Metabolic memories

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CHAPTER 6

General Discussion Perspectives

GENERAL DISCUSSION

In recent years it is becoming increasingly recognized that early life conditions can modulate the balance between health and disease in adulthood. Factors such as nutrient availability and maternal pathological conditions during pregnancy and lactation have been implicated to influence the long-term physiology in the offspring. This often manifests in impaired lipid and(or) glucose homeostasis and an increased risk for disease development in adulthood, particularly of components of the metabolic syndrome - obesity, type II diabetes and cardiovascular disease. The interaction between genetic, epigenetic and environmental factors seems to be crucial for the development of metabolically programmed phenotypes but our understanding of how such relationships are formed and perpetuated into adulthood is far from complete. Oxidative stress is a crucial driver of tissue differentiation during fetal development, while in adulthood it emerges as an important component of the pathophysiology of cardiovascular disease. Epidemiological observations are linking breastfeeding with adult cholesterol metabolism. This observation could be compatible with a potential role for dietary cholesterol intake during infancy in determining parameters of cholesterol metabolism in adults. 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.

Intrauterine oxidative stress conveys protection against Western diet-induced metabolic derangements in adulthood.

Pregnancy complications such as preeclampsia and malnutrition are a frequent cause of fetal growth restriction and increased fetal oxidative stress[53,54,416]. They associate with a higher incidence of the metabolic syndrome in adulthood[54]. Antioxidant supplementation during pregnancy has been associated with a decrease in the risk for preeclampsia together with decreased maternal markers for oxidative stress[417]. The pathophysiological contribution of intrauterine oxidative stress per se on adult offspring health is experimentally difficult to address and has not been investigated. In **Chapter 2** we evaluated the hypothesis that fetal oxidative stress alone would adversely impact adult health. Hence, the impact of intrauterine oxidative stress (IUOx) on the development of adult susceptibility to metabolic syndrome was investigated by using a genetic mouse model, the mitochondrial superoxide dismutase (Sod2)-heterozygous mouse, in which intrauterine oxidative stress was not associated with either IUGR, maternal obesity, diabetes or dyslipidemia[325]. We compared *Sod2*+/+ offspring derived from matings of *Sod2*+/- dams with *Sod2*+/+ males (high intrauterine oxidative stress) to *Sod2*+/+ offspring from *Sod2*+/+ dams with *Sod2*+/- males (controls). To assess the adult life cardiometabolic disease susceptibility

we performed the breeding on the Ldlr-knockout background, a model for diet-induced dyslipidemia[327]. Our data demonstrate that the induced IUOx was not accompanied by differences in offspring body weight at embryonic day 18.5 indicating that this is a rather pure model of oxidative stress. The data obtained from adult offspring, however, showed, that maternally imposed intrauterine oxidative stress (IUOx) provides protection against Western diet-induced gain in adiposity, insulin resistance and dyslipidemia in male, but not in female mice.

The protective effect of IUOx on adult metabolic health is a seemingly counterintuitive finding. Previously, a study in rats found lower body weight and systemic inflammation in 2 months old offspring in response to antioxidant supplementation during pregnancy[418]. In contrast to this study, which exposed the animals to Western diet during pregnancy but not in adult life, we did not provide other stressors than the maternally generated ROS during fetal development. The distinct outcomes might, therefore, be due to species-specific effects or different conditions in the experimental setup. Our own efforts to evaluate the impact of IUOx programming on the physiology of adult offspring not challenged with a Western diet did not reveal differences between the groups.

The original hypothesis for a detrimental effect of IUOx in adult life stems from observations demonstrating that maternal obesity, as well as malnourishment, can aggravate fetal oxidative stress and translate into adult predisposition to type II diabetes[54]. Notably, plasma markers for maternal oxidative stress have been positively correlated with fetal levels of oxidative stress markers[59] and additionally with fetal ghrelin levels[55], the hormone responsible for increase in food intake and fat storage. Along the same line, in vitro studies have implicated ROS in decreasing β-cell propagation and differentiation[58], which may negatively impact pancreatic function in adulthood. Several studies have described beneficial effects of antioxidant supplementation during fetal life on adult metabolism. In both mice and rabbits exposed to maternal hypercholesterolemia, vitamin E treatment during gestation reduces the postnatal susceptibility to atherosclerosis of the offspring[83,419]. Administration of resveratrol, another potent antioxidant, was shown to prevent oxidative stress in pregnant rats fed a low protein diet, and to counteract metabolic dysfunction in their offspring[420]. However, in both studies the oxidative stress was associated with challenging the maternal homeostasis during pregnancy, namely hypercholesterolemia and malnutrition, conceivably resulting in other accompanying effects than simply ROS production. It is likely that factors such as the level of oxidative stress and the specific conditions of its manifestation would determine the role it plays for offspring health. Possibly, both high IUOx and antioxidants may convey beneficial effects for offspring health depending on the initial metabolic context of the fetus, accompanying maternal conditions or the postnatal environment (diet and stressors) encountered by the

offspring. It is thus attractive to evaluate the impact of antioxidant provision using a clean model of gestational oxidative stress, such as the offspring of *Sod2* +/- dams.

While high levels of ROS are detrimental to most cells, at moderate levels ROS have a physiological role in modulating gene expression patterns[60,61], and represent a main driving force for tissue differentiation in early embryonic development. Markedly, ROS accumulation and increased oxidative stress during exercise have been implicated in modulating the activity of key regulators of glucose homeostasis, resulting in an improved metabolic state[66]. The observation that small doses of a stressor i.e. ROS provide biological resistance toward larger successive doses of it is an old concept known as hormesis, which was recently adapted to mitochondrial regulation of cell signaling networks driven by reactive oxygen species and coined mitohormesis[421]. In addition to explaining the beneficial metabolic effects of exercise[65] the concept provides a plausible framework for cardiometabolic protection in response to mildly increased IUOx independent of other changes in maternal physiology. Western diet regimens are associated with higher levels of systemic oxidative stress, hence, offspring pre-conditioned by IUOx are better able to handle the oxidative stress burden of a Western diet challenge. Our study thus contributes to expanding the mitohormesis theory into the field of metabolic programming. Moreover, this notion is also in agreement with the predictive-adaptive response theory[422] for metabolic programming.

Sex-specificity of responses is frequently encountered in metabolic programming studies. The molecular and phenotypic consequences of an adverse intrauterine environment are often more prominent in male offspring[344,345,423]. Since the sex of the embryo can modulate placental size, function and its ability to respond to adverse stimuli[347], sex-specific placental differences have been proposed to contribute to the sexual dimorphism in programming.

Our gene expression data suggest that the protective phenotype in males is conveyed by upregulation of the uncoupling protein 1 (Ucp1) mRNA in white adipocytes and a perceived associated increase in energy expenditure. High levels of Ucp1 expression are usually observed in the thermogenic brown adipose tissue (BAT). However, white adipose tissue (WAT), in response to certain stimuli, can undergo a process known as browning where it acquires the characteristics of BAT, notably the induction of Ucp1 expression[329]. While in females the gene was not upregulated, it is possible that the sex-specificity of the physiological response is caused by differential effects on the level of Ucp1.

A key regulator in the process of WAT browning is the redox-sensitive factor Pgc1α[333], which positively impacts Ucp1 expression. Despite higher Ucp1 mRNA expression in IUOx male mice, it did not associate with differential expression of Pgc1α in the tissue.

This result likely indicates, that a novel, Pgc1α-independent route is responsible for Ucp1 upregulation in our experimental conditions. Such route could possibly involve epigenetic mechanisms as fetal oxidative stress has been implicated as potent activator for chromatin remodeling[69,75]. Moreover, several global changes in histone modifications surrounding the region proximal to Ucp1 promoter have been correlated with its activation during browning of white adipose tissue[424]. The responsiveness of the Ucp1 promoter to 5-aza-deoxycytidine treatment suggests that DNA methylation as well may be involved in the tissue-specific regulation of the gene[425]. Despite that the exact molecular pathways for Ucp1 upregulation remain unidentified, our data provide the basis for future research to specifically address the possible epigenetic link between Ucp1 and early life exposure to oxidative stress.

The long-term health implications of these findings involve reconsidering the notion of an invariably adverse impact of fetal oxidative stress on programming of adult metabolism. It is likely that changes in the redox environment during gestation are indeed important for adult offspring metabolic health. However, whether IUOx exposure proves beneficial or adverse would depend or the presence of confounding maternal conditions as well as the environmental and dietary challenges the offspring encounters in adult life.

Stable milk cholesterol concentration points towards importance of early life cholesterol supply

Serum cholesterol levels, in particular LDL-cholesterol, are strongly related to cardiovascular events in adult life. The role of cholesterol in early life is still less well defined. Exposure to even transient maternal hypercholesterolemia in the first two trimesters of gestation is linked to increased placental transport of cholesterol and accelerated fetal atherosclerotic plaque development[79] while hypocholesterolemia has been associated with fetal growth restriction[80]. As previously discussed, impaired fetal growth correlates with increased risk for adult CVD. 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 and metabolic health. During early postnatal development the role of cholesterol availability for the infant is even less clear. Breast milk contains high levels of cholesterol in contrast to most infant formulas. This is especially relevant regarding the observation that breastfed individuals have lower total plasma and LDL-cholesterol in adulthood than formula-fed individuals[107,108]. The relatively high cholesterol content of breast milk has been suggested to define the long-term cholesterol homeostasis of the offspring. The mechanisms involved in the regulation of milk cholesterol concentration are only partly understood. In **Chapter 3** we addressed the role of maternal hypercholesterolemia during lactation induced by dietary or genetic

means and its effects on cholesterol levels of milk in wild-type mice and in mice genetically deficient in the cholesterol transporters Abcg8 or Ldlr. The milk cholesterol concentration was not affected by genetic inactivation of either of the two candidate genes, or by diet-induced hypercholesterolemia.

Previous research in rodents has indicated that lactation is characterized by increased LDL-cholesterol in the circulation[364] while in humans it is associated with a higher expression of the LDL-receptor in mammary gland alveolar cells[350]. Those observations imply a possible role of LDL particles in contributing to the high cholesterol output into milk during lactation. Our data, however, suggest that the LDL-receptor in mice is not essential for cholesterol uptake into the mammary gland as indicated by the comparable milk cholesterol content between wild-type and knockout animals. Diet-induced hypercholesterolemia increased the amount of mammary gland cholesterol in Ldlr -/- mice, despite the drastic reduction in de novo synthesis in the tissue, which suggests that alternative routes for (V)LDL uptake are active in this case. This conclusion is in agreement with previous data indicating the transfer of ApoB-associated radioactivity across the mammary epithelium in Ldlr-knockout mice[352]. Further research is necessary to assess which alternative receptor(s) for ApoB-containing lipoproteins mediate this effect.

Abcg8 is an apical cholesterol transporter in hepatocytes and enterocytes, where it forms a heterodimer with Abcg5 and facilitates cholesterol export towards bile and the intestinal lumen[168]. Since Abcg5 is also expressed in bovine mammary gland we hypothesized it may be involved in apical cholesterol transport towards milk[356]. However, our data do not support such a role for Abcg8 in mice. Rather, transcriptomic analyses of bovine gene networks active during lactation point that the amount and pattern of cholesterol secretion into bovine milk corresponds with the expression pattern of another ABC transporter – Abca1[426]. Classically Abca1 is responsible for efflux of cholesterol onto lipid poor ApoA1 particles and its ablation is associated with severe depletion of circulating HDL cholesterol levels[205,427].

Milk cholesterol is mainly present as unesterified cholesterol in the milk-fat globule (MFG)-membrane (85-90%) and the remainder as cholesteryl esters in the MFG-core[368,369]. The packaging of the lipid droplets with the MFG membrane, which is essential for their secretion, may therefore translate into rather stable cholesterol content in milk. Thus, aside from apical efflux via a specific transporter cholesterol content in milk may be maintained by the role it plays as a membrane-stabilizing component of the MFG[428].

The present findings of strictly maintained cholesterol concentration in milk under conditions of hypercholesterolemia support the idea of a physiological importance of

stable milk cholesterol supply for the offspring. Since newborns are capable of de novo cholesterol synthesis, they do not critically depend on milk cholesterol for their supply. Infants fed cholesterol-free formula have a compensatory increase of cholesterol synthesis rates compared to breastfed infants[112,113]. It is, therefore, unlikely that milk cholesterol content would be impacting the immediate health of the child. However, in adulthood, individuals who had been breastfed as infant have lower total and pro-atherogenic LDL-cholesterol compared to previously formula-fed individuals[107]. This has led to the hypothesis that early life cholesterol supply can program cholesterol homeostasis in later life. The tightly regulated concentration of milk cholesterol under different dietary and/or genetic conditions of maternal hypercholesterolemia, described in **Chapter 3,** supports the relevance of a stable cholesterol supply for metabolic programming of adult cardiometabolic health.

Gut bacteria are resistant to high intraluminal cholesterol levels

Theoretically, dietary cholesterol could be relevant for the development of intestinal microbiota in early life. Breastfed infants experience a delay in the conversion of cholesterol to coprostanol by intestinal microbiota compared to formula-fed babies, indicating that milk components affect the development of certain bacterial groups with relevance to intestinal cholesterol metabolism[373]. Multiple studies have demonstrated that in adulthood Western diets, rich in fat and cholesterol, can affect cardiometabolic disease susceptibility via the effect they exercise on the composition and function of gut microbiota[375,382,429]. The transition from a plant-based towards a high-fat Western style diet has been shown to modify the gut microbiota by increasing the representatives of *Firmicutes* relative to *Bacteroidetes* in the distal intestine[382]. These changes conceivably contribute to metabolic disease, since the predominance of *Firmicutes* over *Bacteroidetes* has been associated with obesity and metabolic syndrome in mice[383] as well as humans[384]. Similarly, the provision of exclusively high-fat diet (without added cholesterol) is linked to alternations in the gut microbiome composition and changes in microbial metabolism leading to an increased capacity for energy harvest[375]. These observations led us to determine whether cholesterol contributes to such a response from the intestinal bacteria. Research described in **Chapter 4** tested whether in adult mice the exclusive abundance of dietary cholesterol changes the distribution of gut microbial communities with relevance to whole body cholesterol metabolism and cardiometabolic disease susceptibility. To evaluate this *Ldl*-receptor knockout mice, a largely used model for diet-induced dyslipidemia[327], were provided 1.25% high-cholesterol diet for 12 weeks followed by analysis of cecal microbial populations and host cholesterol homeostasis. Our results demonstrate that the composition of abundant bacterial groups in mature mice is remarkably stable despite the considerable changes occurring in host cholesterol metabolism in response to prolonged high-cholesterol

feeding. Several less-abundant and also less well studied groups, however, were differentially regulated. Both high-fat + high-cholesterol and exclusively high-fat diets (without added cholesterol) can elicit a dramatic response in microbial gut populations manifesting in a changed ratio of highly abundant groups such as *Firmicutes* and *Bacteroidetes*[376,397]. Our present data indicate that a proposed impact of dietary cholesterol on this ratio is unlikely. Interestingly, a less abundant group of the *Firmicutes* class, the *Turicibacter* genus, was present in all high-cholesterol fed mice but not detected in the group receiving control diet. An increase in *Turicibacter* was previously found to correspond to the production of caecal butyrate in rats fed a barley-malt based diet with a high-fat content[399]. However, the fractional contribution of *Turicibacter* has also been found to decrease in response to high-fat feeding alone in mice[430] suggesting that *Turicibacter* might be responsive to components in the diet other than fat, i.e. cholesterol. Our results indicate that adding cholesterol to the diet increases *Turicibacter* in the cecum. As previously demonstrated for other colonic bacteria of the *Firmicutes* group[401], this finding projects a possible role of the *Turicibacter* bacterium for assimilation or sequestration of cholesterol. The notion of horizontal gene transfer[431], where bacteria can acquire genetic material from non-parental lineages to develop differential substrate utilization strategies[432], provides possible means. Clearly more research is needed to delineate the pathophysiological importance of these bacteria with low abundance and their potential products for their role in cholesterol metabolism and their potential relevance for the development of cardiometabolic disease.

Certain microbial taxa are involved in bile acid metabolism and can thereby substantially affect the composition of fecal bile acids[433]. Primary bile acids synthesized from cholesterol in hepatocytes reach the intestinal lumen via the bile. In the large intestine unabsorbed bile acids are first deconjugated by the bacterial enzyme bile salt hydrolase; next they are dehydroxylated by bacteria to form the secondary bile acids [396]. Thus, changes in the fecal bile acid composition can provide important cues for an altered microbiota function. However, high-cholesterol diet did not induce any appreciable change in the distribution of fecal secondary bile acids when compared to the control group. The results suggest that the HC diet did not induce regulatory adaptations in microbiota metabolism with respect to bile acid conversion. With our approach limited changes occurring in bacterial cholesterol and bile acid metabolism were observed. A more detailed future study could include an evaluation of genetic networks responding to an increased availability of cholesterol by employing meta-transcriptomic analysis. However, the lack of major detectable changes with our current experimental set up makes it unlikely that such a more elaborate approach would reveal substantial alterations in critical pathways of main bacterial groups. Although dietary cholesterol did not introduce major shifts in the intestinal communities of adult mice, its possible role is still not to be excluded in

the first days of infancy when colonization takes place. To evaluate the contribution of dietary cholesterol from milk future research needs to address this question in animals, in which ideally milk cholesterol levels can be modulated without otherwise impacting the complex composition of breast milk. More research involving e.g. conventionalized germ-free mouse models would be required to address the potential impact that factors other than dietary cholesterol from milk might have on gut microbial populations in relation to host cholesterol metabolism.

Programming of the intestine by early life cholesterol availability

The main finding in **Chapter 3** of stable cholesterol concentrations in milk could have implications for programming of long-term offspring health. Examining this notion, the research detailed in **Chapter 5** tested whether decreasing the bioavailability of milk cholesterol for the offspring during lactation, in similarity to cholesterol-poor formula feeding, conveys long-term metabolic effects on adult cholesterol metabolism. To achieve reduced dietary cholesterol availability during suckling we provided the cholesterol absorption inhibitor ezetimibe to young *Ldlr*-knockout pups via mother's milk. While milk cholesterol content of the dams was not affected, the intestinal cholesterol absorption by the pups was supressed. Our data demonstrate that lowering the availability of dietary cholesterol in this manner results in a reduction in cholesterol absorption which was maintained well into adult life. Human population analyses have revealed that high absorption and low synthesis of cholesterol are associated with greater cardiovascular mortality[117]. The decreased absorption in post-ezetimibe treated mice was associated with upregulation of intestinal cholesterol synthesis in adulthood conceivably leading to comparable plasma cholesterol levels between groups. Previous research, describing lower cholesterol synthesis rates in breastfed infants compared to formula-fed[112], has suggested that breastfeeding plays a role for the establishment of a permanently lower cholesterol synthesis in adulthood which likely translates into lower circulating LDL-C levels and corresponding cardiovascular protection. Our data, however, suggest that at least in mice it is the lower set point for cholesterol absorption that initiates the compensatory increase in synthesis, and a lack of apparent protection against rising plasma cholesterol levels in adulthood. More work in humans is thus required to determine whether dietary cholesterol from milk is beneficial for long-term cardiometabolic health. Hypothetically, the opposing outcomes in mice and human could be related to the fact that cholesterol-related programming events in human may take place predominantly in the liver, where most of the synthesis takes place, while in mice the target organ is the intestine. 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 of the mucosa continues up to 3 weeks postpartum[120,121] making it particularly

sensitive to nutritional impacts.

The reduced cholesterol absorption in post-ezetimibe mice was conveyed by decreased expression of the cholesterol transporter Npc1l1. In mice the protein is present only in the enterocyte, while in human it is also expressed on the canalicular membrane, where it reabsorbs cholesterol from bile. Therefore, differences in the tissue expression patterns of Npc1l1[415] between mice and men may additionally account for the distinction in the homeostatic mechanisms affected.

A study in twins 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. We found no differences in the DNA methylation status in the vicinity of the Npc1l1 promoter that could explain its lower expression in post-eze mice. Instead, reduced Npc1l1 expression was mediated by increased histone H3K9me3 methylation upstream of its promoter, a chromatin mark specifically associated with transcriptionally silenced genes[434,435]. However, the molecular sensors translating cholesterol abundance into epigenetic code are still to be identified.

The early life intervention used in our model to reduce dietary cholesterol availability from milk relies on the administration of ezetimibe to young pups via the dam's milk. Although this was not coupled with any direct effect on Npc1l1 or apparent pathophysiological changes in the pups, an alternative experimental approach could employ fostering offspring by mothers with genetically reduced milk cholesterol content. Such an approach would exclude possible pharmacological effects of ezetimibe. However, this might be more challenging than expected, as indicated by the experimental evidence described in **Chapter 3**, that milk cholesterol levels are robust to manipulation. Nonetheless, by directing us towards the key role that the intestine plays as sensor and integrator of cholesterol metabolism, this research (**Chapter 5**) demonstrates the responsiveness of the enterocyte to metabolic programming stimuli with life-long consequences in mammals. Additionally, our findings may provide insights for the development of novel LDL-cholesterol lowering strategies. Genetic variation at the NPC1L1 locus contributes to low cholesterol absorption and associates with significant cardiovascular disease-related health benefits[414]. The prospect to manipulate adult cholesterol absorption rates with interventions as early as the lactation stage offers attractive options for modulating adult cardiovascular risk. However, it remains to be established to what extent our findings can be extrapolated to the human situation.

CONCLUSION AND OUTLOOK

The key findings of the work described in this thesis extend our current understanding of how early life factors contribute to functional programming of adult (patho)physiology. By targeting different developmental windows we demonstrated the capacity of the young organism to acquire and retain an active metabolic memory of early life conditions, both for oxidative stress and for dietary cholesterol bioavailability. In either of the two models this was reflected in a long-term alteration of the expression levels of key genes maintaining energy and cholesterol homeostasis, respectively. Our work emphasizes the importance of intrauterine oxidative stress and early postnatal nutrition and its ability to induce sustained changes in the epigenetic makeup of the organism. The next steps in continuation of this research would need to target the identification of epigenetically active nutrient sensors, which translate environmental conditions into altered gene expression conveying the adaptive physiologic response. Although species differences can impede the direct translation of outcomes described in this thesis towards the human condition, our efforts provide insights into long-term consequences of early life metabolic challenges. These insights can become the basis for strategic prevention of cardiometabolic disease by intervening in early life, in addition to the present strategies for treating cardiometabolic disease at adult age.