Programming of adult metabolic health

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PROGRAMMING OF ADULT METABOLIC HEALTH

THE ROLES OF DIETARY CHOLESTEROL AND MICROBIOTA IN EARLY LIFE

Mirjam A.M. Lohuis

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CONTENT

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, diabetesand 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 availability7, 9 and the gut microbiota13-15. Nutrient availability can directly affect the development of the organism, as well as indirectly, for example by changing the intestinal microbiota composition16, 17. The intestinal microbiota could influence metabolic development via food processing and generation of specific metabolites15, 18, 19. The two factors, early

life nutrition and the intestinal microbiota composition are first discussed in more detail.

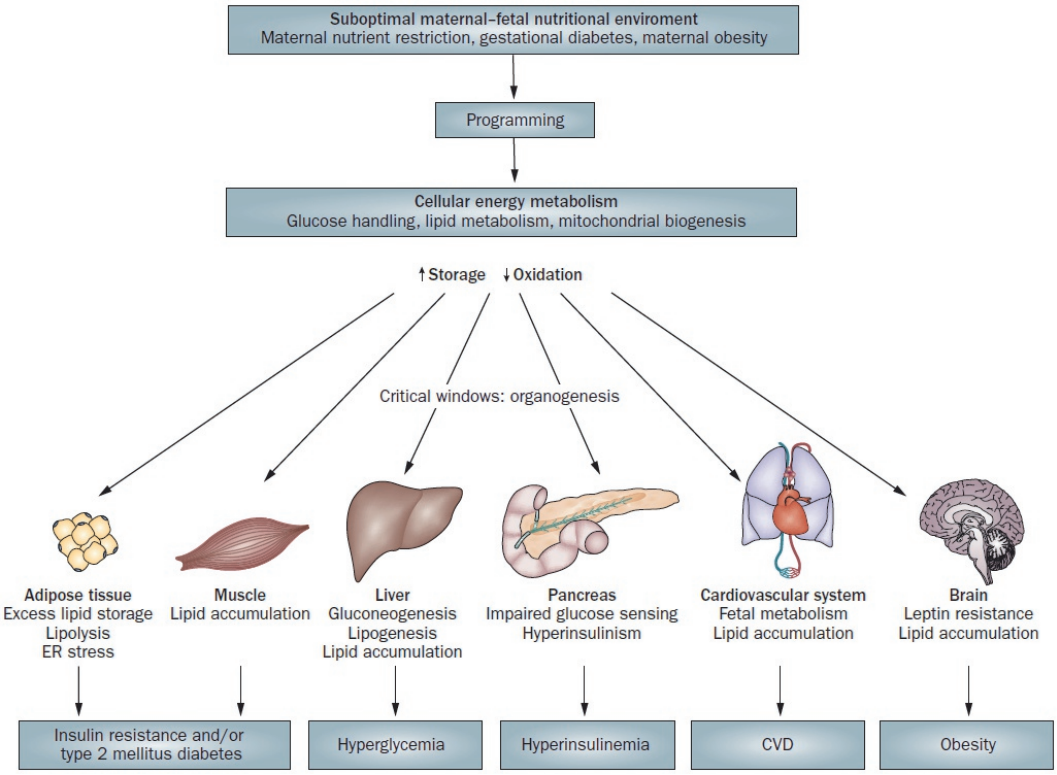

Figure 1. Developmental programming of metabolism predisposing to the metabolic syndrome. Suboptimal environment inducing developmental programming of cellular energy metabolism in favor of lipid storage. Sensitive (critical) windows are determined by the organogenesis occurring at the time. Abbreviations: 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 CVD[21], diabetes and obesity[22, 23]. Some studies demonstrated lower plasma cholesterol and LDL levels in adults that have

been breast-fed as infants[24, 25], while others did not observe this long-term effect on plasma cholesterol[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[26-28]. Cholesterol content in milk is not constant during the lactation period[29], it decreases in consecutive stages from colostrum to mature milk[30].

Cholesterol is an important building block in early life for cell membranes and a precursor for steroid hormones[25] and bile acids[31, 32]. The source of BM cholesterol can be either endogenous from maternal stores, from maternal *de novo* cholesterol synthesis or exogenous via dietary intake[33]. *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[34, 35]. In the blood, cholesterol is transported in lipoproteins such as chylomicrons, very low density lipoproteins (VLDL), LDL and high density lipoproteins (HDL)[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[37]. Several lipoprotein receptors are highly expressed on the mammary gland epithelium, such as LDL-, VLDL- and CD36-receptors[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)[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[44]. Another mechanism of cholesterol transport might be direct secretion of unesterified cholesterol (via ABCG5/8 or ABCA1)[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[45], while five years later Whatley *et al*. (1981)[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.

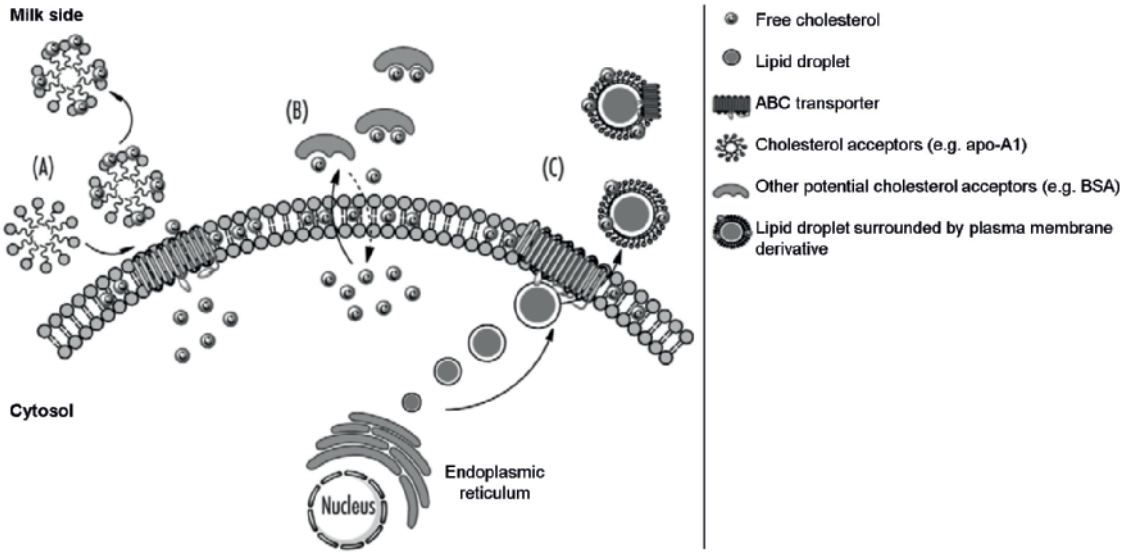

Figure 2. Potential pathways for cholesterol transfer into milk. ABC transporters at the apical plasma membrane mediate the active transfer of cholesterol to lipid-poor apo-A1 (ABCA1) or HDL (ABCG1) (**pathway A**). Alternatively, cholesterol could cross the apical plasma membrane by diffusion following 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)[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[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)[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[49]. Dietary, biliary and TICE-derived cholesterol is partly (re)absorbed by the cholesterol transporter Niemann-Pick C1-Like1 (NPC1L1)[50] into the enterocyte. Subsequently cholesterol is either esterified and packaged into chylomicrons[51], exported by ABCA1 into HDL lipoproteins[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[50] can be counteracted by re-excretion, back into the intestinal lumen, via the intestinal ABCG5/8 transporter complex[53]. Cholesterol (re-)absorption can be inhibited by the drug ezetimibe via inhibition of NPC1L1 internalization[54]. Unabsorbed cholesterol and ~5% 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 *de novo* cholesterol synthesis rates in comparison with formula-fed infants[28, 55-57]. Plasma levels and *de novo* synthesis rates become similar after weaning[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[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[59]. It is unknown whether this effect is limited to reduced cholesterol availability during lactation. Studying the sensitive window (**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[60]. Establishing a healthy intestinal microbiome is important for the offspring, since perturbations during early development may cause metabolic disturbance[61]. The microbial

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 mechanisms66, 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 lipogenesis68. Obese individuals have microbiota compositions with increased energy extraction from food as compared to lean individuals69, 70. The obesity phenotype can be induced when microbiota is transferred from obese mice or humans to lean mice69, 71, 72. The obesity phenotype correlated with differences in microbial metabolite production, microbial bile acid transformation and bile acid-related hepatic gene expression71, 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 parameters74. 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 microbiota75, 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 scarce19. 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 composition64, 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 life13. 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 exposure13. The increased lean and fat mass effect could be reproduced by transfer of the antibiotic-exposed microbiota to young germ-free female mice13. 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 health81.

3.3. Microbiota-cholesterol interactions

The metagenome of the intestinal microbiome encodes for enzymes that differ from enzymes from human and rodent cells[31]. 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[31, 82]. 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[83]. The ratio of cholesterol-to-coprostanol conversion is dependent on the microbiota composition[84] as well as age and sex[83]. 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[68, 85]. Germ-free mice challenged with a high-fat diet show reduced hypercholesterolemia and increased fecal cholesterol excretion compared with conventional mice[85, 86]. Studies in pigs showed that specific bacteria can have a substantial effect on cholesterol metabolism[87, 88]. Administration of *L. ophilus* or *L.casei* with *B. longum* reduced total serum cholesterol in hypercholesterolemic pigs via bile acid modification[87]. 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[88].

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[31, 32]). In humans the primary bile acids are cholic acid (CA) and chenodexoxycholic acid (CDCA), while in mice CDCA is further converted to the muricholic acids αMCA and ßMCA[89]. 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[90]. 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

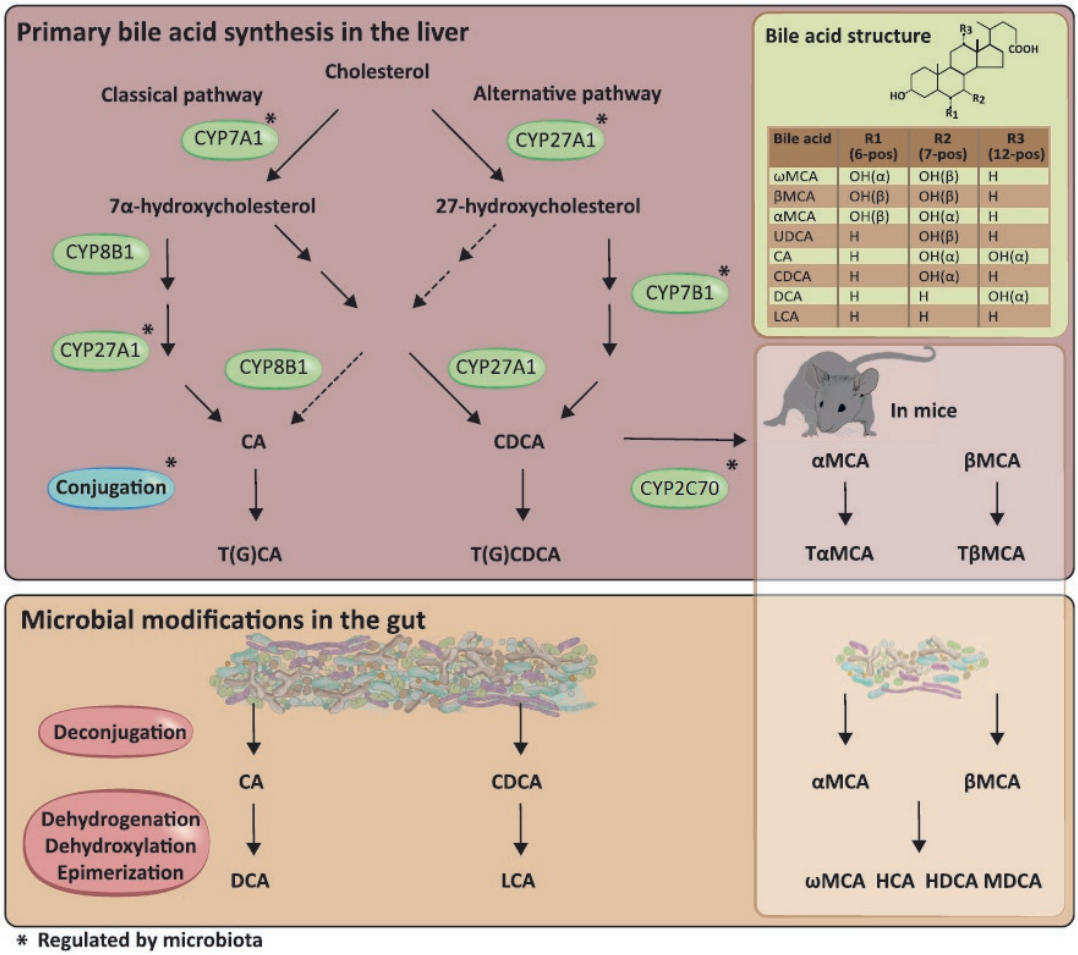

Figure 3. Hepatic bile acid synthesis and microbial conversion. Schematic representation of primary bile acid synthesis pathways in the liver (upper panel) and microbial conversion to secondary bile acids in the intestine (lower panel). Insert top right: table summarizing sites of hydroxylation on steroid nucleus of most common bile acid species. Insert bottom right: murine primary bile acid species that differ from humans. * enzymes or reaction steps regulated by microbiota. G, glycine-conjugated species; T, taurine-conjugated species. Adapted from Wahlstrom *et al.* (2016)[32].

the detergent antimicrobial effect of the bile acid[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[92]. More hydrophobic bile acids such as CA and CDCA are potent activators of FXR, while more hydrophilic bile acids are less potent[89]. Muricholic acids are identified as FXR antagonists[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 *Cyp7a1* and thereby bile acid synthesis[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[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[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/ßMCA (±3:2 ratio)[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[97]. Sex differences in hormones drive sexual dimorphism in microbiota composition[98-101]. Also the bile acid composition shows sex specificity in conventional mice after puberty, but not in germ-free mice[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[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[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.

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[24, 25]. 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[59]. It has remained unclear, however, to what extent the sensitive window would perhaps extent 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[61, 107]. Gut microbiota composition shows sex related differences in humans and mice[98, 101, 104, 108-111]. 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[112-114]. Interestingly, germ-free mice did not show this difference in bile acid composition[102-104]. 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[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[117-119]. Through direct and indirect effects the gut microbiota influences host glucose and lipid metabolism and body composition[32]. Research has shown long-term effects of early life microbiota disturbance[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 **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 **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.

Chapter 2

Milk cholesterol concentration in mice is not affected by high cholesterol diet- or genetically-induced hypercholesterolaemia

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Abstract

SCOPE: Breast milk cholesterol content may imply to affect short- and long-term cholesterol homeostasis in the offspring. However, mechanisms of regulating milk cholesterol concentration are only partly understood.

METHODS AND RESULTS: We used different mouse models to assess the impact of high cholesterol diet (HC)- or genetically-induced hypercholesterolaemia on milk cholesterol content. At day 14 postpartum we determined milk, plasma and tissue lipids in wild type (WT), LDL receptor knockout (*Ldlr*-/-), and ATP-binding cassette transporter G8 knockout (*Abcg8*-/-) mice fed either low- or 0.5% HC diet. In chow-fed mice, plasma cholesterol was higher in *Ldlr-/-* dams compared to WT. HC-feeding increased plasma cholesterol in all three models compared to chow diet. Despite the up to 5-fold change in plasma cholesterol concentration, the genetic and dietary conditions did not affect milk cholesterol levels. To detect possible compensatory changes, we quantified *de novo* cholesterol synthesis in mammary gland and liver, which was strongly reduced in the various hypercholesterolaemic conditions.

CONCLUSIONS: Together, these data suggest that milk cholesterol concentration in mice is not affected by conditions of maternal hypercholesterolaemia and is maintained at stable levels via ABCG8- and LDLR-independent mechanisms. The robustness of milk cholesterol levels might indicate an important physiological function of cholesterol supply to the offspring.

Introduction

Breast milk contains high levels of cholesterol (0.23 - 0.39 mmol/L) in contrast to most infant formulas (0 - 0.10 mmol/L)[26-28]. The relatively high cholesterol concentration in breast milk has been suggested to have a lasting impact on the cholesterol homeostasis of the offspring[26, 120]. Breast-fed offspring has high plasma cholesterol levels in early life, but lower plasma cholesterol in adulthood, compared to formula-fed individuals[24, 26]. The lower plasma cholesterol concentrations in adulthood may relate to long-term cardio-protective effects of breast milk, in accordance with the metabolic programming hypothesis[26, 121]. Additionally, we recently demonstrated that maternal ezetimibe-induced lower dietary cholesterol bioavailability during the lactation period in mice decreases cholesterol absorption in the offspring up to adulthood through decreased intestinal NPC1L1 expression[59].

The mechanisms involved in the regulation of milk cholesterol concentration are only partly understood. With the recent cardiometabolic disease pandemic, dyslipidaemia and disturbances in lipid homeostasis are becoming increasingly common conditions in pregnant and lactating women[122, 123]. Maternal hypercholesterolaemia during gestation has been associated with increased plasma cholesterol in the fetus[124, 125]. However, it remains unclear to what extent maternal hypercholesterolaemia, either caused by genetic or dietary factors, impacts cholesterol transport across the mammary gland and affects cholesterol concentration in milk with possible effects in the offspring.

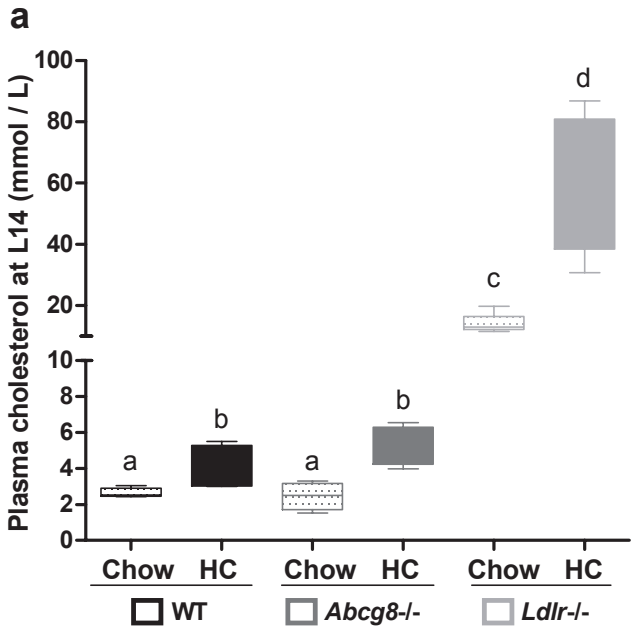
Cholesterol in milk can originate from different sources. The predominant fraction of cholesterol reaches the milk via plasma[35]: either from preformed stores, from dietary origin or from *de novo* synthesis in either the mammary gland epithelium cells[38, 126] or the liver[35]. The detailed transport route by which cholesterol in the circulation is taken up by the mammary gland has not been identified. There have been reports suggesting an ApoB-mediated uptake of cholesterol-containing lipoproteins[127]. Several receptors for uptake of cholesterol-rich apolipoprotein B-containing lipoproteins are abundantly expressed in the mammary epithelial cells, amongst which LDL-, VLDL- and CD36-receptors[38]. Other lipoproteins found in plasma, like the high-density lipoproteins, may serve as an alternative source for cholesterol uptake since scavenger receptors from the CD36 family are also expressed in the mammary epithelium[128]. In addition, mammary gland epithelial cells express cholesterol efflux transporters, such as ATP-binding cassette (ABC) transporters ABCG5/ABCG8, ABCA1, and ABCG1, whose expressions fluctuate depending on lactation stage[39, 41, 129] and could possibly impact cholesterol levels in the milk.

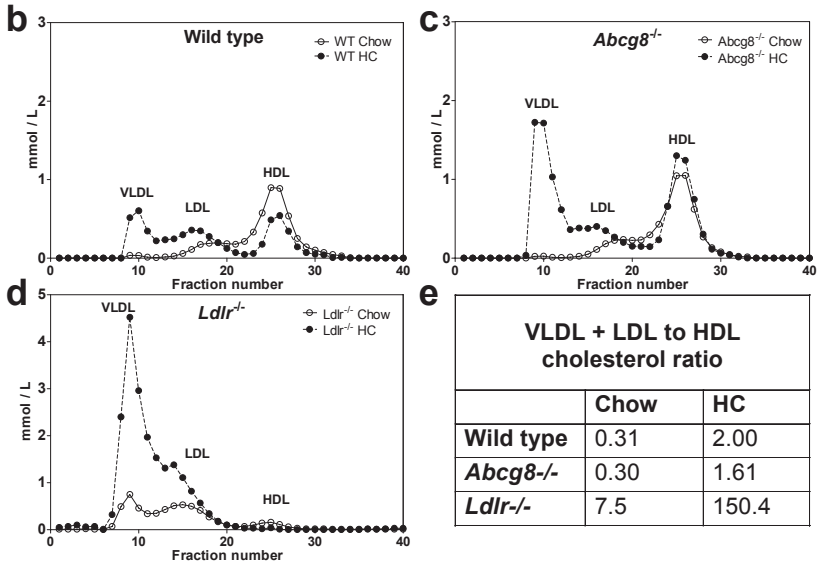
We aimed to address the relationship between maternal hypercholesterolaemia and milk cholesterol concentration in mouse models. We analysed milk cholesterol concentrations in lactating mice with hypercholesterolaemia of different severity, induced by dietary and/or genetic manipulations. The dietary means to manipulate plasma cholesterol concentrations consisted of feeding a high-cholesterol diet (0.5 % w/w), while genetic manipulation involved the ablation of either the *Abcg8* or the *Ldlr* gene. The ABC cassette G8 protein is a cholesterol transporter primarily expressed on the apical membrane of hepatocytes and enterocytes, where it facilitates export of cholesterol[130]. Interestingly, *Abcg8* is also moderately expressed in the lactating bovine mammary gland and in the murine mammary gland, as demonstrated in literature and online databases [41, 129, 131]. The LDL-receptor is the dominant transport protein involved in the uptake of apoB100-containing lipoproteins from the plasma[132], and highly expressed in murine mammary gland[131]. Humans with genetic loss of LDLR function have a severe hypercholesterolaemia that is further increased upon dietary cholesterol exposure[133]. We assessed the potential relevance of cholesterol secretion into milk via the ABCG8 transporter and via mammary gland uptake of cholesterol via the LDL receptor. To assess possible variation in the origin of milk cholesterol in the different models of hypercholesterolaemia, we measured *de novo* cholesterol synthesis in the liver and mammary gland, using deuterated water methodology.

Results

High-cholesterol diet increases plasma and hepatic cholesterol levels

To assess the isolated effect of ABCG8- or LDLR-deficiency we first measured cholesterol levels in plasma of dams on a chow diet. While ABCG8-deficiency did not affect basal plasma cholesterol, the LDLR-deficient dams displayed marked hypercholesterolaemia (5.2-fold change, $p < 0.01$, **Fig. 1a**), mostly due to increased cholesterol levels in LDL and VLDL (**Fig. 1b-d**). Feeding the dams high cholesterol (HC) diet increased the levels of total plasma cholesterol in all models (**Fig. 1a**). The size of the effect reached maximum in the *Ldlr*-/- mice (4.8-fold change, $p < 0.01$) followed by *Abcg8-/-* (2-fold change, $p < 0.05$*)* and wild-type (1.5-fold change, $p < 0.05$). On chow diet, hepatic cholesterol concentration corresponded with the differences in the plasma cholesterol levels: similar levels in wild-type and *Abcg8* knockout mice and 0.6-fold higher in LDLR-deficient mice ($p < 0.01$). The HC diet increased the cholesterol accumulation in the hepatic tissues of all dams ($p < 0.05$, **Fig. 2**). On the HC diet, however, the hepatic cholesterol concentrations did not differ significantly between the three models.





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VLDL + LDL to HDL cholesterol ratio		
	Chow	**HC**
Wild type	0.31	2.00
Abcg8-/-	0.30	1.61
Ldlr-/-	7.5	150.4

Figure 1: Plasma lipids. a) Total plasma cholesterol levels were measured in whole plasma using a commercially available enzymatic assay (WT Chow, n = 5; WT HC, n = 5; *Abcg8* -/- Chow, n = 4; *Abcg8* -/- HC, n = 5; *Ldlr* -/- Chow, n = 8; *Ldlr* -/- HC, n = 5). Data are presented as median and interquartile range (Tukey). Statistical significance was tested with Kruskal-Wallis post-hoc Conover-Inman; non-different groups share a letter. The threshold of significance was $p<0.05$. **b-d)** Cholesterol in lipoprotein fractions following separation by FPLC of pooled plasma samples and **e)** VLDL+LDL to HDL cholesterol ratios calculated from these results (WT Chow, n = 5; WT HC, n = 5; *Abcg8* -/- Chow, n = 4; *Abcg8* -/- HC, n = 5; *Ldlr* -/- Chow, n = 4; *Ldlr* -/- HC, n = 4). □: low cholesterol diet (Chow); ■: high cholesterol diet (HC).

Milk cholesterol levels are independent of plasma, liver and mammary gland cholesterol levels

We then determined whether the hypercholesterolaemia was associated with increased cholesterol content of the mammary glands. On chow diet there were no differences in mammary cholesterol content between genotypes, despite the significantly increased plasma cholesterol levels in *Ldlr-/-* mice (**Fig. 3a**). The HC diet did not increase mammary cholesterol content in the WT mice, in contrast to the *Abcg8* and *Ldlr* knockout mice (+39 %, $p < 0.05$; and +62 %, $p < 0.01$ respectively; **Fig. 3a**). Interestingly, the HC diet-induced hypercholesterolaemia did not affect the milk cholesterol concentrations in any of the three models, with milk cholesterol levels ranging between 1.7-2.3 mM (interquartile range) (**Fig. 3b**).

In order to analyse the possible association between milk cholesterol levels and plasma and mammary gland cholesterol levels and nest size, we performed regression analysis. Nest sizes (range: 2-8 pups) were not correlated with milk cholesterol levels. Cholesterol levels in mammary gland tissue were strongly and positively related to plasma cholesterol levels in WT and *Abcg8-/-* mice, (WT $r^2 = 0.54$, $p = 0.016$; *Abcg8-/-* $r^2 = 0.61$, $p = 0.013$; *Ldlr-/-* $r^2 = 0.51$, $p = 0.0096$). In none of the three groups were plasma and milk cholesterol levels significantly correlated. Ratios of VLDL+LDL to HDL cholesterol as calculated from FPLC fractions (**Fig. 1e**) were also unrelated to milk cholesterol levels.

***De novo* cholesterol synthesis is strongly decreased in high cholesterol-fed mice**

The increased plasma, hepatic and mammary gland cholesterol levels in the hypercholesterolaemic models did not translate into increased milk cholesterol concentrations. We then tested the possibility that the stable concentrations were obtained by suppression of systemic or local cholesterol synthesis. In all chow-fed groups there was *de novo* hepatic and mammary gland cholesterol synthesis (**Fig. 4**). Feeding the HC diet strongly reduced the cholesterol synthesis rate in liver (**Fig. 4a**) and mammary gland (**Fig. 4b**) in all three models.

Next, we used linear regression analysis to assess the possible relationship between the *de novo* synthesis in mammary gland and the milk cholesterol concentration. The milk cholesterol levels did not correlate with the fraction of *de novo* synthesized cholesterol in mammary gland in any of the three groups (WT $r^2 = 0.03$, $p = 0.66$; *Abcg8-/-* $r^2 = 0.07$, $p = 0.51$; *Ldlr-/-* $r^2 = 0.05$, $p = 0.64$).

Discussion

We addressed the relationship between maternal hypercholesterolaemia, induced by dietary or genetic means, and milk cholesterol concentrations in mice. Our data demonstrate that milk cholesterol concentration is not affected by induction of severe hypercholesterolaemia and increased cholesterol levels in liver and mammary gland. Clearly, the ABC-cassette transporter ABCG8 and the LDL receptor do not have a critical role in defining milk cholesterol concentration, since their inactivation did not change it. Our data demonstrate the apparent robustness of milk cholesterol levels, which could support important physiological functions for the offspring.

The milk cholesterol concentration was not affected by genetic inactivation of two candidate genes with a possible role in cholesterol transport towards milk, nor by high cholesterol diet-induced hypercholesterolaemia. This observation indicates that either the gene products are not involved, or that alternative transporting mechanisms ensure redundancy in the supply of cholesterol destined for secretion into the milk.

The hypothesis that the LDL receptor is involved in milk cholesterol transport was based on findings describing an association between lactation and increased mammary gland expression of *LDLR* in human subjects[38] and high LDLR

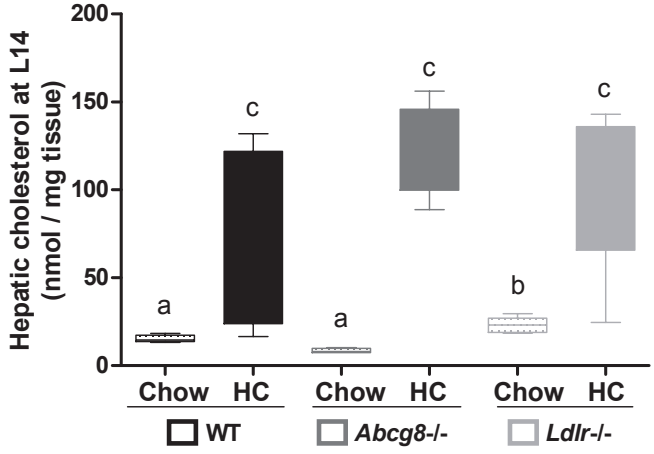

Figure 2: Hepatic cholesterol levels. Hepatic lipids were extracted according to Bligh & Dyer and measured by gas chromatography (WT Chow, n = 5; WT HC, n = 5; *Abcg8* -/- Chow, n = 4; *Abcg8* -/- HC, n = 5; *Ldlr* -/- Chow, n = 8; *Ldlr* -/- HC, n = 5). Data are presented as median and interquartile range (Tukey). □: low cholesterol diet (Chow); ■: high cholesterol diet (HC). Statistical significance was tested with Kruskal-Wallis post-hoc Conover-Inman; non-different groups share a letter. The threshold of significance was $p < 0.05$.

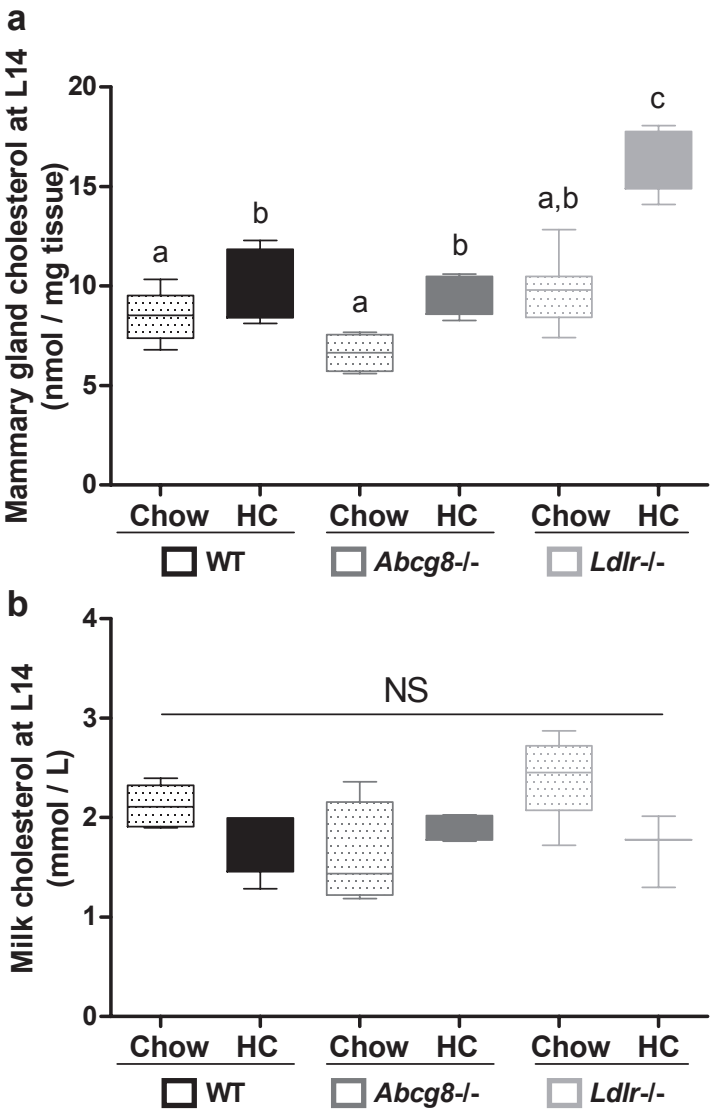

Figure 3: Mammary gland and milk cholesterol. a) The lipid content of mammary tissue was extracted according to Bligh & Dyer and measured by gas chromatography (WT Chow, n = 5; WT HC, n = 5; *Abcg8* -/- Chow, n = 4; *Abcg8* -/- HC, n = 5; *Ldlr* -/- Chow, n = 8; *Ldlr* -/- HC, n = 5). **b)** Milk samples were obtained after i.p. injection with 1 IU oxytocin by using a modified electric human breast pump. Milk lipids were extracted according to Bligh & Dyer and cholesterol was quantified by gas chromatography (WT Chow, n = 5; WT HC, n = 5; *Abcg8* -/- Chow, n = 4; *Abcg8* -/- HC, n = 4; *Ldlr* -/- Chow, n = 6; *Ldlr* -/- HC, n = 3). Data are presented as median and interquartile range (Tukey). □: low cholesterol diet (Chow); ■: high cholesterol diet (HC). Statistical significance was assessed with Kruskal-Wallis post-hoc Conover-Inman test; non-different groups share a letter. The threshold of significance was $p < 0.05$.

expression in the murine mammary gland[131]. In addition, lactation in rodents is characterized by an increase in circulating LDL[134], compatible with a role for the low-density lipoproteins as a source for milk cholesterol. Our data indicate that uptake of cholesterol by the mammary gland can be conducted quantitatively by LDLR-independent mechanism(s). We cannot exclude that an alternative, LDLR-independent mechanism involves an alternative receptor for LDL uptake. In support of this notion, radioactivity studies in mice have shown the transfer of ApoB100 across the mammary epithelium towards the milk to take place at the same extent in both wild-type and LDLR-deficient mice[127]. Possibly VLDL and LRP receptors[135], or even CD36[136] can substitute for LDLR-deficiency. The hypothesis that ABCG8 is involved in milk cholesterol transport rests on the increased expression levels of the heterodimer ABCG5/ABCG8 in lactating bovine mammary glands[41, 129], and appreciable expression of ABCG8 in murine mammary gland[131]. In hepatocytes and intestinal epithelial cells the ABCG5/ABCG8 dimer is expressed at the apical membrane[130] where it is essential for the export of free cholesterol towards the bile and intestinal lumen,

respectively[137]. Our data, however, does not support a critically important role for ABCG8 in the process of cholesterol efflux across the mammary gland epithelium. The unchanged plasma cholesterol levels in Abcg8-/- mice on chow may be related to the fact that the diet used contained no cholesterol. Apparently neither the LDL receptor nor ABCG8 is crucial for cholesterol transport towards milk in our experimental setup. In order to further explore the mechanistic effects of genetic ablation of LDLR and ABCG8 on milk production, additional studies in an *in vitro* model would be helpful. Unfortunately, however, there is no established reliable *in vitro* system for lactating mammary gland cells available to study alveolar mammary gland epithelial cell cholesterol transfer[138].

De novo cholesterol synthesis has been shown to contribute to milk cholesterol[35]. For the dams, cholesterol demand is increased during lactation, corresponding with increased expression of cholesterol synthesis genes in both liver and mammary glands of bovines, rodents and humans[34, 38, 126]. We found 12-fold higher fractional cholesterol synthesis rates in liver compared to mammary gland, which is in agreement with previous studies demonstrating a larger contribution to milk cholesterol originating from hepatic than from mammary synthesis[35]. The lower mammary gland cholesterol synthesis compared with hepatic synthesis also corresponds to the expression levels of the *Hmgcr* gene in the two tissues, encoding for the rate-limiting enzyme of cholesterol synthesis[126].

In each of the three murine genotypes, dietary cholesterol supplementation strongly decreased *de novo* cholesterol synthesis in liver and in mammary gland, similarly to observations in rats[139]. The decreased *de novo* synthesis rates in

liver and mammary gland, however, did not decrease milk cholesterol levels. The cholesterol synthesis rate is apparently not a critical driver for the amount of cholesterol secreted into milk. Rather, it seems that milk cholesterol concentration is robust and "protected" against profound hypercholesterolaemia despite strongly increased tissue cholesterol levels. In addition, comparable to respective

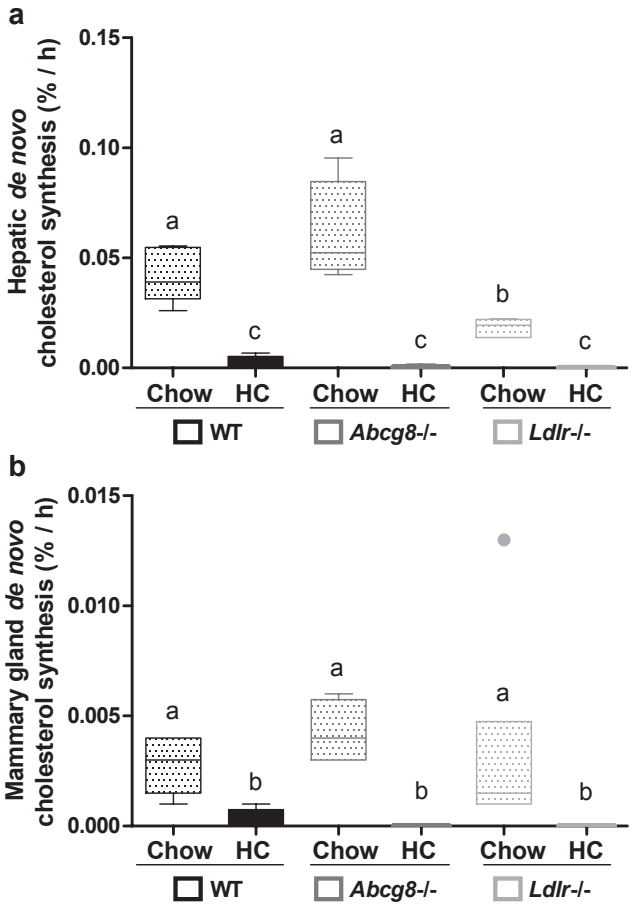

Figure 4: ***De novo*** **cholesterol synthesis.** On L14 the dams received deuterium water i.p. one hour before harvesting the organs. The mammary gland was milked 10 minutes before harvesting. The fraction of deuterium-incorporated cholesterol in liver and mammary gland was assessed using isotope ratio mass spectrometry (IRMS). **a)** De novo cholesterol synthesis in the liver (% / h). **b)** De novo cholesterol synthesis in the mammary gland (% / h). (WT Chow, n = 5; WT HC, n = 4; *Abcg8* -/- Chow, n = 4; *Abcg8* -/- HC, n = 5; *Ldlr* -/- Chow, n = 6; *Ldlr* -/- HC, n = 4). Data are presented as median and interquartile range (Tukey). Statistical significance was tested with Kruskal-Wallis post-hoc Conover-Inman; non-different groups share a letter. □: low cholesterol diet (Chow); ■: high cholesterol diet (HC). The threshold of significance was $p < 0.05$.

compensatory changes in other organs such as the liver, also in the mammary gland cholesterol synthesis decreased in response to dietary cholesterol feeding.

The use of whole-body inactivation of specific genes, as utilized in this study, is comprehensively associated with systemic changes in cholesterol metabolism and apolipoprotein balance. Employing mammary gland-specific genetic models would exclude the influence of hepatic or intestinal deficiency in our mice. However, the present lack of influence on milk cholesterol concentration in whole body-knockouts does not support the possibility that organ-specific inactivation would greatly affect milk cholesterol concentrations. We would like to hypothesize on the physiological explanation(s) of the present findings.

First, it is tempting to speculate that the apparent robustness of the cholesterol concentration in milk relates to physiological importance in milk secretion. The importance of a stable milk cholesterol concentration could relate to the process of secretion of milk lipids, in particular triglycerides. Within the alveolar cells of the mammary gland the secretory lipids are shaped in single phospholipid layer-wrapped lipid droplets. During exocytosis the lipid droplets acquire an additional cholesterol-rich phospholipid bilayer, resulting in the formation of the milk-fat globule (MFG)[140]. Milk cholesterol is mainly present as unesterified cholesterol in the MFG-membrane (85-90 %) and the other part as cholesteryl esters in the MFG-core[141, 142]. The packaging of the lipid droplets with the MFG membrane, which is essential for their secretion, may therefore translate into a rather stable cholesterol content in milk, based on its role as an emulsion-stabilizing component as part of the MFG-membrane.

Second, the robust cholesterol concentration in milk could also underline the hypothesized physiological function of milk cholesterol for later health of the offspring. In contrast to breast milk, the fat globules of common infant milk formula are smaller in size and differ in composition, being coated with milk proteins instead of a phospholipid and cholesterol-rich membrane[143, 144]. Indeed, infant formulas hardly contain cholesterol[28]. Cholesterol in early life is not considered an essential dietary component since infants are capable of *de novo* cholesterol synthesis, and thus do not critically depend on milk for their cholesterol supply. As expected, infants fed cholesterol-free formula have increased cholesterol synthesis rates compared to breast-fed infants[145]. Interestingly, however, adult individuals who had been breast-fed as infant have lower total and pro-atherogenic LDL-cholesterol compared to previously formula-fed subjects[28]. This has led to the hypothesis that early life cholesterol supply can program cholesterol homeostasis in later life. In support of this notion, we recently reported indications that dietary cholesterol availability in early life of mice determines the set-point for cholesterol absorption efficiency at adult age[59]. The rather strictly regulated concentration

of milk cholesterol found in this study could support the relevance of a stable cholesterol supply for its programming importance.

Third, a stable supply of dietary cholesterol could theoretically be relevant for the development of intestinal microbiota in early life. The cholesterol synthesis rate, the biliary cholesterol secretion and the fecal cholesterol excretion have all been shown to correlate with abundance of certain bacterial taxa in hamsters[146]. Additionally, conversion of cholesterol to the neutral sterol coprostanol by the intestinal microbiota is delayed in breast-fed infants[147], indicating inhibited growth of certain bacterial groups. Yet, a recent study by ourselves in (adult) LDLR-deficient mice does not support the concept that dietary cholesterol has a substantial impact on shaping the intestinal microbiota, since neither the composition nor the functionality of the intestinal microbiota were affected even after prolonged dietary cholesterol exposure[148].

In conclusion, our results clearly demonstrate that milk cholesterol levels are resistant to maternal high cholesterol diet- and genetically-induced hypercholesterolaemia in mice. We speculate that the robust maintenance of stable milk cholesterol levels may serve relevant physiological functions in the offspring, such as programming of long-term health benefits. Further research however, is required to firmly establish such cause-effect relationships.

Materials and methods

Animal studies. Female C57BL6/J (n = 10), *Ldlr* knockout[149] (n = 13) and *Abcg8* knockout[130, 150] (n = 9) mice were housed in temperature controlled-conditions with 12:12 light dark cycles and maintained on chow diet (RMH-B, ABDiets, Woerden, Netherlands) with listed specified ingredients: wheat, meat meal, yellow dent corn, whole oats, wheat middlings, alfalfa, soya oil, dried yeast, dicalcium phosphate, calcium carbonate, NaCl, dl-methionine, vitamins and trace elements. Breeding was initiated between 8-12 weeks of age. Due to accumulation of dietary xenosterols, *Abcg8-/-* mice are infertile, which is relieved upon ezetimibe treatment[151]. Therefore, in order to facilitate fertilization, *Abcg8-/-* females were pre-treated for 3 weeks with 0.005 % ezetimibe provided via the food, which was removed from the diet once pregnancy was confirmed in accordance with Solca *et al*.[151]. The rest of the models were fed chow until E18, when half of the mice received 0.5 % cholesterol diet. Lactation day 1 (L1) was considered the day at which pups were born. On L14 the dams were injected i.p. with 2.3 mL 99 % $^{2}H_{2}O$ (deuterium oxide) per 100 g BW, containing 0.9 % NaCl. After 50 minutes, milk was collected for 10 minutes (details see below) directly followed by termination and harvesting of blood, liver, and mammary glands. All animal experiments were approved by the ethical committee for animal experimentation at

the University of Groningen and performed in accordance with relevant guidelines and regulations.

Milk collection. At lactation day 14[152], the dams were separated from the pups for 3 hours followed by i.p. injection of 1IU oxytocin (Synthocinon®, Sigma-Tau Industrie Farmaceutiche Riunite, Rome, Italy). Milk samples were collected continuously for 10 minutes from the mammary gland of isoflurane-anesthetized mouse with the aid of a modified human electric breast pump (Calypso, Ardo Medical AG, Unterägeri, Switzerland). The samples were initially preserved at 4 °C during collection and further stored at -80 °C until use.

Determination of milk cholesterol. Thawed milk samples were homogenized by continuous vortexing. 25 to 50 µL of milk sample was subjected to lipid extraction according to Bligh & Dyer[153]. Unesterified cholesterol was subsequently derivatized to cholesteryl acetate and quantified by gas chromatography, using 5-alpha cholestane as internal standard[154].

Total plasma cholesterol and lipoprotein profiles. Total plasma cholesterol was measured enzymatically using a commercially available kit (Roche Diagnostics GmbH, Mannheim, Germany). Lipoprotein fractions of pooled plasma samples (n = 3-5) were separated via fast protein liquid chromatography gel filtration using a superose 6 column (GE Healthcare, Little Chalfont, UK) as published[155]. Samples were chromatographed at a flow rate of 0.5 ml/min, and lipoprotein fractions of 500 µl each were collected. Individual fractions were assayed for cholesterol concentrations using a commercial kit (Roche Diagnostics GmbH, Mannheim, Germany).

Hepatic and mammary gland total cholesterol quantification. Liver and mammary gland tissues were homogenized using RNAse free-beads and the TissueLyser LT system (Qiagen GmbH, Hilden, Germany). Lipids were extracted according to Bligh & Dyer[153]. Cholesterol was de-esterified according to Ichihara *et al*.[156]. Free cholesterol underwent acetylation followed by quantification using gas chromatography (GC, Agilent 6890, Amstelveen, the Netherlands)[154].

Organ-specific de novo cholesterol synthesis. Liver- and mammary gland-specific *de novo* cholesterol synthesis was quantified using the deuterium incorporation method[157]. Briefly, at L14 the dams were injected i.p. with deuterated water (2.3 mL/100 g BW, 99 % $^{2}H_{2}O$, 0.9 % NaCl) and terminated after 60 minutes by cardiac puncture[158]. We used a non-injected control mouse for determining the number of hydrogen atoms incorporated in a single newly synthesized cholesterol molecule as measured by GC-MS. Following lipid extraction and de-esterification, the abundance of deuterium-substituted hydrogen atoms was

determined by isotope ratio mass spectrometry (IRMS). Synthesis rates were determined as previously published[159].

Statistical analysis. The significance of dietary influence within the different genotypes and the analysis of variance between genotypes in the same dietary condition was performed with Kruskal-Wallis followed by a multiple comparisons adjustment using Conover-Inman test. P-values below 0.05 were considered significant.

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

Testing, data acquisition and analysis, and drafting the article were performed by L.G.D. and M.A.M.L.. Statistical analysis was performed under the supervision of V.W.B.. U.J.F.T. and H.J.V. were responsible for the conception, design, and supervision of the study, interpretation of data, and critical article revision for important intellectual content. All contributing authors gave final approval for the version to be published.

Additional information

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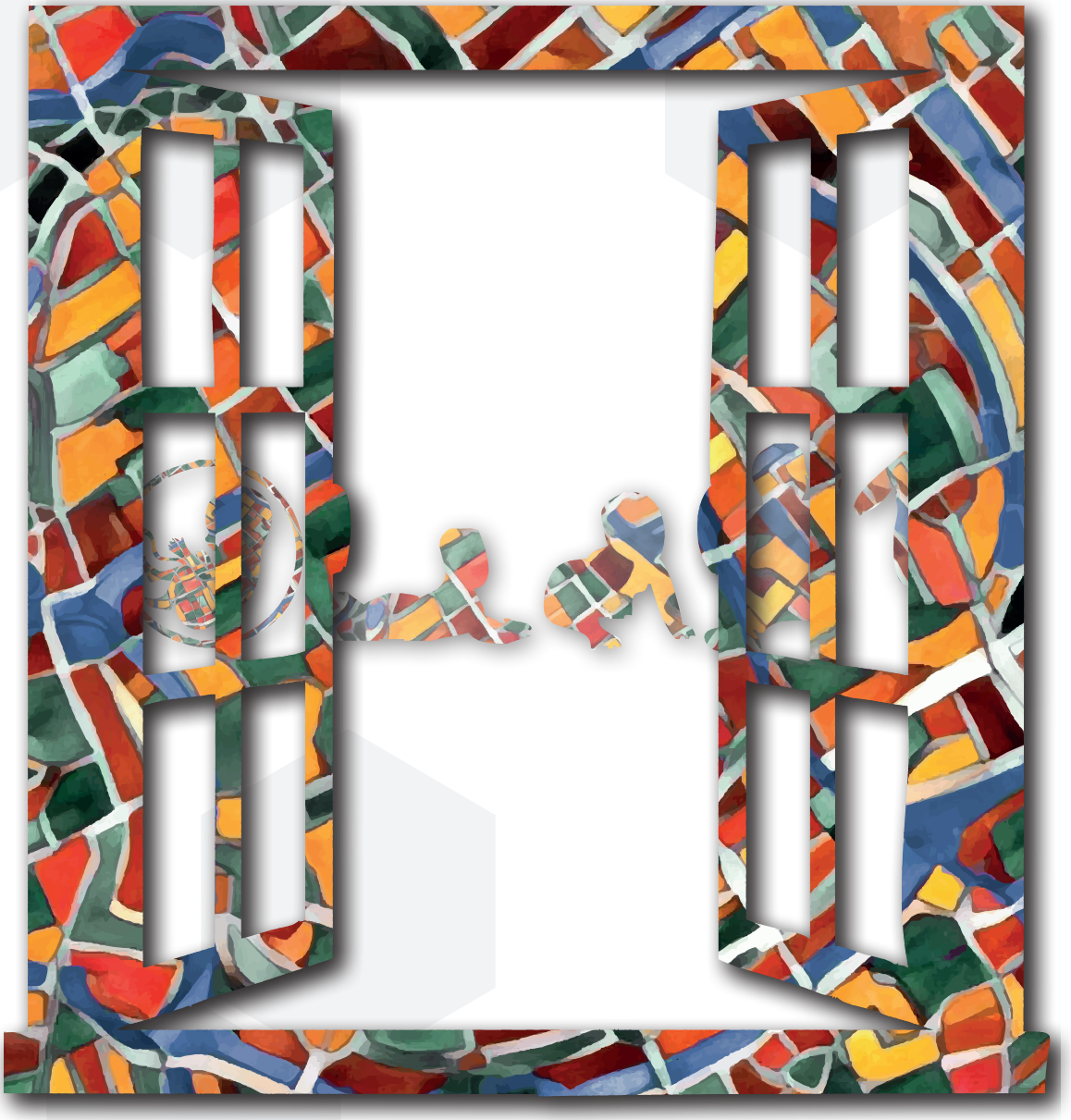
Competing interests.

The authors declare that they have no competing interests.



Chapter 3

Programming of intestinal cholesterol absorption in mice: sensitive window of ezetimibe treatment

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Manuscript in preparation

Abstract

SCOPE: Maternal administration of the cholesterol absorption inhibiting drug ezetimibe during the lactation period reduces cholesterol absorption in the offspring up into adulthood via epigenetic programming. Developmental programming of metabolic processes is known to have sensitive windows. We now determined whether the sensitive window, in which ezetimibe programs cholesterol absorption, extends beyond the lactation period.

METHODS AND RESULTS: We administered ezetimibe to C57BL6/JOlaHsd mice via the food (0.005 w/w %) for three weeks after weaning and compared these mice to non-treated mice. Cholesterol absorption was measured during and at six weeks after ezetimibe treatment using dual-isotope methodology. Feces was collected during, and at two and six weeks after ezetimibe administration to determine sterol excretion.
During its administration, ezetimibe decreased cholesterol absorption (-78 %, $p = 0.004$) and increased fecal NS excretion (+ 301%, $p = 0.004$), compared to controls. Two and six weeks after ezetimibe administration, however, fecal NS excretion was similar to the control group. At 6 weeks after ezetimibe administration, neither cholesterol absorption differed significantly from control mice ($p = 0.33$), nor did plasma cholesterol levels, lipoprotein profiles, or intestinal NPC1L1 mRNA expression.

CONCLUSIONS: This study demonstrates that in mice the sensitive window for ezetimibe-induced programming of cholesterol absorption does not extend beyond the lactation period.

Introduction

Cholesterol absorption is related to both cholesterol gallstone disease[160] and atherosclerotic cardiovascular disease (CVD)[161]. These conditions are responsible for substantial morbidity and, in case of CVD, also mortality[162]. Higher cholesterol absorption results in hypercholesterolemia[163], which is a well-established risk factor for atherosclerosis development[164]. Cholesterol is absorbed by the enterocytes of the small intestine via the Niemann-Pick C1-Like1 (NPC1L1) protein[54]. Intestinal cholesterol absorption can be attenuated by inhibition of NPC1L1 function, for example by an inactivating mutation[165] or the drug ezetimibe[161, 163]. Inhibiting cholesterol absorption with ezetimibe[50] reduces hypercholesterolemia[163], increases fecal neutral sterol (NS) excretion, decreases plasma cholesterol levels[166] and induces an overall improved plasma lipoprotein profile[161]. The clinical relevance of ezetimibe's mechanism has recently been illustrated by the IMPROVE-IT trial, where adding ezetimibe to statin therapy resulted in incremental LDL cholesterol lowering and consequently a further significant CVD risk reduction in a secondary prevention setting[167]. These observations emphasize the metabolic impact of cholesterol absorption, its potency to be inhibited by ezetimibe and its association with metabolic health.

Bile is responsible for the micellization of lipids, such as cholesterol, to make them accessible for absorption. The hydrophobicity of the BA pool positively correlates with cholesterol absorption and is determined by the individual bile acid species[168]. Alpha- and β-muricholic acids are considerably hydrophilic and potent inhibitors of cholesterol absorption as well as stimulators of BA synthesis[94, 168, 169]. Disposal of cholesterol occurs quantitatively via fecal excretion of cholesterol or bile acids (BA), after hepatic conversion of cholesterol to BA[170].

Cholesterol levels in breast milk are high, relative to those in infant formula[26]. The high breast milk cholesterol concentration has been suggested to have a lasting beneficial impact on cholesterol homeostasis of the offspring[26, 120]. This phenomenon is consistent with the so called Barker hypothesis, which states that the *in utero* and infant environment permanently changes the development and function of the body, thereby 'programming' adult disease risk[121]. Whether the epidemiologically beneficial effects of breast milk are solely attributable to the substantial differences in cholesterol content between breast milk and infant formula or if other factors are in play is not exactly known.

Recently, we demonstrated that in mice the lactation period is a sensitive window to program intestinal cholesterol absorption of the offspring. Decreasing the dietary cholesterol availability during lactation by exposing the offspring to ezetimibe via the maternal milk programmed a decreased cholesterol absorption

in adulthood via epigenetic reduction of intestinal *Npc1l1* expression[59]. This observation demonstrated the ability of the murine intestine to sense nutritional conditions in early life and to retain an active long-term metabolic memory. Noah *et al.* (2011)[171] postulated that intestinal development in mice is completed at weaning and that substantial epigenetic changes are only likely to occur during this period. On the other hand, Pacha *et al.* (2000)[172] concluded that intestinal development extends beyond weaning, adapting to the change of milk to solid food intake post-weaning. Therefore regulation of these metabolic processes up into adulthood might not only be affected by environmental signals during lactation but possibly also when present during the post-lactation period. An extended sensitive window beyond lactation would indicate an extended period in which strategies can be imposed to beneficially manipulate long-term cholesterol absorption and, indirectly, cardiovascular health.

In the present study we set out to determine whether the sensitive window to program long-term cholesterol absorption extends beyond lactation. We exposed murine male offspring to the drug ezetimibe via solid, cholesterol-containing food during three weeks after weaning. During ezetimibe exposure and six weeks later, possible changes in dietary cholesterol absorption, as well as other cholesterol and bile acid homeostasis parameters were determined to evaluate the sensitive window of ezetimibe-induced programming of cholesterol absorption.

Results

Direct effect of ezetimibe on cholesterol absorption

Figure 1A shows the experimental setup of the study. Throughout the study, the body weight was similar between the ezetimibe-treatment and control group (**Fig. 1B**). At the age of 6 weeks, after three weeks of control or ezetimibe diet, fat and lean mass did not differ between the two groups (**Fig. 1C**). Six weeks after ezetimibe exposure, daily food intake, liver weight, intestinal length, and epididymal fat were not affected by the prior ezetimibe treatment (data not shown). At 2-3 weeks after weaning, cholesterol absorption was profoundly lower in the ezetimibe group compared to controls (-78%, $p = 0.004$; **Fig. 1D**). In agreement with lower cholesterol absorption, the excretion of NS was higher in the ezetimibe group (+301%, $p = 0.004$; **Fig. 1E**).

Ezetimibe treatment lowered fecal BA excretion compared to the control group (-68 %, $p = 0.004$; **Fig. 1F**). Fecal BA composition analysis demonstrates that lower fecal BA excretion is predominantly due to lower αMCA and βMCA excretion in the ezetimibe-receiving mice (**Fig. 1G**).

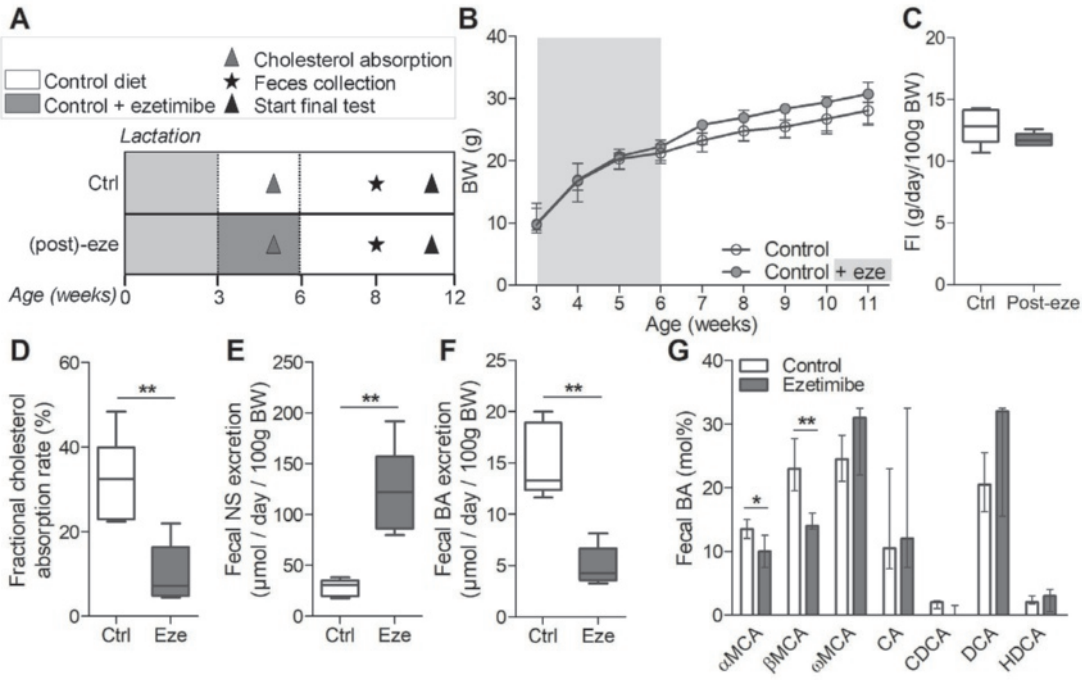

Figure 1: Cholesterol and bile acids during the ezetimibe diet. (A) Simplified schematic of the experimental setup. **(B)** Body weight development (median + range). At the age of 5-6 weeks during ezetimibe treatment: **(C)** Fat and lean body mass, **(D)** Fractional cholesterol absorption, **(E)** Fecal NS excretion and **(F)** Fecal bile acid excretion. **(G)** Fecal BA composition at five weeks age.

Post-treatment effects of ezetimibe on cholesterol absorption and on fecal cholesterol and BA excretion

In our previous study on ezetimibe treatment during the lactation period, the cholesterol absorption in the offspring remained lower, up to 21 weeks after the ezetimibe treatment [59]. In the present experiment, treatment with ezetimibe during 3 weeks post-lactation, did not affect fecal NS or fecal BA excretion at 2 weeks after the last ezetimibe administration (**Fig. 2**), indicating that the sensitive window to program cholesterol absorption does not extend beyond lactation.

At age 11-12 weeks, 5-6 weeks after the last ezetimibe intake, the fractional cholesterol absorption was similar in previously ezetimibe-treated and control mice (**Fig. 3A**). However, a trend for increased fecal total NS excretion was seen in the post-eze group (**Fig. 3B**, p = 0.052). Further compositional analysis of fecal neutral sterols, indicated that fecal cholesterol excretion was significantly higher in the post-eze mice (+37 %, p = 0.0087, data not shown) while coprostanol and dihydroxycholesterol did not differ. Plasma cholesterol was similar between the

groups (**Fig. 3C**), as well as lipoprotein distribution (data not shown). Biliary cholesterol was not significantly different between control and post-eze mice (**Fig. 3D**). *De novo* cholesterol synthesis, as measured from the appearance of labelled cholesterol in the plasma, was below the detection limit of 1% in both groups and could therefore not be used for reliable calculations.

Transintestinal cholesterol excretion after ezetimibe exposure

The cholesterol present in the intestinal lumen originates from the diet, from biliary secretion, and from trans-intestinal cholesterol excretion (TICE) [173]. The intestinal cholesterol balance can be estimated based on the net cholesterol transport across the intestine: the difference between fecal NS excretion and the sum of dietary and biliary cholesterol influx into the intestine (**Fig. 3E**). The amount of cholesterol secreted via the feces did not exceed the dietary and biliary influx, indicating that absolute cholesterol absorption may exceed TICE (**Fig. 3F-G**).

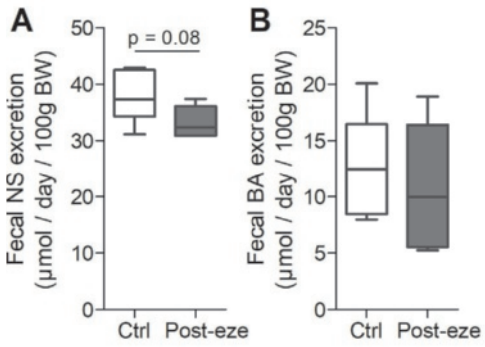

Figure 2: Fecal cholesterol and bile acid excretion two weeks after ezetimibe exposure. (A) Fecal NS excretion and **(B)** BA excretion at the age of 8 weeks, two weeks after the intervention diet with added ezetimibe.

Bile acids after ezetimibe exposure

At 6 weeks after ezetimibe intake, total plasma BA levels were not affected by the ezetimibe treatment early in life, neither was the bile flow (**Fig. 4A**; **Fig. 4B**, p = 0.14). BA secretion in the bile as well as BA excretion in the feces appeared unaffected (**Fig. 4C**, **4E**). The composition of fecal BA was virtually identical between the groups (data not shown), as depicted by the equal hydrophobicity index (**Fig. 4D**).

Gene expression after ezetimibe exposure

To assess the possible effect of early life ezetimibe exposure on expression of genes related to cholesterol homeostasis we measured relative gene expression levels in the proximal ileum (**Fig. 5**). *Npc1l1* expression appeared to be unaffected by post-

lactation ezetimibe treatment. *Hmgcr*, the gene encoding the rate-limiting enzyme of cholesterol synthesis, was also not affected. The protein encoded by *Abcg8* facilitates transport of sterols into the bile or back into the intestinal lumen. *Abcg8* did not differ between the groups, neither did *Abca1*, encoding for a cholesterol efflux transporter. *Srebp2*, required for lipid homeostasis and *Ldlr*, the low density lipoprotein receptor were expressed at equal level in control and post-ezetimibe treated mice.

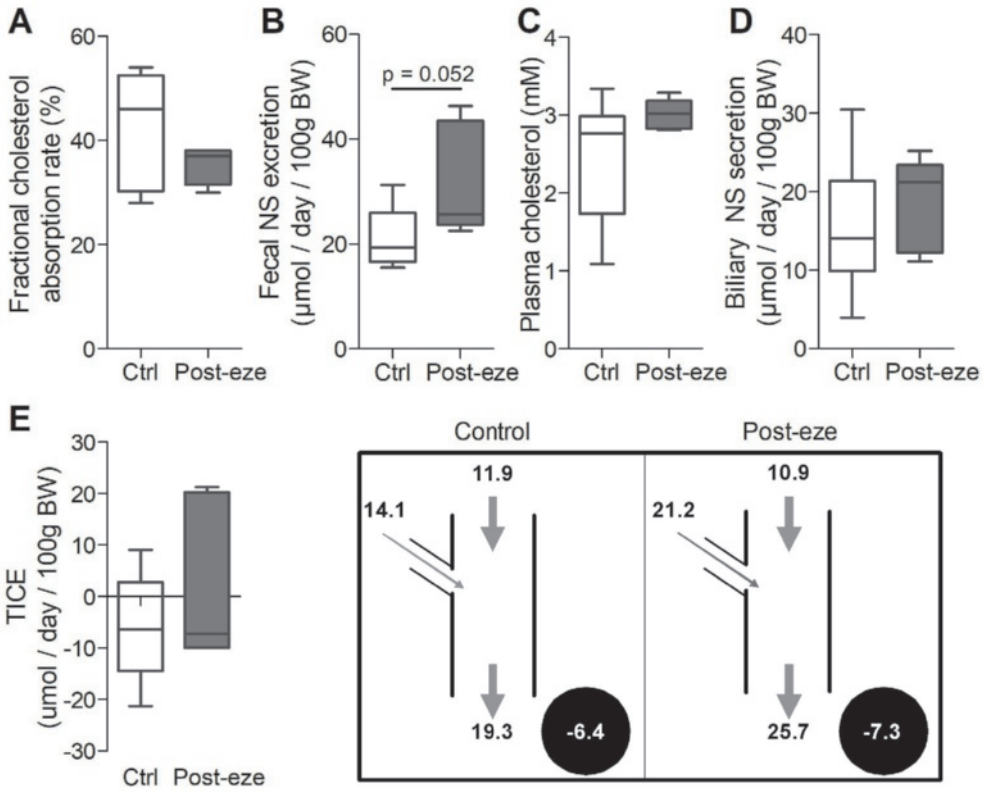

Figure 3: Cholesterol metabolism at six weeks after ezetimibe exposure. Parameters at 11-12 weeks of age, six weeks after the intervention diet. **(A)** Fractional cholesterol absorption. **(B)** Fecal NS excretion. **(C)** Plasma cholesterol. **(D)** Biliary cholesterol secretion. **(E)** Intestinal cholesterol balance = fecal NS excretion – (cholesterol intake + biliary NS). **(F)** Intestinal balance. **(G)** Schematic representation of intestinal net cholesterol and fecal neutral sterol fluxes (median values in µmol/day/100g BW).

Discussion

We investigated whether the sensitive window to program long-term the intestinal absorption efficacy of cholesterol by ezetimibe was extended beyond the lactation period in mice. Our results, however, indicate that there is no metabolic memory of ezetimibe treatment during a three weeks post-lactation period, whereas ezetimibe during the lactation period epigenetically lowered adult cholesterol absorption [59].

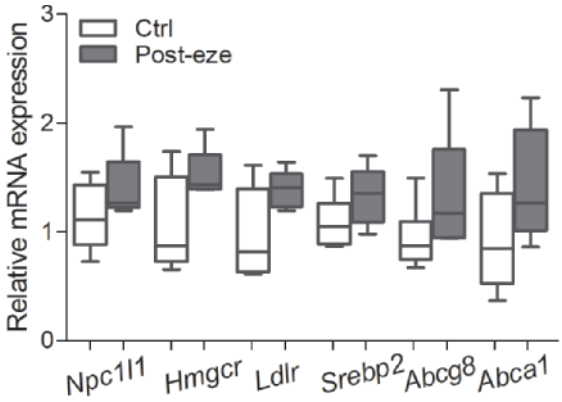

Figure 4: Bile acid parameters six weeks post-programming diet. Bile acid parameters in control and post-ezetimibe mice six weeks after ezetimibe administration. **(A)** Total bile acid concentration in plasma. **(B)** Hepatic bile flow. **(C)** BA secretion in hepatic bile. **(D)** Hydrophobicity index of bile. **(E)** Fecal BA excretion.

Upon administration immediately after weaning, ezetimibe significantly decreased cholesterol absorption with a corresponding increase in fecal NS excretion, similar to earlier reports in adolescent to adult male mice [50, 54, 86, 166, 174] and humans [48, 166, 175]. Our data indicate that within 6 weeks after the last ezetimibe administration, the effects on cholesterol absorption have almost completely disappeared: the fractional cholesterol absorption, plasma cholesterol levels and lipoprotein profiles are similar between the previously ezetimibe-receiving group and the control group. The only difference that we observed at 6 weeks after treatment (but not at 2 weeks after treatment) was a trend for increased fecal NS excretion in the previously ezetimibe-treated group. This suggests that although fractional cholesterol absorption and intestinal *Npc1l1* expression were not affected, ezetimibe may have shaped long-term cholesterol homeostasis. Ezetimibe is capable of changing the gut microbiota composition, specifically it may increase *Lactobacillus spp.* [176]. Increased abundance of specific *Lactobacillus spp*. has been linked to lower serum cholesterol [177], elevated BSH-activity in the feces [178], increased fecal cholesterol and BA excretion [87, 177, 179], and hepatic and intestinal expression of cholesterol homeostasis genes [176]. Thus, although the sensitive window for programming cholesterol absorption does not extend beyond

lactation, the data indicates some long-term effects on (microbiota)metabolism might occur.

Cholesterol disposal from the body occurs quantitatively by direct secretion of cholesterol into the intestine or via hepatic conversion of cholesterol into bile acids and subsequent excretion into the feces [170]. Interestingly, fecal BA excretion was decreased during ezetimibe treatment ('acute' effect) and similar to the control group 2 and 6 weeks after treatment. This acute effect of ezetimibe administration was not reported in earlier studies in mice and humans in which ezetimibe either increased or did not affect fecal BA excretion [166, 174, 175, 180]. The observed decrease in fecal BA could be attributed to reduced levels of α- and β-muricholic acids. Alpha- and β-muricholic acids are considerably hydrophilic and have been characterized as FXR antagonists [93]. Thereby, muricholic acids can inhibit cholesterol absorption and stimulate BA synthesis [94, 168, 169]. It is difficult to explain the observed decrease in fecal BA with the current knowledge and data.

Our data seem to support the hypothesis by Noah *et al.*, stating that murine intestinal development is completed at weaning [171]. During lactation intestinal cells still develop, including the intestinal stem cells which give rise to the enterocytes for the rest of the lifetime. Enterocytes have an absorptive function, including absorption and further metabolism of cholesterol [81, 171]. During lactation, the intestinal stem cells are under influence of many epigenetic changes which may affect long-term intestinal stem cell function and consequent metabolic health [81]. However, other studies indicate changes in enterocytes upon weaning due to the transition from fluid to solid nutrition [172].

Our data do not provide indications that manipulation of intestinal cholesterol absorption beyond the murine lactation period affects long-term cholesterol

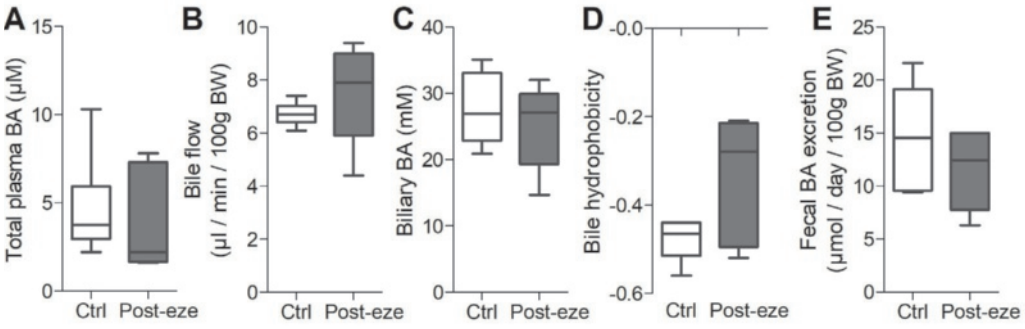

Figure 5: Intestinal gene expression levels six weeks post-programming diet. mRNA expression levels of cholesterol transport-related genes in the proximal ileum of control and post-ezetimibe treated mice at the age of 12 weeks, relative to *36B4*.

absorption. It remains to be determined whether the "shutting off" of the sensitive window is related to age or development or, rather to the switch from milk to solid food. If the latter is important, it may be possible that prolongation of the lactation period such as physiological in the human situation, may still impose a window of opportunity for programming. To explore the dynamics of the window of intestinal plasticity, the epigenetic changes in intestinal stem cells could be determined in a study with different pre- and post-lactation duration of liquid nutrition similar to breast milk composition. If long-term programming of cholesterol homeostasis in early life is linked to epigenetic modification of the *Npc1l1* gene, translation of murine data should take into account that the *Npc1l1* expression pattern is different in humans. Human *NPC1L1* is not only expressed in the small intestine but also in the liver, where the protein reduces biliary cholesterol secretion [181]. Therefore, ezetimibe in humans increases endogenous cholesterol excretion via the bile [48] and may thus have a different long-term outcome.

Apart from the time of ezetimibe administration, several differences should be taken into account when comparing this study to our previous work (2017) [59]. First, we used males instead of females. Research shows that there are many sex differences in metabolism and also with respect to the risk of disease due to early life events [13, 99, 182]. In adult humans, ezetimibe treatment with or without statin achieves better lipid-lowering effects in men than in women [183, 184]. This difference is possibly related to genetic variations in the *NPC1L1* gene [185]. Second, instead of LDL-receptor knockout mice we used wild-type C57BL/6JOlaHsd mice whose lipoprotein profile is more distinct from humans. Yet, cholesterol exposure via maternal high-cholesterol diet during (pre-)gestation and lactation can program lipoprotein parameters in C57BL/6J mouse offspring up into adulthood [186, 187], indicating that nutritional cues such as cholesterol in early life does have the ability to program metabolism in this strain.

Taken together, our data suggests that the sensitive window for programming adult cholesterol absorption by ezetimibe does not extend beyond lactation in male mice, although ezetimibe does affect cholesterol disposal possibly via the microbiota. If the same is true for humans, adapting long-term cholesterol absorption to improve cardiovascular health should occur before the end of lactation.

Materials & Methods

Animal studies. We used C57BL/6JOlaHsd mice (Envigo) which were group-housed in temperature-controlled conditions with 12:12 light dark cycles. For breeding, males were

kept on AIN-93M+0.15% cholesterol and females on AIN-93G+0.15% cholesterol diet (Research Diets, New Brunswick, USA). Nests were standardized to n = 6 with 4 males + 2 females on postnatal day 1-3. Offspring was weaned into individual cages and divided into two dietary groups for three weeks: AIN-93G+0.15% cholesterol (control, n = 6) and control + 0.005% Ezetimibe (Ezetrol (R); Eze, n = 5). Cholesterol absorption was measured at 5-6 weeks of age. Feces were collected at 5, 8 and 11 weeks of age. From six to 12 weeks of age all mice received AIN-93M+0.11% cholesterol (Research Diets, New Brunswick, USA). We repeatedly measured body weight and at 6 and 11 weeks of age body composition analysis was performed by nuclear magnetic resonance (NMR) for small animals (Minispec LF90 Body Composition Analyzer; Bruker, Germany). At age 11-12 weeks, we assessed the following cholesterol homeostasis parameters: dietary cholesterol intake, intestinal absorption, *de novo* synthesis, biliary secretion rate, and finally, bile composition and fecal excretion rate. Gallbladder cannulation was performed at 12 weeks to collect hepatic bile as described previously [154]. Briefly, bile was cannulated for 20 minutes under Hypnorm (fentanyl/fluanisone; 1 mL/kg) and diazepam (10 mg/kg) anesthesia using a humidified incubator to maintain body temperature. After cannulation, blood was obtained via heart puncture, the mice were sacrificed and adipose tissue, liver and intestine were excised and snap-frozen in liquid nitrogen. All animal experiments were approved by the ethical committee for animal experimentation (IACUC) at the University of Groningen and performed in accordance with the Dutch National Law on Animal Experimentation and international guidelines on animal experimentation. A schematic of the detailed set-up of the study is shown in **Figure 1A**.

Fractional Intestinal Cholesterol Absorption. Fractional cholesterol absorption was measured using the plasma dual-isotope ratio method as described previously [59]. Briefly, at the end of the dark phase the non-fasted 10.5-week-old animals were injected intravenously with 0.3 mg ^{5}D-cholesterol dissolved in Intralipid (20%; Fresenius Kabi, Den Bosch, The Netherlands) and orally gavaged with 0.6 mg ^{7}D-cholesterol dissolved in medium-chain triglyceride oil. Blood spots from the tail were collected on filter paper before and after administration of the isotopes at 3, 6, 12, and 24 hours for the first day, and after that every 24 hours for the next consecutive 7 days. Cholesterol was extracted from blood spots, followed by analysis by GC-MS. Briefly, the calculation of fractional cholesterol absorption was based on the decay curves of ^{5}D (intravenously) and ^{7}D-cholesterol (oral) in plasma after their correction with the administered dose: F*a* = (area under the label enrichment curve curve$_{oral}$/area under the label enrichment curve curve$_{intravenous}$ × dose$_{intravenous}$/dose$_{oral}$) × 100.

Cholesterol synthesis and balance. Fractional cholesterol synthesis was determined by Mass Isotopomer Distribution Analysis (MIDA) using 13C-acetate (Isotec, Miamisburg, OH,

USA) as labeled precursor as described previously [159]. Transintestinal cholesterol excretion (TICE) was calculated as dietary cholesterol intake + biliary cholesterol secretion – fecal neutral sterol excretion.

Measurement of cholesterol, lipoprotein profiles and bile acids in plasma and bile. Total plasma cholesterol was measured enzymatically using a commercially available kit (Roche Diagnostics GmbH, Mannheim, Germany). Lipoprotein fractions of pooled plasma samples (n = 5-6) were separated via fast protein liquid chromatography (FPLC) gel filtration using a superose 6 column (GE Healthcare, Little Chalfont, UK) as published [Wiersma 2009]. Samples were chromatographed at a flow rate of 0.5 mL/min, and lipoprotein fractions of 500 µl each were collected. Individual fractions were assayed for cholesterol concentrations as described above. Biliary and plasma bile acid (BA) concentrations were determined using liquid-chromatography mass spectrometry (LCMS) as described previously [188]. Neutral sterols (NS) were extracted from bile according to Bligh and Dyer [153] followed by derivatization in BSTFA, pyridine, and TMCS (5:5:0.1), re-dissolving in heptane with 1% BSTFA, and measurement by GC. Total bile acids from bile were measured using an enzymatic fluorescent assay according to Mashige et al. [189].

Cecum analysis. Bacterial DNA of the available cecum content was isolated, subsequently measured using MiSeq sequencing of the amplified 16S rRNA genes and analyzed by the QIIME and ARB method as described in Heida *et al.* [190].

Fecal neutral sterols and bile acids. Fecal samples from individually housed mice collected over 24 hours were dried, weighed and ground to powder. NS and BA profiles were determined using gas-liquid chromatography as published [154, 155]. Briefly, 50 mg feces was saponified in the presence of 1 mL alkaline methanol (1:3 NaOH:methanol) by heating for 2 hours at 80°C. The NS were extracted with petroleum ether, derivatized in a mixture of N,O-bis-trifluoroacetamide (BSTFA), pyridine, and trimethylchlorosilance (TMCS) in a ratio of 5:5:0.1, re-dissolved in heptane containing 1% BSTFA, and measured by gas chromatography (GC). After NS extraction, total bile acids were extracted from the aqueous phase using SepPak C18 cartridges (Waters, Dublin, Ireland), methylated, and after derivatization with BSTFA, pyridine, and TMCS, were measured by GC. The same methodology was applied for the determination of cholesterol content using a 50 mg aliquot of the animal food; by knowing the food intake, the dietary cholesterol intake could be calculated.

Gene expression. Proximal duodenum mRNA was extracted using TriReagent (Sigma) and quantified with a Nanodrop ND-100UV-vis spectrometer (NanoDrop Technologies Wilmington DE). cDNA was made from 1µg of RNA using reagents from Invitrogen

(Carlsbad CA). Primers were synthesized by Eurogentec (Seraing, Belgium). Real-time PCR was performed using an ABI Prism 7700 machine (Applied Biosystems, Damstadt Germany). mRNA expression levels of individual genes were calculated relative to the housekeeping gene *36b4*.

Statistical analysis. The statistical analysis was performed with GraphPad Prism 5 Software using Mann-Whitney U Test. P-values below 0.05 were considered significant (* $p < 0.05$; ** $p < 0.01$). Graphs are made with GraphPad Prism 5; using the Tukey method for plotting the box ((25^{th} - 75^{th} percentiles), whiskers (1.5 IQR) and outliers (>1.5 IQR) unless stated otherwise. Open symbols = control group (n = 6); grey symbols = post-ezetimibe group (n = 5).

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Author contributions

Testing, data acquisition and analysis, and drafting the article were performed by M.A.M.L.. U.J.F.T. and H.J.V. were responsible for the conception, design, and supervision of the study, interpretation of data, and critical article revision for important intellectual content. All contributing authors gave final approval for the version to be published.

Additional information

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Competing interests.

The authors declare that they have no competing interests.

Plasma bile acid dynamics after conventionalization of germ-free mice with opposite sex microbiota

Chapter 4

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Submitted

Abstract

SCOPE: The bile acid (BA) pool composition is partly determined by the intestinal microbiota. BA composition differs between males and females in conventional mice, but not in germ-free (GF) mice. We tested the hypothesis that the sex differences in BA composition correlate with sex differences in intestinal microbiota. GF C57BL/6OlaHsd males and females were inoculated at 12 weeks of age with a cecum microbiota pool from either male or female donors.

METHODS AND RESULTS: Plasma was sampled before conventionalization and at 1, 2, 3 days and 10 weeks post-conventionalization for determination of BA concentration and composition. Mice were then sacrificed and cecum microbiota composition was analyzed.
GF males and females showed a similar plasma BA composition, but differed in the plasma BA concentration (240 % higher in females, $p = 0.0017$). At 2 and 3 days post-conventionalization plasma BA concentration was higher in male and female recipients of the female compared to the male microbiota donor pool. At 10 weeks after conventionalization plasma BA levels and composition were largely independent of the donor, and, interestingly, also similar between male and female recipients. Hepatic and ileal gene expression as well as microbiota composition did not differ notably between the sexes at 10 weeks after conventionalization.

CONCLUSIONS: Our data indicate that the sex of the microbiota donor influences plasma BA concentration and composition after conventionalization of GF mice on the short term, but not in the long term. The lack of difference in BA composition 10 weeks after microbiota inoculation may be related to the germ-free state of these mice till 12 weeks of age. We hypothesize that the host (epi)genome determines the microbiota composition and secondary to that, the BA metabolism and composition.

Introduction

Bile acids (BA) play an important role in the metabolic system. BA not only aid in fat and vitamin absorption but also induce metabolic signaling pathways via the nuclear receptor farnesoid X receptor (FXR) and the G protein-coupled bile acid receptor 1 (TGR5). Both FXR and TGR5 provide feedback signals on BA synthesis and impact glucose and lipid metabolism as well as energy expenditure[191, 192]. Metabolism, bile acids (BA) and microbiota are associated in an FXR-dependent and sex-specific manner[105]. In general, metabolism is regulated differently in males and females[97]. Specifically, BA composition and the serum metabolome differ considerably between conventional male and female mice[102-104, 114, 193] and humans[112, 113]. However, such a sexual dimorphism is hardly observed in mice lacking a functional microbiota[102-104]. This suggests a sex-related role for intestinal bacteria in BA composition. Interestingly, microbiota composition also shows sex-specific differences in post-puberty mice[98, 104, 108, 109] and humans [101, 108, 110, 111], presumably mediated at least partly by differences in circulating hormone levels[98-101]. In addition, also BA transporter and BA synthesis enzymes such as *Cyp7a1* exhibit sex-dependent differential expression levels[114]. The sex-differences in both microbiota composition and bile acids conceivably contribute to the variance in metabolism and metabolic disease risk between males and females[104, 106].

Intestinal bacteria alter BA composition by deconjugation of taurine or glycine with the bile salt hydrolase (BSH) enzyme, and subsequent conversion of primary BAs into secondary BAs in the intestine prior to their reabsorption into the enterohepatic cycle[32, 93, 119]. These conversions regulate BA signaling and thereby affect multiple metabolic pathways[117-119]. The amount and type of BA modification depend on the bacteria and their enzymes present in the intestine. In return, taurine-conjugated BA and the hydrophobicity of BAs affect their bactericidal activity and thus intestinal microbiota composition[118, 194]. BAs influence microbiota composition by stimulating growth of BA-metabolizing bacteria and inhibiting growth of other genera or species via anti-bacterial properties[32, 117, 118].

Whether a sex-specific BA composition develops based on a respective sex-specific microbiota composition has not been elucidated. Therefore, we assessed in the present study the effect of sex of the microbiota donor on the changes in plasma BA composition upon conventionalization of GF male and female mice. The effect of the sex of the microbiota donor was measured sequentially up to 10 weeks after conventionalization. Additionally, long-term effects of conventionalization on cecum microbiota composition and expression of BA-regulated genes were assessed.

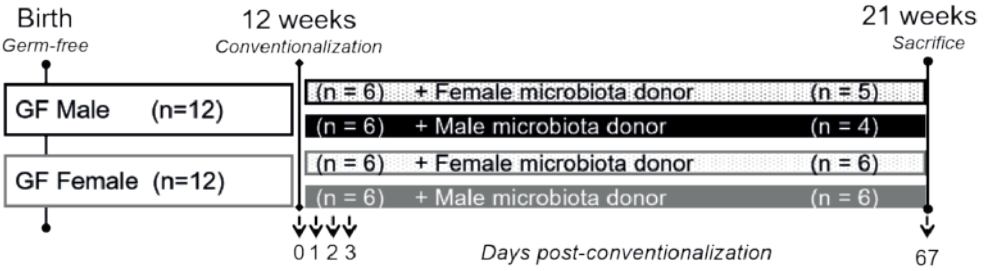

Figure 1: Experimental setup of conventionalization. Scheme of the experimental setup. 12 week old male and female GF mice received a single fecal inoculation of a diluted cecum microbiota donor pool of the same or opposing sex. Plasma samples were taken from the tail right before and 1, 2, 3 days and 10 weeks after conventionalization. Body weight and food intake were also monitored before and 10 weeks post-conventionalization. At 10 weeks post-conventionalization mice were sacrificed and blood, liver and cecum content was collected for further analysis.

Figure 2: Absolute plasma BA levels and BA composition before and post-conventionalization. Total **(A-D)** and relative **(D-F)** BA levels in plasma before **(A,E)**, 3 days post- **(B,F)**, and 10 weeks **(C,G)** post-conventionalization. BA species levels in plasma before **(H)**, 3 days post- **(I)**, and 10 weeks post-conventionalization **(J)**.Black = male; grey = female; n = 4-6 / group. Open bar = female donor; closed bar = male donor. * Figure displayed on next page.

Results

Bile acid levels and composition

Prior to conventionalization, at the age of 12 weeks (**Fig. 1A**), GF males had a higher body weight than GF females ($p = 0.0003$) and food intake corrected for body weight did not differ between the sexes. At 10 weeks post-conventionalization body weight was still higher in male recipients ($p < 0.0001$) but not liver weight corrected for body weight. These parameters were not affected by the sex of the microbiota donor. Female mice had increased food intake compared to males ($p = 0.036$) and recipients of the male microbiota donor pool showed a trend towards higher food intake ($p = 0.058$) compared to recipients of the female microbiota donor pool.

Total plasma BA concentration was significantly higher in GF females (**Fig. 2A**; $p = 0.0017$), predominantly due to higher absolute levels of TßMCA, TαMCA and TCA (**Fig. 2H**). Although absolute BA levels varied between the sexes, the relative BA pool composition was almost the same (**Fig. 2E**). GF females showed slightly higher TαMCA and TCDCA percentage (data not shown, $p = 0.0001$). Three days after conventionalization, total plasma BA levels were higher in recipients of the

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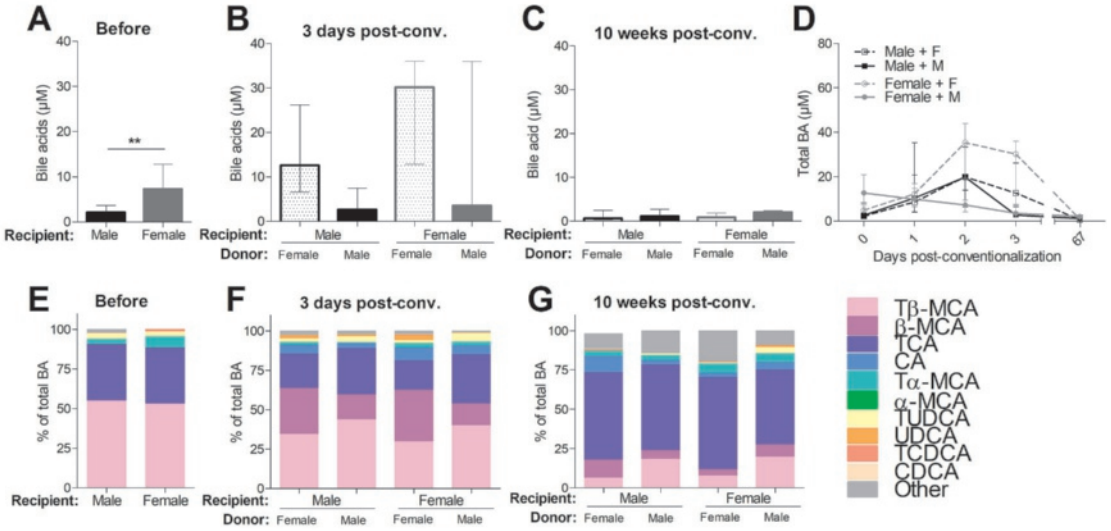

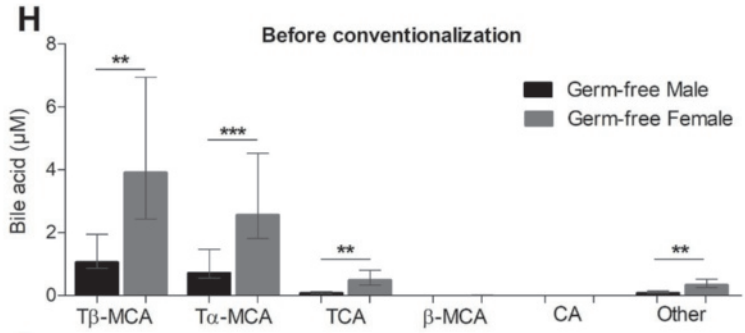

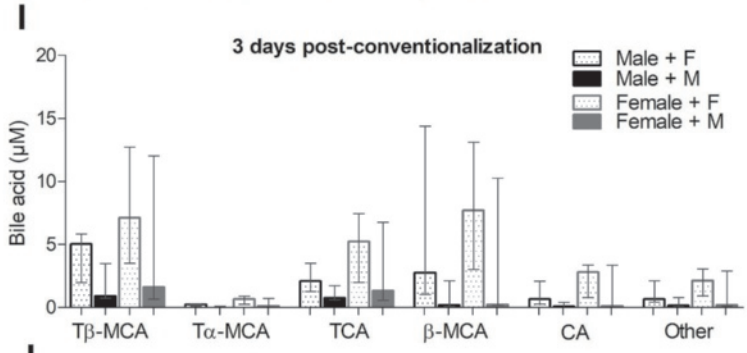

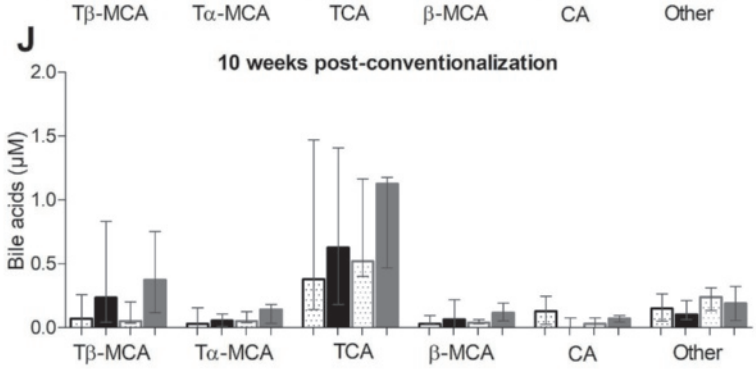

female microbiota donor pool (**Fig. 2B,D**; r = 0.56, p = 0.011), again predominantly by higher ßMCA and CA species (**Fig. 2I**). However, this donor sex effect on total BA level had disappeared at 10 weeks post-conventionalization (**Fig. 2C**, p = 0.436). Not only did the total amount of plasma BAs decrease substantially between 3 days and 10 weeks after conventionalization (**Fig. 2C, D**), but also the effect of the sex of the recipient on total plasma BA levels seen in GF mice was not present any longer at this time point (**Fig. 2C,J**). The relative BA composition was similar between all groups, predominantly consisting of TCA and muricholic acids (**Fig. 2F, J**).

Influence of microbiota on BA deconjugation and conversion over time

Before conventionalization, the plasma BA pool contained, as expected, exclusively conjugated primary BAs in both males and females (**Fig. 3A,B**). After conventionalization, primary BA levels increased in all groups except for

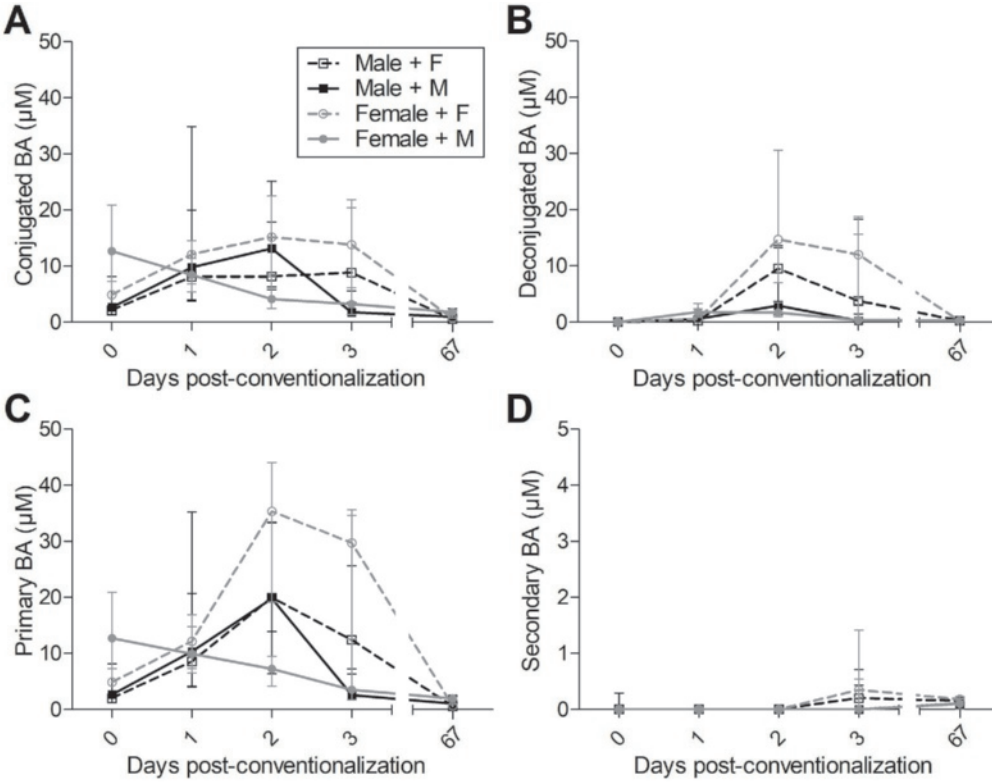

Figure 3: Absolute plasma BA changes before and after conventionalization. Change of BA levels from before to 10 weeks post-conventionalization. Conjugated **(A)**, deconjugated **(B)**, primary **(C)**, and secondary **(D)** BAs. Black = male; grey = female; n = 4-6 / group. Open symbol/striped line = female donor; closed symbol/ striped line = male donor.

female mice with a male microbiota donor (**Fig. 3C**). Primary BA levels were higher in recipients of a female microbiota donor on day two and three post-conventionalization (r_s = 0.54, p = 0.014 and r_s = 0.56, p = 0.011, respectively).

Deconjugated BAs appeared within 24 hours post-conventionalization, with no difference between groups, but absolute levels were again higher in female recipients (**Fig. 3B**; r_s = 0.46, p = 0.041). Two and three days post-conventionalization the level of deconjugated BAs was higher in mice with a female microbiota donor (r_s = 0.62, p = 0.005 and r_s = 0.57, p = 0.008, respectively). Conjugated BA levels slightly increased up to two days and had decreased after 10 weeks (**Fig. 3A**). Secondary BAs only appeared after 72 hours (**Fig. 3D**), and their levels were higher in mice with a female microbiota donor (r_s = 0.45, p = 0.045). Thus, before and one day post-conventionalization plasma BA levels depend on the sex of the recipient, while two and three days post-conventionalization, BA levels depend on the sex of the microbiota donor. Ten weeks after conventionalization, the BA levels are independent of both the sex of the donor and the sex of the recipient.

The relative amounts of individual BA species determine the hydrophobicity of the BA pool (**Fig. 4A**). GF mice showed a hydrophobicity index of -0.48 (-0.45 – -0.49). The hydrophobicity hardly changed within the first three days after conventionalization. In more detail, three days post-conventionalization recipients of a male microbiota donor showed a lower relative abundance of βMCA (p = 0.024), UDCA (p = 0.017), TCDCA (p = 0.041) and CDCA (p = 0.046) (**Fig. 4B**) and higher relative abundance of TCA (p = 0.046) and TUDCA (p = 0.030). Additionally, females had higher TαMCA (p = 0.019). After 10 weeks, the BA pool had turned more hydrophobic (range: 0.14 – -0.40) and this did not correlate with the sex of the donor (r_s = 0.47, p = 0.082) nor with that of the recipient (r_s = 0.10, p = 0.703). However, TβMCA and TUDCA abundance was increased in mice with a male microbiota donor, while TαMCA was higher in females (**Fig. 4C**).

Hepatic and ileal gene expression

To determine the long-term influence of the sex of the microbiota donor and the sex of the recipient on gene expression we analyzed several BA metabolism related genes in liver and distal ileum at 10 weeks after conventionalization (**Fig. 5**). From all genes tested, only hepatic *Cyp8b1* mRNA expression was affected by the sex of the donor, with slightly lower levels in recipients of the male microbiota donor (**Fig. 5A**; p = 0.016). Female mice showed marginally higher *Ntcp* expression (p = 0.005). Other hepatic gene (*Fxr*, *Cyp7a1*, *Cyp27a1* and *Bsep*) and distal ileum genes (*Fxr*, *Fgf15*, *Asbt*, *Ostα*) showed similar expression between all groups (**Fig. 5B**).

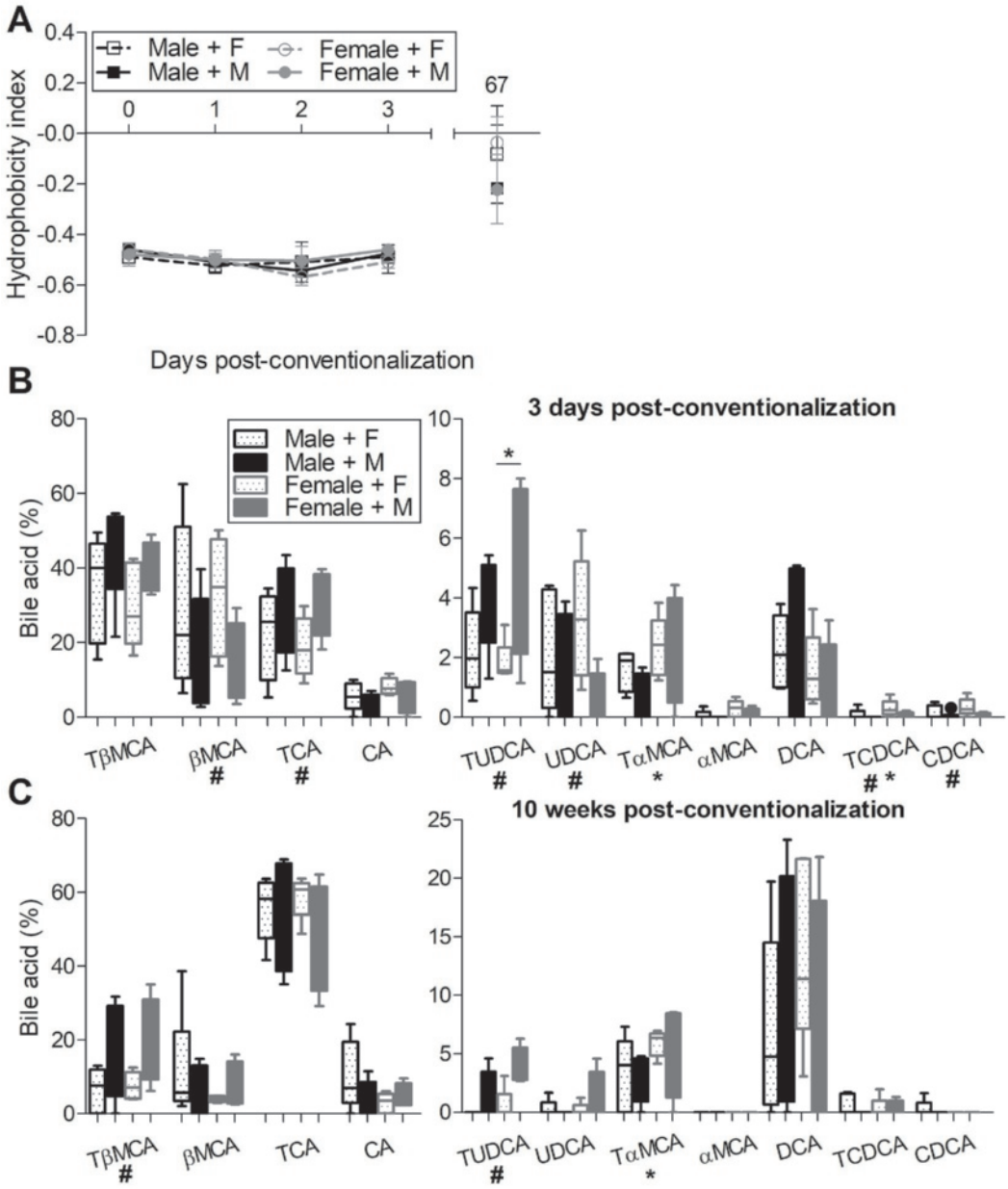

Figure 4: Relative plasma BA levels and hydrophobicity. Change in BA hydrophobicity index from before to 10 weeks after conventionalization **(A)**. Specific relative BA levels 3 days **(B)** and 10 weeks after conventionalization **(C)**. # sex of donor effect; * sex of recipient effect. Black = male; grey = female; n = 3-6 / group. Open bar = female donor; closed bar = male donor. $^{*/\#}p < 0.05$.

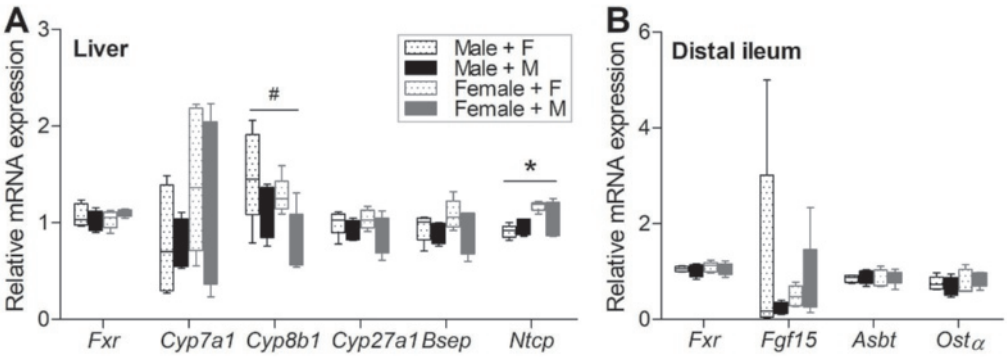

Figure 5: Hepatic and distal ileum gene expression. Gene expression in liver **(A)** and distal ileum **(B)** at 10 weeks post-conventionalization. # sex of donor effect; * sex of recipient effect; $^{*/\#}p < 0.05$. Black = male; grey = female; n = 3-6 / group. Open bar = female donor; closed bar = male donor.

Cecum microbiota composition post-conventionalization

To compare microbiota differences between the male and female microbiota donor pools and the conventionalized groups, we determined cecum microbiota composition by 16S rRNA-gene sequencing (**Fig. 6A**). The microbiota composition of the male and female microbiota donor pool used for conventionalization differed in *Muribaculaceae* (*S24-7*; 39.6% vs 28.1%, respectively), *Lactobacillaceae* (1.4% vs 6.0%, respectively), and *Bacteroidaceae* (0.7% vs 8.6%, respectively). Bray-Curtis dissimilarity-based principal coordinates analysis (PCoA) was performed on 16S rRNA gene sequencing data of the cecum microbiota composition of the recipients 10 weeks after conventionalization. This revealed that overall microbiota composition was similar between the conventionalized groups (**Fig. 6B**). The samples do not form clusters and 67 % of the variance could be explained by PCo1 (46 %) and PCo2 (21 %).

Correlation analysis showed that the Shannon Index neither correlated with the sex of the recipient nor with the microbiota donor (data not shown). At the family level, *Clostridiaceae* and *Lachnospiraceae* were enriched in female recipients (**Fig. 6C**; r = 0.53, p = 0.02 and r = 0.44, p = 0.03, respectively). *Coriobacteriaceae*, *Proteobacteria* phylum bacteria and *Helicobacteraceae* showed lower relative abundance in recipients of male donor microbiota (**Fig. 6C**). Thus, sex-specific differences observed in the donor microbiota pools were not reestablished 10 weeks after microbiota transfer. Absolute abundance of plasma bile acids correlated with the relative abundance of several bacterial families (**Fig. 6C**). βMCA and its conjugated form correlated with enrichment of many bacterial families.

Discussion

In the present study we tested the development of the BA composition upon conventionalization of GF mice, and the role that the sex of the microbiota donor plays in determining plasma BA levels and composition over time. Our data demonstrate that GF males and females conventionalized with cecum microbiota from the same or opposing sex on the long-term do not significantly differ in their plasma BA nor microbiota composition. Both intestinal microbiota and BA are important players in metabolic homeostasis[106]. Sexual dimorphism is apparent in either of the two and conceivably contributes to explain the variation in metabolism and metabolic disease risk between males and females[98, 101-104, 108-111, 114]. Our findings strongly suggest that a sexual dimorphic BA composition, as previously described in conventional mice, is not reestablished within 10 weeks after conventionalization of GF mice indicating that host factors in early life are important to shape the composition of the microbiota.

Before conventionalization of GF mice we found higher plasma BA levels in females, but a similar BA composition. Both findings were also seen in previous studies on GF mice, with the GF BA pool predominantly consisting of TβMCA and TCA[102, 103]. A likely mechanistic explanation for these observations comes from work demonstrating that the primary BA TβMCA is an FXR antagonist[93]. Higher levels of TβMCA, as seen in GF mice, specifically in GF females, result in less CYP7A1 inhibition and thus increased BA synthesis and pool size. Additionally, BA reabsorption via ASBT is stimulated in GF mice via TβMCA-mediated FXR inhibition, further contributing to an increased BA pool size[94].

The microbiota composition differences found in the male and female microbiota donor pool used for conventionalization was partly consistent with data from C57BL/6 microbiota sex-differences obtained in earlier studies[98, 105, 195]. An increased level of *Muribaculaceae* (*S24-7*) in the male microbiota donor pool and *Bacteroidaceae* and *Lactobacillaceae* in the female donor pool is in agreement with C57BL/6 sex differences in data from Sheng *et al*. (2017)[105]. Also, Org *et al*. (2016)[98] found lower relative abundance of *Bacteroides* (*Bacteroidaceae* family) in 16 week old C57BL/6 males. In contrast to our data, three strains of

Figure 6: Microbiota composition. Microbiota composition at the family level in the female and male microbiota donor pool and in recipients 10 weeks post-conventionalization **(A)**. Bray-Curtis dissimilarity-based principal coordinates analysis (PCoA) was performed on 16S rRNA gene sequencing data; the first two coordinates are shown (representing 67% of the total variance) **(B)**. Significant ($p < 0.05$) spearman correlations between absolute plasma bile acid levels and relative cecum microbiota family abundance 10 weeks post-conventionalization (male = 0; female = 1) **(C)**. n = 4 – 5 / group. * Figure displayed on next page.

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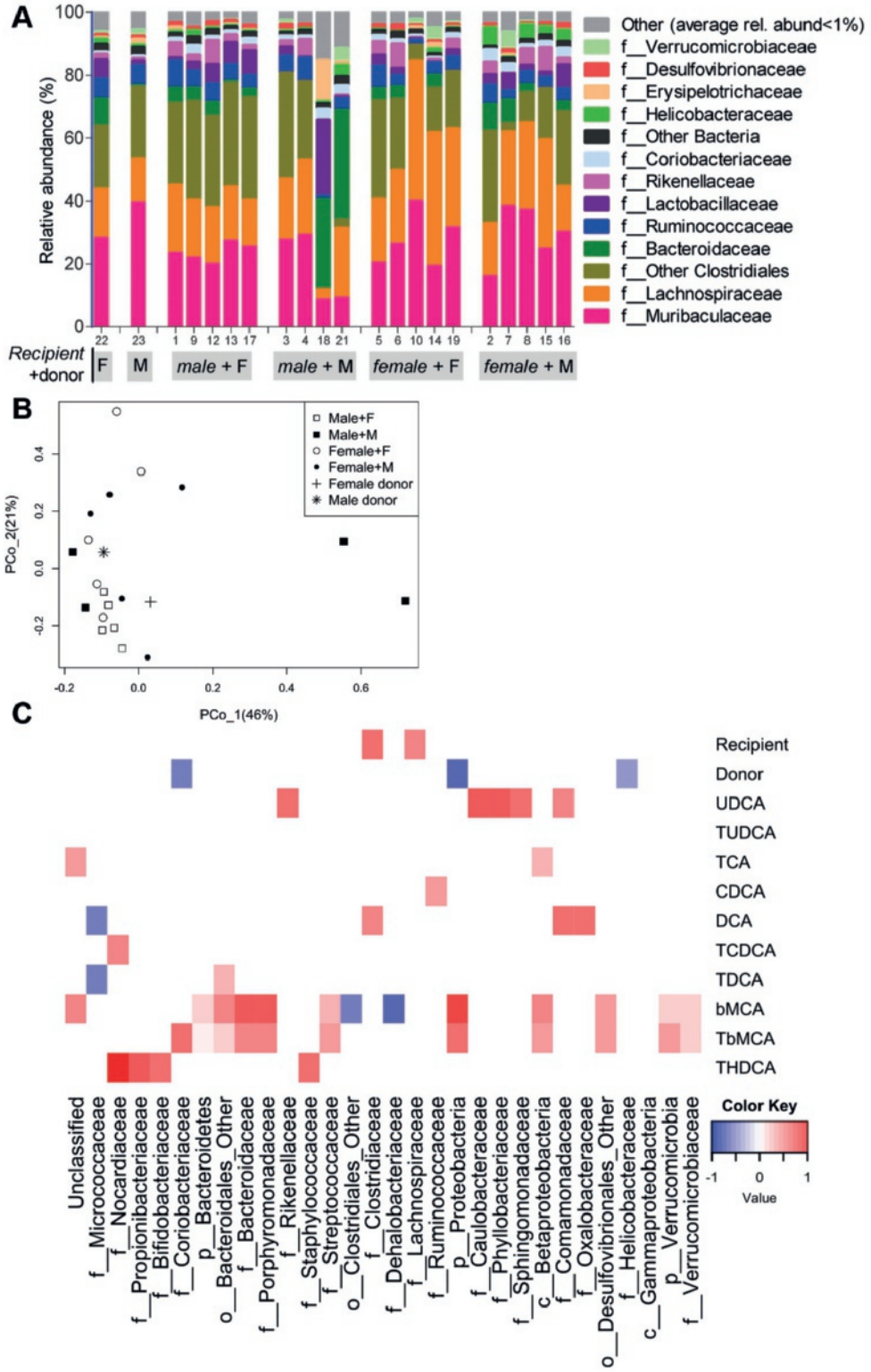

Lactobacillus and *Bacteroides* (*Lactobacillaceae* and *Bacteroidaceae* family) were enriched in 11 to 23 week old C57BL/6 males compared to females[195]. Some other bacterial genera previously indicated to be differentially present in male and female microbiota of C57BL/6 mice, such as *Coprococcus*[98], *Clostridium* and *Enterococcus*[195], were not different in our male and female microbiota donor pool (genus level data not shown). Gilliland and colleagues (2018)[196] observed that the cecum of GF mice which received cecal content, was first dominated by bacteria distinct from that in the transferred cecal content. Seven to 21 days later, the cecum microbiota was equal to that of the donor. This suggests that in the first days post-conventionalization, the changes in BA composition are a reflection of the fast developing intestinal microbiota composition and their respective enzymes. Thereby, specific intestinal bacteria determine the deconjugation and type of modification of the conjugated primary BAs. The developing microbiota in the first days after conventionalization may also explain the substantial variation in bile acid levels between the mice.

Upon conventionalization of the GF mice with a microbiota donor of the same or opposing sex, the BAs in the plasma of conventionalized mice show that deconjugation of intestinal BAs readily occurs within the first 24 hours. Activity of the deconjugating enzyme BSH has been demonstrated in the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Clostridium*, and *Bacteroides*. Indeed, recipients of the female microbiota donor pool, where *Lactobacillus* and *Bacteroides* were present in higher relative abundance, showed quicker appearance of deconjugated BA in plasma than recipients of the male microbiota donor pool. Also in humans, the female gut microbiota has been shown to have elevated BSH abundance[197].

Secondary BAs appeared in plasma only after 72 hours, indicating a delay in conversion of deconjugated primary BAs into secondary BAs. Removal of taurine/glycine is required for the most important conversion 7α/ß-hydroxylation, but not for oxidation and epimerization[91, 198]. Release of taurine or glycine upon deconjugation can stimulate growth of specific aerobic and anaerobic bacteria[199] with enzymes for conversion of BAs. Additionally, the metabolic conversion of primary to secondary BA is restricted to a limited intestinal bacterial group of anaerobic bacteria, in human colonic flora only 0.0001% of total bacteria, including the genera *Clostridium*, *Bacteroides*, and *Eubacterium*[82, 91]. The mainly obligate anaerobic bacteria needed for producing secondary BA might not survive the harvest and conventionalization processes, thus delaying the appearance of secondary BA in the plasma of the conventionalized germ-free mice.

Almost 10 weeks after conventionalization there were little differences between conventionalized male and female mice. In parallel, absolute and relative abundance of BAs as well as the hydrophobicity of the BA pool were similar

between the groups. An interesting explanation to explore for this unexpected lack of sex differences is that the mice in this study were germ-free up to 12 weeks of age, which may have caused differences in the development of regulatory pathways of BA metabolism as compared to conventionally-grown mice. In an earlier study we demonstrated that bile flow and bile acid secretion in adulthood are affected by a germ-free gestation and lactation period[200]. However, Wang *et al*. (2016)[201] conventionalized 8-10 week old male and female germ-free C57BL/6J mice by oral gavage with stool from a 25-year old man and found many microbiota diversity and species differences within 7 days. Their data suggests that germ-free mice of this age display sex differences in intestinal handling of incoming microbiota on short term, but this study did not investigate microbiota composition beyond one week after conventionalization, nor interplay between microbiota and bile acids. ßMCA and its conjugated form positively correlated with several bacterial families, but none of these families were detected to correlate with this bile acid in a previous study[105]. This discrepancy could be related to differences in diet, since diet and microbiota also strongly interact[202].

Sex differences in serum BA levels and composition of conventional mice become more prominent later in life[104, 114], likely due to post-puberty hormone levels[98, 106, 114]. We cannot exclude that the lack of substantial differences found in this study may be related to the relative young age of the microbiota donors (12 weeks). Interestingly, transfer of microbiota can also affect sex hormone levels in the recipient, temporarily resulting in more testosterone in female mice and a changed serum metabolome[104]. An elevated opposite-sex hormone level in conventionalized mice might reduce differences between conventionalized males and females.

Fecal microbiota transfer (FMT) as a method to treat metabolic diseases and infections is increasingly investigated and in cases of recurrent *C.difficile* infection quite effective[203]. However, treatment of *C.difficile* infection with FMT was associated with a lower success rate in females[204]. Sex-differences in the microbiota-host interaction reveal that the response to FMT and other live biotherapeutics is likely different between the sexes[106].

In summary, we are, to the best of our knowledge, the first to describe the dynamics of BA changes after conventionalization of GF mice. Our findings demonstrate that sex differences in the microbiota composition of a fecal donor do not have lasting influences on the biliary system when given to a young germ-free host of the same or opposing sex. More work is required to explore the underlying molecular basis of these observations. In the meantime, future studies should take the recipient sex into account when searching for treatment options of disorders involving an altered microbiome.

Materials and methods

Animal studies. GF C57BL/6JOlaHsd mice were group-housed in isolators and fed autoclaved GF diet (ssniff® R/M-H autoclavable, V1534-3, ssniff Specialdiäten GmbH, Germany). The GF status was confirmed at monthly intervals by incubator swap cultures. At 12 weeks of age, tail blood was taken and six male and six female mice received one 60 µL oral gavage of cecum content donor pool of either the same or opposing sex. Subsequently, all mice were individually housed in temperature-controlled conditions with 12:12 light dark cycles and continued to be fed the autoclaved GF diet. The microbiota donor pool consisted of cecum content of either five male or five female mice of 12 weeks age, acclimatized to the GF diet for four weeks prior to sacrifice. Cecum content was collected, 24x diluted in PBS and 20% glycerol, aliquoted in cryogenic vials and stored at -80^{0}C until conventionalization. Tail blood was taken before and one, two, and three days post-conventionalization. We repeatedly measured body weight and food intake and collected feces. 67 days post-conventionalization, blood was obtained via heart puncture, the mice were sacrificed and liver and intestine were excised and snap-frozen in liquid nitrogen. All animal experiments were approved by the ethical committee for animal experimentation (IACUC) at the University of Groningen and performed in accordance with the Dutch National Law on Animal Experimentation and international guidelines on animal experimentation. A schematic of the detailed set-up of the study is shown in **Figure 1**.

Plasma BAs. Plasma BA concentrations were determined using liquid-chromatography mass spectrometry (LCMS) as described previously[103].

Cecum microbiota analysis. Bacterial DNA of the available cecum content was isolated and processed for the 16S rRNA-gene sequencing using the Illumina Miseq platform as described in Heida *et al.* (2016)[190].

Gene expression analysis. Hepatic and distal ileum mRNA was extracted using TriReagent (Sigma) and quantified with a Nanodrop ND-100UV-vis spectrometer (NanoDrop Technologies Wilmington DE). cDNA was made from 1µg of RNA using reagents from Invitrogen (Carlsbad CA). Primers were synthesized by Eurogentec (Seraing, Belgium). Real-time PCR was performed using an ABI Prism 7700 machine (AppliedBiosystems, Damstadt Germany). mRNA expression levels of individual genes were calculated relative to the housekeeping gene cyclophilin.

Statistics. The statistical analysis was performed with GraphPad Prism 5 Software. Statistical significance was tested with two-way ANOVA post-hoc Bonferroni. Graphs are made with GraphPad Prism 5; using the Tukey method for plotting the box ((25th - 75th

percentiles), whiskers (1.5 IQR) and outliers (>1.5 IQR) or median plus interquartile range. Spearman correlation analysis on bile acid levels over time was performed using SPSS. Statistical analysis of the MiSeq 16S rRNA-gene data was done using the R package "stats" and "vegan" to investigate the microbial community and its correlations with bile acids.

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Author contributions

M.A.M.L. was responsible for data acquisition and analysis and drafting the article; P.L. and H.J.M.H. were responsible for analysis and supervision of the MiSeq analysis and drafting the microbiota figures; and U.J.F.T. and H.J.V. were responsible for the conception, design, and supervision of the study, interpretation of data, and critical article revision for important intellectual content. All contributing authors gave final approval for the version to be published.

Additional information

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Competing interests.

The authors declare that they have no competing interests.

Absence of intestinal microbiota during gestation and lactation does not alter the metabolic response to a Western-type diet in adulthood

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Chapter 5

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Abstract

SCOPE: Microbiota composition in early life has been implied to affect the risk to develop obesity in adulthood. It is unclear whether this risk is due to long-lasting microbiome-induced changes in host metabolism. We aimed to identify whether the presence or total absence of early-life microbiota affects host metabolism in adulthood.

METHODS AND RESULTS: We compared the effects of a germ-free (Former GF) versus conventional (Conv) status during gestation and lactation on the metabolic status in adult offspring. Upon conventionalization at weaning, all mice were metabolically challenged with a Western-type diet (WTD) at 10 weeks age. Between age 10 and 30 weeks, a former GF status did not notably affect overall body weight gain, cholesterol metabolism, glucose tolerance or insulin sensitivity at adult age. However, Former GF mice had lower bile flow and bile acid secretion in adulthood, but similar bile acid composition.

CONCLUSIONS: A germ-free status during gestation and lactation does not substantially affect key parameters of the metabolic status before 10 weeks of age on chow diet or in adulthood following a WTD challenge. These data imply that microbiota in early life does not critically affect adult metabolic plasticity.

Introduction

Early-life environmental conditions have been implied in health risks in adulthood. One of the classic examples has become the epidemiological research by Barker *et al*.[121]. Barker *et al*. have shown that conditions in fetal life are related to adult cardiovascular health risk. There is a growing interest in the potential long-lasting role of the intestinal microbiota in early life[205].

Microbiota and microbiota-derived metabolites in early life can affect the development and function of the metabolic system[205], also in humans[14, 206, 207]. A study in mice has shown that antibiotics in early life transiently changed microbiota composition, leading to increased adiposity in adulthood[13]. This phenotype could be reproduced by microbiota transfer to germ-free mice, suggesting that adult adiposity may be programmed by the early-life microbiota[13]. Other studies in rodents demonstrated that the presence of intestinal microbiota, its composition, and their specific enzymes affect bile acid, cholesterol and lipid metabolism as well as body weight, and glucose and insulin tolerance[85, 208-211]. Cholesterol conversion into bile acids is an important method for maintaining cholesterol homeostasis. Bile acid receptors are involved in pathways of bile acid synthesis and transport, choleresis, glucose homeostasis and lipid metabolism[212]. Modification of bile acids by the gut microbiota changes the signaling properties of bile acids and thereby possibly whole-body physiology[117]. Diet can play an important role in steering this microbiota-regulated host metabolism[213].

In the present study, we determined the effects of an extreme situation in early-life microbiota, namely a germ-free condition confined to the gestation and lactation period, on metabolism in adulthood. We compared this condition to conventional mice (i.e. with normal exposure to microbiota in early life). To determine potential effects on long-term metabolic adaptations, we characterized key metabolic parameters, including cholesterol and bile acid levels, on chow diet and after challenging the metabolic system with a Western-type Diet (WTD). Since sexual dimorphism in metabolic programming has been demonstrated[13, 99, 182], we studied male and female mice separately.

Results

Body weight development: effects of former GF status and sex

At weaning, germ-free and conventional mice received an oral gavage with an inoculum from age-matched male donors (Former GF and Conv group, respectively). At 24h post-gavage, predominantly conjugated primary bile acids

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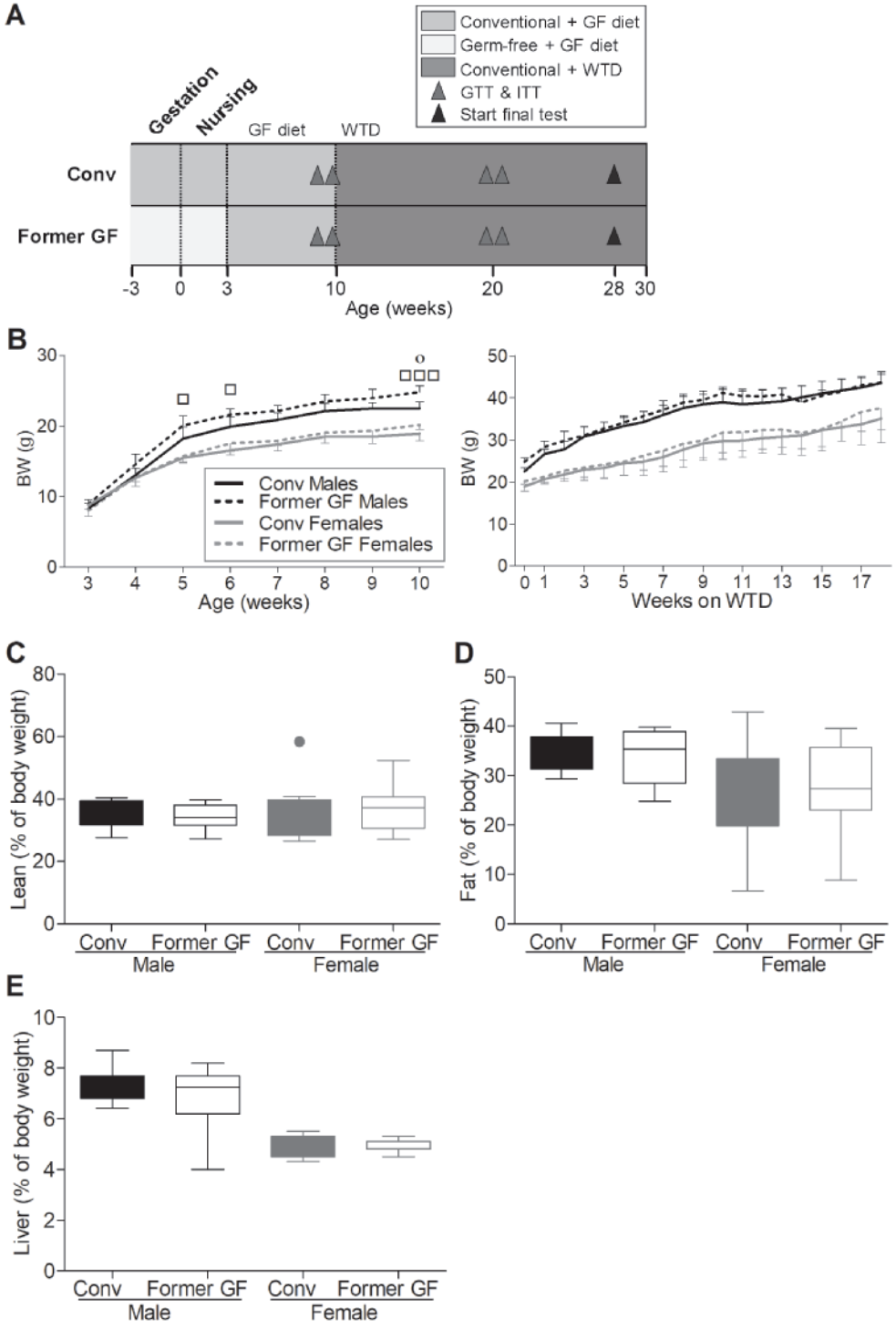

were present in the feces of the Former GF group but not in the Conv group (Supporting Information Figure S1A-B), in accordance with the previous germ-free state of the Former GF mice.

The former GF status resulted in an elevated body weight in males throughout the post-weaning period, up to the switch to the WTD at the age of 10 weeks (Figure 1A-B). The body weight of Former GF females was also slightly higher than Conv females, although this only reached significance at 10 weeks of age. From 10-30 weeks of age body weight was not significantly different between Former GF and Conv mice (Figure 1B). Body composition, measured 12 weeks after the start of the WTD, was not different between Former GF and Conv mice, but body fat was higher in males compared to females (Figure 1C; Figure 1D, $p < 0.05$).

At sacrifice, after correction for body weight, liver weight, food intake and fecal production were not affected by absence of microbiota in early life, but liver weight was higher in males compared to females ($p < 0.01$; Figure 1E), and food intake and fecal production were higher in females than males (+36 %, $p = 0.002$; +9%, $p = 0.002$, respectively; Supporting Information Figure S2A-B). Additionally, the former GF status resulted in lower food intake in females (-25 %) but not in males (interaction, $p = 0.01$).

No effects of the former GF status on glucose and insulin tolerance

To determine whether early-life absence of microbiota has long-lasting effects on glucose and insulin homeostasis, we performed a glucose and insulin tolerance test before the WTD challenge (at 9-10 weeks age) and 10 weeks after the start of the WTD (at 20-21 weeks age). Despite the significant difference in body weight before the dietary challenge the glucose and insulin tolerance were similar between Conv and Former GF mice at 10 weeks of age (Supporting Information Figure S3). Also after 10 weeks on the WTD, the glucose and insulin tolerance were not significantly affected by the former GF status in male nor female mice (AUC NS) (Figure 2).

Figure 1: Basic parameters. A) Experimental setup. **B)** Body weight development. Between male groups = □; between female groups = ○; □/○ $p < 0.05$; □□□ $p < 0.0001$. At 3-10wk of age: Males: microbiota effect, $p = 0.003$; Females: interaction (time affects BW differently, $p = 0.004$. At 10-28wk of age: Males: interaction, $p = 0.010$. **C)** Lean mass; and **D)** fat mass at the age of 23 weeks; sex effect, $p = 0.006$ and $p = 0.002$, respectively. **E)**. Liver weight per 100g body weight at sacrifice; sex effect: $p < 0.0001$. Straight line/closed box = conventional (black = male, n = 7; grey = female, n = 9); dotted line/open box = Former GF (black = male, n = 8; grey = female, n = 7). *Figure displayed on previous page.

No effects of the former GF status on plasma cholesterol and hepatic lipid levels

We determined the effect of the germ-free gestation and lactation on lipid profiles in plasma and liver at adult age. Plasma cholesterol and hepatic triglyceride levels were lower in female mice, independent of whether or not they had been exposed to a GF status in early life (Conv: $p < 0.01$; Former GF: $p < 0.05$; Figure 3A,C). The elevated plasma cholesterol in males was due to higher cholesterol levels in HDL and in IDL/LDL-size particles (Figure 3B). Hepatic total and free cholesterol as well as phospholipid levels were similar in all groups (Figure 3D-F).

Liver, bile and feces parameters: effects of former GF status and sex

To determine whether bacterial absence in early life can also influence bile acid metabolism in adult life we analyzed hepatic and fecal bile parameters. The former GF status significantly decreased bile flow in both males and females (Figure 4A;

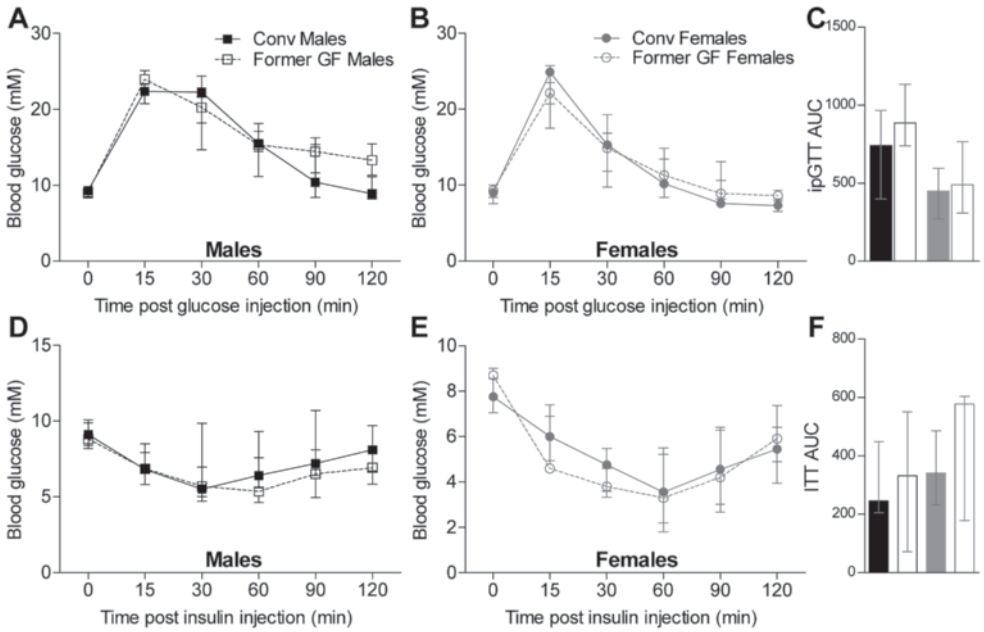

Figure 2: Glucose and insulin tolerance on the WTD. Intraperitoneal glucose (ipGTT) and insulin tolerance tests (ITT) were performed around 20-21week age, after 10 weeks on the WTD. Blood glucose levels were measured after ip glucose injection (2.5 g / kg body weight) in males **(A)** and females **(B)**. Blood glucose levels were measured after ip insulin injection (0.5U / kg body weight) in males **(D)** and females **(E)**. Area under the curve was calculated for ipGTT **(C)** and ITT **(D)**. Black = conventional; grey = Former GF; n = 7 – 9 / group.

-20 % and -13 %, $p < 0.05$ each, respectively). Also biliary bile acid secretion rate was decreased in Former GF mice (Figure 4B; males -40%, n.s.; females -26%, $p < 0.05$). Overall biliary bile acid composition was not substantially different between the groups (Figure 4C). However, biliary bile acid concentration was slightly higher in females, with increased levels of Tα-MCA, TUDCA, and TCDCA (Supporting Information Figure S4; +190 %, +138 %, +196 %, respectively). Fecal bile acid excretion was significantly higher in female mice, without an effect of the former GF status (Figure 4D; +87 %, $p < 0.0001$).

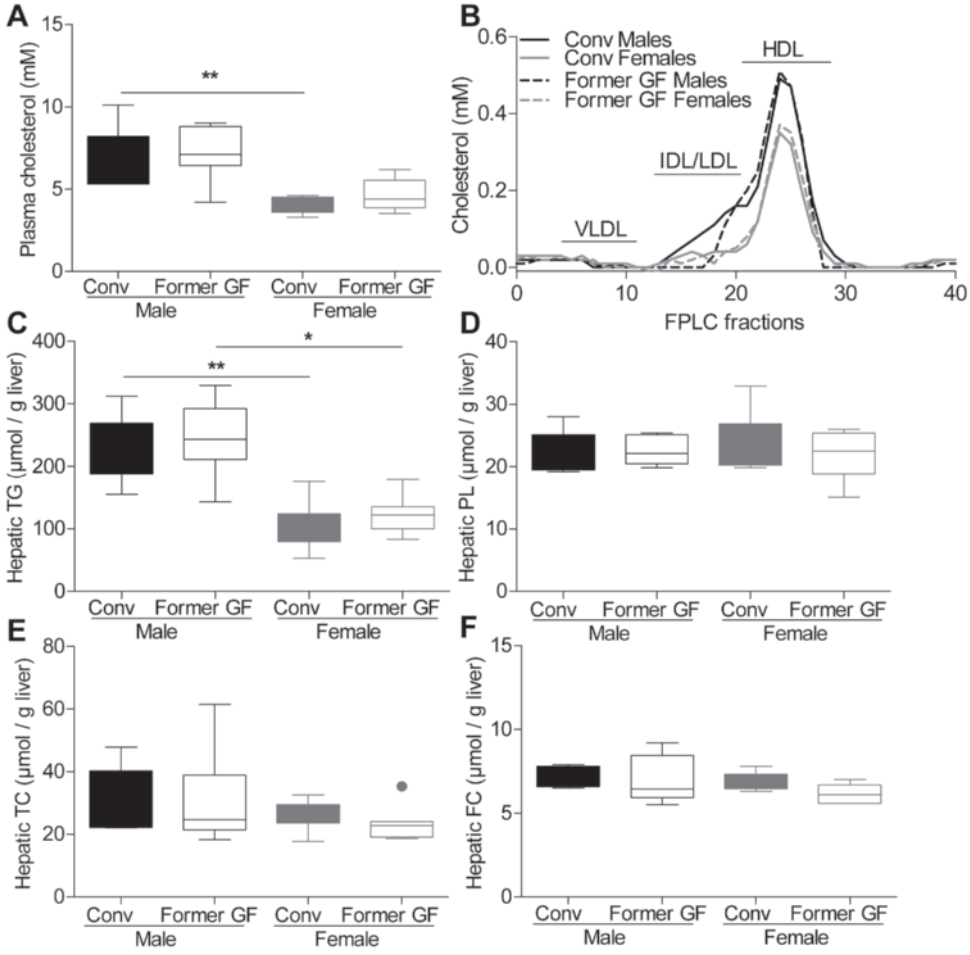

Figure 3: Lipid parameters in adulthood. A) Total plasma cholesterol levels. **B)** Cholesterol in lipoprotein fractions. Hepatic **C)** triglycerides (TG), **D)** phospholipids (PL), **E)** total cholesterol (TC) and **F)** free cholesterol (FC). Straight line/closed box = conventional; dotted line/open box = Former GF; black = male; grey = female; n = 6 – 9 / group).

We determined gene expression of bile acid-, cholesterol- and lipoprotein-related genes in liver and distal ileum (Figure 4E). Several sex-dependent differences were observed: the bile acid synthesis enzyme *Cyp7a1* was increased in females ($p = 0.005$), while *Cyp8b1* and *Cyp27a1* ($p = 0.007$ and $p = 0.004$) as well as the HDL-receptor *Srb1* ($p < 0.001$) were decreased compared to males. The former GF status increased hepatic *Ldlr* gene expression ($p = 0.023$). No differences were observed for *Abcg5*, *Abcg8*, *Bsep*, *Fxr*, or *Hmgcr*. In the distal ileum, the bile acid-responsive gene *Fgf15* was non-significantly decreased in Former GF mice ($p = 0.065$). The mRNA expression of the bile acid transporter *Asbt* was neither affected by sex nor early-life microbiota status.

Cholesterol fluxes at adult age: effects of former GF status and sex

Since bile acid metabolism relates to cholesterol homeostasis, we also determined the effect of the early-life GF status on cholesterol metabolism. Dietary cholesterol intake, as calculated from food intake, was decreased in Former GF females but not in males, compared to the Conv mice (-25 %, $p < 0.05$, Figure 5A). The former GF status significantly decreased cholesterol secretion via the bile in males (Figure 5B: -52 %, $p < 0.01$) but not in females (interaction, $p = 0.015$). Excretion of cholesterol via the feces in the form of neutral sterols was lower in all female compared to male mice, and not affected by the former GF status (Figure 5C, -29 %, $p = 0.0002$). The net cholesterol transport across the intestine is the difference between fecal NS excretion and the sum of dietary and biliary cholesterol influx into the intestine. In agreement with previous studies, the amount of cholesterol secreted via the feces exceeded the dietary and biliary influx, indicating net transintestinal cholesterol excretion (TICE) (Figure 5D). Net TICE was higher in females than males (+237 %, $p < 0.0001$) and lower in Former GF females than Conv females (interaction, $p = 0.05$; -44 %, $p < 0.05$).

The fractional cholesterol absorption was higher in females than in males (Figure 5E; +66 %, $p < 0.0001$), without an effect of the former GF status. The former GF status did not significantly affect the fractional cholesterol synthesis in both males and females (Figure 5F; interaction $p = 0.029$: males + 45 %, females -10 %).

Adult microbiota is not affected by the former GF status

To assess possible long-term influences of the early-life microbiota status on adult microbiota composition, we determined cecum microbiota composition of the inoculum and after conventionalization at 30 weeks of age (Figure 6). The Shannon index showed higher diversity in the original microbiota composition of the inoculum as compared to the composition at sacrifice (data not shown).

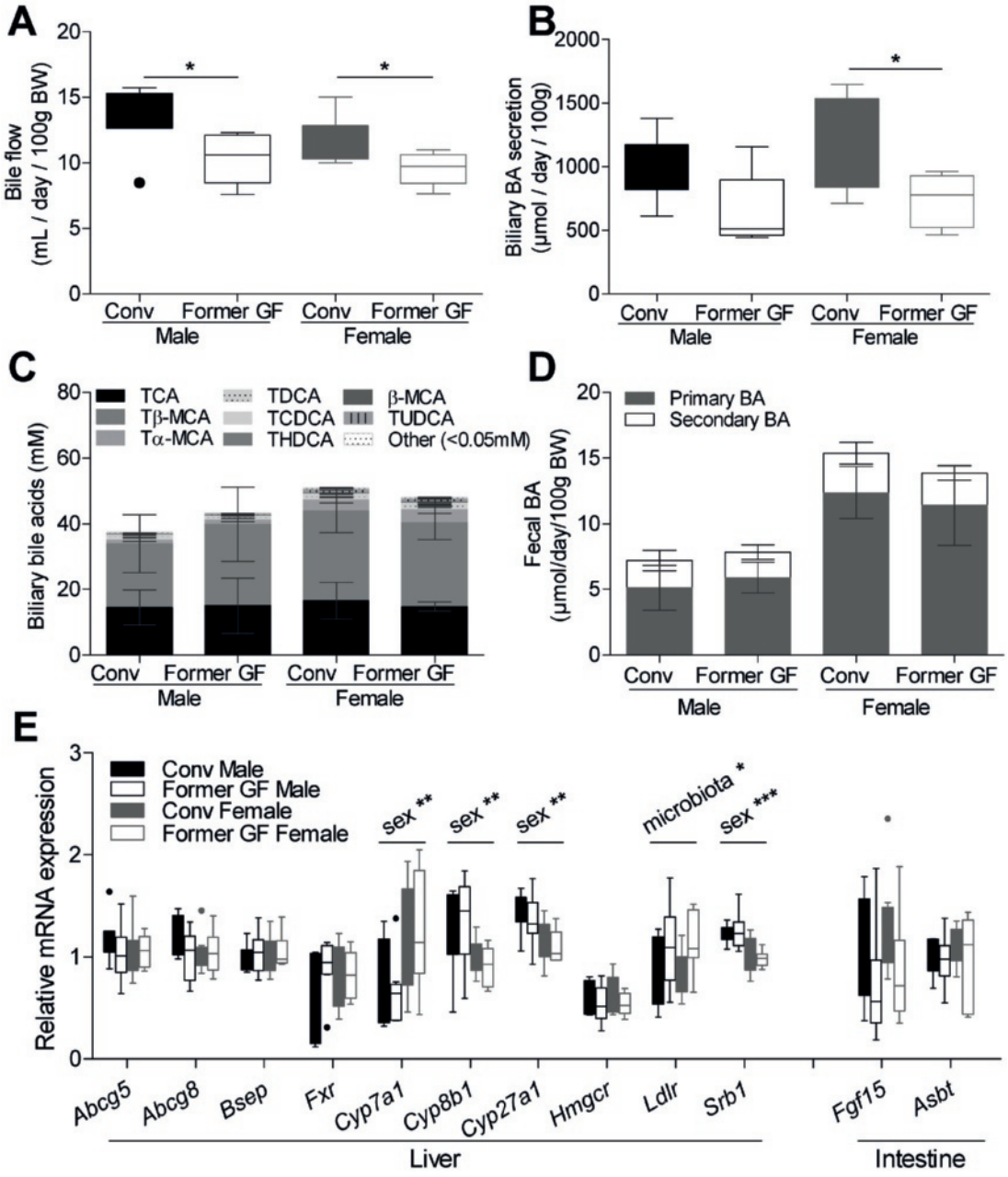

Figure 4: Hepatic and fecal bile acids and gene expression. A) Hepatic bile; microbiota effect: p = 0.0006. **B)** Total biliary BA; microbiota effect: p = 0.001. **C)** Biliary BA composition. **D)** Fecal BA; sex effect: p < 0.0001. **E)** Relative mRNA expression in liver and distal ileum (ratio versus cyclophilin). Straight line/closed box = conventional; dotted line/open box = Former GF; black = male; grey = female; n = 6 – 9 / group).

The number of operational taxonomic units (OTU) was also higher in the inoculum (data not shown). Principal component analysis of the cecal microbiota composition at sacrifice revealed that overall microbiota composition was similar between the groups (Supporting Information Figure S5). At the family level, most variation could be explained by PC1 (81 %) and PC2 (8 %). Correlations between specific bacterial species levels and metabolic parameters were also analyzed. Apart from slightly higher abundance of two *Clostridiales*-order families: *Clostridiaceae* and *Peptostreptococcaceae* in Former GF mice ($p = 0.003$, data not shown), no strong associations could be identified (data not shown).

Discussion

We determined in mice the effect of a germ-free gestation and lactation period on adult metabolic parameters after a challenge with a WTD. Our approach to compare former germ-free to conventional mice provided the opportunity to determine possible long-lasting metabolic effects of early life absence of microbiota. Also, this model excluded possible indirect influences from for example antibiotics, which have been used in previous studies on long-term effects of microbiota modulation[13, 80]. Our data do not indicate major effects of a GF period during gestation and lactation on adult body weight and composition nor on glucose or insulin sensitivity. The early-life GF status reduced bile flow and bile acid secretion in adult male and female mice, but did not majorly affect bile acid composition or cecal microbiota composition. Therefore, direct microbiota-mediated effects as well as microbiota-derived products in early life may not be of major relevance for adult metabolic homeostasis.

A previous study reported programming effects of early-life antibiotic treatment in mice[13]. Due to low-dose penicillin exposure from the end of gestation until weaning, the microbiota composition transiently changed and these mice developed increased body fat as compared to non-exposed control mice[13]. This increased adiposity phenotype could be reproduced by transferring the low-dose penicillin-exposed microbiota to GF mice, suggesting that early-life microbiota composition has the potential to affect adult body physiology. Both in that and in our present study, male mice developed increased body weight until the chow diet was replaced by either a high fat or Western-type diet. We cannot exclude that the diets used in these studies were overriding the observed subtle body weight effects, consistent with the sensitivity of C57BL/6J males to an ad libitum high-fat diet[214]. To examine this possibility, a control group of mice remaining on a chow diet could elucidate this, but this has not been performed so far. After the

switch to the WTD, the body weight became similar between the Former GF and Conv males. These results are comparable to findings of Bäckhed *et al*.[68], who demonstrated that GF males conventionalized at 7-10-weeks of age caught up on body fat and lowered chow consumption to the level of conventional males within

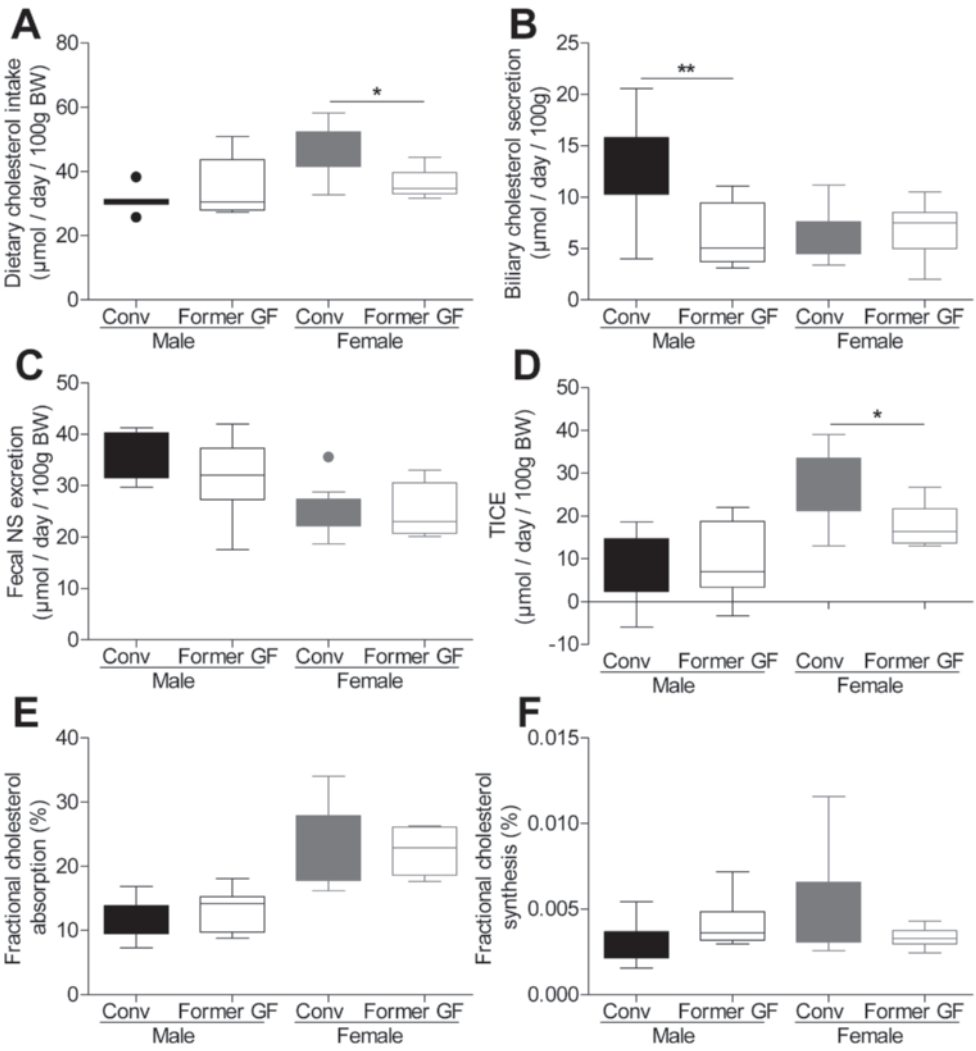

Figure 5: Cholesterol homeostasis. A) Dietary cholesterol intake; interaction effect: p = 0.012. **B)** Biliary cholesterol; interaction effect: p = 0.015. **C)** Fecal NS; sex effect: p = 0.0002. **D)** Net cholesterol transport is the result of Fecal NS excretion – (Dietary cholesterol intake + biliary cholesterol secretion); interaction effect: p = 0.046. **E)** Fractional cholesterol absorption; sex effect: p < 0.0001. **F)** Fractional cholesterol synthesis; interaction effect 0.029. Closed box = conventional; open box = Former GF; black = male; grey = female; n = 6 – 9 / group).

14 days. Females were not investigated in that study. In our study, females exposed to a former GF condition increased neither body weight nor body composition. In contrast, low-dose penicillin-exposed females acquired increased total, lean and fat mass[13]. Antibiotic administration reduces and changes the diversity of the microbiota composition, but may still allow for indirect microbiota effects[215]. The Former GF offspring was not exposed to microbiota or microbiota-derived products of any kind until weaning and thus the presence of indirect microbiota effects can be excluded[216]. Furthermore, besides killing bacteria, antibiotics also affect mitochondrial and thus energy metabolism[217]. Thus, antibiotic exposure during development may induce mitochondrial changes and thereby long-lasting effects on metabolism.

Mice in this study were fed a diet specifically designed for germ-free mice. These germ-free diets contain extra nutrients to compensate for the lack of nutrients normally produced by the microbiota. Fortified diets are supplemented with vitamins that would otherwise not be available in the diet, which prevents vitamin-deficiency and in some cases lethality in the germ-free mouse[218].

The results of this study did not show an effect of the former GF status on glucose and insulin tolerance later in life. Although the IQR was significant, particularly for the ITT, neither glucose tolerance nor insulin tolerance correlated with body weight or epididymal fat. The effect of early-life microbiota absence on glucose and insulin homeostasis, compared to the conventional condition, has not been tested before as far as we know. A recent study determined that a combination of pre- and probiotics in early life can protect against disturbances in glucose homeostasis in adulthood[219], suggesting a role for early-life microbiota. Several studies investigated the direct effect of a GF status or (early-life) antibiotics on glucose and insulin tolerance. GF male mice have improved glucose and insulin tolerance as compared to GF males conventionalized at birth or two weeks before measurement, however, this was not compared to regular conventional mice[68, 220]. Early-life and adult antibiotic treatment in mice improved glucose tolerance presumably via altering microbiota and LPS exposure, but this effect did not last beyond the antibiotic treatment[221, 222]. Early-life antibiotics in a swine model induced a minimal decrease in glucose tolerance via short-chain fatty acid signaling and pancreatic development, together with only a transient change in microbiota composition[223]. The inconsistencies in findings may be due to the model used, including organism and the start, type and duration of microbiota change in early life. Our study does indicate, however, that the complete absence of microbiota during early life has no long-lasting effects on body weight, body composition, or glucose and insulin metabolism, upon a challenge with a WTD.

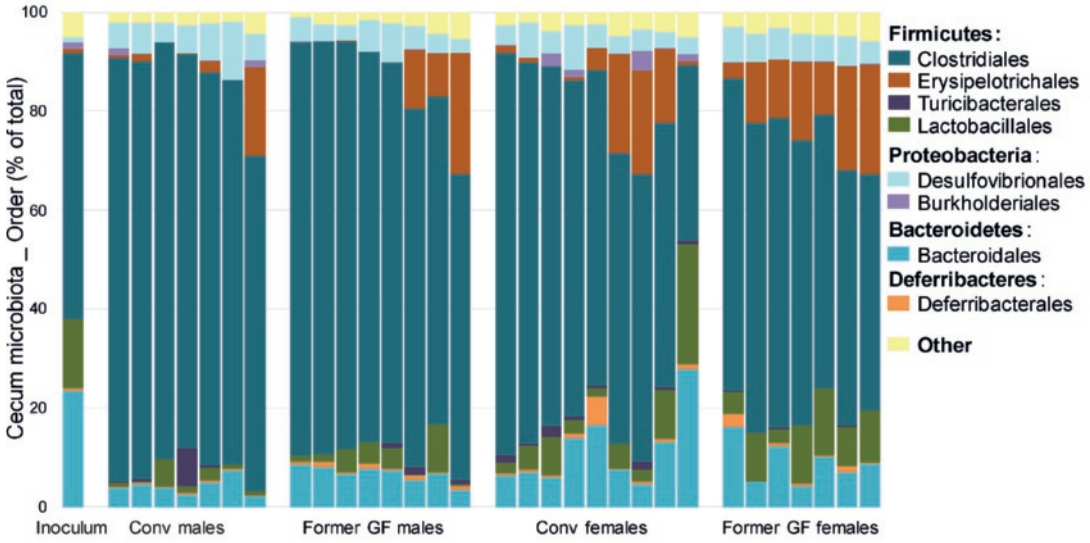

Figure 6: Cecum microbiota composition in adulthood and of inoculum. Cecum microbiota composition in adulthood and of the original inoculum from MiSeq data at order level. Conv male, n = 7; Conv female, n = 9; Former GF male, n = 8; Former GF female, n = 7. Data are presented as % of total.

One rather unanticipated finding was the reduced bile flow and bile acid secretion in Former GF mice. We thought of several possible explanations for this result. First, bacteria can influence the bile acid composition and thereby also the bile acid-dependent bile flow[224]. Out *et al*.[188] demonstrated that gut microbiota can inhibit ASBT-dependent enterohepatic recycling of bile acids, with increased bile flow in antibiotic treated mice. However, this cannot explain the lower bile flow in the Former GF mice, since intestinal microbiota composition and ileal *Asbt* expression were similar between Conv and Former GF mice. The only carry-over effect in adulthood of the former GF status was the abundance of the families *Clostridiaceae* and *Peptostreptococcaceae*. Yet, no apparent link between these levels and bile parameters can be found with current measurements and knowledge. Second, food intake is known to lead to bile secretion[225]. Food intake and biliary BA secretion were exclusively lower in Former GF females, not in males, when corrected for body weight. Therefore, food intake does likely not explain the decreased bile flow that was observed in both Former GF males and females. Third, bile acids promote *Fgf15* expression via ileal *Fxr* to inhibit hepatic bile acid synthesis. *Fgf15* expression in the distal ileum was indeed slightly decreased in Former GF mice, but the difference did not reach statistical significance. Also, the expression of hepatic bile acid synthesis genes (*Cyp7a1, Cyp8b1, and Cyp27a1*) was not affected by the former GF status. The present analyses do not provide a likely

explanation regarding the observed long-term effect of the former GF status on bile flow and bile secretion by the former GF status. We believe that bile acid kinetics, allowing the quantitation of the bile acid pool size and synthesis, may prove to be helpful to elucidate this.

Metabolic development and control of homeostasis is fundamentally different between males and females[97]. Consequently, sex affects the basic levels and the impact of early-life environment on adult metabolic parameters such as body weight, food intake and plasma glucose[226-228]. Therefore, we performed our studies separately in male and female mice. Indeed, we observed many sex differences, demonstrating that males and females can adapt differentially to (absence of) early-life microbiota. Also, it indicates that long-term effects are likely to be subjected to sex-specificity, which should be taken into account in future long-term effect studies.

We conclude that the long-term metabolic effects of a germ-free gestation and lactation period on body composition as well as glucose and insulin tolerance are minimal in mice, even when challenged with a WTD. However, discrete effects on bile acid and cholesterol metabolism were identified. Our findings indicate that, under these circumstances, microbiota in early life is not necessary to achieve a healthy metabolic phenotype in adulthood. While absence of microbiota in early life did not profoundly affect adult health, qualitative differences of early-life microbiota composition might still have a role in modulating long-term metabolic health. To further delineate possible long-term effects of qualitative early-life microbiota composition, one would need to consider a possible critical window of plasticity, type of dietary challenge and sex-specificity.

Materials and methods

Animal studies. We used germ-free C57BL/6JOlaHsd mice to produce conventional parents. Germ-free mice were conventionalized with inoculum (as described below), 5 weeks before mating for breeding and group-housed in individually ventilated cage (IVC) cages. Germ-free mice were group-housed in isolators. The germ-free status was confirmed at monthly intervals by incubator swap cultures. Breeding was initiated between 8-12 weeks of age. At weaning (postnatal day 20-24), male and female offspring originating from the germ-free (Former GF) and the conventional (Conv) group were conventionalized with aliquots of the same inoculum (as detailed below). From then on, all mice were individually housed in IVC cages, in temperature-controlled conditions with 12:12 light dark cycles, and kept on autoclaved germ-free diet (ssniff® R/M-H autoclavable, V1534-3, ssniff Specialdiäten GmbH, Germany). From age 10-30 weeks, mice were challenged with a WTD (42% kcal

from fat; 0.2% cholesterol) (TD.88137, Envigo, Mucedola, Italy). We weekly measured body weight and performed intraperitoneal glucose and insulin tolerance tests (ipGTT and ITT) before and after the dietary challenge. For the final test at age 28-30 weeks, we assessed the following cholesterol homeostasis parameters: daily dietary food intake, intestinal absorption, *de novo* synthesis, biliary secretion rate, and finally, bile composition and feces production. Gallbladder cannulation was performed before sacrifice to collect hepatic bile as described previously [154]. Briefly, bile was cannulated for 20 minutes under Hypnorm (fentanyl/fluanisone; 1 mL/kg) and diazepam (10 mg/kg) anesthesia using a humidified incubator to maintain body temperature. After cannulation, blood was obtained via heart puncture, the mice were sacrificed and fat, liver and intestine were excised and snap-frozen in liquid nitrogen. All animal experiments were approved by the ethics committee for animal experimentation (IACUC) at the University of Groningen and performed in accordance with relevant guidelines and regulations. A schematic of the detailed set-up of the study is shown in Figure 1A.

Dosage information. The inoculum consisted of the cecum content of 10 C57BL/6JOlaHsd males of 3-4 weeks of age, which was collected in PBS, mixed and diluted 10 times in 20% glycerol and aliquoted in cryogenic vials for storage at -80°C. For conventionalization, aliquots were thawed, diluted up to 16x, filtered (pluriStrainer® 200 μm, pluriSelect), and 50μL was administered once via oral gavage. Microbiota composition of the inoculum is shown in **Figure 6**.

ipGTT and ITT. The intraperitoneal glucose tolerance test was performed before the dietary challenge at 8 weeks, and 11 weeks on the WTD at 21 weeks age. After 6 h food deprivation, the mice were injected with 2.5 g/kg D-glucose. The intraperitoneal insulin tolerance test was performed before the dietary challenge at 9 weeks of age, and after 10 weeks on the WTD at 20 weeks of age. After 4 h food deprivation, the mice were injected with 0.5 U/kg insulin (Novorapid, Insulin Aspart DS6M700). Blood glucose concentrations were analyzed from tail vein samples before and 15, 30, 60, 90, and 120 minutes after injection using OneTouch Ultra glucose strips (Life Scan, Milpitas, CA, USA).

DEXA. At 22 weeks of age, after 12 weeks on the WTD, body composition analysis was performed by dual energy x-ray absorptiometry (pDEXA, Norland-Stratec, Norland Medical Systems Inc., Basingstoke, Hampshire, UK). During the procedure the animals were anesthetized with isoflurane for a total of 15 minutes. Fat and lean body mass were calculated based on the automated bone mass density evaluation (0.06 g / cm3).

Fractional Intestinal Cholesterol Absorption. Fractional cholesterol absorption was measured using the plasma dual-isotope ratio method as described previously[59]. Briefly, at

the end of the dark phase the non-fasted 28-week-old animals were injected intravenously with 0.3 mg ^{5}D-cholesterol dissolved in Intralipid (20%; Fresenius Kabi, Den Bosch, The Netherlands) and orally gavaged with 0.6 mg ^{7}D-cholesterol dissolved in medium-chain triglyceride oil. Blood spots from the tail were collected on filter paper before and after administration of the isotopes at 3, 6, 12, and 24 hours for the first day, and after that every 24 hours for the next consecutive 7 days. Cholesterol was extracted from blood spots, followed by analysis by GC-MS. Briefly, the calculation of the fractional cholesterol absorption was based on the decay curves of ^{5}D (intravenously) and ^{7}D-cholesterol (oral) in plasma: after their correction with the administered dose: F*a* = (area under the label enrichment curve curve$_{oral}$/area under the label enrichment curve curve$_{intravenous}$ × dose$_{intravenous}$/dose$_{oral}$) × 100.

Cholesterol synthesis and balance. Fractional cholesterol synthesis was determined by Mass Isotopomer Distribution Analysis (MIDA) using 13C-acetate (Isotec, Miamisburg, OH, USA) as labeled precursor as described previously[159]. Transintestinal cholesterol excretion (TICE) was measured as dietary cholesterol intake (as calculated from food intake) + biliary cholesterol secretion – fecal neutral sterol excretion.

Measurement of cholesterol, lipoprotein profiles and bile acids in plasma and bile. Total plasma cholesterol was measured enzymatically using a commercially available kit (Roche Diagnostics GmbH, Mannheim, Germany). Lipoprotein fractions of pooled plasma samples (n = 6-9) were separated via fast protein liquid chromatography (FPLC) gel filtration using a superose 6 column (GE Healthcare, Little Chalfont, UK) as published[154]. Samples were chromatographed at a flow rate of 0.5 mL/min, and lipoprotein fractions of 500 µl each were collected. Individual fractions were assayed for cholesterol concentrations as described above. Biliary and plasma bile acid (BA) concentrations were determined using liquid-chromatography mass spectrometry (LCMS) as described previously[188]. Neutral sterols (NS) were extracted from bile according to Bligh and Dyer[153] followed by derivatization in N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), pyridine, and trimethylchlorosilane (TMCS) (5:5:0.1), re-dissolving in heptane with 1% BSTFA, and measurement by GC. Total bile acids from bile were measured using an enzymatic fluorescent assay according to Mashige *et al*.[189].

Hepatic lipids. Hepatic tissue was homogenized using RNAse free beads and the TissueLyser LT system (Qiagen GmbH, Hilden, Germany). Lipids were extracted according to Bligh & Dyer[153]. Cholesterol was de-esterified according to Ichihara *et al*.[156]. Lipids were redissolved in water containing 2% Triton X-100. Triglycerides were measured with colorimetric commercially available kits (Roche Diagnostics GmbH, Mannheim, Germany). Free cholesterol underwent acetylation followed by quantification using gas

chromatography (GC, Agilent 6890, Amstelveen, the Netherlands)[154]. Phospholipids were determined by measuring the phosphorus content of lipid extracts after perchloric acid treatment[229].

Cecum microbiota analysis. Bacterial DNA of the available cecum content was isolated, subsequently measured using MiSeq sequencing of the amplified 16*S* rRNA genes and analyzed by the QIIME and ARB method as described in Heida *et al.*[190].

Fecal neutral sterols and bile acids. Fecal samples from individually housed mice collected over 24 hours were dried, weighed and ground to powder. NS and BA profiles were determined using gas-liquid chromatography as published[154, 155]. Briefly, 50 mg feces was saponified in the presence of 1 mL alkaline methanol (1:3 NaOH:methanol) by heating for 2 hours at 80°C. The NS were extracted with petroleum ether, derivatized in a mixture of BSTFA, pyridine, and TMCS in a ratio of 5:5:0.1, re-dissolved in heptane containing 1% BSTFA, and measured by gas chromatography (GC). After NS extraction, total bile acids were extracted from the aqueous phase using SepPak C18 cartridges (Waters, Dublin, Ireland), methylated, and after derivatization with BSTFA, pyridine, and TMCS, were measured by GC. The same methodology was applied for the determination of cholesterol content using a 50 mg aliquot of the animal food; by knowing the food intake, the dietary cholesterol intake could be calculated.

Gene expression. Hepatic and distal ileum mRNA was extracted using TriReagent (Sigma) and quantified with a Nanodrop ND-100UV-vis spectrometer (NanoDrop Technologies Wilmington DE). cDNA was made from 1µg of RNA using reagents from Invitrogen (Carlsbad CA). Primers were synthesized by Eurogentec (Seraing, Belgium). Real-time PCR was performed using an ABI Prism 7700 machine (Applied Biosystems, Damstadt Germany). mRNA expression levels of individual genes were calculated relative to the housekeeping gene cyclophilin for hepatic genes, 36B4 for ileal genes.

Statistical analysis. The statistical analysis was performed with GraphPad Prism 5 Software. Body weight is shown as mean + SD and the significance of body weight increase over time between Former GF and Conv mice was analyzed using repeated measurements two-way ANOVA. GTT and ITT is shown as medians + interquartile range (IQR). Significance was analyzed measuring the area under the curve followed by Kruskal-Wallis post-hoc Dunn's multiple comparison test. Statistical analysis on plasma parameters was performed with Kruskal-Wallis post-hoc Dunn's multiple comparisons. For body composition, liver, cholesterol and bile parameters, data was analyzed using two-way ANOVA post-hoc Bonferroni. "Sex effect" refers to male versus female; "microbiota effect" to Conv versus Former GF; "interaction" is whether males and females react similarly to the former GF

status. P-values below 0.05 were considered significant. Graphs are made with GraphPad Prism 5; using the Tukey boxplot ((25^{th} - 75^{th} percentiles) + whiskers (1.5 IQR) and outliers (>1.5 IQR)) or mean + SD (Fig 4C-D). Principal component analysis (PCA) with both the QIIME data at family level and the ARB data was performed. Contamination found via the ARB method was distracted from the QIIME data values.

Acknowledgements & Author contributions

M.A.M.L. was responsible for data acquisition and analysis and drafting the article; C.C.N.W. was responsible for data acquisition and analysis; and U.T. and H.J.V. were responsible for the conception, design, and supervision of the study, interpretation of data, and critical article revision for important intellectual content. Rick Havinga, Rima H. Mistry, Renze Boverhof, Martijn Koehorst, Theo S. Boer, Theo H. van Dijk, and Carien Bus-Spoor are kindly acknowledged for expert technical assistance. The authors gave final approval for the version to be published.

Additional information

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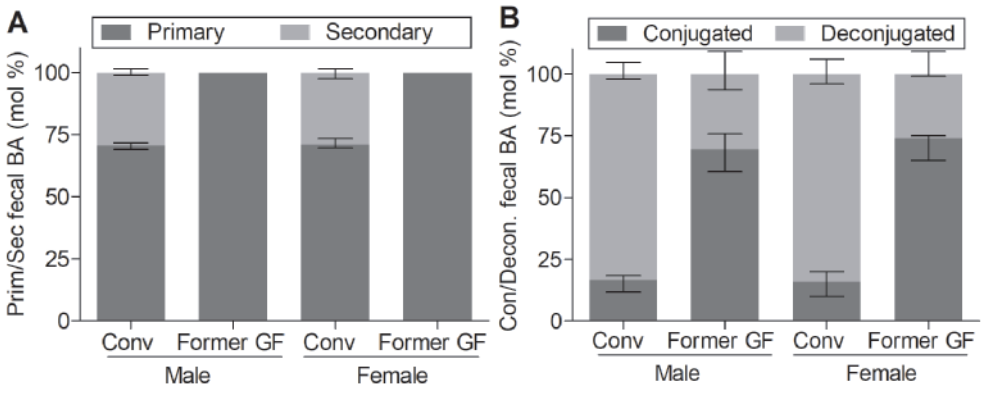
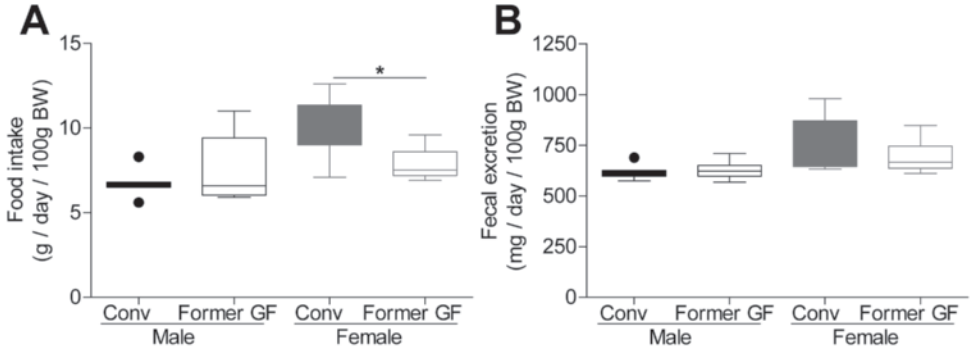
Conflict of interest.

The authors declared that they have no competing interests.

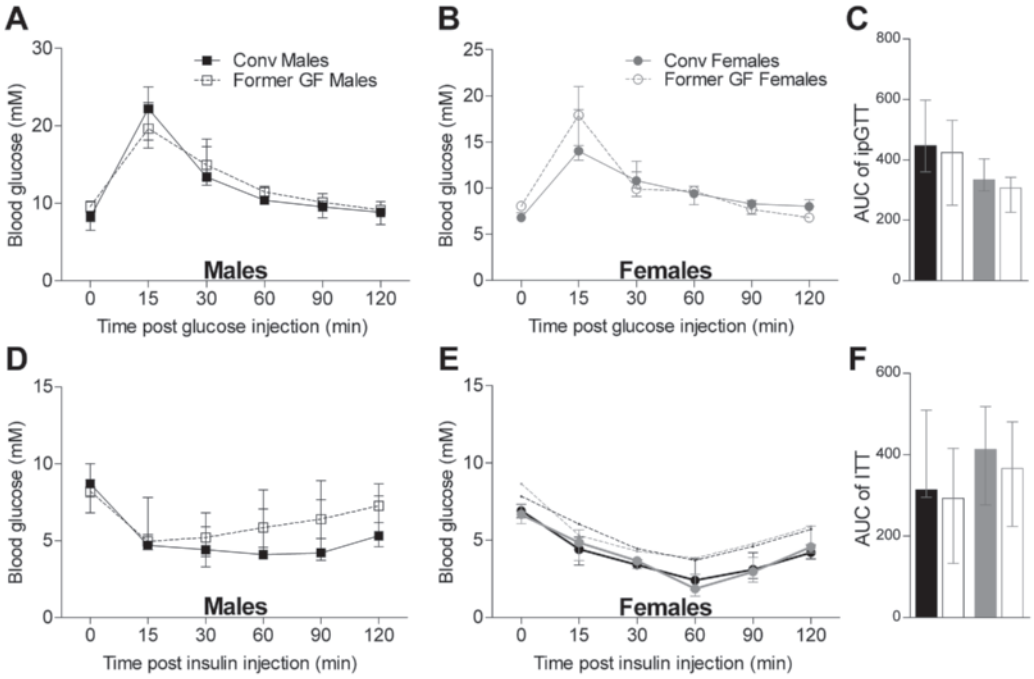
Supplementary Figures



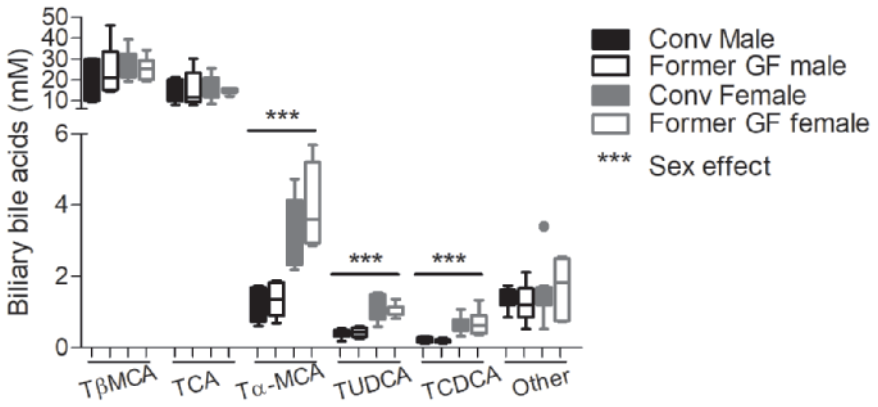
Supplementary Figure 1: Fecal bile acid composition post-conventionalization. Fecal bile acid composition (mol %) in the 24 hours post-conventionalization period, as measured by GC. Primary and secondary BA **(A)** and conjugated and deconjugated BA **(B)**.



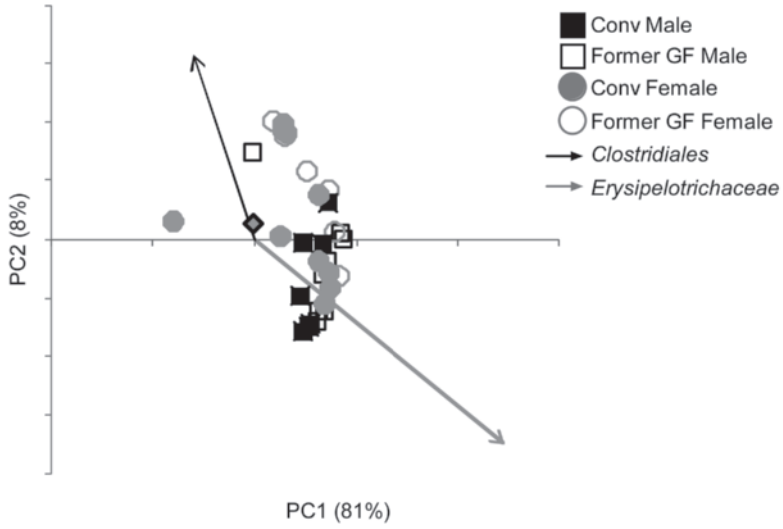
Supplementary Figure 2: Food intake and fecal production. Food intake at 30 weeks of age **(A)** shows an interaction ($p = 0.013$) and a sex effect ($p = 0.002$) after correction for body weight. Fecal production at 30 weeks of age **(B)** shows a sex effect ($p = 0.002$). Closed box = conventional; open box = Former GF; black = male; grey = female; n = 6 – 9 / group).



Supplementary figure 3: Glucose and insulin tolerance at 10 weeks age. An intraperitoneal glucose tolerance test (ipGTT) was performed before the dietary challenge in males **(A)** and females **(B)**. An insulin tolerance test (ITT) was performed before the dietary challenge in males **(D)** and females **(E)**. Area under the curve for GTT **(C)** and ITT **(F)**. Black = conventional; grey = Former GF; n = 5 – 9 / group.



Supplementary figure 4: Biliary bile acids. Bile acid concentration in hepatic bile at termination. Black = conventional; grey = Former GF; n = 5 – 9 / group. *** sex effect by two-way ANOVA post-hoc Bonferroni statistical analysis.



Supplementary figure 5: Principal component analysis. Principal component analysis (PCA) on the family level. Principal component (PC)1 (81%) and PC2 (8%), represented on the x- and y-axis, respectively, highlight some of the main differences between Former GF and Conv males and females. PC1 is mainly determined by *Clostridiales*, and PC2 by *Clostridiales* and *Erysipelotrichaceae*. Black = conventional; grey = Former GF; n = 7 – 9 / group.

General Discussion

Chapter 6

This thesis aimed to determine how specific interventions targeted at cholesterol metabolism and microbiota in early life affect the risk to develop features of the metabolic syndrome later in life. The Barker hypothesis proposes that environmental influences that impair fetal and neonatal growth and development may increases the risk for chronic disease in adulthood[121, 230, 231]. According to the hypothesis, the fetus and newborn child attempt to adapt to restrictive environmental conditions, what may be counterproductive when the restrictions are overcome in later life. These adaptations occur via epigenome modifications that alter gene expression and metabolism and these changes may predispose to disease in adulthood[6]. Epigenetic changes can also occur throughout the lifetime of an individual in response to environmental exposure and can influence the response to later life events. However, adaptive programming is most likely to occur during critical developmental periods for the specific event/trait *in utero* and post-delivery[232], so-called sensitive windows.

Animal models were used in this study to perform and standardize interventions that are not permitted or feasible in humans and to standardize genetic backgrounds. Thereby, experimental metabolic results can be linked to exact environmental cues where this is often not possible in humans. Ageing in mice takes weeks to months versus years to decades in humans, what makes the mouse a more feasible model to study effects occurring during a lifetime. Adaptive programming to early life environment can occur via epigenetic and metabolic modifications. To identify mechanisms that induce these modifications a standardized (epigenetic) background is especially important, since epigenetic markers can be transferred across generations[233]. Adaptive programming effects may occur in early life due to microbiota-related signaling. The germ-free mouse model was used for this thesis to investigate more fundamental mechanisms underlying possible microbiota-induced long-term effects on the metabolic system.

Regulation of breast milk cholesterol

Breast milk (BM) contains high levels of cholesterol (0.23–0.39 mmol/L) in contrast to most infant formulas (IF; 0–0.10 mmol/L). BM has been suggested to have a lasting impact on cholesterol homeostasis of the offspring. A prior study has noted that cholesterol availability during lactation has a programming effect on intestinal cholesterol metabolism in adult mice[59]. Therefore, it is important to investigate how BM cholesterol levels are influenced by maternal plasma cholesterol levels. Interestingly, BM cholesterol levels appeared stable despite up to 5-fold increased plasma cholesterol levels in mice, upon induction by a high-cholesterol diet or by genetic inactivation of either the Low-Density Lipoprotein (LDL) Receptor (LDLR) or ATP-binding cassette sub-family G member 8 (ABCG8)

(**Chapter 2**). This outcome is contrary to that of Tsuduki *et al*. (2016)[234] who found that high maternal cholesterol intake (0.2%) during lactation increased milk cholesterol levels in mice and promoted fatty liver development in the offspring via increased hepatic lipoprotein influx. This inconsistency with our milk cholesterol data may be due to the different methods to collect the milk. In **Chapter 2** we obtained the milk using a BM pump at lactation day 14, while the other study harvested milk from the stomach of offspring at unknown age. We speculate that our method reflects better the native milk composition than collecting milk from the stomach of the offspring. The increased lipoprotein influx in the offspring of their study might be related to a change in other milk lipid parameters.

Possibly, the offspring benefits from a stable milk cholesterol supply during the lactation period to induce positive metabolic consequences. Cholesterol is important during rapid growth in early life. It is needed for synthesis of bile acids, hormones, cell membranes, lipoproteins, vitamin D and myelin[26, 235]. During the third trimester of gestation, fetal cholesterol is for 20-40% provided via the maternal circulation through placental transport[236-239]. The remaining cholesterol of the fetal pool originates from *de novo* synthesis by the fetus[240]. In a healthy pregnancy, maternal total and LDL-cholesterol (LDL-C) in plasma increase during the pregnancy[241]. Maternal *hyper*cholesterolemia affects offspring serum cholesterol differently when exposure occurs during pregnancy than when offspring is exposed during lactation. Maternal *hyper*cholesterolemia or elevated LDL-C during pregnancy due to genetic or dietary influence has been associated with elevated LDL-C levels in plasma and/or atherosclerosis development of the offspring in both humans[242, 243] and mice[244, 245]. In hamsters, feeding a high cholesterol diet throughout pre-pregnancy, gestation and lactation resulted in increased serum and hepatic cholesterol and increased hepatic LDLR and HMGCR expression in the offspring[187]. However, since exposure also occurred during the pre-lactation period, these effects cannot be related to high cholesterol exposure during lactation alone. Rats fed a high cholesterol-high fat (HCHF) diet during lactation demonstrated decreased HMGCR and increased CYP7A1 activity (the rate-limiting enzymes in cholesterol and bile acid synthesis, respectively), and unaltered serum cholesterol in adult offspring, but only when also challenged with a HCHF diet post-weaning[246]. The previous findings in hamsters and rats in combination with the data in **chapter 2** indicate that there is no increased transfer of cholesterol via BM during lactation in hypercholesterolemic mice. Unchanged BM cholesterol levels suggest that abovementioned atherosclerotic effects in mice and humans in association with maternal *hyper*cholesterolemia may be restricted to increased cholesterol transfer during pregnancy, to possibly non-cholesterol

lipid transfer during lactation altering lipoprotein metabolism, or to species differences.

A rather robust BM cholesterol level under widely varying maternal serum cholesterol levels may be related to the physiology of the milk production process, specifically to the subcellular processes in the mammary gland involved in lipid secretion into the milk. Lipid droplets in the ER are wrapped in a monolayer of the endoplasmic reticulum membrane forming the milk fat globule (MFG) in the cytosol, and subsequently packaged in a bilayer of the apical plasma membrane when the droplets are pinched off from the lactating cell. The majority of milk cholesterol is located in the MFG-membrane (MFGM)[47]. The MFGM contains liquid ordered (*Lo*) and liquid disordered (*Ld*) phases[44]. The *Lo* phase is rigid and consists of cholesterol and milk sphingomyelin (SM)[44]. Membrane fluidity is determined by the ratio of cholesterol : SM and amount of *Lo* phases[44, 247]. Since the MFGM originates from the plasma membrane, milk cholesterol levels might be bound by fluidity constraints of the plasma membrane. The cholesterol and cholesterol synthesis enzymes amount in MFGs is negatively related to the size of the MFG, suggesting that they may play a role in lipid droplet growth or secretion[248]. The MFG size distribution varies during the day and lactation stages and ranges from 1 to 10μm[44, 47, 143]. Structural differences in size distribution and MFG(M) composition may affect MFG digestion[247] and may be of nutritional significance for the infant[247, 249, 250].

Importance of cholesterol supply in early life

The stable milk cholesterol levels found in this thesis do not directly indicate its metabolic relevance and neither implies that cholesterol should be added to IF in order to achieve long-term positive effects. There are some data which could support the concept that BM factors other than cholesterol content account for these effects. Firstly, supplementation of cholesterol to IF in infants up to 12-months [145] did not mimick plasma cholesterol levels of breast-fed infants nor did it reduce fractional cholesterol synthesis rates[55, 56, 145]. Adding MFGM, which also contain cholesterol, to IF has yielded serum cholesterol levels closer to those of infants fed BM[251]. Additionally, MFGM-supplemented IF reduced the incidence of infectious morbidity and improved cognitive performance in infants at two years of age[251]. Since MFGM consists of a multitude of components, it seems likely that these results are not or, at least, not exclusively related to the cholesterol component. Milk SM is known to inhibit cholesterol absorption and, together with other BM components, it shapes gut microbiota development and modifies the immune system[250]. Secondly, ezetimibe-induced decreased cholesterol availability during lactation in mice, similar to cholesterol-poor IF feeding, epigenetically

programmed decreased intestinal cholesterol absorption and increased synthesis into adulthood[59]. In humans, on the other hand, decreased cholesterol absorption and increased synthesis, have been linked to glucose and insulin values, specifically to insulin resistance[252-254]. The reverse condition, namely high cholesterol absorption and low cholesterol synthesis, is more often seen in patients with CVD and hemodialysis patients at risk for cardiovascular mortality than in healthy controls[255, 256]. These last findings imply that low cholesterol availability during lactation may be beneficial for the long-term health of the offspring with regard to cholesterol homeostasis and CVD risk, but detrimental for insulin resistance. Nonetheless, these associations are based on an established disease condition in adult humans. The mouse model described by Dimova *et al*. (2017)[59] could be used to investigate what a life-long decrease of cholesterol absorption, induced by ezetimibe in early life, does for the risk to develop metabolic syndrome.

Sensitive window for programming cholesterol absorption in men and mice

The lactation period is a sensitive window for programming later life metabolism with regard to cholesterol and glucose homeostasis[12, 59]. In **chapter 3** we determined whether the sensitive window for adaptive programming of adult cholesterol homeostasis extends beyond the physiological lactation period in mice (18-19 days). Ezetimibe exposure during lactation reduced cholesterol absorption in mice via an epigenetic memory that decreased Npc1l1 expression[59]. However, this adaptive programming did not occur upon ezetimibe treatment between postnatal days 21 and 42. Thus, at least in mice, the sensitive window to program long-term reduced cholesterol absorption does not extend beyond the physiological lactation period. Human and mouse intestine are at different intestinal developmental stages during lactation[257]. While human small intestinal crypt-villus structure is mostly completed at birth, this maturation in rodent intestine, as well as differentiation of absorptive cells into typical absorptive cell morphology is only completed around weaning[171, 257-259]. The murine intestine may therefore be less sensitive to nutritional impact on epigenetic modifications after lactation than during lactation[171]. Also, the switch from fluid to solid food might trigger intestinal changes[172] and thereby terminate the sensitive window. Weaning in rodents initiates crypt cell proliferation in the small intestine and accelerated proliferation of enterocytes[172], but also rapidly alters composition of the microbiota[257]. The difference in intestinal maturation stage likely makes the lactation period a sensitive window for nutritional cues in rodents but this may be less apparent in humans.

Besides differences in intestinal maturation, mice and humans also have a different *Npc1l1* expression pattern which may contribute to the distinction in which mechanisms of cholesterol homeostasis are affected. In humans, *Npc1l1* is not only expressed in the intestine but also in the liver where it re-absorbs cholesterol that has been secreted into the biliary canaliculus. Accordingly, ezetimibe administration to humans leads to even higher fecal cholesterol excretion than in mice, which mainly consists of endogenous cholesterol[48, 166]. Ezetimibe treatment and naturally occurring mutations that disrupt NPC1L1 function in humans are each associated with reduced cholesterol absorption, LDL-cholesterol levels and CVD risk in humans[165, 260, 261]. Long-term decrease of NPC1L1 function by either natural variation or ezetimibe treatment increases the risk of gallstone disease[261]. Therefore, it is questionable whether life-long programming of decreased cholesterol absorption is advisable for adult human health. By inference from the studies on reduced bioavailability of cholesterol by ezetimibe, one could speculate that cholesterol-poor IF could program long-term decreased cholesterol uptake. Yet, it seems too early at this moment for firm conclusions in this respect. In future investigations, cholesterol absorption and synthesis and CVD parameters will need to be determined in adult mice and humans fed with cholesterol-rich or cholesterol-poor IF in early life.

Microbiota-induced adaptive programming

Cholesterol is the precursor for the synthesis of bile acids, which are involved in lipid and vitamin absorption, biliary secretion of lipids and signaling of multiple metabolic processes[262]. Treatment of 6 week old mice with the cholesterol absorption inhibitor ezetimibe decreased fecal bile acid excretion, in contrast to earlier findings (**Chapter 3**). Bile acids can be modified by microbial enzymes, which influences their interaction with the host physiology, such as bile acid synthesis rate and intestinal reabsorption rate of bile acids[118]. The intestinal microbiota may have been modified by ezetimibe[86, 176]. Although the effect of altered microbiota on bile acid composition was not reported by Catry (2015)[176] and Zhong (2015)[86], it is reasonable to assume that the changed microbiota may subsequently affect bile acid composition and excretion, as is observed in this thesis. Two studies in this thesis were designed to unravel metabolic consequences of early life microbiota-related events, mainly in respect to bile acid and cholesterol metabolism. Gene expression and metabolism are substantially different in males and females[8, 99] and this difference depends on the presence of the microbiota[103, 263]. Early life nutritional exposures impact long-term metabolic health differently in males and females[8]. Thus, determination of sexual dimorphism was included in the microbiota studies.

The sex of the microbiota donor shaped the plasma bile acid composition during the first days after conventionalization of germ-free males and females (**chapter 4**). Thereafter however, the plasma bile acid composition was largely independent of the donor sex and similar between male and female recipients. These findings were rather unanticipated, since conventional mice have a sex-specific bile acid composition[102-104, 114, 193] as have humans[112, 113]. The disappearance of the sex-specific bile acid composition after conventionalization of the germ free mice may be attributable to the germ-free state of these mice until 12 weeks of age and possible adaptive programming due to the absence of microbiota in these first 12 weeks of life. Male and female mice that had been germ-free up to weaning had a decreased bile flow and bile acid secretion at adult age. These findings indicate that life-long changes may be established by the absence of microbiota-related signaling during murine gestation and lactation (**Chapter 5**). These observations could be related to the complex interplay between microbiota and sex-specific gene expression and metabolism. A previous study in germ-free mice demonstrated diminished sex-specific gene expression, showing more male-like hepatic gene expression in females and more female-like hepatic gene expression in males[263]. The microbiota produces metabolites and stimulates ghrelin secretion, an appetite and energy homeostasis regulating hormone, in a sex-specific manner[263]. Together these molecules sustain sexual dimorphic gene expression and metabolism, likely by stimulating growth hormone (GH) secretion and sexual maturation. In absence of microbiota and microbiota-derived metabolites, reduced gene expression differences between the sexes are likely caused by altered sexual maturation and GH secretion. Sex specific microbiota composition becomes more apparent with increasing age[104]. Possibly, absence of exposure to microbiota until after puberty differentially programs the conventional regulation of hepatic bile acid metabolism, thereby moderating sex-specific metabolism and sex-specific interaction of metabolism with microbiota and bile acids (**Chapter 4**). Interestingly, microbiota transfer from males increases testosterone levels in female mice with a consequently altered serum metabolome more similar to the male metabolome, presumably via the androgen receptor[104]. It is therefore tempting to speculate that this increase in male sex hormone levels in conventionalized female mice moderates sexual differences usually observed between conventional males and females. Both reduced stimulation of sex-specific metabolism in germ-free mice up to 12 weeks of age, and the increase in male hormone levels in females receiving microbiota from a male donor, may contribute to the sexual indifference observed in bile acid and microbiota composition (**Chapter 4**). A further study with more focus on male-female metabolic differences and sex hormone levels in adult conventionalized mice is therefore suggested. Mice in the studies of **Chapter 5**

were gavaged at the age of 3 weeks, with male microbiota donors of the same age. At this age microbiota composition does not yet show sex-specific differences[104]. Therefore, a lack of sex-specific differences may be less apparent in these studies (**Chapter 5**). More work is needed to investigate the underlying signaling pathways that cause sex differences in the microbiota and bile acid composition. Additionally, it seems warranted to determine whether a sensitive window exists for microbiota-stimulated programming of male-female differences, in analogy to the presently described serum bile acid composition. Clinically, the metabolic consequences may be of relevance for fecal transfer therapies where a recipient has a different age and sex compared with the microbiota donor. Although murine studies indicate that the sex of the microbiota donor impacts metabolism of steroids and hormones[104, 264], the necessity of matching donor and recipient sex data in human studies has not (yet) been demonstrated[203, 265].

Rodent-specific bile acids

Rodents and humans differ considerably in bile acid composition and metabolism[32, 89]. In humans, the hepatic alternative bile acid synthesis pathway ends with chenodeoxycholic acid (CDCA). In mice, however, CDCA is further modified into hydrophilic muricholic bile acids[32, 266]. Muricholic acids inhibit cholesterol absorption[168, 169] and stimulate bile acid synthesis via their antagonistic activity on farnesoid X receptor (FXR) in the intestine[93, 94]. Conventionalization of germ-free mice reduces muricholic acid levels, thereby alleviating FXR inhibition[93] and likely increasing cholesterol absorption[86, 168, 169]. Differences between mouse and human microbiota, their interaction with bile acids and subsequent feedback on bile acid synthesis pathways as well as cholesterol absorption should be taken into account when translating data. Using animals models with a more human-like microbiota and bile acid composition would aid in solving questions regarding the sexual dimorphism in microbiota and bile acid composition, their interactions and their effects on the metabolic system. The responsible enzyme for CDCA- and UDCA-modification into rodent-specific muricholic acids is cytochrome P450 2c70 (CYP2C70)[267]. Genetic *Cyp2c70* inactivation in mice induces a more humanized bile acid pool, as characterized by absence of muricholic acids and their derivatives and high concentrations of CDCA and UDCA[89, 267]. Studies in this *Cyp2c70* knockout mouse model, together with a more humanized microbiota composition[268, 269] could provide more worthwhile information on human conditions, such as the effects of bile acid signaling on the development of features of the metabolic syndrome. Although this model would better represent the human bile acid signaling to study interactions with the microbiota and general metabolism, dissimilarities in

microbiota composition as well as the diet, the physiology of the intestinal tract and the host genetics still would have to be considered[270, 271].

Interplay between microbiota, diet and host genetics

Interestingly, the absence of microbiota during gestation and lactation did not induce substantial effects on key metabolic parameters in adulthood (**Chapter 5**). Microbiota composition in early life has frequently been implied to affect the risk on many conditions at adult age, including the risk to develop obesity[205-207, 272, 273]. It is still unclear to what extent the microbiota programs long-term the metabolic system[182] and/or if the microbiota composition itself is programmed[274]. Acute effects of microbiota on the host are abundant, involving modification of bile acids, gene expression and metabolism[31, 275]. Bile acid metabolism, and thereby indirectly also cholesterol metabolism, can be altered by intestinal bacteria via bile salt hydrolase activity (BSH), that hydrolyzes conjugated bile acids to deconjugated bile acids[119, 276]. The increase in deconjugated bile acids is suggested to lead to increased ABCG5/8 activity[276, 277], presumably via FXR activation followed by downregulation of small heterodimer partner (SHP) and upregulation of liver X receptor LXR[276]. LXR stimulates ABCG5/8, resulting in lower cholesterol absorption and increased biliary cholesterol secretion[276]. However, these microbiota effects are due to direct interaction of bacterial BSH enzyme with bile acids. Although it is known that the early life environment, such as maternal nutrition, cross-fostering, breastfeeding and (maternal) antibiotics, may shape the microbiota permanently[63, 77, 80, 272], insight in long-term effects as a consequence of a transient aberrant microbiota is scarce. It has been demonstrated that early life antibiotics in a swine model can affect short chain fatty acid signaling and glucose metabolism up until weeks after cessation of antibiotic use despite only a short-term transient effect on the microbiota composition[223]. Antibiotics impact on the microbiome's adaptability to diet, diversity, composition and metagenomics content[278, 279]. The impact of antibiotic treatment increases with the type, duration and dosage of the antibiotic used[278]. It has remained unclear, however, whether the long-term effects on metabolism are due to (transient) microbiome changes or to other antibiotic-induced changes. Transiently aberrant microbiota may process food, sterols and bile acids differently and thereby affect their signaling properties[216]. Indeed, metabolic parameters are dependent on the microbiota, but also on the host genetics and diet[202, 216]. *Vice versa*, the diet as well as previous environment and host genetics shape microbiota composition[202]. Moreover, the microbiome, diet and host genetics all interact in development of the metabolic syndrome[202]. Microbiome-derived metabolic signaling is absent in germ-free mice, while antibiotic-treated mice still harbor microbiota, which may explain the different

long-term metabolic effects seen in response to the different approaches[13, 200, 280]. Studies with qualitative differences of early-life microbiota composition (such as the contribution of BSH-producing bacteria[276]) in genetic identical mice on the same diet could further delineate possible long-term effects on the metabolic system. Additionally, effects should be determined separately in males and females, given that also sex differences are present in the interaction between microbiota, diet, host metabolism and consequently disease susceptibility[106, 263, 280]. Expanding our knowledge on these individual factors and their interactions will increase the ability to develop personalized treatments for metabolic syndrome-associated risks and diseases, because each case is unique due to environmental, sex, genetic and epigenomic differences.

CONCLUSIONS

In conclusion, the studies described in this thesis have increased our understanding of how early life factors during defined sensitive windows may contribute to physiology and metabolism later in life. The first part of this thesis demonstrates that BM cholesterol levels are independent from maternal genetic or diet-induced *hyper*cholesterolemia. Although these robust cholesterol levels in BM can be essential for adult cholesterol homeostasis, the sensitive programming window of its bioavailability appears to close after weaning in our mouse models. Understanding the relationship between the sensitive window for cholesterol supply and life-long human metabolic health is of importance since adult health appears to be partly dependent on early life environment. The second part of this thesis has shed light on the interplay between microbiota and bile acid and cholesterol metabolism. The next steps in continuation of this research should focus on further elucidation of the specific pathways of this interplay. In this frame, our results indicate that sex specificity should always be taken into account. Since direct translation of outcomes described in this thesis to the human condition can be hampered by species differences, future efforts would be expected to benefit from more humanized compositions of both microbiota and bile acids. If the present findings in rodents can be confirmed in humans, novel strategies for the prevention of metabolic syndrome-related disease in early life can be developed instead of, or in addition to, treating the condition at adult age.

Chapter 7

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ENGLISH SUMMARY

The obesity epidemic

The number of overweight and obese people (Body mass index (BMI) higher than 25 or 30 kg/m^2) has increased considerably in recent years. Obesity is becoming increasingly common early in life, which is worrying because it is associated with obesity in later life and metabolic diseases in early and late life. Obesity occurs when the build-up and breakdown of substances, also known as *metabolism*, is not in balance. Obesity increases the risk of health problems such as the *metabolic syndrome*. The metabolic syndrome is characterized by increased blood pressure, increased blood sugar levels and disturbed blood lipid values (such as increased cholesterol: *hypercholesterolaemia*). Someone with metabolic syndrome has an increased risk of developing type 2 diabetes and cardiovascular disease. Treatment and lifestyle advice appear to be insufficient to counteract the increase in the number of people with obesity and metabolic syndrome. That is why prevention and new therapies are of great importance. This first requires more knowledge of the causes and mechanisms of the development of obesity and metabolic syndrome.

Causes of obesity and metabolic syndrome

Well-known causes of obesity are genetic predisposition and factors such as food intake and little exercise. In recent years, scientific research has shown that environmental factors during critical periods of (organ) development at an early age can play a role in the risk of developing obesity and metabolic diseases in later life. The scientist David Barker called this concept in 1990 the "*Barker hypothesis*", now also known as "*Developmental Origins of Health and Disease*" (DOHaD). Since the Barker hypothesis in 1990, studies have shown that both fetal and postnatal growth are *critical/sensitive periods* of development in which adulthood health and disease can be determined.

Research suggests some mechanisms by which early-life conditions can affect health later in life, including irreversible changes in organ and tissue structure, changed standard values for hormone concentrations, and *epigenetic regulation of gene expression* (also called '*programming*'). Epigenetics means "around the DNA" and signifies 'markings' around the DNA that can turn genes in the DNA 'on' or 'off'. For example, 'on' means that the gene that codes for an enzyme can be made by the cell. Environmental conditions, especially at an early age, can influence these markings on the DNA and thus regulate the 'on' and 'off' state of genes at a later

age. The effect on adult age depends on the type, size, duration and moment of the (alternative) environmental conditions and the organ system that is affected.

Programming by early-life food and microbiota

Observational studies have shown that adults breast-fed at an early age have a lower risk of obesity and metabolic diseases than bottle-fed adults. Many differences between breastfeeding and bottle feeding could be responsible for this, such as the composition of the milk and contact with the mother. Obesity and other metabolic diseases are also often associated with a disturbed intestinal flora (*microbiota*). It is unclear whether this disturbed microbiota is the cause or consequence of the metabolic disease. In addition to nutrition, also intestinal bacteria influence the metabolism of the host. It is possible that intestinal bacteria already at an early age influence the metabolism of the host temporarily or permanently.

In this thesis, ***we have aimed to identify short- and long-term consequences of early-life interventions in dietary cholesterol supply (Chapters 2 & 3) and microbiota presence (Chapters 4 & 5) on weight gain and metabolism later in life.*** We have used the mouse as an animal model to investigate our hypotheses.

Cholesterol in breast milk

Breast milk contains high concentrations of cholesterol compared to bottle milk (infant formula). The difference in cholesterol concentration between breast milk and infant formula may be associated with the beneficial health effects seen in adults who were breastfed as a child. During development, cholesterol is an important building block for cell membranes and cholesterol is used to make hormones and bile acids. In addition to food as a source of cholesterol, the body also makes cholesterol itself, mainly in the liver but also in other organs such as the mammary glands. During lactation, maternal cholesterol synthesis is increased to meet the high cholesterol requirement for milk production. Cholesterol transport in the blood takes place in, among other things, fat globules (also known as the "bad" LDL cholesterol and the "good" HDL cholesterol). Exactly how cholesterol travels from the blood, to the mammary gland and to the milk is not entirely clear. It is also unknown how milk cholesterol concentrations are precisely regulated.

In **Chapter 2** we examined whether hypercholesterolaemia in the mother mouse causes a higher cholesterol concentration in breast milk. We caused hypercholesterolemia by means of a high cholesterol diet and/or genetic inactivation of genes involved in cholesterol transport (Low-density lipoprotein receptor (LDLR) and the cholesterol transporter ATP-binding cassette G8

(ABCG8)). The results showed that cholesterol levels in breast milk remained constant despite up to 5-fold elevated cholesterol levels in maternal blood and despite the absence of cholesterol transport-related genes. LDLR and ABCG8 were not critical proteins to maintain breast milk cholesterol levels in mice. Upon maternal plasma cholesterol increase, hepatic and mammary gland de novo cholesterol synthesis decreased to undetectable rates. The decreased cholesterol synthesis is possibly a compensatory mechanism against milk cholesterol increase. These results may indicate that the constant cholesterol concentration in milk 1) is restricted to the milk formation process because of the function of cholesterol in the milk fat globules and/or 2) is important for the development of the offspring.

The sensitive period for programming cholesterol absorption

Our group has previously demonstrated that reduced cholesterol absorption in the offspring during lactation programs reduced cholesterol absorption in adulthood in mice. Decreased cholesterol absorption during lactation was caused by the administration of ezetimibe to the offspring through the maternal breast milk. Ezetimibe is a drug that inhibits cholesterol absorption in the intestines and is used in adults to lower cholesterol levels in the blood. This programming effect of decreased cholesterol absorption occurred via reduced expression of the intestinal cholesterol transporter Niemann-Pick C1-Like 1 (NPC1L1). In **Chapter 3**, we have tested in mice the hypothesis that the sensitive window in which long-term cholesterol absorption efficiency can be programmed, extends beyond lactation. However, we observed that the reduced cholesterol absorption during administration of ezetimibe, for three weeks after lactation via the diet, disappeared as soon as ezetimibe administration stopped. We conclude from this that the sensitive period for programming cholesterol absorption stops after the lactation period. That means that preventive measures or therapies to adapt long-term cholesterol metabolism are probably the most effective during lactation. Together, the results of Chapters 2 and 3 do not directly indicate the metabolic relevance of breast milk cholesterol, nor does it imply that cholesterol should be added to infant formula to achieve similar long-term beneficial health effects as breast milk. The life-long metabolic risk of lower cholesterol availability during lactation on adult metabolism can be investigated in a mouse model with ezetimibe as described above.

Cholesterol excretion and the role of bile salts

More cholesterol excretion is beneficial against hypercholesterolemia to reduce the risk of cardiovascular disease and metabolic syndrome. Cholesterol excretion can occur via the liver/bile route or via direct excretion of cholesterol from the

bloodstream to the intestinal lumen and then to the stool. The latter process is called "Trans-Intestinal Cholesterol Excretion" (TICE) and is important in mice. In the liver/bile route, cholesterol in the liver is converted into bile acids and then stored in the gallbladder. After eating a (fatty) meal, the bile (consisting among other things of bile salts and cholesterol) is excreted in the small intestine. There, bile salts function as soaps that dissolve fat particles from food. This makes it easier for the fat particles, including cholesterol and vitamins, to be digested and absorbed. In the last part of the small intestine, bile acids are reabsorbed and transported through the circulation back to the liver, where they can be reused. Due to this efficient process in the *enterohepatic circulation*, only ~ 5% of the bile acids per round is lost with the stool. The production of new bile acids is closely regulated in response to various signals, including signals from bile acids in the intestines. In addition to their soap function, bile acids can also influence metabolism because they can bind to cell surface receptors. These receptors trigger a cascade of reactions that regulate the synthesis of bile acids but also the metabolism of sugars and fats. Therefore bile acids can possibly be used for the treatment of a disrupted metabolism.

The interaction between bile acids and intestinal bacteria

The human microbiota consists of more than 1000 different species, most of which reside in the colon. The intestines are reasonably sterile until birth, although the fetus in the womb may be exposed to bacterial-related signaling from the mother's intestines. During and after birth the baby comes into contact with bacteria from the environment and the microbiota develops in 1-2 years. This development is highly dependent on environmental influences such as contact with parents, food intake, environment and antibiotic exposure before and after birth, and therefore every creature has a unique microbiota composition. There is increasing evidence that bacteria are important in early life for the development of the intestines and the metabolic system.

Bacteria in the intestines process bile acids, giving them different properties and changing their influence on metabolism. In addition to bile acids, bacteria process food components and produce short-chain fatty acids (SCFA) and other metabolites. These metabolites alter the local metabolic signaling and are also absorbed and circulated to the liver and many other places in the body to influence metabolism there. Two studies in this thesis (**Chapters 4** and **5**) have been designed to unravel metabolic consequences of early-life microbiota-related events, mainly in respect to bile acid and cholesterol metabolism.

Sex-specific influence of the microbiota on the bile acid composition

Gene expression and metabolism are considerably different in men and women and this difference depends on the presence of the microbiota. Moreover, nutritional exposures in early life impact long-term metabolic health differently in males and females. Investigating and understanding gender differences is therefore very important if you want to use the information to develop prevention measures and therapies. Thus, determination of sexual dimorphism was included in the microbiota studies.

Bile acid composition and total concentration differ in male and female mice that contain microbiota (*conventional mice*), but not in mice without microbiota (*germ-free mice*). This suggests a role for intestinal microbiota in regulating sex differences in bile acid composition. Indeed, microbiota composition varies between males and females in both men and mice. **Chapter 4** describes bile acid dynamics in male and female germ-free mice after these mice received cecum microbiota (stool or *feces*) from the same or opposing sex (this fecal transplantation in germ-free mice is called *conventionalization*). As expected, plasma bile acid levels were higher in recipients of female microbiota. However, this effect was only observed during the first three days after conventionalization. Ten weeks later, bile acid levels and composition were similar between male and female mice conventionalized with microbiota from either males or females. We hypothesize that the germ-free start of life (up to 12 weeks, the "young-adult" mouse age) of the conventionalized mice may have programmed the metabolic system. A germ-free start may influence how microbiota later in life can affect the bile acid metabolism of the host. More work is needed to investigate underlying mechanisms that cause sex dependencies on the bile acid composition in humans and conventional mice. Future studies should take into account the translation of data from mice to humans. Unlike humans, mice have a few mouse-specific (muri) bile acids. Deficiency of the enzyme that converts bile acids to muricholic acids (CYP2C70) will give mice a more human-like bile acid profile. The sex-specific metabolic effects after introducing foreign feces (including microbiota and bile acids) into ones intestinal system could be of clinical interest for fecal transfer therapies in metabolic diseases.

Long term effects of absence of microbiota at an early age

Presence of bacteria in the intestines has major implications on the metabolic system, as demonstrated by the dynamic bile acid profile after germ-free mice were exposed to microbiota in **Chapter 4**. In humans, a germ-free status during pregnancy and lactation is impossible, which is why we used a germ-free mouse model to investigate the long-term effects of microbiota (or its absence) on the

metabolic system. We hypothesized that a germ-free pregnancy and lactation would induce a different regulation of gene expression from mice exposed to microbiota during this developmental phase, resulting in distinct adult metabolism.

In **Chapter 5**, we have explained in more detail the consequences of microbiota absence during pregnancy and lactation on metabolic parameters later in life in mice, when they were metabolically challenged with a Western-type diet. Surprisingly, absence of microbiota in early life followed by microbiota introduction (fecal transplantation) at weaning had no effect on adult body weight gain, cholesterol metabolism, glucose tolerance or insulin sensitivity. A germ-free period in early life did result in lower bile flow (volume and bile acid concentration) to the intestines compared to conventionally-raised mice. These findings imply that microbiota in early life does not critically affect adult metabolic plasticity. However, data from humans and mice have shown that antibiotic exposure in early life can have long-lasting metabolic effects. Antibiotics diminish the amount and diversity of the microbiota community temporary. A transient change in microbiota may induce different metabolic signals from the non-antibiotic situation and may possibly program adult metabolism. Studies with qualitative differences of early-life microbiota composition could further elucidate possible long-term effects on the metabolic system.

Conclusion and future posibilities

Together, the research in this thesis provides valuable insights into how early-life factors such as cholesterol supply and microbiota presence during defined sensitive windows may contribute to physiology and metabolism later in life. Since direct translation of outcomes described in this thesis to the human condition can be hampered by species differences, future efforts are expected to benefit from more humanized compositions of both microbiota and bile acids. If the present findings in rodents can be confirmed in humans, novel strategies for the prevention of metabolic syndrome-related diseases in early life can be developed instead of, or in addition to, treating the condition at adult age.

NEDERLANDSE SAMENVATTING

De obesitas epidemie

Het aantal mensen met overgewicht of obesitas (Body mass index (BMI) hoger dan 25 of 30 kg/m^2) is de laatste jaren aanzienlijk toegenomen. Steeds vaker komt obesitas vroeg in het leven voor, wat zorgwekkend is omdat dit geassocieerd wordt met obesitas op latere leeftijd en metabole ziekten op jonge en late leeftijd. Overgewicht ontstaat wanneer de opbouw en afbraak van stoffen, ook wel *metabolisme* genoemd, niet in balans is. Obesitas vergroot het risico op gezondheidsproblemen zoals het *metabool syndroom*. Het metabool syndroom wordt gekenmerkt door een verhoogde bloeddruk, verhoogde bloedsuikerspiegel en verstoorde bloedvet waarden (zoals verhoogd cholesterolgehalte: *hypercholesterolemie*). Iemand met metabool syndroom heeft verhoogd risico op het ontwikkelen van diabetes type 2 en hart- en vaatziekten. Behandeling en leefstijladviezen blijken onvoldoende te werken om de toename in het aantal mensen met obesitas en metabool syndroom tegen te gaan. Daarom zijn preventie strategieën en nieuwe therapieën van groot belang. Hiervoor is het belangrijk eerst meer kennis te vergaren van de oorzaken en mechanismen van het ontstaan van obesitas en metabool syndroom.

Oorzaken van obesitas en metabool syndroom

Alom bekende oorzaken van obesitas zijn een genetische aanleg en factoren als teveel voedselinname en weinig lichaamsbeweging. De laatste jaren heeft wetenschappelijk onderzoek aangetoond dat omgevingsfactoren tijdens kritische perioden van (orgaan)ontwikkeling op vroege leeftijd een rol kunnen spelen in het risico op de ontwikkeling van obesitas en metabole ziekten op latere leeftijd. De wetenschapper David Barker noemde dit concept in 1990 de "*Barker hypothesis*", nu ook bekend als "*Developmental Origins of Health and Disease*" (DOHaD). Sinds de Barker hypothesis in 1990 hebben studies aangetoond dat zowel de foetale als de postnatale tijd *kritische perioden* van ontwikkeling zijn, waarin gezondheid en ziekte op latere leeftijd bepaald kunnen worden.

Onderzoek suggereert enkele mechanismen waarop omstandigheden in het vroege leven invloed kunnen hebben op gezondheid later in het leven, waaronder irreversibele veranderingen in orgaan en weefselstructuur, veranderde standaardwaarden voor hormoonconcentraties en *epigenetische regulatie van genexpressie* (ook wel '*programmering'* genoemd). Epigenetica betekend 'rondom het DNA' en gaat over 'markeringen' rondom het DNA die genen in het DNA 'aan'

of 'uit' kunnen zetten. 'Aan' betekent dat het gen, dat codeert voor bijvoorbeeld een enzym, aangemaakt kan worden door de cel. Levensomstandigheden, vooral op vroege leeftijd, kunnen deze markeringen op het DNA beïnvloeden en daarmee op latere leeftijd het 'aan' en 'uit' zetten van genen reguleren. Het effect op latere leeftijd is afhankelijk van het type, de omvang, de tijdsduur en het moment van de (alternatieve) levensomstandigheden en van het orgaan systeem dat beïnvloed wordt.

Programmering door voeding en microbiota

Observationele studies hebben aangetoond dat volwassenen die borstvoeding kregen als kind minder risico hebben op obesitas en metabole ziekten dan volwassenen die flesvoeding kregen toen ze jong waren. Vele verschillen tussen borstvoeding en flesvoeding zouden hiervoor verantwoordelijk kunnen zijn, zoals de samenstelling van de melk en het contact met de moeder. Obesitas en andere metabole ziekten worden ook vaak in verband gebracht met een verstoorde darmflora (*microbiota*). Het is onduidelijk of deze verstoorde microbiota oorzaak of gevolg is van de metabole ziekte. Behalve voeding beïnvloeden darmbacteriën namelijk ook het metabolisme van de gastheer waarin ze verblijven. Het is mogelijk dat darmbacteriën al op vroege leeftijd het metabolisme van de gastheer tijdelijk of permanent beïnvloeden.

In dit proefschrift hebben we ons gericht op ***het identificeren van korte- en lange termijn effecten van de beschikbaarheid van cholesterol (Hoofdstuk 2 & 3) en de microbiota (Hoofdstuk 4 & 5) in het vroege leven op gewichtstoename en metabolisme in het latere leven.*** Onze hypothesen hebben we onderzocht in muizen.

Cholesterol in moedermelk

Moedermelk bevat hoge concentraties cholesterol in vergelijking tot flesvoeding. Het verschil in cholesterol concentraties in moedermelk en flesvoeding is mogelijk geassocieerd met de voordelige gezondheidseffecten gezien in volwassen die borstvoeding kregen als kind. Tijdens de ontwikkeling is cholesterol een belangrijk bouwblok voor celmembranen en wordt cholesterol gebruikt om hormonen en galzouten van te maken. Behalve voeding als bron van cholesterol maakt het lichaam ook zelf cholesterol, voornamelijk in de lever maar bijvoorbeeld ook in de borstklieren. Tijdens de lactatie is de cholesterolsynthese in het lichaam van de moeder verhoogd om te voldoen aan de grote cholesterolbehoefte voor melkproductie. Cholesterol transport in het bloed vindt plaats in onder andere vetbolletjes (ook wel bekend als het 'slechte' LDL-cholesterol en het 'goede' HDL-

cholesterol). Hoe cholesterol precies vanuit het bloed de borstklier in komt en hoe het vanuit daar naar de melk gaat is niet helemaal duidelijk. Tevens is onbekend hoe de cholesterol concentraties in melk precies gereguleerd worden.

In **hoofdstuk 2** hebben we gekeken of een te hoog cholesterol gehalte (hypercholesterolemie) in de moedermuis zorgt voor een hogere cholesterol concentratie in de moedermelk. Hypercholesterolemie veroorzaakten we door middel van een hoger cholesterol gehalte in het dieet van de moedermuis en/of inactivatie van genen betrokken bij cholesterol transport. De resultaten toonden aan dat cholesterolconcentraties in de moedermelk constant bleven ondanks tot 5-voudig verhoogde cholesterol waarden in het bloed van de moeder en ondanks de afwezigheid van de cholesterol transport-gerelateerde genen. Bij hypercholesterolemie ontstaan door een hoog cholesterol dieet verminderde de cholesterolsynthese in de lever en de borstklieren tot onmeetbare waarden. Deze verlaagde cholesterolsynthese is mogelijk een compensatie mechanisme tegen verhoging van cholesterol in melk. Hieruit concluderen wij dat de constante cholesterol concentratie in melk 1) voortkomt uit het melkvormingsproces en door de functie van cholesterol in de melkvetbolletjes en/of 2) belangrijk is voor de ontwikkeling van de nakomeling.

De kritische periode voor het programmeren van cholesterol absorptie

Onze onderzoeksgroep heeft voorheen in muizen aangetoond dat verminderde cholesterol opname (absorptie) in de nakomeling tijdens de lactatie leidt tot verminderde cholesterol absorptie op volwassen leeftijd. Verminderde cholesterol absorptie tijdens de lactatie werd veroorzaakt door nakomelingen ezetimibe toe te dienen via de moedermelk. Ezetimibe is een medicijn dat de cholesterol absorptie in de darm remt en bij volwassen mensen gebruikt wordt om de cholesterol concentratie in het bloed te verlagen. De programmering van verlaagde cholesterol absorptie op latere leeftijd gebeurde via verminderde expressie van de cholesterol absorptie transporter in de darm (NPC1L1). In **hoofdstuk 3** hebben we in muizen onderzocht of de kritische periode waarin cholesterol absorptie tot op latere leeftijd geprogrammeerd kan worden, tot voorbij de lactatie periode gaat. We zagen echter dat de verminderde cholesterol absorptie tijdens toediening van ezetimibe via het dieet gedurende drie weken na de lactatie verdween zodra ezetimibe toediening stopte. Hieruit concluderen wij dat de kritische periode voor het programmeren van cholesterol absorptie stopt na de lactatie periode. Dat betekent dat preventie maatregelen of therapieën voor cholesterol metabolisme waarschijnlijk het meest effectief zijn tijdens de lactatie. De resultaten van hoofdstuk 2 en 3 samen wijzen niet direct op de metabolische relevantie van moedermelk cholesterol en het impliceert evenmin dat cholesterol toegevoegd

moet worden aan flesvoeding om dezelfde gunstige gezondheidseffecten als moedermelk te bereiken. Het levenslange risico van lagere cholesterol beschikbaarheid tijdens de lactatie op het metabolisme op volwassen leeftijd kan worden onderzocht in een muismodel met ezetimibe zoals hierboven beschreven.

Cholesteroluitscheiding en de rol van galzouten

Meer cholesterol uitscheiding bij hypercholesterolemie is goed om het risico op hart- en vaatziekten en metabool syndroom te verlagen. Cholesterol uitscheiding kan via de lever/gal-route of via directe uitscheiding van cholesterol vanuit de bloedbaan naar de darmholte en vervolgens naar de ontlasting. Het laatste proces wordt "Trans-Intestinale Cholesterol Excretie" (TICE) genoemd en is belangrijk in muizen. In de lever/gal-route wordt cholesterol in de lever omgezet in galzouten en vervolgens opgeslagen in de galblaas. Na het eten van een (vette) maaltijd wordt de gal (bestaande uit onder andere galzouten en cholesterol) uitgescheiden in de dunne darm. Daar functioneren galzouten als zepen die vetdeeltjes uit de voeding oplossen. Hierdoor kunnen de vetdeeltjes, waaronder ook cholesterol, en vitaminen gemakkelijker verteerd en opgenomen worden. In het laatste deel van de dunne darm worden galzouten heropgenomen en terug getransporteerd naar de lever, waar ze hergebruikt kunnen worden. Door dit efficiënte proces gaat slechts ~5% van de galzouten per ronde verloren met de ontlasting. De productie van nieuwe galzouten wordt nauw gereguleerd in reactie op verschillende signalen, waaronder signalen van galzouten in de darm. Behalve de zeep-functie, kunnen galzouten ook het metabolisme beïnvloeden doordat ze kunnen binden aan receptoren aan het oppervlak van cellen. Deze receptoren brengen een cascade aan reacties teweeg die de aanmaak van galzouten reguleert maar ook het metabolisme van suikers en vetten. Daarom zijn galzouten een mogelijk aangrijpingspunt voor het behandelen van een ontregeld metabolisme.

De interactie tussen galzouten en darmbacteriën

De humane microbiota bestaat uit meer dan 1000 verschillende soorten bacteriën waarvan de meeste in het colon, de dikke darm verblijven. Tot aan de geboorte zijn de darmen nog redelijk bacterievrij, hoewel de foetus in de baarmoeder mogelijk blootgesteld wordt aan bacterie-gerelateerde signalering vanuit de darmen van de moeder. Tijdens en na de geboorte komt de baby in contact met bacteriën uit de omgeving en ontwikkelt de microbiota zich in 1-2 jaren. Deze ontwikkeling is sterk afhankelijk van omgevingsinvloeden zoals het contact met de ouders, voeding, omgeving en antibiotica en daarom krijgt ieder wezen een unieke samenstelling. Er zijn steeds meer aanwijzingen dat bacteriën in het vroege leven belangrijk zijn voor de ontwikkeling van de darmen en het metabole systeem.

Bacteriën in de darmen bewerken galzouten waardoor deze andere eigenschappen krijgen en hun invloed op het metabolisme verandert. Naast galzouten bewerken bacteriën voedselcomponenten en produceren daaruit korte-keten vetzuren (SCFA) en andere stoffen. Deze stoffen veranderen de lokale metabole signalering en worden ook geabsorbeerd en circuleren naar de lever en veel andere plaatsen in het lichaam om daar het metabolisme te beïnvloeden. Twee studies in dit proefschrift (**Hoofdstuk 4** en **5**) beschrijven de metabole gevolgen van microbiota-gerelateerde gebeurtenissen in het vroege leven, voornamelijk met betrekking tot het metabolisme van galzouten en cholesterol.

Sekse specifieke invloed van de microbiota op de galzoutsamenstelling

Genexpressie en metabolisme zijn aanzienlijk verschillend bij mannen en vrouwen en dit verschil is afhankelijk van de aanwezigheid van de microbiota. Ook voedingsstoffen in het vroege leven beïnvloeden de metabole gezondheid op de lange termijn anders bij mannen en vrouwen. Het onderzoeken en begrijpen van sekse verschillen is daarom erg belangrijk als men de informatie wil gebruiken om preventiemaatregelen en therapieën te ontwikkelen. Daarom zijn sekse specifieke verschillen meegenomen in de microbiota-onderzoeken in dit proefschrift.

De galzoutsamenstelling en totale concentraties verschillen in mannetjes en vrouwtjes muizen die microbiota bevatten (*conventionele* muizen), maar niet in muizen zonder microbiota (*bacterievrije* muizen). Dit suggereert een rol voor intestinale microbiota bij het reguleren van sekseverschillen in de samenstelling van galzouten. Behalve de galzoutsamenstelling verschilt inderdaad ook de microbiota tussen mannen en vrouwen bij zowel mensen als muizen. **Hoofdstuk 4** beschrijft de galzoutdynamiek in mannelijke en vrouwelijke bacterievrije muizen nadat deze muizen microbiota (poep) uit de dikke darm hadden gekregen van muizen met hetzelfde óf tegenovergestelde geslacht (deze poeptransplantatie in bacterievrije muizen noemen we *conventionalisering*). Zoals verwacht waren de plasmaconcentraties van galzouten hoger bij ontvangers van vrouwelijke microbiota, maar dit effect werd alleen de eerste drie dagen na toedienen van de microbiota waargenomen. Tien weken later waren de galzoutconcentraties en -samenstelling vergelijkbaar tussen mannelijke en vrouwelijke muizen die microbiota afkomstig van mannetjes ofwel van vrouwtjes hadden gekregen. Wij veronderstellen dat een bacterievrije start van het leven (tot 12 weken, de 'jong-volwassen' muisleeftijd) het metabole systeem kan hebben geprogrammeerd. Een bacterievrije start beïnvloedt mogelijk hoe microbiota later in het leven het galzoutmetabolisme van de gastheer kan beïnvloeden. Er is meer werk nodig om het onderliggende mechanisme te onderzoeken dat sekseverschillen in de galzoutsamenstelling veroorzaakt bij mensen en conventionele muizen.

Toekomstige onderzoeken moeten rekening houden met de vertaling van gegevens van muizen naar mensen. In tegenstelling tot mensen hebben muizen een paar muis-specifieke (muri-) galzouten. Afwezigheid van het enzym dat omzetting van galzouten in muri-galzouten veroorzaakt, zal muizen een meer humane galzoutsamenstelling geven. De sekse specifieke metabole effecten na inbrengen van ontlasting van een vreemde (met daarin darmbacteriën en galzouten) in het darmstelsel van een patiënt kunnen van klinisch belang zijn voor het toepassen van poeptransplantaties bij metabole ziektes.

Lange termijn effecten van afwezigheid van microbiota op vroege leeftijd

Aanwezigheid van bacteriën in de darmen heeft grote gevolgen voor het metabole systeem, zoals aangetoond door het dynamische galzoutprofiel nadat bacterievrije muizen in **Hoofdstuk 4** aan microbiota waren blootgesteld. Bij de mens is een bacterievrije status tijdens de zwangerschap en lactatie onmogelijk en daarom hebben wij een bacterievrij muismodel gebruikt om te onderzoeken wat voor lange termijn effecten microbiota (of de afwezigheid daarvan) op het metabole systeem heeft. Wij veronderstelden dat een bacterievrije zwangerschap en lactatie zou leiden tot een andere regulatie van genexpressie dan bij muizen die blootgesteld zijn aan bacteriën tijdens deze ontwikkelingsfase, resulterend in een afwijkend metabolisme op volwassen leeftijd.

In **Hoofdstuk 5** hebben we de gevolgen van afwezigheid van microbiota tijdens zwangerschap en lactatie bij muizen op metabole parameters later in het leven getest, waarbij het metabolisme van de muizen werd geprikkeld met een Westers dieet. Verrassend genoeg had de afwezigheid van microbiota in het vroege leven, gevolgd door een poeptransplantatie bij het spenen, geen effect op de volwassen lichaamsgewichtstoename, cholesterolmetabolisme, glucosetolerantie of insulinegevoeligheid. Een bacterievrije periode in het vroege leven resulteerde wel in een verminderde galuitscheiding (volume en concentratie galzouten) met minder galzouten naar de darmen vergeleken met conventioneel opgevoede muizen. Deze bevindingen impliceren dat microbiota in het vroege leven de metabole plasticiteit van volwassenen niet kritisch beïnvloedt. Gegevens van mensen en muizen hebben echter aangetoond dat antibiotica blootstelling in het vroege leven langdurige metabole effecten kan hebben. Antibiotica vermindert tijdelijk de hoeveelheid en diversiteit van de microbiota. Een tijdelijke verandering in de microbiota kan verschillende metabole signalen en mogelijk programmering van het volwassen metabolisme veroorzaken. Toekomstige studies met kwalitatieve verschillen in de microbiota samenstelling op vroege leeftijd zouden mogelijke lange termijn effecten op het metabole systeem verder kunnen ophelderen.

Conclusie & mogelijkheden voor de toekomst

Het verrichte onderzoek in dit proefschrift biedt waardevolle inzichten in hoe vroege levensomstandigheden, zoals cholesterol beschikbaarheid in de darmen en aanwezigheid van microbiota tijdens gedefinieerde kritische perioden, op latere leeftijd kunnen bijdragen aan fysiologie en metabolisme. Omdat directe vertaling van resultaten beschreven in dit proefschrift naar de menselijke conditie kan worden belemmerd door soortverschillen, verwacht ik dat toekomstige onderzoeken met diermodellen zullen profiteren van meer gehumaniseerde samenstellingen van zowel microbiota als galzouten. Als de huidige bevindingen in knaagdieren kunnen worden bevestigd bij mensen, kunnen nieuwe strategieën voor de preventie van metabool syndroom-gerelateerde ziekte in het vroege leven worden ontwikkeld in plaats van, of in aanvulling op, de behandeling van de aandoening op volwassen leeftijd.

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Lieve lezer / Dear reader,

Nu ik dit schrijf, is morgen exact vijf jaar geleden dat ik mijn officiële eerste dag als PhD student begon, en de dag dat ik mijn boekje eindelijk naar de drukker kan sturen. Deze laatste pagina's wil ik graag gebruiken om mijn begeleiders, collega's, vrienden en familie te bedanken. Jullie hebben mij geholpen, gemotiveerd, gesteund en vermaakt, of je daar nou een grote of kleine rol in speelde, je had een belangrijke rol voor mij bij het onderzoek en de afgelopen jaren in Groningen en daarbuiten. Sowieso allemaal hartelijk bedankt (ook als ik je naam niet specifiek noem, maar ik ga mijn best doen)!

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Liefs,
Mirjam

BIOGRAPHY

Mirjam Lohuis was born on November 30th, 1989 in Groot Agelo, the Netherlands. After graduating from the Twents Carmel College locatie De Thij in Oldenzaal in 2008, she started her bachelor in Biomedical Sciences at the Utrecht University. During her bachelor degree she was secretary of the Drama Committee and of the Foreign Excursion Biomedical Students Utrecht Foundation where she has helped to organize an educational trip to the UK. In her third year she mentored 12 new students to help them accustom to their new study.

From 2011 to early 2014, she did a master 'Infection and Immunity'. Within the master she did an internship in the department of Medical Microbiology – Virology at the Utrecht University under the supervision of Dr. R.J. (Robert-Jan) Lebbink and Prof.dr. E.J.H.J. (Emmanuel) Wiertz. There she validated and characterized new host genes involved in virus-mediated down-regulation of MHC class I heavy chain molecules in immune cells. Then she moved to San Francisco, to do her second internship under supervision of Prof.dr. Tippi Mackenzie at the department of Pediatric Surgery, concerning maternal T cell awareness of the fetus after fetal intervention. Back in the Netherlands she finished her master with her thesis on the topic "Intestinal colonization in premature and very low birth weight infants: influencing factors and NEC" to graduate in July 2014.

In May 2014, Mirjam started her PhD in Groningen under supervision of Prof. dr. Henkjan J. Verkade and Dr. Uwe J.F. Tietge at the Department of Pediatrics. In her project Mirjam investigated the role of dietary cholesterol availability and intestinal microbiota presence in early life on the metabolic health in adulthood. Next to her research, she was a member of the Graduate School of Medical Science PhD Council to arrange lectures and fight for (future) PhD rights. During her PhD she discovered her enthusiasm and joy in supervising several bachelor and master students.

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