General Discussion
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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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) 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. During the third trimester of gestation, fetal cholesterol is for 20-40% provided via the maternal circulation through placental transport. The remaining cholesterol of the fetal pool originates from de novo synthesis by the fetus. In a healthy pregnancy, maternal total and LDL-cholesterol (LDL-C) in plasma increase during the pregnancy. Maternal hypercholesterolemia affects offspring serum cholesterol differently when exposure occurs during pregnancy than when offspring is exposed during lactation. Maternal hypercholesterolemia 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 and mice. 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. 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. 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 hypercholesterolemia 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)\textsuperscript{47}. The MFGM contains liquid ordered (\textit{Lo}) and liquid disordered (\textit{Ld}) phases\textsuperscript{44}. The \textit{Lo} phase is rigid and consists of cholesterol and milk sphingomyelin (SM)\textsuperscript{44}. Membrane fluidity is determined by the ratio of cholesterol : SM and amount of \textit{Lo} phases\textsuperscript{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\textsuperscript{248}. The MFG size distribution varies during the day and lactation stages and ranges from 1 to 10\textmu m\textsuperscript{44, 47, 143}. Structural differences in size distribution and MFG(M) composition may affect MFG digestion\textsuperscript{247} and may be of nutritional significance for the infant\textsuperscript{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\textsuperscript{145} did not mimic plasma cholesterol levels of breast-fed infants nor did it reduce fractional cholesterol synthesis rates\textsuperscript{55, 56, 145}. Adding MFGM, which also contain cholesterol, to IF has yielded serum cholesterol levels closer to those of infants fed BM\textsuperscript{251}. Additionally, MFGM-supplemented IF reduced the incidence of infectious morbidity and improved cognitive performance in infants at two years of age\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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 \textit{et al.} (2017)\textsuperscript{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\textsuperscript{12, 59}. In \textbf{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\textsuperscript{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\textsuperscript{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\textsuperscript{171, 257-259}. The murine intestine may therefore be less sensitive to nutritional impact on epigenetic modifications after lactation than during lactation\textsuperscript{171}. Also, the switch from fluid to solid food might trigger intestinal changes\textsuperscript{172} and thereby terminate the sensitive window. Weaning in rodents initiates crypt cell proliferation in the small intestine and accelerated proliferation of enterocytes\textsuperscript{172}, but also rapidly alters composition of the microbiota\textsuperscript{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\(^{93, 263}\). 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 modifies 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\textsuperscript{104}. Therefore, a lack of sex-specific differences may be less apparent in these studies (\textit{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\textsuperscript{104, 264}, the necessity of matching donor and recipient sex data in human studies has not (yet) been demonstrated\textsuperscript{203, 265}.

\textbf{Rodent-specific bile acids}

Rodents and humans differ considerably in bile acid composition and metabolism\textsuperscript{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\textsuperscript{32, 266}. Muricholic acids inhibit cholesterol absorption\textsuperscript{168, 169} and stimulate bile acid synthesis via their antagonistic activity on farnesoid X receptor (FXR) in the intestine\textsuperscript{93, 94}. Conventionalization of germ-free mice reduces muricholic acid levels, thereby alleviating FXR inhibition\textsuperscript{93} and likely increasing cholesterol absorption\textsuperscript{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)\textsuperscript{267}. Genetic \textit{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\textsuperscript{89, 267}. Studies in this \textit{Cyp2c70} knockout mouse model, together with a more humanized microbiota composition\textsuperscript{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\textsuperscript{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 (\textit{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\textsuperscript{205-207, 272, 273}. It is still unclear to what extent the microbiota programs long-term the metabolic system\textsuperscript{182} and/or if the microbiota composition itself is programmed\textsuperscript{274}. Acute effects of microbiota on the host are abundant, involving modification of bile acids, gene expression and metabolism\textsuperscript{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\textsuperscript{119, 276}. The increase in deconjugated bile acids is suggested to lead to increased ABCG5/8 activity\textsuperscript{276, 277}, presumably via FXR activation followed by downregulation of small heterodimer partner (SHP) and upregulation of liver X receptor LXR\textsuperscript{276}. LXR stimulates ABCG5/8, resulting in lower cholesterol absorption and increased biliary cholesterol secretion\textsuperscript{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\textsuperscript{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\textsuperscript{223}. Antibiotics impact on the microbiome’s adaptability to diet, diversity, composition and metagenomics content\textsuperscript{278, 279}. The impact of antibiotic treatment increases with the type, duration and dosage of the antibiotic used\textsuperscript{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\textsuperscript{216}. Indeed, metabolic parameters are dependent on the microbiota, but also on the host genetics and diet\textsuperscript{202, 216}. \textit{Vice versa}, the diet as well as previous environment and host genetics shape microbiota composition\textsuperscript{202}. Moreover, the microbiome, diet and host genetics all interact in development of the metabolic syndrome\textsuperscript{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\textsuperscript{13, 200, 280}. Studies with qualitative differences of early-life microbiota composition (such as the contribution of BSH-producing bacteria\textsuperscript{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\textsuperscript{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 hypercholesterolemia. 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.