Metabolic memories

Dimova, Lidiya Georgieva

CHAPTER 1

General Introduction
Scope of the thesis

GENERAL INTRODUCTION

In 2010 nearly 37% of all reported deaths worldwide were caused by ischemic heart disease, stroke or type-two diabetes, the main manifestations of a complex array of pathologies commonly known as cardiometabolic disease. According to the World Health Organisation's estimates, this percentage is projected to increase globally to 52% by 2020 [1]. The dramatic rise of these numbers propels us to look for contributing factors that may lay beyond the classical lifestyle-associated risks as unhealthy diet, lack of physical activity or chronic stress. The idea that in addition to these, the increase in susceptibility to cardiometabolic disease is predetermined and conveyed in part by the mechanisms of metabolic programming, becomes prominent.

This introduction will first summarize our current understanding of factors that initiate and drive metabolic programming processes and their role for the offspring during the different developmental windows by focusing on pre- and postnatal programming mechanisms. Disturbances in cholesterol and lipid metabolism, leading to increased incidence of cardiometabolic disease, represent a major consequence from early life metabolic programming. Thus, the second part of the introduction will discuss the physiology of cholesterol metabolism and the main molecular players involved in maintaining the balance between cardiometabolic health and disease. Finally, the aims and scope of this thesis will be presented.

I. WHAT IS METABOLIC PROGRAMMING?

Currently, the term metabolic programming is used as the focus of a field continuously expanding its boundaries to cover new phenotypical relationships and mechanisms. Metabolic programming refers to those phenomena where early life events (environmental, genetic) exert a lasting impact on physiological outcomes in adulthood. A nutritional, hormonal or environmental insult once applied in a sensitive ontogenetic window, when the developmental plasticity of the organism is high, can change it in a way that a challenge met in adulthood can easily shift the balance between health and disease (**Figure 1**). Historically, one of the most prominent epidemiological observations links undernutrition during gestation with smaller size at birth, which correlates with increased total plasma and LDL-cholesterol[2]. This translates into increased risk of death from coronary heart disease in adulthood[3]. Further studies of undernutrition during pregnancy have demonstrated that it could affect not only cholesterol metabolism of the infant but may also result in impaired glucose tolerance and insulin resistance, especially if the newborn encounters nutritional abundance in later life[4]. Such observations give rise

to the thrifty-phenotype hypothesis[5], which is related but not identical to the concept of metabolic programming and is based on the notion that due to fetal adaptations persevered into adulthood, a mismatch between poor fetal environment and nutritionally rich postnatal life leaves the organism unprepared and is thus metabolically unfavorable.

Earlier views supported the idea that during fetal ontogenesis the growth and development of different tissues could be impaired by nutritional insufficiencies or inadequate oxygen supply, which would result in underdevelopment of the tissue type, growing fastest at the time of the insult[6]. Those adaptations in the structure and physiology of the fetus, however, might become disadvantageous in adulthood resulting in increased risk for chronic disease. Such notion may explain the reduced number of nephrons observed in infants with a background of intrauterine growth restriction (IUGR). This early life adaptation leads to the development of hypertension in adulthood, an accompanying feature and risk factor for cardiac disease[7]. According to Barker[6], in times of intrauterine nutrient deprivation, the fetus may divert blood flow towards more demanding organs like the brain, at the expense of an underdevelopment of organs such as the liver, which is the key determinant of overall cholesterol homeostasis. Therefore, hepatic structural underdevelopment may result in persistent cholesterol metabolism changes leading to an increased LDL-cholesterol in adulthood. Such structural programming of the liver has been speculated to take place in a model of restricted protein during gestation, resulting in changed activities of key hepatic enzymes leading to an increase in gluconeogenesis[8]. The proposed mechanism includes shifts in the relative size of liver substructures, resulting in a larger pre-portal zone[9].

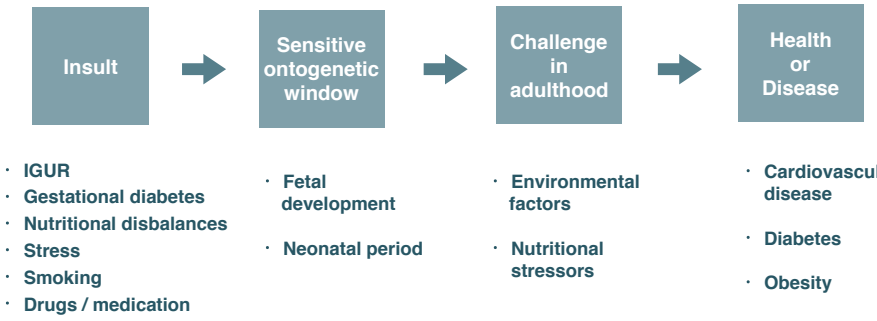

Figure 1: The metabolic programming paradigm. The alignment of several key components may turn the balance from health to disease. An insult applied to an insult-sensitive ontogenetic window may induce such changes in the young organism as to modify its ability to respond to a later life challenge adequately.

A more recent concept, however, attributes the main mechanism of programming to persistent changes in the epigenetic makeup of the young organism, which influence the life-long expression pattern of genetic networks governing metabolism. DNA methylation, histone modifications as well as mechanisms involving non-coding RNAs have been implicated in models of fetal malnutrition or hormonal disparity[10-12]. Notably, a strong association was found between exposure of pregnant women to famine and reduced levels of DNA methylation at the IGF2 promoter in their adult offspring[13]. The growing number of mechanistic insights linking early life insult-factors to critical ontogenetic windows and adulthood phenotype development are currently establishing the view that a combination of case-specific mechanisms involving both epigenetic events and preserved structural-physiological changes from early life are contributing to the adulthood outcomes. This notion is strongly supported by reports demonstrating the epigenetically mediated transgenerational transmission of metabolically programmed phenotypes, which previously have been correlated with small birth size or organ underdevelopment[14,15]. Since both DNA methylation and histone modifications can be permanently altered via availability levels of dietary components, nutrient balance during critical periods of early life and the recognition of epigenetically active sensors for suboptimal biochemical milieu during development, become essential points of investigation.

WHEN DOES METABOLIC PROGRAMMING OCCUR?

While exposure to a suboptimal environment in early life may lead to adult dysregulation of metabolism, it is likely that the mechanisms responsible and the systems affected may vary with the timing of the exposure. A prompt example comes from the Dutch famine during the second World War (1944-1945). Food limitation in early gestation was associated with adult obesity in men[16], whereas famine in late gestation was linked to hypertension[17]. Moreover, prenatal famine exposure has been related to persistent changes in DNA methylation patterns at specific loci, which happens in a sex-specific way and largely depends on the timing of exposure[18]. It is, therefore, probable that different components of the cardiometabolic disease, e.g. pathologies related to obesity and type II diabetes or alternatively alterations of cholesterol metabolism as well as atherogenesis, are programmable with increased sensitivity during distinct ontogenetic windows.

1. PRENATAL PROGRAMMING OF COMPONENTS OF THE CARDIOMETABOLIC SYNDROME

Early life is characterized by high developmental plasticity and fast tissue growth, which is dependent on nutrient availability. During mammalian development, the environment of the fetus is predominantly determined by maternal physiology. It can impact the fetus directly via substrate availability or indirectly via changes in placental properties. Structurally, the placenta consists of a maternal and a fetal side. During its formation, the outer surface of the chorion projects into the uterine wall forming chorionic villi populated by trophoblasts. They are eventually connected to the maternal circulation via the spiral arteries of the endometrium, which create the intervillous space, where gas diffusion and nutrient exchange between the fetus and the maternal circulation is possible. In order to affect the fetus, any disturbance in maternal metabolism has to be transferred across the placenta.

1.1 Placenta: the interpreter of maternal homeostasis

Many fetal adaptations to placental dysfunction have been associated with increased susceptibility to disease in adulthood. For example, IUGR is not only linked to undernutrition but is sometimes the result of an increase in placental vascular resistance[19]. Moreover, a thicker placental exchange barrier often results in fetal hypoxia, which in rats has been shown to affect cardiomyocyte development ultimately resulting in increased sensitivity to ischemia in adulthood[20]. Maternal under- or malnutrition during gestation can affect not only the size of the placenta by inhibiting proliferation[21] but also its morphology and ability to transport micro- and macronutrients by affecting gene expression of placental nutrient transporters[22]. Caloric restriction in pregnant baboons resulted in downregulation of placental amino acid transporters, which precedes the development of IUGR[23]. In mice, it associates with changes in the epigenetic signature of the placenta leading to promoter hypermethylation and reduced expression of glucose transporter Glut3[24]. Besides caloric, also macronutrient restriction can modulate placental gene expression. In rats, limitation of protein during gestation upregulated the expression of placental genes involved in cholesterol transport towards the fetus[25]. In addition, low-protein diet was shown to induce changes not only in placental genes but also in the fetal expression of LXRα, achieved via altered DNA methylation at its promoter[26].

Maternal malnutrition can also affect micronutrient availability through the placenta. Low vitamin B12 levels combined with high maternal folate levels associate with increased insulin resistance in the offspring[27]. Similarly, Zn2+ deficiency during

pregnancy has been shown to disturb leptin production and sensing in the placenta[28]. This can ultimately translate into decreased sensitivity to leptin in adulthood and increased risk for compensatory weight gain and obesity.

Worldwide, however, maternal overnutrition and obesity nowadays present a greater challenge than famine to both mother and developing fetus. This is even more tangible in cases where increased caloric intake during pregnancy is combined with a decrease in micronutrient density. Maternal obesity can lead to increased glucose transport across the placenta, thereby exposing the fetus to excessive nutrient supply resulting in increased body weight at birth[29]. In similarity to smaller for gestational age infants, higher birth weight is associated with increased risk of adult obesity and cardiometabolic disease[2,30,31]. Higher levels of fetal exposure to circulating glucose are also common in gestational diabetes which similarly leads to fetal macrosomia, triggered by altered placental transport of glucose and fatty acids[32]. This effect on fetal growth is also promoted by the increased fetal insulin secretion in response to the high levels of maternal glucose. Correspondingly, excessive weight gain during pregnancy has been proposed to alter placental function and induce macrosomia by affecting the mTOR and insulin signaling axes in placenta[33]. The stress-related signaling between mother and fetus can also be affected in response to high-fat diet. High circulating levels of glucocorticoids, and decreased expression of placental 11β-hydroxy steroid dehydrogenase-2 (11β-HSD2), which protects the fetus from overexposure to cortisol, were described in pregnant mice fed high-fat diet. Interestingly, IUGR has been implicated in epigenetic silencing of the 11β-HSD2 gene, involving decreased histone methylation at its promoter[34]. Thus, interruption of the feto-placental barrier could provide a plausible explanation for epidemiological observations linking prenatal stress to components of the metabolic syndrome.

1.2 The impact of programming on insulin resistance

Considering the fetal origin of adulthood predisposition to insulin resistance and type II diabetes, maternal hyperglycemia stands out as one of the most prominent fetal insults triggering this adult phenotype. In man, there is a strong correlation between maternal plasma glucose levels in gestation and the increase in fasting glucose levels of the offspring at adulthood[35]. Research in pregnant women shows that both gestational diabetes and type I diabetes during pregnancy associate with reduced insulin sensitivity and decreased pancreatic β-cell function in their adult offspring36. The effect is more exaggerated in late gestation when the fetus starts producing its own insulin in response to the maternal hyperglycemia. This leads to fetal hyperinsulinemia, increased transport of amino acids across the placenta, accelerated growth and fetal macrosomia. A common outcome in cases

of extreme maternal hyperglycemia is fetal hyperinsulinemia and consequently hypertrophy of fetal β-cells. However, animal studies showed that by the end of gestation they become exhausted, thereby leading to fetal hypoinsulinemia and resultant growth restriction[37]. Experiments in the insulin resistant *Lir*-knockout mice have revealed lower β-cell proliferation and reduced islet number in offspring exposed to maternal hyperinsulinemia and transient hyperglycemia[38]. This suggests that even subclinical dysregulation in maternal glucose homeostasis can permanently disrupt the endocrine function of the pancreas and hence increase the risk for pathology in the progeny. Interestingly, several genome-wide DNA methylation studies recently demonstrated that insulin secretion from human pancreatic β-cells could be regulated epigenetically via differential methylation[39], and that gestational diabetes interferes with DNA methylation patterns of offspring genes involved in metabolic disease pathways[40]. Notably, the expression of one of the key transcription factors involved in islet development, Pdx1, appears to be suppressed in growth-restricted fetuses by a series of progressively established histone modifications at its promoter region during the different stages of development. This process ultimately results in CpG island methylation at the Pdx1 promoter in adulthood, which translates into subsequent β-cell dysfunction[41]. Together these findings suggest a highly plausible role for epigenetics in the intrauterine predisposition to insulin resistance and type II diabetes in adulthood.

1.3 The impact of programming on body composition

As mentioned, dysregulation of glucose homeostasis during gestation has prominent effects also on body composition, associating with either fetal micro- or macrosomia, manifesting in increased adult adiposity[30,42]. Maternal diet composition has also been shown to have effects on neonatal adiposity, which associates with increased intake of total fat and carbohydrates but not protein[43]. A study in British men shows that a history of a lower for gestational age birth weight seems to be a good predictor for a higher percentage of fat mass and less lean body mass compared to individuals born larger for gestational age[44]. There are strong indications that impaired fetal growth, measured by birth weight, may have a gender-specific effect on body composition as it appears to be related to central fat distribution in male and decreased bone and fat-free mass in female individuals[45]. It seems, however, that in adolescence weight gain during childhood is a more important determinant of body composition than birth size[46]. Initially, it was considered that the exposure to a compromised intrauterine environment permanently affects the number of adipocytes, which respond to nutritional abundance in adulthood by increasing in size. However, more recent data point towards a more dynamic regulation of adipogenesis[47] which suggests a possible role of an early life set point for leptin resistance[48,49]. In addition to total fat and lean body mass, animal studies in rats have demonstrated a negative

impact of early life overnutrition on the functional development of interscapular brown adipose tissue (BAT)[50], which was accompanied by hyperglycemia and hyperinsulinemia. This suggests that impairing energy expenditure via reduced thermogenesis facilitates the development of an obesogenic, insulin-resistant phenotype. On the other hand, protein restriction during pregnancy in rats was recently shown to have a positive effect on BAT activity, thereby protecting the adult offspring against diet-induced obesity and insulin resistance[51]. Based on human population studies it has been speculated that differences in obesity susceptibility between ethnic groups may have its origins in the fetal programming of BAT development as a result of evolutionary adaptation to cold climates[52]. The exact mechanisms for activation in either case, however, remain to be elucidated.

1.4 Role of oxidative stress in metabolic programming

As stated above, the placenta seems to be both the target and a source for pathological insults reaching the fetus. Thus, it is necessary to emphasize, that maternal pathologies and complications during pregnancy can initiate a cascade of pathophysiological responses in the placenta each one of which can serve as an independent insult with a possible impact on the developmental programming of the fetus.

For example, one common denominator of pregnancy complications like fetal growth restriction, preeclampsia, and gestational diabetes, are the increased levels of oxidative stress for the fetus (**Figure 2**). Markedly, the pathogenesis of preeclampsia, a condition of maternal hypertension during gestation, and a well-established intrauterine insult linked to fetal programming, has been consistently assigned to oxidative stress[53]. It has been demonstrated that maternal obesity, as well as malnourishment, can aggravate fetal oxidative stress supposedly translating into adult predisposition to type II diabetes[54]. Notably, plasma markers for maternal oxidative stress have been positively correlated with fetal ghrelin levels[55], which is the main appetite-stimulating hormone with effects on increasing food intake and fat storage. Others have revealed a correlation between maternal hyperglycemia, as indicated by the glycemic biomarker HbA(1c)[56] and umbilical cord oxidative stress levels[57]. In vitro, increased levels of exposure to reactive oxygen species (ROS) have a lipotoxic effect and impair β-cell propagation and differentiation[58], which could directly translate into decreased pancreatic function in adulthood. Meanwhile, others have inferred that maternal oxidative stress can be transferred across the placenta, as strong correlations have been found between maternal and fetal levels of antioxidants and oxidative stress markers[59].

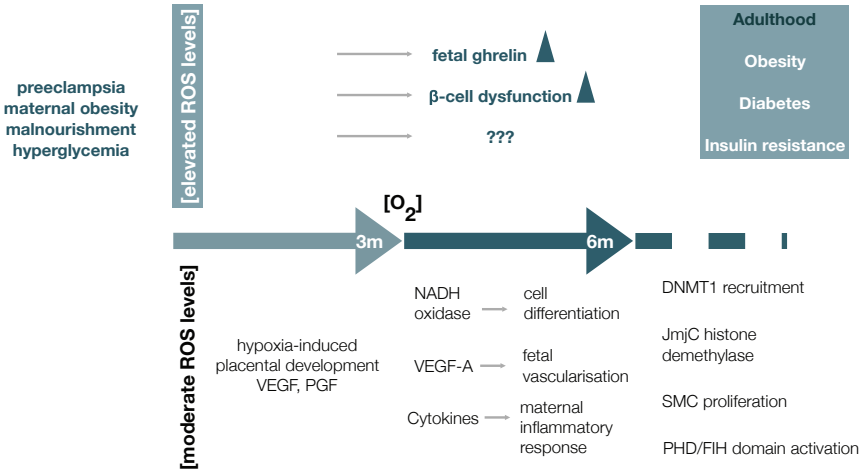

Figure 2: Fetal redox balance is important for variety of processes during normal gestation, from placental development to fetal vascularisation, differentiation and regulation of gene activity. However, multiple maternal pathologies associate with elevated oxidative stress and have been linked to functional changes in fetal metabolism with life-long unfavorable consequences.

Redox balance has a physiological role in modulating gene expression and cell signaling cascades[60] with ROS presenting as a main driving force for tissue differentiation in early embryonic development. During pregnancy, the first trimester is characterized by low oxygen pressure and generally hypoxic conditions, which stimulate placental development and vascularization[61]. A main role in this process plays the hypoxia-inducible factor 1-α (HIF1α), which regulates trophoblast differentiation and is essential for normal placental development and embryo survival[62]. Its most fundamental role, however, is to control oxygen homeostasis, which modulates the expression and activity of HIF-family members. In normal oxygen conditions, HIF1α has a short half-life being the subject of proteasomal degradation. During hypoxia, however, the protein is stabilized and can migrate to the nucleus and stimulate the transcription of genes involved in the low-oxygen response. These include vascular endothelial and erythropoietic growth factors, glucose transporters and glycolytic enzymes[63]. Loss of Hif1a leads to an abnormal development of the myocardium[64], thereby suggesting the possibility for a link between dysregulation of the activity of HIF due to oxidative stress and future development of cardiovascular disease (CVD). The later stages of gestation, as result of fetal vascularization, are characterized by an increase in the partial oxygen pressure and accordingly activation of enzymatic (superoxide dismutase, catalase) and non-enzymatic (glutathione, peroxiredoxin, thioredoxin) antioxidant systems. Their role is to prevent oxidative stress in the cells caused by ROS and resulting in lipid, protein oxidation, and DNA damage. The role of superoxide dismutase 2 (SOD2) is especially important as it is

localized in the mitochondria, where the electron transport chain is a main source of ROS. At moderate levels, however, ROS seem to have a positive impact on health, as they aid protecting the organism against larger subsequent doses of the stressor, a concept known as "mitohormesis"[65]. Under this hallmark, increased levels of oxidative stress in mitochondria have been mechanistically linked to some of the beneficial effects of aerobic exercise[66] such as amelioration of insulin resistance. This process largely takes place in mitochondria and involves the redox-sensitive transcription factor, PGC-1α, which is also especially important in the second half of gestation. By promoting mitochondrial biogenesis and respiration, the activity of PGC-1α results in increased oxygen consumption, leading to a decrease in intracellular O2 availability, which in turn stabilizes the protein levels of HIF-1α[67]. Experimental evidence indicates, however, that HIF-1α is transcriptionally upregulated in response to increased oxidative stress, which is mediated by a functional NFκB site, located at its promoter[68].

Reactive oxygen species can modulate gene expression during development also by interfering with the establishment of epigenetic marks. H2O2 exposure in vitro attenuates the activity of JmjC domain-containing histone demethylases and class I/II histone deacetylase (HDAC), which results in global changes of gene expression patterns[69]. DNA methylation patterns are especially susceptible to alteration in a pro-oxidative environment. Replacement of guanine with its oxidized form, 8-hydroxyguanosine (8-OHdG) can alter the methylation state of neighboring cytosines[70] and prevent binding of DNA methyltransferases (DNMTs), which maintain inherent DNA methylation. In stem cells, an altered epigenetic state can be perpetuated through self-renewal and maturation until terminally differentiated cells develop in adulthood. ROS can also induce global epigenetic changes because antioxidant synthesis pathways and the DNA and histone methylation machinery are sharing common substrate metabolites, e.g. S-adenosylmethionine[71]. This emphasizes the strong influence metabolism can exercise on epigenetics via nutrient and substrate availability. Plausible targets of ROS-inducible epigenetic regulation with impact on cardiovascular and metabolic disease risk include the endothelial nitric oxide synthase (eNOS)[72], arginase 2 (Arg2)[73] and the estrogen receptor α[74]. The current challenge is to link in vivo fetal oxidative stress exposure with specific target sites of epigenetic imprinting in the offspring. A prompt example comes from an animal study in rats. During gestational hypoxia the expression of PKCε in fetal hearts is downregulated due to methylation of specific CpG sites at the promoter of the gene[75]. This finding points toward a possible link between intrauterine redox conditions and adult CVD predisposition since PKCε has a role in maintaining myocardial resistance against ischemic injuries[76].

1.5 Maternal hypercholesterolemia and programming of cholesterol metabolism

Cholesterol is another main factor potentially contributing to fetal programming, and a dominating risk factor for adult cardiovascular disease. During early embryonic development cholesterol is necessary for the activation of sonic hedgehog, an essential morphogen involved in organogenesis and neural system patterning[77]. By being a main constituent of the lipid rafts, where cell signaling cascades usually originate, it has an important role in the processes of tissue proliferation and differentiation. Cholesterol can be synthesized by the fetus, but can also be derived from the maternal plasma cholesterol pool mainly via lipoprotein-mediated placental transport[78]. The presence of a strong correlation between maternal plasma cholesterol levels and fetal cholesterol which lasts until the end of the second trimester[79], suggests that especially in the early stages of gestation maternal cholesterol metabolism has a strong impact on the developing fetus. Interestingly, low maternal plasma cholesterol levels have been associated with fetal growth restriction[80] and as well as compensatory changes in the human placental expression of the cholesterol uptake transporters LDL-receptor (LDLR) and the scavenger receptor B1 (SRB1)[81]. Further, an American cohort study of smaller for gestational age infants found an association between birth weight and plasma cholesterol levels after birth[82]. Thus, maternal plasma cholesterol levels have the potential to modulate fetal cholesterol metabolism and likely indirectly fetal growth, which can be reflected in later changes of adult cholesterol homeostasis. Contrary to the impact of low maternal cholesterol during pregnancy, maternal hypercholesterolemia, even if only transient, is able to accelerate the development of human fetal atherosclerotic plaques[79]. In a study with hypercholesterolemic rabbits, this atherogenic effect was prevented by provision of lipid-lowering therapy or antioxidants, which implicates high levels of maternal cholesterol and lipid peroxidation as main potential causative factors[83]. Further research in Ldlr-knockout mice demonstrated that the impact of maternal hypercholesterolemia during gestation and lactation is preserved into adulthood, resulting in increased aortic root plaque area even in offspring fed non-atherogenic chow diet. Importantly, the phenotype was accompanied by changes in gene expression patterns of morphologically normal abdominal aorta[84].

Certainly, the results of these studies are supporting the concept that early life cholesterol exposure associates with adult risks for cardiovascular disease. The mechanisms behind this relationship remain elusive, although epigenetic programming of key regulators of cholesterol and lipid metabolism provides plausible means. LXR is a key regulator, which senses not only cholesterol abundance but also the oxidative state by its ability to bind oxysterols. Upon activation, it induces the expression of genes involved in lowering cellular cholesterol levels such as cholesterol efflux transporters, lipogenic genes, and, in rodents, bile acid synthesis enzymes. Interestingly, hypermethylation at the promoter of

LXRα has been described as the aftermath of intrauterine protein restriction in mice[26]. Further studies have indicated that the altered epigenetic state of LXR promoter upon malnutrition can be detected in gametes of male F1 and F2 mice, thereby carrying the programming effect into the following generations[14]. However, the impact of maternal-fetal cholesterol levels on possible long-term epigenetic modulation of LXR activity has not been mechanistically investigated. On the other hand, it is worth mentioning that epigenetic changes occur in response to maternal metabolic state at the promoter of the cholesterol efflux transporter ABCA1[85]. This suggests that the epigenetic modulation of genes conveying programming effects with respect to cholesterol metabolism could be widespread, occur on several levels and involve not only master transcriptional regulators but also single players in cholesterol transport or intracellular trafficking.

2. POSTNATAL METABOLIC PROGRAMMING

At birth, many developmental processes are still far from complete. The brain continues to go through extensive synapse formation and myelination well into infancy and childhood[86]. Part of neural development consists of establishment of hypothalamic circuitries and the distribution of hypothalamic leptin receptors[87], which may bear a possible long term-effect into adulthood. Hepatocytes undergo proliferation, which in humans takes place in the first 2 years of life[88]. During this time the liver retains its high developmental plasticity. Therefore, another important aspect to be considered is the extent to which intrauterine programming is continued in the critical postpartum period, with the initiation of lactation and the increasing exposure of the infant to external environmental factors.

2.1 Associations between breastfeeding and adult health

The nutrition administered early postpartum seems to be of particular importance as multiple studies have investigated the impact of breastfeeding on the prevalence of obesity[89,90], type I[91] and II[92] diabetes mellitus, immune dysfunction[93,94], neural development[95] and cardiovascular disease[96,97,98]. On the overall, breastfed individuals seem to have an advantage, being at a lesser risk for adulthood morbidity compared to formula-fed individuals, although some studies have reported a lack of difference[99] or only a marginal difference[100] in the outcomes, and thus this is a still ongoing debate.

2.2. Differences between breast milk and formula

Milk synthesis and secretion in the mammary gland takes part in several phases during which the composition of milk varies considerably. The first secretion from the gland is

called colostrum, and it gradually evolves into that of mature milk in order to meet the changing needs of the baby during growth and maturation. This is reflected in the ratios of protein and fat content changing over the course of lactation[101]. The protein content of milk forms a gradient decreasing from colostrum to mature milk, which is generally lower than in cow-milk based formula. At the same time, lipid concentrations of colostrum and transitional milk are lower than the ones usually found in formula but similar to lipid concentrations in mature milk[102]. These differences in protein and fat content between human breast milk and different standard infant formulas have been proposed to contribute to the accelerated postnatal growth of bottle-fed babies, which is argued against as predisposing to childhood obesity[103]. Additionally, in terms of composition breast milk stands against commercial formulas in that it contains a complex mixture of human hormones and growth factors, immunoglobulins, oligosaccharides and non-essential fatty acids, and it can populate the infant's intestine with beneficial microbial populations, directly or indirectly[104]

2.3 Cholesterol and programming of cholesterol metabolism

Another important compositional difference between infant formula and breast milk is their cholesterol content. Human milk contains 10 to 15 mg/dL cholesterol[105], while in cow milk-based formula this number is 10 times lower[106] and in soy-based products virtually zero. This difference is especially relevant with respect to the observation that breastfed individuals have lower total plasma and LDL-cholesterol in adulthood than formula-fed individuals[107,108]. In animal models formula feeding has been shown to alter hepatic gene expression and cholesterol homeostasis in the neonate[109] indicating that also a programming effect on cholesterol metabolism could be plausible.

During infancy, the higher dietary exposure to cholesterol from milk results in increased total plasma cholesterol levels and LDL-cholesterol of breastfed infants compared to formula fed ones[110,111]. In response to the infant hypercholesterolemia, feedback regulatory mechanisms for sterol synthesis are activated which manifest in compensatory reduction in the rates of fractional cholesterol synthesis seen at 4[112] and 18 months of age[113]. This effect has been speculated to play a role in the establishment of a lower set point for cholesterol synthesis in adulthood, thereby leading to lower total and LDL-cholesterol and being protective against adult CVD. Further population analyses have shown that the initial difference in cholesterol homeostasis between breastfed and formula-fed infants disappears during childhood and adolescence[114,115], just to come back later with the opposite trend - higher plasma cholesterol in adult individuals previously fed formula[113]. Although persistent changes in the cholesterol synthesis rates have been speculated to play a role in the establishment of this phenotype, there is a general lack of mechanistic evidence supporting this hypothesis. Human population studies, however,

have demonstrated that independent of LDL-cholesterol levels, alterations in cholesterol homeostasis, particularly combinations of high cholesterol absorption and low endogenous synthesis, are associated with increased cardiovascular risk[116]. There is a bimodality in the general population with respect to cholesterol absorption and synthesis: individuals with low cholesterol absorption generally have a high cholesterol synthesis profile, or vice versa. This might have therapeutic relevance, since a difference in response to statin therapy has been indicated dependent on the cholesterol absorption/synthesis balance[117]. Thus metabolic programming of this balance established in infancy may have direct implications for CVD risk in adulthood. Interestingly, a study in twins has demonstrated that close to 30% of the epigenome is significantly changed between birth and 18 months of age, with a large fraction of the genes associated with lipid metabolism[118]. This makes it highly plausible that epigenetic processes take root in infancy and affect adult physiology and disease risks.

2.4 Programming of the intestinal metabolism

It seems that the small intestine of the infant could be particularly vulnerable to epigenetic programming initiated by early life diet as proliferation occurring at the crypts is twice as active as in the adult[119]. While the maturation of the mucosal barrier is ongoing, the infant intestine has still a relatively high permeability, which allows macromolecules from the food to be introduced into the infant circulation. This includes maternal growth factors and immunoglobulins coming from breast milk but could also include allergens, hence predisposing towards food sensitivities in later life. It is generally accepted that the maturity of the mucosal barrier at birth is dependent on the length of gestation. In rodents, as species with a shorter gestation period, postnatal maturation continues up to 3 weeks postpartum[120,121] and includes substitution of the cell populations covering the villi, accompanied by rapid proliferation and migration in the intestinal crypt. Studies performed in healthy infants demonstrated that despite the lack of effect on the villus area, formula-fed subjects had a 30% increase in the crypt depth and mitotic cell count per crypt compared to breastfed[122]. Similar, but more pronounced results have been obtained from rodents[123,124]. It can be concluded that factors found in the early postnatal diet may have an important role in postnatal maturation of the small intestine and hence induce a programming effect possibly reflected in adult absorption capacity of certain dietary components with negative connotation to cardiometabolic disease e.g. cholesterol and fat.

2.5. Programming by other components or qualities of milk

Long-chain polyunsaturated fatty acids (PUFAs) are other components of early life nutrition with potential impact on long-term health. They have an important role for neural development in addition to cardiovascular health and inflammation

and it is generally considered that a lower *ω-6:ω-3* ratio is more favorable. Maternal supplementation with *ω*-3 during pregnancy has been implicated with beneficial effects in offspring e.g. alleviating adult metabolic dysfunction[125], while *ω*-3 deprivation during pregnancy and lactation has been linked to adverse effects on neurogenesis caused by epigenetic events[126]. Experiments in mice have shown that postpartum manipulation of maternal diet is reflected in milk composition and can lead to an increase in the amount of *ω*-3 found in the brain of the offspring[127]. In rats, supplementation with *ω*-3-rich flaxseed oil during lactation has been shown to reduce body fat in the suckling pups[128], while in mice the effect on reduced adiposity was preserved at 12 weeks of age[129]. However, a couple of recent randomized controlled human trials testing the long-term benefits of a low *ω*-6:*ω*-3 ratio and *ω*-3 postpartum supplementation in the offspring found no difference in body composition[130] or cardiovascular health parameters[131] at 5 and 9 years of age. Whether certain effects of early life PUFA supplementation may manifest beyond this point in humans remains to be established.

Several studies have indicated that possible programming effect can be conveyed not only by the amount of certain nutrients in the early life diet but also by the form in which they are presented. The physical structure of lipids in milk, attributed to lipid droplet size and phospholipid coating, have been suggested to influence the process of diet-induced fat accumulation and adipocyte hypertrophia[132-134]. Therefore, the optimization of future infant formula faces the challenge to not only component-match breast milk but also to structurally simulate the presentation of components with potential biological activity.

The first few weeks postpartum are the period when bacterial colonization takes place in the intestine. Several studies have indicated that the maternal gut microbiota can be transferred to the infant and prime its intestinal microbial population both during and post delivery via an entero-mammary route[135]. Breastfeeding promotes a gut microbiome abundant in *Bifidobacterium* and *Lactobacillus*, which in adults have been shown to be beneficial, whereas formula-fed babies have a more diverse colonization. Along with providing nutritional value for the infant, breast milk has to present substrates for the growing bacterial community[136]. The diverse group of human milk oligosaccharides (HMOs) has particularly attracted attention due to their beneficial role for the infant as prebiotics[137,138] and their large quantity in breast milk. Meanwhile, *Lactobacillus* and *Bifidobacterium* have the gene clusters necessary for HMO utilization. Further, the development of potential pathogens e.g. enteropathogenic *E. coli* and *Helicobacter pylori* is suppressed by the ability of HMOs to act as anti-adhesive antimicrobials[139,140]. Supplementation with galacto- and fructo- oligosaccharides, on the other hand, has been shown to promote different *Bifidobacterium* species to the levels observed in breastfed

infants[141]. Lacking or aberrant microbiota composition in early life may impact the risk of subsequent disease, as demonstrated by animal experiments[142,143]. Antibiotic treatment during pregnancy and lactation was shown to increase the weight gain following a high-fat diet challenge in adulthood by increasing energy harvest[143]. Likewise, low doses of penicillin administered postpartum induced a permanent shift in microbial populations, thereby amplifying the obesogenic effects of high-fat feeding later on by affecting hepatic gene expression, metabolic hormone levels, and visceral fat accumulation[142].

2.6 Accelerated growth theory

A postpartum increase in energy harvest from food, whether caused by gut microbial activity or energy-dense infant nutrition, could also be linked to accelerated growth patterns in infancy. First described among preterm and smaller for gestational age infants, lately, postpartum accelerated growth has been the subject of debate as a potential factor impacting long-term health. In rats non-confounded by fetal growth retardation, overfeeding during lactation associated with adult hypercholesterolemia and insulin resistance[144]. In humans, slower postpartum growth independent of birth weight was associated with improved endothelial function and lower CVD risk in adolescents[103]. Currently, the notion that accelerated growth in infancy increases the risk for cardiometabolic dysfunction later in life pervades the field. The implications of these findings are that enhancing infant growth rate by the provision of nutrient-dense early life diets might have more detrimental effects in the long term and should be the subject of reconsideration.

II. REGULATION OF CHOLESTEROL HOMEOSTASIS

Cholesterol is a lipid with a negative public reputation, notorious because of the strong correlation between high levels of cholesterol in the blood and the incidence of cardiovascular diseases. However, its physiological role is far more pervasive. Cholesterol is crucial for maintaining optimal membrane fluidity; being particularly enriched in the lipid rafts where it facilitates clustering of signal transduction proteins and maintains membrane fusion dynamics, which are central to a variety of biological processes. In addition, it serves as a precursor of steroid hormones that regulate an array of organismal functions. In the liver, cholesterol undergoes a multi-step oxidation process to be converted to bile acids, which aid in the secretion of lipids as well as endogenous and exogenous compounds into the bile and solubilize dietary fats to enhance their absorption from the intestine. Thus cholesterol is an essential molecule and along with being obtained by the diet, it is readily derived by endogenous synthesis in all animal cells. To avoid elevating the risk for cardiovascular complications the balance of cholesterol in the body is

promptly maintained by the dynamics of three interrelated processes: intestinal cholesterol absorption, endogenous synthesis and disposal of cholesterol by fecal excretion either as cholesterol of as bile acids.

1. Cholesterol absorption and fate in the enterocyte

The main sites for cholesterol absorption are the duodenum and the proximal jejunum of the small intestine[145]. Dietary cholesterol is subjected to emulsification and subsequent integration into bile salt-phospholipid micelles. Depending on the dietary source, cholesterol can be present as esterified or free cholesterol. Pancreatic cholesterol esterase disrupts the covalent bond between cholesterol and the fatty acid, thereby releasing free cholesterol, which is essential for its absorption[146]. Unesterified cholesterol can interact with transporters in the brush border membrane of the enterocyte where absorption of dietary fat and cholesterol is initiated. The main cholesterol transporter is the Niemann-Pick C1-like protein 1 (NPC1L1). While in the mouse it is mostly expressed in the proximal parts of the small intestine[147], in humans NPC1L1 is also present at high levels in hepatocytes, where it mediates reabsorption of cholesterol secreted into bile[148]. NPC1L1 was identified as the molecular target of the dietary cholesterol absorption inhibitor ezetimibe[149] after the finding that NPC1L1-knockout mice and ezetimibe-treated wild-type animals have a comparable reduction in cholesterol absorption and plasma cholesterol levels[147].

NPC1L1 is a polytopic transmembrane protein with a sterol-sensing domain (SSD), bearing high structural resemblance to the SSD of hydroxymethylglutaryl CoA reductase (HMGR) and the SREBP cleavage-activating protein (SCAP)[147,150]. In NPC1L1 it is responsible for protein translocation from the apical cell membrane to the intracellular endosome compartment in response to cholesterol abundance. Binding of cholesterol to NPC1L1 promotes the formation of cholesterol-rich microdomains featuring the lipid raft proteins flotillin-1 and flotillin-2[151]. A concomitant conformational change in the C-terminus of NPC1L1 allows the complex to interact with the clathrin adaptor Numb, which is essential for endocytosis via the clathrin/AP2 pathway[152]. Ezetimibe inhibits absorption by preventing the sterol-induced internalization of the complex which tethers NPC1L1 at the plasma membrane[149,153]. As a result of this cycling, in the absence of cholesterol NPC1L1 is localized at the brush-border membrane, while in high-cholesterol diet conditions its characteristic location is in the endosomal compartment[154]. Treatment with ezetimibe initiates a response by LXR- and SREBP2-regulated genes, which counteracts the depletion of cholesterol levels in the enterocyte[155]. This includes the activation of a complex network involving transcriptional and post-translational control aiming to increase sterol synthesis by HMGR, to elevate uptake by LDL-receptors, while

reducing the expression of IDOL (inducible degrader of LDLR) and ABCG5/8 efflux transporter.

The transcriptional control of NPC1L1 itself is more poorly understood. While cholesterol-rich diets promote strong downregulation of the gene[147], suggesting that its expression is sensitive to intestinal cholesterol uptake, the levels of both NPC1L1 mRNA and protein remain unaltered upon treatment with ezetimibe[155]. Several in vitro studies have proposed a possible regulation of NPC1L1 by SREBP2. A response element binding SREBP2 has been described at the promoter of the gene in both human hepatocytes[156] and Caco2-cells[13], which induces a dose-dependent expression of NPC1L1 upon introduction of a SREBP2 expression vector. The nonlinearity of the response to ezetimibe- and cholesterol-triggered SREBP2-control implies that NPC1L1 might be the target of several layers of transcriptional regulation. A role for LXR in this regulation has been suggested based on the reduced cholesterol absorption seen in wild-type mice treated with a LXR-agonist, while LXR α/β knockout animals do not show this effect[157]. It is likely, though, that this is due to induced ABCG5/8 expression and enhanced excretion of cholesterol back to the intestinal lumen, reflected in seemingly decreased net absorption. Later, LXR activators were found to be able to downregulate NPC1L1, concomitant with ABCA1 upregulation, in both the polarized Caco2/TC7 cell line and in mice[158]. PPARα has also been suggested to play a role in the regulation of NPC1L1, since mice treated with the PPARα agonist WY14,643, independent of dietary cholesterol content, demonstrated reduced levels of intestinal cholesterol absorption, while the effect was absent in PPARα knockout mice[159]. A similar response was induced also in mice treated with fenofibrate, which resulted in a 40-60% reduction in NPC1L1 mRNA and protein levels in proximal small intestine[160]. However, others found no difference in NPC1L1 expression levels after PPARα stimulation with several ligands, including WY14,643 and fenofibrate[158], despite the strong induction of classical PPARα -target such as PDK4. In even stronger contrast, a later study described a drastic reduction in NPC1L1 mRNA and protein levels in HepG2 cells transfected with PPARα siRNA, thereby suggesting a positive influence from PPARα activity on the expression of NPC1L1[161]. In addition, several other response elements have been found in the vicinity of the NPC1L1 promoter, including the orphan nuclear receptors HNF4α and HNF1α binding sites. While HNF4α was shown to be interacting with SREBP2 in regulating sterol synthesis and uptake, knockdowns of the gene in HepG2 cells resulted in a 20-30% reduction in NPC1L1 mRNA levels[162] and abolished its cholesterol-dependent regulation; also, HNF4α stimulates the transcriptional activation of the NPC1L1 promoter together with SREBP2 but not alone. Later studies in HuH7 cells, however, did not observe changes in NPC1L1 expression upon transfection with HNF4α siRNA, nor found a

synergistic action between SREBP2 and HNF4α; instead, they identified HNF1α as a positive transcriptional regulator of NPC1L1[156]. Finally, PPARβ/δ activation has also been associated with reduced cholesterol absorption and transcriptional downregulation of NPC1L1 in mice[163]. Recently, the role of epigenetics in regulating NPC1L1 expression has become more prominent, as hypermethylation of specific CpG positions in the proximal region of the human promoter were described as responsible for the drastically lower expression of NPC1L1 in distal compared to proximal intestine[164].

Next to cholesterol uptake from the enterocyte, NPC1L1 is also responsible, although with lower efficiency, for the uptake of plant sterols[147]. This is suggested by studies showing a reduction in the levels of plant sterols in the plasma of sitosterolemic patients[165] and mice[166] upon treatment with ezetimibe or genetic ablation of Npc1l1[167]. The main discrimination between cholesterol and phytosterols, however, happens at the level of the heterodimeric ABC-transporter ABCG5/G8, which is also located at the brush border membrane[168] and facilitates the export of plant-derived sterols back into the intestinal lumen[169,170]. In addition, the ABCG5/G8 heterodimer also pumps back unesterified cholesterol into the intestinal lumen. Mutations in either of the half-transporters lead to sitosterolemia, a rare condition accompanied by accumulation of plant sterols and cholesterol, which predispose to premature atherosclerosis[169]. The opposing roles of NPC1L1 and ABCG5/G8 become even more evident from their action in liver in humans. While both are located at the canalicular membrane of the hepatocyte, ABCG5/G8 is actively engaged in cholesterol secretion into bile[171], while NPC1L1 binds free cholesterol from the biliary compartment and moves it back to the endoplasmic reticulum of the hepatocyte[172]. Ablation of the ABCG5/G8 system in mice leads to a reduction in biliary cholesterol output and susceptibility to diet-induced hypercholesterolemia[173], while its overexpression results in elevated cholesterol and plant sterols in bile and reduced dietary sterol absorption[174]. Additionally, the ABCG5/G8 transporter is suggested to play a role in triglyceride catabolism[175] and to contribute to the transintestinal route for cholesterol elimination (TICE)[176].

Following uptake by NPC1L1, free cholesterol is re-esterified within the membranes of the endoplasmic compartment; an action mainly performed by acetyl-CoA acetyltransferase 2 (ACAT2)[177], which is essential for further transport of cholesterol into the circulation via chylomicrons. Along the intestinal axis ACAT2 has an expression profile similar to that of NPC1L1[149] and likewise its deficiency results in a considerable reduction of cholesterol absorption from cholesterol-rich diets, and resistance against diet-induced hypercholesterolemia[178,179]. In the endoplasmic reticulum, the microsomal triglyceride transfer protein (MTP)[180] mediates the formation of the nascent chylomicron by transferring lipids onto ApoB48, an action accompanied by recruitment of

phospholipids to cover as a monolayer the hydrophobic core. Intestinal MTP deficiency results in reduced cholesterol absorption and low plasma triglyceride levels which trigger a compensatory increase in hepatic lipogenesis leading to triglyceride accumulation in the liver[181,182]. Similar to their roles in intestinal chylomicron assembly, ACAT2 and MTP are essential for VLDL assembly in hepatocytes.

Enterocytes also expresses ABCA1 and APOA1, which are key components of the HDL assembly line[183] and unesterified cholesterol can leave the enterocyte via ABCA1-mediated basolateral efflux onto nascent pre-β-HDL particles[184]. However, multiple mechanisms in the enterocyte maintain the balance between free- and esterified cholesterol in favor of the ester[185], which translates into higher cholesterol flux via chylomicrons compared to intestinal HDL.

2. Lipoproteins and cholesterol transport in the body

The chylomicrons formed in the intestine enter the circulation via the lymphatic system. They are relatively large lipoprotein particles, with a hydrophobic core, composed of triglycerides and cholesterol esters. ApoB is the essential structural protein of chylomicrons, VLDL, IDL and LDL which is the reason that they are together commonly referred to as ApoB-containing lipoproteins[186]. The human intestine, however, produces a shorter form of the protein, APOB48, which is result of a post-transcriptional editing with the participation of the intestine-specific factor, APOBEC[187]. In mice, Apobec is also expressed by hepatocytes[188]. As a result of this truncation, APOB48 lacks the C-terminal domain necessary for interaction with the LDLR[189]. Besides APOB48, nascent chylomicrons also contain APOA1 and APOA4[190]. Once in the blood compartment, the chylomicrons obtain also APOC2 and APOC3. APOC2 is an important activator of the lipoprotein lipase (LPL)[191]. LPL initiates hydrolysis of the triglyceride core and release of free fatty acids, which are taken up into the peripheral tissues. The main protein for fatty acid uptake in the tissues is the scavenger receptor CD36, which can also bind large lipoprotein ligands and enhance LPL efficiency[192]. Upon this lipolysis, chylomicrons decrease in diameter and are converted into chylomicron remnants, which are subjected to hepatic clearance. In contrast, APOC3 inhibits LPL activity, and interferes with chylomicron clearance by hepatic APOE receptors, thereby promoting hypertriglyceridemia[193]. Following lipolysis, the chylomicron remnants acquire ApoE which is secreted by hepatocytes and abundant in the hepatic sinusoids[194]. This ensures their ability to interact with hepatic LDLR and LRP and their clearance from the circulation[195].

The liver is the main site of assembly for the second biggest lipoprotein particle, the VLDL. In contrast to the chylomicrons, which are secreted in the postprandial stage,

VLDL is constitutively released from the liver directly into the circulation. In humans, their signature protein is APOB100, while in rodents the liver synthesizes both ApoB100 and ApoB48[188]. In similarity to the chylomicron, their biogenesis takes place in the ER and encompasses a 2-stage process. First, MTP partially lipidates APOB100 with both polar and neutral lipids; this stabilizes the protein and results in a primordial VLDL particle[196]. Next, cytosolic triglyceride-rich particles fuse with the primordial entity in a process likely mediated by both MTP and the protein CIDEB[197]. Structurally, VLDLs are similar to chylomicrons in that they contain APOC and APOE proteins[198], and are thus a subject of the same interactions with LPL. In addition to LPL activity, VLDL may also dispose of triglycerides due to the activity of the cholesteryl ester transfer protein (CETP), which mediates the transfer of triglycerides to HDL in exchange of cholesteryl esters[199]. This process is absent in rodents since they do not express CETP[200]. These enzymes are responsible for the removal of triglycerides from VLDL and its subsequent transformation into smaller, cholesterol-rich particles. Roughly, half of all VLDL remnants that display both APOB100 and APOE on the surface are cleared with increased efficiency by hepatic LDLR and LRP. The rest gives rise to the LDL-subclass of lipoproteins, which functions to deliver cholesterol to the tissues. LDLs are pro-atherogenic, and their level in plasma is strongly correlated with cardiovascular mortality. The atherosclerotic process is initiated by subendothelial retention of APOB-containing lipoproteins and their subsequent modification within the intima[201]. The successful reduction of cardiovascular risk that comes with the use of statins is attributed to their ability to lower plasma LDL-cholesterol[202].

The third main class of lipoproteins is HDL, which comprises a diverse group ranging in size and composition. Their main structural protein is APOA1, followed by APOA2 and a constellation of other proteins[203] involved in a variety of processes including coagulation, inflammatory responses, lipid metabolism, etc. ApoA1 is expressed in both liver and intestine and these organs represent the main source of HDL, contributing 70% and 30% to the HDL pool in mice, respectively[184,204]. APOA1 undergoes lipidation with phospholipids and free cholesterol by the ABC transporter ABCA1, a process which releases a lipid-poor pre-β-HDL particle into the circulation[205]. In the blood compartment, pre-β-HDL interact with lecithin:cholesterol acyltransferase (LCAT), which transesterifies lecithin and free cholesterol to produce cholesteryl esters that accumulate in the core of the particle, thereby transforming it into mature, lipid-rich α-HDL[206]. These larger HDL particles can interact with other cholesterol transporters such as the unidirectional efflux transporter ABCG1 and the bidirectional scavenger receptor SRB1, and can thus obtain free cholesterol from them[207,208]. Amongst other cell types, both proteins are expressed in macrophage foam cells where they facilitate the

removal of unesterified cholesterol from the atherosclerotic plaque. The pathway, by which excess extrahepatic cholesterol is removed from the circulation via HDL-mediated delivery to the liver and its subsequent excretion into bile and feces, is classically known as reverse cholesterol transport (RCT)[209]. HDL-cholesterol can enter the hepatic cholesterol pool via selective uptake mediated by SRB1[210,211] or alternatively by endocytosis involving APOA1 recognition by the P2Y13 receptor[212] on the basolateral membrane of the hepatocyte. Consequently, the HDL-derived cholesteryl esters are hydrolyzed to free cholesterol and fatty acids by the hepatic cholesteryl ester hydrolase[213]. The derived free cholesterol can then be used for the synthesis of bile acids, or it can be directly excreted into the bile via the ABCG5/G8 efflux transporter. The biliary bile acid secretion, however, is the main driving force for free cholesterol efflux into the canalicular space mediated by ABCG5/G8, as mixed micelles composed of bile acids and phospholipids act as acceptors for cholesterol[214]. Essential for the process are the ABC transporters ABCB4 and ABCB11. The bile salt export pump ABCB11, also known as BSEP, mediates the active excretion of bile acids into the canalicular lumen[215], where they form simple micelles. Simultaneously ABCB4, also known as MDR2, acts as a floppase, translocating phosphatidylcholine to the outer layer of the canalicular membrane[216] from where they are incorporated into bile acid micelles converting them into mixed micelles. Ablation of Abcb4 drastically reduces biliary cholesterol secretion in mice[217,218] while its overexpression in HEK293T cells lead to toxicity[219] demonstrating its important role in maintaining membrane stability. Abcb11-transgenic mice were initially shown to have increased biliary output of bile acids[220.] However, later kinetic experiments could not replicate these findings, instead implicated the protein in a more complex cross talk with the intestine which determines the enterohepatic circulation rate[221]. Deficiency of Abcb11, on the other hand, causes cholestasis[222]. Further studies have demonstrated that cholesterol can also be excreted into bile in an Abcg5/g8 independent way[223] in a process largely mediated by canalicular Srb1[224,225].

3. Hepatic cholesterol metabolism

Liver is the central organ for the regulation of cholesterol metabolism. On the basolateral side of the hepatocyte VLDL, LDL and LRP receptors are expressed which are responsible for the uptake of APOB-containing lipoproteins[195,186,198] from plasma. At the same time basolateral SRB1 receptors mediate the selective uptake of HDL-cholesterol[210] in addition to its holoparticle endocytosis regulated by P2Y13 [226-228]. In humans, free cholesterol is taken up from the biliary compartment by apical NPC1L1[229,230]. Lastly, cholesterol is also *de novo* synthesized at a considerable extent, which leads to the formation of several distinct pools of either free- or esterified cholesterol. The

activity of hepatic ACAT2 converts free cholesterol into esters[231], which can either be stored or assembled with triglycerides into VLDL and secreted into the circulation. Free cholesterol, on the other hand, can be either secreted into VLDL or into the bile, either as cholesterol or, after conversion, as bile acids. The hepatocyte has thus to orchestrate the distribution of cholesterol from several sources to a number of destinations, a process that is tightly controlled and involves both metabolic and structural compartmentalization which includes hepatic zonal heterogeneity[232]. Earlier studies have shown that biliary cholesterol is preferentially obtained via the HDL-uptake pathway[233], while reduction of plasma ApoB-cholesterol translates into lower hepatic bile acid synthesis without affecting biliary cholesterol levels[234]. Accordingly, LDL-derived cholesterol has been suggested to undergo selective esterification and be shunted towards VLDL and HDL assembly, without necessarily passing through the central pool and affecting hepatic endogenous synthesis[235]. This is a notion that may explain previous observations dissociating the response of hepatic cholesterol synthesis and LDLR activity to increased hepatic LDL uptake in conditions of hypercholesterolemia[236-238].

De novo cholesterol is synthesized from acetyl-CoA in a long chain of reactions encompassing four distinct stages: i) condensation of acetyl-CoA to mevalonate; ii) conversion of mevalonate to activated isoprenes, iii) six units of which condense to form the 30-carbon squalene; iv) lastly, conversion of squalene into a four-ringed steroid nucleus, which undergoes several side chain modifications to finally become the 27-carbon containing cholesterol. The total energetic cost for each cholesterol molecule is accounted for by the hydrolysis of 18 phosphoanhydride bonds, which renders cholesterol synthesis energetically an expensive process. *De novo* cholesterol synthesis is tightly regulated, and although every mammalian cell produces a certain amount of cholesterol, the demands of the peripheral tissues are mainly covered by cholesterol originating from the liver and intestine[239]. Nearly half of the cholesterol in the squirrel monkey body is derived from the diet, and the rest is *de novo* synthesized[240]. High dietary cholesterol intake is capable of suppressing hepatic cholesterol synthesis, without affecting the cholesterol synthesis rates in the intestine or other tissues tested[241]. In rats, 50% of the newly synthesized cholesterol within one hour after the administration of 3H-water, was assigned to a hepatic origin, followed by 24% coming from the small intestine[242]. Later experiments showed a direct correlation between the amount of newly synthesized cholesterol in liver and the presence of label in the plasma compartment while such correlation was not detected in the rest of the tissues[243]. In other species such as monkeys, rabbits, hamsters and guinea pigs the relative contribution of hepatic synthesis to the whole body cholesterol pool is much smaller, ranging from 16-40%[243]. Those species have a larger relative amount of cholesterol synthesized in the small intestine, which is also considered to be the case in man[239,244].

Similarly, the lactating mammary gland is capable of producing and secreting large amounts of cholesterol into milk[245,246].

4. Regulation of cholesterol metabolism

The second step of the biosynthetic pathway, the synthesis of mevalonate, catalyzed by hydroxymethylglutaryl-CoA reductase (HMGR), determines the rate of cholesterol synthesis. HMGR is the enzyme inhibited by statins, which successfully leads to a reduction in pro-atherogenic LDL-cholesterol and overall cardiovascular risk. However, the mechanism of action of statins in vivo was recently challenged, as they were shown to actually increase hepatic cholesterol synthesis in mice[247].

4.1 SREBP

The expression of HMGR is controlled by the intracellular levels of cholesterol through a feedback loop involving a sterol-regulatory element (SRE) in its promoter. Several transcription factors from the sterol regulatory element binding protein-family (SREBPs) have high affinity towards conserved SRE genomic elements and stimulate transcription of genes involved in lipid synthesis. Those include the SREBP1a, SREBP1c, and SREBP2. The first two isoforms are related to fatty acid synthesis, while SREBP2 controls cholesterol synthesis and uptake. Unlike other transcription factors, SREBPs are inserted during synthesis into the ER membrane in an inactive form, which is bound to a sterol-sensing SREBP-cleavage activating protein (SCAP). Abundance of cholesterol induces a SCAP conformation, which allows it to bind with high affinity to integral ER membrane proteins from the INSIG-family[248]. In mammalian cells, they are present in two isoforms, and their main role is to act as an ER-anchor, preventing the escape of the SREBP2-SCAP complex from the endoplasmic reticulum, by interfering with the formation of SCAP-COPII vesicles, which bud towards Golgi[249]. INSIGs can also interact with sterol-bound HMGR and target it for ubiquitination[250], thereby reducing its lifetime activity. Depletion of cholesterol releases the interaction between INSIG and SCAP, which results in SREBP2 translocation to the cis –Golgi. There, two consecutive cleavages by proteases SP1 and SP2 release the N-terminal biologically active domain to proceed to the nucleus and activate target gene expression. The genes inducible by SREBP2 promote increase of cellular cholesterol levels and include all the genes from the cholesterol biosynthetic pathway, as well as receptors for cholesterol uptake, i.e. LDLR, its regulator PCSK9, transporters NPC1L1, ABCA1 and a number of others[251,252]. Interestingly, the INSIG1-gene is also among the transcriptional targets of SREBP2[253], reflecting an important mechanism for self-regulation. Abundance of cholesterol results in low INSIG expression, and accordingly increased numbers of SCAP-SREBP

complexes can translocate to Golgi. Induced INSIG expression by the released nuclear SREBP (nSREBP) consequently promotes retention of SREBP in the ER. Of note, the proximal promoter region of SREBP-1c and SREBP2 isoforms contain an SRE sequence which translates into a positive feedback regulatory loop[254]. This explains the high levels of SREBP2 mRNA in animals overexpressing the nuclear form of the factor[255]. In addition, within the intronic region of the SREBP1/2 gene system, recently the presence of a non-coding RNA, miR33a/b, was discovered, the expression of which negatively regulates cholesterol efflux and fatty acid oxidation[256,257].

Transcriptional control over the SREBP-system is exercised by at least three different mechanisms. First, there is a self-perpetuating increase in expression due to the SRE in the promoter of SREBP1c and SREBP2. Second, SREBP1c is demonstrated as a target gene of LXRα/β[157], which allows induction of fatty acid biosynthesis triggered by increased levels of free cholesterol in the cell. This ensures sufficient substrate fatty acids for the esterification of cholesterol thus preparing it for storage or VLDL assembly in the liver. Third, the expression and activity of all SREBP isoforms are suppressed by the protein deacetylase SIRT6. Transgenic mice overexpressing Sirt6 have reduced LDL-cholesterol and plasma triglycerides[258,] which was achieved by reduced expression of the Srebp-cluster combined with increased retention of the Srebp-precursors in the ER.

4.2. PPARs

Activation of members of the peroxisome proliferator-activated receptors (PPAR) family plays an important role in the metabolism of multiple lipids, their distribution in the body via lipoproteins, fat storage and energy metabolism. Acting as ligand-activated nuclear transcription factors, their effects are cell-type specific and depend on the interaction with a transcriptional partner, the retinoid X-receptor (RXR), and the binding of co-activators with tissue-specific expression patterns. The PPAR-family is represented by three isoforms, PPARα, PPARβ/δ, and PPARγ, which all have different tissue expression profiles, ligand specificities and physiological effects. Due to their large ligand-binding pocket, some of the natural ligands for PPAR activation include big lipophilic molecules such as the ω-3 fatty acids (DHA and EPA), and certain eicosanoids. Multiple synthetic agonists have been developed as well, amongst which are the fibrates and thiazolidinediones developed for treatment of dyslipidemia and diabetes.

PPARα is predominantly expressed in liver, heart and skeletal muscles, where fatty acid oxidation takes place at significant rates. It mediates the physiological response to fasting by stimulating fatty acid catabolism and gluconeogenesis. Naturally, native ligands for PPARα are oxidized fatty acids, amongst which the oxidized forms of DHA and EPA are especially potent, shown to reduce inflammatory responses and prevent steatosis

in a PPARα-dependant fashion[259,260]. Ablation of PPARα in mice results in severe hepatic steatosis[261], while its activation by the synthetic agonist fenofibrate alleviates the condition[262]. In addition, PPARα-stimulated reduction in ApoC3 expression results in lowering of the triglyceride-rich lipoprotein fraction in plasma. This is concomitant with upregulation of APOA1 and consequently contributes to an increase in HDL[263,264]. Moreover, in transgenic mice expressing CETP under the control of its native human promoter, provision of fenofibrates dose-dependently increased HDL levels by suppressing the mRNA expression of CETP[265]. Fenofibrate also induced formation of larger HDL-particles via increased expression of ABCG1[266]. Therefore, PPARα activation is associated with improved cholesterol clearance from the circulation and atheroprotection.

PPARγ is most abundant in brown and white adipocytes, where together with its co-activator PGC1α[267], it enhances adipogenesis, lipid synthesis and adaptive thermogenesis. Its target genes facilitate lipolysis (LPL), fatty acid uptake by the adipocyte (CD36) and fatty acid synthesis (see review by Anghel et al.[268]). By controling the release of adiponectin and leptin and preventing excessive lipid accumulation in other tissues, PPARγ controls insulin sensitivity[269,270]. Natural ligands include long-chain polyunsatured (DHA, EPA) as well as monounsatured fatty acids, supplementation with which increased PPARγ expression and lowered markers of inflammation in adipocytes[271]. Pharmacological activation of PPARγ is conveyed by the thiazolidinediones, which is a group of compounds developed as insulin sensitizers, acting by reducing insulin-stimulated glucose uptake[272]. However, in the plasma compartment other compounds of this class seem to impact differently on the various lipoprotein fractions. For example pioglitazone reduces plasma triglycerides and free fatty acids, and increases cholesterol within the HDL-fraction, without affecting the ApoB-compartment, whereas rosiglitazone increases cholesterol in all lipoprotein fractions[273]. This is associated with increased cardiovascular mortality characteristics for rosiglitazone[274], while pioglitazone-patients showed a reduction in cardiovascular events[275].

PPARβ/δ is ubiquitously expressed and, similar to PPARα, it facilitates fatty acid oxidation and energy expenditure. Knockout mice challenged with a high-fat diet display decreased expression of UCP1 and reduced energy expenditure resulting in obesity, while transgenic mice are resistant to obesity[276]. In the macrophage PPARβ/δ activation stimulates the expression of the LPL inhibitor angiopoietin-like protein 4 (Angptl4)[277] and thereby reduces uptake of fatty acids from VLDL. Interestingly, macrophages lacking PPARβ/δ, have increased expression of the VLDL-receptor, which suggests a negative regulation by PPARβ/δ. Thus, the activity of PPARβ/δ decreases cellular lipid accumulation by preventing fatty acid uptake and inducing their oxidation. Along with that, it decreases cholesterol accumulation by increasing the expression of ABCA1 and

ABCG1 and accelerating cholesterol efflux towards HDL[278,279]. However, in various other peripheral tissues, PPARβ/δ has been implicated with a multitude of functions, some of which form potential links between cancer development and lipid metabolism.

4.3 LXR

LXR is another major regulator of cholesterol metabolism, which similarly to PPARβ/δ is dependent on RXR binding, neccessary for its activity[280]. LXR and PPARβ/δ can also be viewed as nuclear receptors with somewhat complementary actions, as they facilitate the depletion of lipid levels in the cell. LXR is present in two isoform LXRα and LXRβ with similar structure and ligand preference but different tissue distribution; LXRα is mostly found in liver, intestine, adipose tissue, macrophages and adrenals, whereas LXRβ is ubiquitously expressed. In mice the effect of LXR deletion is pro-atherogenic, resulting in a drastic decrease of total-body cholesterol excretion[281]. At the same time agonists lead to an increase in HDL-cholesterol, reverse cholsterol transport as well as transintestinal cholesterol elimination in mice, and is thus atheroprotective[282,283]. LXR can serve as cellular sterol sensor as its activity depends on the concentration of oxisterols and free cholesterol, which are its natural ligands. A large array of cholesterol-related genes are subject to LXR regulation, including central regulators such as the SREBP-system. In the intestine, LXR activation results in suppressed expression of NPC1L1 which limits apical cholesterol absorption[158]. This action is combined with upregulation of the inducible degrader of LDLR (IDOL), which decreases uptake from ApoB and ApoE-containing lipoproteins[284]. At the same time, excretion of sterols into the intestinal lumen is increased due to upregulation of the ABCG5/G8 transporter[283]. LXR stimulates also the basolateral expression of the ABC transporter ABCA1, which facilitates increased biogenesis of HDL[285], while the membrane localization of the bidirectional cholesterol receptor SRB1 is suppressed[286]. Further, CETP is another LXR target upregulated upon treatment with LXR ligands[200,287]. A potential drawback of hepatic LXR activation would be mediated by its effect on SREBP1c[157], as this induces *de novo* lipogenesis resulting in severe lipid accumulation associated with the pathogenenis of non-alcoholic fatty liver disease (NAFLD). Importantly, at least in rodents the conversion of cholesterol to bile acids is also under the control of LXR, as it upregulates the bile acid synthesis enzyme CYP7A1[288].

Bile acids are synthesized from cholesterol via two parallel metabolic pathways, the so-called classical or neutral pathway, which accounts for 75% of the production, and its alternative, the acidic pathway; their end-products are respectively cholic (CA) and chenodeoxycholic acid (CDCA). The most important enzymes of their synthesis are positively regulated by LXR in response to increased cellular free cholesterol and oxysterols. Those include CYP7A1, which starts the classical biosynthetic route, and

CYP27A1, which catalyses the first reaction of the alternative pathway[289,290]. The two routes are linked by intermediate compounds, for which the availability is determined by CYP8B1. In this way the acitivity of CYP8B1 can alter the bile acid pool composition, while CYP7A1 and CYP27A1 determine its size. One major difference between human and mice, is that in rodents most of the CDCA is converted to α-muricholic (αMCA) and β-muricholic acid (βMCA), which are more hydrophilic in nature. Before secretion into bile, hepatic conjugation takes place, coupling either glycine (human) or taurine (rodents) to the respective bile acids thereby decreasing their hydrophobicity. Once in bile, bile acids form mixed micelles with phospholipids and cholesterol, after which they enter the intestine. There they aid dietary fat emulsification and solubilization and act as antimicrobials preventing bacterial overgrowth in the proximal intestine[291]. Conjugated bile acids are preferentially taken up in the terminal ileum by the Na-dependent bile acid transporter ASBT and returned to the liver via the portal vein. The cycling of bile acids in the axis liver-intestine-liver is termed entero-hepatic circulation and accounts for the 95% reabsorption rate for primary bile acids. Bacterial deconjugation done by species secreting the enzyme bile salt hydrolase (BSH) inhibits the process of reabsorption by ASBT[292]. Those species include members of *Lactobacteria, Bifidobacteria, Bacteroidetes* and *Clostridium*[293]. As a result deconjugated bile salts enter the colon where they can be further modified by the intestinal microbiota. Two of those main modifications are oxidation and 7α-dehydroxylation. Oxidation is mainly done by bacteria, expressing hydroxysteroid dehydrogenases (HSDHs) which are abundant in species from the *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* phyla[294]. While the reaction is reversible, it can result in epimerization and formation of iso-secondary bile acids. An example is iso-DCA, which has been shown to promote the growth of *Bacteroidetes*[295], thus assigning a role for microbial HSDHs as a potent regulator of gut microbial composition. 7α-dehydroxylation, on the other hand, is the result from several reactions performed by a relatively small number of species mostly belonging to *Clostridium* and *Eubacterium*. During the process CA is converted to deoxycholic acid (DCA), and CDCA to lithocholic acid (LCA). Analogously, in mice αMCA and βMCA are converted to murideoxycholic acid, while ωMCA is the result of βMCA epimerization[296]. As a consequence of microbial modification the bile acid pool becomes more diverse as well as more hydrophobic. A small portion of the secondary bile acid pool gets passively reabsorbed in the colon, which allows them to enter the circulation and act as signaling molecules.

2.2.4 FXR

Already in the small intestine, uptake of primary bile salts in the enterocyte can indirectly induce changes in hepatic metabolism of bile acids by interacting with the farnesoid X-receptor (FXR). Uptake of bile acids in the ileal enterocytes stimulates FXR

to induce the production of fibroblast growth factor 19 (FGF19; rodent homologue Fgf15), which can reach the liver via the portal circulation. In the hepatocyte FGF19 triggers a signal transduction cascade resulting in reduced transcription of CYP71A[297]. In addition, BA may affect hepatic synthesis directly via FXR activation in the liver. In the hepatocyte, bile acid-activated FXR induces the expression of the LRH1-inhibitor SHP. As a result LRH1 cannot initiate the transcription of CYP7A1, thereby leading to inhibition of hepatic bile acid synthesis[298]. Thus, bile acids can self-regulate their own synthesis by inducing two different action arms. The strongest natural agonists for FXR are CDCA, followed by CA, DCA and LCA. However, certain secondary bile acids like UDCA or the murine taurine-conjugated α- and β-MCA, may competitively bind to FXR without activating it, thus functioning as antagonists[299,300]. Since biliary cholesterol secretion is dependent on hepatic bile acid formation and export, such inhibition of FXR, indirectly leads to a reduction in biliary cholesterol output[301]. FXR has also been shown to induce the expression of PPARα, which translates into increased lipolysis and fatty acid oxidation[302]. The effects of FXR signalling, however are still a subject of ambiguity, since several knockout mouse models have demonstrated resistance against diet-induced obesity[303] and atheroprotection[304] associated with the lack of FXR. The discrepancy in these and other results[305,306] might be explained by the interaction of dietary macronutrients, genetic background, microbiota and probably differential effects of hepatic and intestinal FXR activation[305,307], all questions of current investigation.

III. SCOPE AND AIM OF THE THESIS

Both epidemiological and animal research support the now widely adopted idea that exposure to a suboptimal environment during critical developmental windows, such as the early prenatal and neonatal life, can have a long lasting impact on the metabolic health of an individual. Cholesterol and lipid homeostasis pathways might be especially sensitive to such impacts, where early life metabolic imbalances translate into an altered susceptibility towards cardiometabolic disease. However, insights into how development of an adult phenotype depends on the interaction between an early insult and the critical window of its application are still limited. Moreover, little is known about the mechanisms translating the impact of a particular insult into an altered physiological state in adulthood. One reason for these current limitations is the unavailability of intelligible preclinical models, providing sufficient time resolution and triggers for the response. Therefore, **the aim of this thesis was to develop and characterize animal models that allow for assessing the impact of single isolated factors in early life, namely oxidative stress and cholesterol, on the programming of cardiometabolic disease.** Within this frame,

the regulation of breast milk cholesterol content and the impact of dietary cholesterol on the intestinal microbiota were additionally investigated.

Chapter 2 focuses on the development a pre-clinical model where the effects of intrauterine oxidative stress were isolated from other programming factors with influence on adult pathophysiology. A heterozygous breeding scheme was employed to generate wild-type offspring, originating from a Sod2-heterozygous intrauterine environment associated with oxidative stress (IUOx). The effects of isolated intrauterine oxidative stress on the development of components of cardiometabolic disease in adult offspring mice challenged with Western diet were evaluated.

We also aimed to develop a preclinical model for postnatal metabolic programming based on manipulation of early life cholesterol availability. Infant formula milk, in contrast to breast milk, contains little to no cholesterol. This difference in cholesterol content has been hypothesized to play a role in mediating (some of) the protective effects of breastfeeding against adult CVD risk. However, the factors determining the cholesterol concentration of mother milk and its regulation are largely unknown. **Chapter 3** evaluated the relationship between maternal hypercholesterolemia during lactation and milk cholesterol content. The early postnatal period is also when microbial colonization takes place in the gut, a process modulated by native milk components such as the human milk oligosaccharides (HMOs), which provide substrate for the growing bacterial communities. Similarly, part of the beneficial effects of breastfeeding could be conveyed by cholesterol-induced adaptation in the microbiome.

In adults bacterial dysbiosis in the intestine been implicated in mediating some of the adverse metabolic effects of Western (high-fat, high-cholesterol) and high-fat (only saturated fat, no cholesterol) diet. In **Chapter 4** we investigated whether the cholesterol component of Western diet alone can also elicit changes in the gut microbial distribution and metabolism with the potential to impact host pathophysiology.

Finally, the long-term effects of a reduced cholesterol exposure during lactation (**Chapter 5**) were tested in a pharmacological model, where instead of manipulating milk cholesterol concentration in the dam the absorption of cholesterol from the milk in the pups was blocked by administering ezetimibe during the lactation window. This resulted in a scenario closely mimicking the human situation, where cholesterol-poor formula associates in lower total and LDL-cholesterol in infancy.

The implications of these findings and our interpretation of the underlying mechanisms are being discussed in **Chapter 6**.