Programming of adult metabolic health
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Chapter 1

General Introduction &
Scope of this thesis
1. Background

Non-communicable diseases (NCD) account for more than 70% of all deaths worldwide, of which almost half are due to cardiovascular diseases (CVD) and diabetes\(^1\). Among the risk factors contributing to NCDs are an unhealthy diet and physical inactivity. This may result in increased blood pressure, high circulating levels of glucose and fat, and overweight or obesity. These conditions combined are known as the metabolic syndrome\(^2\). The obesity prevalence is a global health problem in both children and adults: it tripled in the last 40 years and is still rising\(^3\).

The risk to develop NCDs might originate in early life. Events during intrauterine or early postnatal life may alter the response to an environmental challenge in the future, thereby possibly increasing the risk of disease later in life, including development of obesity, diabetes and CVD\(^4-6\). The long-term health consequence of early life events is also referred to as ‘developmental programming’ and may affect various body systems and processes\(^6,7\). The influence of events in early life, and the occurrence and manifestation of metabolic diseases differ between males and females and thus sex differences should be taken into consideration for prevention, diagnosis and therapy\(^8\).

From many environmental events it is unclear when and how they may program the risk on metabolic syndrome later in life. Pre- and postnatal nutrient availability during critical developmental periods can program long-lasting changes in gene expression, resulting in altered organ function and growth\(^9\). The long-lasting memory of early life events may occur via epigenetic modifications in chromatin structure and DNA methylation that induce changes in regulation of gene expression\(^10\). Programming has a specific window of sensitivity, which differs depending on the metabolic trait and organism, but is thought to occur predominantly during intrauterine and early postnatal development (Figure 1)\(^11,12\). This thesis describes several studies in model systems related to long-term metabolic effects of a certain early life event.

1.1 Early life environmental factors influencing development

Two environmental factors that conceivably have the potential to program metabolism at adult age are nutrient availability\(^7,9\) and the gut microbiota\(^13-15\). Nutrient availability can directly affect the development of the organism, as well as indirectly, for example by changing the intestinal microbiota composition\(^16,17\). The intestinal microbiota could influence metabolic development via food processing and generation of specific metabolites\(^15,18,19\). The two factors, early
life nutrition and the intestinal microbiota composition are first discussed in more detail.

Figure 1. Developmental programming of metabolism predisposing to the metabolic syndrome. Suboptimal environment inducing developmental programming of cellular energy metabolism in favor of lipid storage. Sensitive (critical) windows are determined by the organogenesis occurring at the time. Abbreviations: ER, endoplasmic reticulum; CVD, cardiovascular disease. Obtained from Symonds et al. (2009)20.

2. Breast milk

2.1 Regulation of breast milk cholesterol
The first source of nutrients for the newborn is either breast milk (BM) or infant milk formula (IMF). The BM exposure during the postnatal period is a potential sensitive window for programming. BM feeding has been associated with long-term health benefits such as lower risk of CVD21, diabetes and obesity22, 23. Some studies demonstrated lower plasma cholesterol and LDL levels in adults that have
been breast-fed as infants\textsuperscript{24, 25}, while others did not observe this long-term effect on plasma cholesterol\textsuperscript{23}. Despite the attempts to produce IMF with a composition as close as possible to that of BM, current infant milk formulas still differ in many aspects from BM. One of the differences is the presence of cholesterol in BM at relatively high levels: 0.23-0.39 mmol/L in human BM versus 0-0.10 mmol/L in IMF\textsuperscript{26-28}. Cholesterol content in milk is not constant during the lactation period\textsuperscript{29}, it decreases in consecutive stages from colostrum to mature milk\textsuperscript{30}.

Cholesterol is an important building block in early life for cell membranes and a precursor for steroid hormones\textsuperscript{25} and bile acids\textsuperscript{31, 32}. The source of BM cholesterol can be either endogenous from maternal stores, from maternal \textit{de novo} cholesterol synthesis or exogenous via dietary intake\textsuperscript{33}. \textit{De novo} cholesterol synthesis in both the liver and the mammary gland are increased during lactation to meet the high cholesterol demand for milk production\textsuperscript{34, 35}. In the blood, cholesterol is transported in lipoproteins such as chylomicrons, very low density lipoproteins (VLDL), LDL and high density lipoproteins (HDL)\textsuperscript{36}. The actual process by which cholesterol enters the milk from the maternal plasma has been elucidated to a lesser extent. There are several proposed mechanisms for active and passive cholesterol transport from the blood to the milk. Active cholesterol transport may occur via membrane cholesterol transporters, by receptor mediated endocytosis and by passive transport via diffusion\textsuperscript{37}. Several lipoprotein receptors are highly expressed on the mammary gland epithelium, such as LDL-, VLDL- and CD36-receptors\textsuperscript{38}. Membrane cholesterol transporters are expressed on the basolateral and apical side of the lactating mammary gland and members of the ABC transporter family (A1, G1, G5, and G8) are also found on the plasma membrane surrounding milk fat globules (MFG)\textsuperscript{39-43}. These cholesterol transporters could play a role in importing or exporting cholesterol from the mammary gland across the basolateral and across the apical membrane, i.e. transport into milk. Milk is formed by the formation of lipid droplets (with cholesterol esters inside) within the secretory pathway, enclosed by a monolayer derived from the ER membrane. The milk fat globules (MFG) are secreted into the milk by taking a part of the double-layer plasma membrane (Figure 2). The resulting, secreted tri-layer MFG membrane is rich in unesterified cholesterol\textsuperscript{44}. Another mechanism of cholesterol transport might be direct secretion of unesterified cholesterol (via ABCG5/8 or ABCA1)\textsuperscript{25}.

The effect of the maternal conditions, such as hypercholesterolemia, due to dietary or genetic means, on cholesterol content in BM has remained unclear. A study conducted in 1976 showed no effect of blood cholesterol levels on concentration of human milk cholesterol\textsuperscript{45}, while five years later Whatley \textit{et al.} (1981)\textsuperscript{46} demonstrated a 2-fold higher milk cholesterol level in rabbits with 100-
fold increase in plasma cholesterol with no change in TG and protein content. More insight into the effects of maternal hypercholesterolemia and cholesterol transporters on BM cholesterol levels would be of interest. If controlled manipulation of BM cholesterol content is feasible, long-term metabolic effects of altered cholesterol availability in a natural setting in early life could be determined.

Figure 2. Potential pathways for cholesterol transfer into milk. ABC transporters at the apical plasma membrane mediate the active transfer of cholesterol to lipid-poor apo-A1 (ABCA1) or HDL (ABCG1) (pathway A). Alternatively, cholesterol could cross the apical plasma membrane by diffusion following the concentration gradient and attach to potential cholesterol acceptors, such as BSA (pathway B). Milk fat globule secretion (pathway C), includes formation of small lipid droplets in the endoplasmic reticulum that then migrate towards the apical membrane as they mature. At the apical membrane, lipid droplets are surrounded by the plasma membrane and then pinched off into the milk. Obtained from Albrecht et al. (2013)\textsuperscript{25}.

2.2 Effects of intestinal cholesterol availability
Relative to body weight, daily cholesterol intake in breast-fed infants is about six times higher than consumption in adulthood\textsuperscript{47}. Intake of dietary cholesterol has various metabolic effects. Upon drinking IMF or BM, gallbladder bile is secreted to aid in fat and vitamin absorption. BM cholesterol consists mainly of free cholesterol and for 5-15\% of cholesterol esters, which need to be hydrolyzed to free cholesterol for solubilization. In adults, the majority of free cholesterol entering the intestine comes from bile and trans-intestinal cholesterol excretion (TICE)\textsuperscript{48}. Whether the same is true in infants is unknown. Bile contains bile acids (produced by hepatic
cholesterol conversion), cholesterol and phospholipids. The detergent function of biliary bile acids allows the formation of micelles which makes intestinal fats (such as cholesterol) transportable for absorption by the enterocytes\textsuperscript{49}. Dietary, biliary and TICE-derived cholesterol is partly (re)absorbed by the cholesterol transporter Niemann-Pick C1-Like1 (NPC1L1)\textsuperscript{50} into the enterocyte. Subsequently cholesterol is either esterified and packaged into chylomicrons\textsuperscript{51}, exported by ABCA1 into HDL lipoproteins\textsuperscript{52}, or re-secreted into the intestinal lumen by ABCG5 and G8. After secretion across the basolateral membrane, the absorbed cholesterol can be delivered to the liver or to peripheral tissues. The intestinal cholesterol uptake by NPC1L1\textsuperscript{50} can be counteracted by re-excretion, back into the intestinal lumen, via the intestinal ABCG5/8 transporter complex\textsuperscript{53}. Cholesterol (re-)absorption can be inhibited by the drug ezetimibe via inhibition of NPC1L1 internalization\textsuperscript{54}. Unabsorbed cholesterol and \textasciitilde5\% of the bile acids which are not reabsorbed per cycle will be excreted as respectively neutral sterols (NS) and bile acids via the feces.

The relatively high cholesterol intake in breast-fed infants has been associated with increased plasma total cholesterol and LDL-levels and decreased \textit{de novo} cholesterol synthesis rates in comparison with formula-fed infants\textsuperscript{28, 55-57}. Plasma levels and \textit{de novo} synthesis rates become similar after weaning\textsuperscript{55, 56}. Finally, adults that have been breast-fed as infants, show slightly lower levels of total and LDL-cholesterol in plasma, compared with those fed with IMF\textsuperscript{24, 25, 57, 58}. A recent study in mice demonstrated that decreased availability of BM cholesterol by maternally administered ezetimibe epigenetically programmed decreased NPC1L1 expression in adulthood, resulting in decreased cholesterol absorption but increased synthesis in adult life\textsuperscript{59}. It is unknown whether this effect is limited to reduced cholesterol availability during lactation. Studying the sensitive window (\textbf{Figure 1}) of programming adult cholesterol absorption could provide the information for the timing to develop potential preventive intervention strategies against CVD risks.

3. Intestinal microbiome

3.1. Microbiota establishment
Positive health effects associated with long breastfeeding duration, such as decreased need for antibiotics after weaning and lower BMI, have been related to the intestinal microbiota, since these associations were not present in infants with antibiotic exposure before weaning or short breastfeeding duration\textsuperscript{60}. Establishing a healthy intestinal microbiome is important for the offspring, since perturbations during early development may cause metabolic disturbance\textsuperscript{61}. The microbial
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colonization of a neonate is affected by the composition of its nutrition, such as oligosaccharide content and composition. Specific human milk oligosaccharides have been associated with a microbiota-dependent improved lean body mass gain and liver metabolism capable of utilizing nutrients for anabolism\(^62\). The composition of the intestinal microbiome is also influenced by genetic factors of the host, antibiotic exposure, and the transfer of microbiota from the mother and the environment before, during, and after delivery\(^63-65\).

3.2. Microbial programming of metabolic health

Bacteria in the gut could affect the host metabolic system via direct and indirect biological mechanisms\(^66, 67\). Mice without a microbiome (germ-free mice) on a Western-type diet are less prone to weight gain than mice with a microbiome (conventional mice)\(^68\). The weight gain in conventional mice is related to a microbiota-dependent increase in dietary energy extraction from the food and a stimulation of lipogenesis\(^68\). Obese individuals have microbiota compositions with increased energy extraction from food as compared to lean individuals\(^69, 70\). The obesity phenotype can be induced when microbiota is transferred from obese mice or humans to lean mice\(^69, 71, 72\). The obesity phenotype correlated with differences in microbial metabolite production, microbial bile acid transformation and bile acid-related hepatic gene expression\(^71, 73\), demonstrating that altered microbiota can change the metabolic state. Vice versa, both fecal transfer from lean mice and antibiotic treatment can diminish diet-induced metabolic syndrome parameters\(^74\). Also in humans transferring microbiota from lean individuals to individuals with metabolic syndrome transiently improves metabolic syndrome parameters such as insulin sensitivity and metabolites produced by the microbiota\(^75, 76\). These data demonstrate the important direct role for the microbiota on host metabolism, and the metabolic consequences when the microbiota composition is disturbed.

Research on (epigenetic) programming of long-term metabolic homeostasis by the microbiota in early life is scarce\(^19\). Most research focuses on programmed microbiota (environmental effects that program/affect long-term microbial composition)\(^77, 78\), and on direct or long-term effects of permanently altered microbiota composition\(^64, 65, 79, 80\). Investigating the role of microbiota in early life on the function of the host metabolic system in the long-term would aid in understanding the mechanisms of microbiota-host interaction. Microbiota interactions in early life do appear critical for metabolism later in life\(^13\). Short-term antibiotic exposure in mice during the end of gestation and during lactation changed the microbiota composition transiently, but had long-term metabolic consequences which were similar to those observed upon prolongation of the
antibiotic exposure. The increased lean and fat mass effect could be reproduced by transfer of the antibiotic-exposed microbiota to young germ-free female mice. Body weight increase differed between male and female mice, indicating that sexual dimorphism also plays a role. These data suggest that antibiotic-induced metabolic changes can be conveyed by microbiota and that the sensitive window for these changes rests in early development up to lactation. Lactation is a critical period for epigenetic development in intestinal stem cells, likely guided and facilitated by the microbiota, and possibly affecting long-term metabolic health.

3.3. Microbiota-cholesterol interactions
The metagenome of the intestinal microbiome encodes for enzymes that differ from enzymes from human and rodent cells. Specific microbial enzymes convert and produce metabolites that would otherwise not be available to the host, such as short chain fatty acids (SCFA) from food, neutral sterols (NS) from cholesterol, and secondary bile acids from primary bile acids. Cholesterol can be (re)absorbed, but coprostanol, the main NS produced, is a poorly absorbed sterol in the human intestine and thus excreted into the feces. The ratio of cholesterol-to-coprostanol conversion is dependent on the microbiota composition as well as age and sex. Studies in germ-free mice have demonstrated that absence of gut microbiota alters cholesterol metabolism and protects against diet-induced weight gain and insulin resistance. Germ-free mice challenged with a high-fat diet show reduced hypercholesterolemia and increased fecal cholesterol excretion compared with conventional mice. Studies in pigs showed that specific bacteria can have a substantial effect on cholesterol metabolism. Administration of L. ophulus or L. casei with B. longum reduced total serum cholesterol in hypercholesterolemic pigs via bile acid modification. Administration of the bacterium L. rhamnosus altered microbiome composition, increased SCFA production, increased hepatic 3-Hydroxy-3-Methylglutaryl-CoA Reductase (Hmgcr) and Ldlr expression and reduced levels of plasma cholesterol.

3.4. Microbiota-bile acid interactions
Hepatic cholesterol can be converted into primary bile acids, starting with 7α-hydroxylation by the rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1) or sterol-27-hydroxylase (CYP27A1) and oxysterol 7α-hydroxylase (CYP7B1) (Figure 3) (As reviewed in). In humans the primary bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA), while in mice CDCA is further converted to the muricholic acids αMCA and βMCA. Bile acid conjugation with the amino acids glycine (predominantly in humans) or taurine (predominantly in mice) enhances the hydrophilicity and the functional detergent properties
of the molecule at the pH in the small intestine. Bile acids are secreted in bile, together with cholesterol and phospholipids, into the duodenum. Bile acids can damage bacterial cell membranes and induce both bacterial and mammalian DNA and protein damage. The bacterial enzyme *bile salt hydrolase* (BSH) can deconjugate the taurine or glycine from the bile acids. This reaction provides the bacterium with nitrogen, sulphur and carbon atoms and simultaneously reduces...
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the detergent antimicrobial effect of the bile acid\textsuperscript{91}. Deconjugated bile acids can be further modified into secondary bile acids by microbial enzymes, can enter the enterohepatic circulation upon reabsorption by the host in the distal small intestine or, passively, in the colon, and return to the liver for re-conjugation. Alternatively, bile acids may escape reabsorption and thus be excreted via the feces.

Secondary bile acids have different physicochemical properties than primary bile acids. Their increased hydrophobicity is associated with a higher detergent activity and thus cytotoxicity for the host. Besides their antimicrobial function, bile acids are also signaling molecules. Bile acids can activate the farnesoid X receptor (FXR) and takeda G receptor 5 (TGR5), thereby regulating lipid, glucose and energy homeostasis\textsuperscript{92}. More hydrophobic bile acids such as CA and CDCA are potent activators of FXR, while more hydrophilic bile acids are less potent\textsuperscript{89}. Muricholic acids are identified as FXR antagonists\textsuperscript{93, 94}. Activation of intestinal FXR induces the production and secretion of fibroblast growth factor 15/19 (FGF15 in mice, FGF19 in humans), which can activate the hepatic FGF receptor 4 to inhibit \textit{Cyp7a1} and thereby bile acid synthesis\textsuperscript{92}. Bile acid related signaling can also lead to changes in lipid and lipoprotein metabolism, glucose homeostasis, energy expenditure and bacterial growth (as reviewed in\textsuperscript{95}).

Bile acids affect cholesterol homeostasis directly in the intestine. Hydrophobic bile acids (such as CA) stimulate cholesterol absorption, while hydrophilic bile acids (such as TMCA) inhibit cholesterol absorption\textsuperscript{96}. The human bile acid pool consists of mainly CA:CDCA:DCA (±2:2:1 ratio) and is more hydrophobic and has been linked to gallstone formation, in contrast to the hydrophilic murine bile acid pool which consists mainly of CA:αMCA/βMCA (±3:2 ratio)\textsuperscript{96}.

4. Sexual dimorphism

Metabolism is differentially regulated in males and females due to genetics, pre-pubertal testosterone-induced programming and sex hormone signaling after puberty\textsuperscript{97}. Sex differences in hormones drive sexual dimorphism in microbiota composition\textsuperscript{98-101}. Also the bile acid composition shows sex specificity in conventional mice after puberty, but not in germ-free mice\textsuperscript{102-104}. This indicates that there might be a sex-specific role for the microbiota in forming the bile acid composition. Indeed, there is an interaction between bile acids, microbiota and metabolism and this interaction is FXR-dependent and sex-specific\textsuperscript{105}. In conclusion, dysbiosis of the microbiota, as induced by diet, antibiotics or other interventions, can trigger NCD which manifest differently in males and females (as
reviewed in\textsuperscript{8,106}). Finally, in chapter 6 we discuss the most relevant findings of this thesis and our interpretation of underlying mechanisms in early life programming of adult metabolic responses, as well as proposed future steps.
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5. Scope of the thesis

Nutrition and microbiota are of great importance for early life development and have been implicated in the risk to develop metabolic syndrome-related disease later in life. The overlapping scope of this thesis is: “How do specific interventions in nutrition and microbiota in early life affect the risk to develop metabolic syndrome symptoms later in life?”. The intervention strategies in this thesis focus on the early life stage, more specifically gestation, lactation or early post-lactation. To address the scope and to be able to study long-term effects on the whole organism we used mouse models.

Cholesterol intake in early life is high when infants are breast-fed. Little is known, however, about the regulation of BM cholesterol levels. BM intake is considered beneficial for long-term metabolic health and possibly limits cardiovascular disease risk. Since the basic relationship between maternal cholesterol levels and BM cholesterol remains unclear, we set out to determine the origin and regulation of murine milk cholesterol levels (chapter 2). We determined the relationship between BM cholesterol content in different models of maternal hypercholesterolemia, induced by dietary means and/or genetic manipulation.

The stable cholesterol levels in breastmilk found in chapter 2 may indicate a role for cholesterol in offspring development. A former study has shown that a drug-imposed decrease in cholesterol bioavailability during lactation epigenetically decreased cholesterol absorption up to adulthood. It has remained unclear, however, to what extent the sensitive window would perhaps extend to the early post-weaning period. In chapter 3 we investigated whether the sensitive window for programming decreased cholesterol absorption extends beyond the lactation period by decreasing cholesterol availability during the first three weeks post-weaning.

As discussed above, the intestinal microbiome constitutes another factor in early life that influences the metabolic system. Gut microbiota composition shows sex related differences in humans and mice. In the distal small intestine and colon, bacterial enzymes can deconjugate and convert bile acids into unconjugated, secondary bile acids. Like microbiota composition, bile acid composition also shows sexual dimorphism in humans and mice. Interestingly, germ-free mice did not show this difference in bile acid composition. In chapter 4 we investigated how the sex of a microbiota donor affected bile acid dynamics in murine hosts of the same or opposing sex.
As stated, the microbiota composition constitutes an environmental factor that conceivably influences early life metabolic development. Microbiota colonization starts in utero and its development is affected by genetics, early life nutrition, and other environmental factors such as antibiotic exposure\textsuperscript{115, 116}. Intestinal bacteria can have several effects on the host: they convert bile acids and thereby influence bile acid signalling and they produce specific metabolites from available nutrients in the intestines\textsuperscript{117-119}. Through direct and indirect effects the gut microbiota influences host glucose and lipid metabolism and body composition\textsuperscript{32}. Research has shown long-term effects of early life microbiota disturbance\textsuperscript{13, 116}. An extreme manipulation in early life microbiota influence would be the complete absence of a microbiome. To assess the potential effects of this extreme manipulation on metabolic programming, we determined in \textbf{chapter 5 the effect of early life absence of microbiota on metabolic parameters later in life, during a dietary challenge with Western-type diet in adulthood.}

Finally, in \textbf{chapter 6} we discuss the most relevant findings of this thesis and our interpretation of underlying mechanisms in early life programming of adult metabolic responses, as well as proposed future steps.