [18,20].

**Liver Function** - In addition to structural changes, there have been numerous reports that liver functionality is affected by maternal malnutrition. In rats partially deprived of protein during pregnancy, gluconeogenesis and hepatic glucose handling in offspring are altered compared to controls [20]. Glucose output from lactate is increased in maternal low-protein offspring, which is related to the difference in glucose handling. One possible mechanism is increased glucogenesis due to the greater absolute phosphoenolpyruvate carboxykinase (PEPCK) activity found in these livers [20]. Additionally, several animal models of maternal over-nutrition indicate mitochondrial abnormalities in the liver of offspring. Bruce and colleagues reported that the activity of the hepatic mitochondrial electron transport chain (ETC) enzyme complex (I,II/III, and IV) is reduced in offspring of high fat-fed mothers [18]. In the progression from NAFLD to non-alcoholic steatohepatitis (NASH), inflammatory pathways, which are also affected by fetal programming, are important. In that context, it has recently been reported that offspring from over-nourished dams showed increased Kupffer cell numbers with impaired phagocytic function and raised reduced oxygen species (ROS) synthesis together with reduced natural killer T cells and raised interleukin 12 and interleukin 18 levels [26]. Even though many of these animal models use different diets and different strategies, all accumulate fat in the liver and liver functionality seems to be altered in one way or the other (Table 1).

**Lipotoxicity** - Excessive hepatic fat storage has been shown in many animal models of fetal programming. While hepatic fat accumulation is not necessarily malignant, it is often associated with insulin resistance [27]. Models of maternal restriction, over-nutrition and glucocorticoid exposure show increased body fat and altered hepatic lipid metabolism in offspring, accompanied by accumulating triglycerides and cholesterol, characteristics of hepatic steatosis [16,18,19,28].

It is still unclear what is underlying the increased lipid accumulation. Impaired fat oxidative capacity (impaired mitochondrial function) may be of importance; on the other hand, several animal models report a lipogenic transcriptome signature early in the development of the liver [22,29]. Nonhuman primates of over-nourished mothers show signs of NAFLD beginning in the early third trimester, including hepatic inflammation, oxidative stress, triglyceride accumulation and gluconeogenic
gene activation [29]. This is associated with PPAR gamma coactivator 1 alpha (PGC1α) deacetylation and increased hepatocyte nuclear factor 4 alpha (HNF4α) expression in the fetal liver, suggesting an important early mechanism by which excess lipids may reprogram hepatic lipid and glucose metabolism in the liver. In this study, the elevation of hepatic triglyceride levels persisted until adolescence with a 2-fold increase in percent body fat. Another study performed in rats found increased percent liver weight and enlarged hepatocytes and lipid accumulation in livers of offspring at weaning; it is suggested that exposure to maternal overweight programs systemic changes in insulin and adiponectin levels and alteration of genes involved in carbohydrate metabolism, lipid biosynthesis and fatty acid catabolism. Interestingly, sterol regulatory element-binding protein 1 (SREBP1) was increased and identified as a common regulator of the altered genes, and in addition a decrease of PPAR-α-AMPK signaling was indicated [22].

In contrast, Krasnow and colleagues found no differences in triglyceride accumulation and hepatic inflammation in newborn mice. However, they reported an increase in fat mass in offspring from mothers fed a high-fat diet [30].

Currently, most of the research points towards programming of multiple aspects of energy-balance regulation in the offspring during gestational exposure to malnutrition. Therefore, an early change in a lipogenic pathway could be a cause in the development of NAFLD, because the transcriptome is already altered very early in life.

As stated earlier, the pathophysiological and metabolic mechanisms of fetal programming are still under investigation. Possible mechanisms might involve epigenetic regulation as well as placental adaptations during important developmental phases.

Epigenetics

During the past decade, the role of epigenetics in the pathogenesis of disease has been increasingly recognized. Epigenetics is defined as heritable changes in gene activity and expression that occur without alterations in the DNA sequence. Epigenetic mechanisms include chromatin architecture, histone modifications and DNA methylation. In short genomic DNA is packaged with histones to form complexes called chromatin, chromatin can be divided into two different states; euchromatin and heterochromatin. Euchromatin represents the active state where the DNA is accessible, conversely heterochromatin represents the inactive state where the DNA is packaged into highly condensed forms that are inaccessible to transcription factors or chromatin-associated proteins. Histone modifications are critical for regulating
chromatin structure and function. Acetylation and methylation of lysines at histone tails are the most common modifications at the level of the histone. Hyperacetylation of histones is positively correlated with actively transcribed genes, in contrast histone methylation is associated with either transcriptional activation, inactivation or silent genomic regions. One of the best documented forms of epigenetic regulation is DNA methylation. DNA methylation is a stable and heritable element of epigenetic regulation and occurs by covalent modification of the fifth carbon in the cytosine base. In vertebrates DNA methylation occurs almost exclusively in cytosine-guanine (CpG) dinucleotides, which are often clustered into CpG islands, and subsequently influence transcriptional gene expression. All these modifications are crucial for packaging and interpreting the genome under the influence of physiological factors, and therefore it is considered a bridge between genotype and phenotype.

In the developing embryo, virtually all DNA is modified by the addition of a methyl group to the 5' position of cytosine residues which precede a guanine residue, a so-called CpG dinucleotide. As development and differentiation takes place and specific genes are recruited for expression in certain cell types, these nucleotides become demethylated and potentially associated with an open chromatin configuration. The lifetime pattern of methylation is established for the majority of genes by the end of this differentiation process, and DNA replication results in faithful copying of these methylation patterns. The period during which the cellular methylation pattern is established may be susceptible to various fetal (maternal) insults and is therefore a good candidate mechanism for fetal programming.

In humans, it has been shown that people prenatally exposed to famine during the Dutch hunger winter have less DNA methylation of the IGF2 gene compared with their unexposed, same sex siblings[31].

However, the specific role of epigenetics in the pathogenesis of NAFLD is largely unknown. A recent study in human patients showed a tight interaction between the presence of NAFLD and hepatic DNA methylation of PGC1A and mitochondrial transcription factor A (TFAM) promoters[32].

In rodents, numerous studies focus on the consequences of maternal nutrition on the liver epigenome. Promoters of nuclear receptors are relatively well-studied candidates for differential methylation. Lillicrop and colleagues characterized changes in methylation and expression of the glucocorticoid receptor (GR) and peroxisome proliferator-activated receptor (Ppar) alpha [33,34]. Van Straten et al. made similar observations for the liver X receptor alpha (LXRa), amongst 200 other loci [35]. It seems plausible that changes in these key factors have long-term consequences for the regulation of metabolism, especially under challenging nutritional conditions.
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On the other nutritional extreme, two recent reports showed that maternal high fat diet may alter DNA methylation and gene expression in the offspring. First, maternal high fat feeding reduces methylation and increased expression of the cyclin-dependent kinase inhibitor 1A (Cdkn1a) during neonatal liver development [36]. This alteration is responsible for changing hepatic proliferation and liver size, two aspects that are compatible with the development of a fatty liver phenotype [18]. The second report demonstrated that in offspring with increased serum glucose and liver triglyceride levels, hepatic Wnt1 (wingless-type MMTV integration site family, member 1) activity is affected through histone modifications [37].

The placenta

The placenta plays an essential role in the metabolic programming of adult diseases, since nutrients are transmitted to the fetus by the placenta. The placenta responds to alterations in maternal metabolism by changing its structure and/or function, consequently this influences circulation of nutrients and placental functionality. The potential role of altered placental functionality in the metabolic programming of adult diseases have been extensively reviewed by others [38].

Human pregnancies in women with type 1 diabetes and pregnancies complicated with gestational diabetes have an increased risk for fetal macrosomia, which has been shown to be associated with altered placental transport. Likewise, woman who are overweight or obese at the start of pregnancy are more likely to display altered placental nutrient transport.

In addition animal studies provide strong evidence that maternal nutrition affects placenta nutrient transport, and therefore fetal development. Several mouse studies report that a high fat diet in pregnant mice leads to fetal overgrowth, in part by altered placental function. Li et al report altered placental vasculature and inflammatory response, seen by changes in Hif-1a, tnfα, vegf, il6, nf-kb and CD31 [39]. Besides changes by maternal diet, there is a placental divergence between the sexes. Most studies reporting placental changes by maternal diet, report sex differences [40-42]. There are several reports showing that after a maternal dietary exposure there are only a few genes found in common in the placenta samples between males and females, implying a clear sexual dimorphism in the response of the placenta to environmental influences between the sexes.
SCOPE OF THIS THESIS

Human and animal studies have shown that prenatal and neonatal conditions can exert long lasting changes in physiological function and affect the risk to develop metabolic diseases at adult age, e.g. obesity, cardiovascular diseases and type 2 diabetes.

Over the past decades, the mean body mass index of adults has increased, including that of women at child bearing age. A number of epidemiological studies have shown a relationship between maternal obesity and nutritional intake is associated with an increased risk in infant mortality and neonatal adiposity. Yet, the underlying mechanism(s) between the early nutritional environment and the physiological consequences at adult life are largely unknown.

The aim of the research described in this thesis was to characterize the immediate and late physiological consequences of maternal western diet during the early developmental phases of the offspring. We first developed and characterized an animal model (Chapter 2). The long-term physiological consequences of the maternal western diet during important developmental phases was analyzed with respect to a predisposition toward the development of NAFLD in the male offspring at adult age. Assessment of early environment effects on processes at adult age can be hampered by a multitude of interfering factors during growing up. In chapter 3 we addressed this potential distortion by performing analyses on pre-weaning offspring to elucidate the direct molecular impact of the maternal diet on the liver of the offspring. Importantly we also considered the potential sex-specificity of the effects. We determined the immediate effects of maternal western diet on both the molecular and the physiological outcome of young offspring, using microarray analyses. In chapter 4, we studied the the placental adaptations to maternal western nutrition in male and female offspring, in order get insights in in utero mechanisms of maternal programming.

To discriminate maternal programming effects from factors influencing disease development during growing up, we assessed genome wide changes in DNA methylations from livers of offspring at 2 and at 32 weeks of age (Chapter 5). We concentrated on epigenetic changes, based on a number of recent studies that indicated that epigenetic mechanism play a role in long-term programming. We aimed to make a distinction between early epigenetic alterations in the liver that are conserved into adulthood and later events influencing the liver phenotype seen in the long term development of the offspring.

In chapter 6, we investigated the causative effects of methylation and expression
patterns in early human chorion villi placental biopsies on small for gestational age pregnancy outcomes. These selected genes have been published to correlate with being born small for gestational age in term placenta.

Finally, in chapter 7 we discuss the most relevant findings of this thesis, and the implications for our present understanding of the mechanism and the proposed future steps.

Energy starvation during storage. Consequently, the storage stability of the yeast is increased. A disadvantage of a period of nitrogen starvation is that it also causes a partial loss of the fermentative capacity. However, the loss of fermentative capacity after carbon starvation is much more severe than after nitrogen starvation [257, 259, 305]. It is clear that the production of yeast is a complex process consisting of different stages, which should simultaneously optimize fermentative capacity and storage stability.