Fetal and neonatal environment

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FETAL AND NEONATAL ENVIRONMENT: EFFECTS ON BILE ACID AND LIPID METABOLISM

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CHAPTER

1

INTRODUCTION, AIM AND OUTLINE OF THE THESIS

GENERAL INTRODUCTION

Lipids are water-insoluble molecules with a diversity of biological roles. Lipids are indispensible in eukaryotic cells for cell membrane structure and function, as a source of energy, and as ligands of specific transcription factors. Their various functions can be illustrated by the biological significance of one well known lipid; cholesterol. Cholesterol is a structural component of plasma and organelle membranes (1). Cholesterol is the precursor for biologically important molecules such as steroid hormones and bile acids. Additionally, cholesterol activates Sonic Hedgehog (2), a signalling protein which is essential for embryonic development. The importance of cholesterol for normal development is emphasized by the devastating effects of inborn errors in cholesterol synthesis, such as the Smith-Lemli-Opitz syndrome (3). On the other hand, elevated plasma concentrations of cholesterol, particularly low-density lipoprotein cholesterol (LDL-c) is a well known risk factor in the development of cardiovascular disease, similar to increased plasma concentrations of another lipid, triglycerides, (4). As both shortage and excess of lipids disrupt normal growth and development, lipid metabolism needs to be carefully balanced.

METABOLIC PROGRAMMING

Environmental factors during gestation, such as nutrional conditions, have been related to the susceptibility to chronic diseases later in life. A U-shaped relationship exists between birth weight and increased susceptibility to develop adult disease, as both fetal undernutrition and fetal overnutrition increase the risk to develop features of the metabolic syndrome. Epidemiological studies showed associations between impaired fetal nutrition and an increased vulnerability to develop components of the metabolic syndrome such as hypertension, diabetes, cardiovascular disease and obesity (5-15). In addition, epidemiological studies showed a similar inverse relationship between size at birth, as a marker of impaired fetal nutrition, and dyslipidemia in adulthood. Dyslipidemia is strongly related to atherosclerosis, obesity, diabetes and fatty liver disease (16, 17). A small abdominal circumference at birth is associated with raised serum concentrations of total and low-density lipoprotein cholesterol (LDL-c) and (the LDL-c associated) apolipoprotein B at the age of about fifty years (5). People exposed to famine in early gestation, due to the Dutch hunger winter in 1944, display a more atherogenic lipid profile when compared to people that were not exposed to famine in their fetal period (18). The Hertfordshire study in which a cohort of men and woman born between 1911 and 1930 in England was studied in their sixties, showed that reduced fetal growth (reflected by birth weight) was associated with higher plasma triglyceride levels and lower high-density lipoprotein levels in adulthood (7). Already shortly after birth, infants that are small-for-gestational age (SGA) display a more atherogenic plasma lipid profile (higher total cholesterol, triglycerides and LDL-c) compared with normal weight controls (19). Additionally, SGA newborn

infants displayed significant maximum aortic thickening with hypertriglyceridemia compared with normal weight controls suggesting that fetal nutrition might predispose to increased risk of later cardiovascular disease (20).

The epidemiological association between fetal environment and/or size at birth and adult disease has lead to several hypotheses on the developmental origins of health and disease. The term "Programming" was first defined by Lucas, as reflecting the scenario by which a stimulus or insult operating at a critical or sensitive period of growth and development results in long term consequences for the structure or function of the organism. The important underlying idea is that the programming event can only occur during a specific window of sensitivity (21, 22). Hales and Barker described the "thrifty phenotype" hypothesis which suggests that fetal adaptations in answer to a nutritionally deprived environment may permanently reset or program fetal metabolism in order to optimize its chances of survival in what it expects to be a poor nutritional environment. This adaptive response is beneficial for the organism when placed in a nutrional poor fetal environment but becomes harmful in a nutritional rich postnatal environment (21). Epidemiological studies have also related postnatal nutrition (human milk versus formula) to the susceptibility in the development of cardiovascular disease, hypertension (23, 24) and dyslipidemia (14, 25). Although the epidemiological relationships between early events and adult disease have become convincingly strong, the biological mechanisms behind these programming effects have remained largely unclear. To gain insight in the mechanisms behind metabolic programming would allow for the development of preventive strategies to target the negative long-term consequences of early nutrition.

METABOLIC PROGRAMMING AND ANIMAL MODELS

To unravel the pathophysiological mechanisms behind fetal programming, a variety of animal models implying food restriction or the manipulation of specific nutrients during gestation has been developed (26, 27). An increasing number of experimental studies using different strategies in a variety of animal species demonstrated fetal programming effects (26, 27). An established model used in rat and mice to investigate the mechanisms behind programming effects, consists of maternal protein restriction during gestation. Studies in rodents show that maternal protein restriction during gestation leads to features of the metabolic syndrome in the adult offspring, such as obesity, hypertension and diabetes (28-34). As stated before, the pathophysiological mechanisms behind metabolic programming are largely unknown. One proposed mechanism is that of epigenetic modification. This hypothesis implies that early life environmental conditions can cause stable alterations in gene expression potential that persist throughout life, without modifying DNA sequence. For example; adult individuals that were prenatally exposed to famine during the Dutch Hunger Winter in

1944-45 had less DNA methylation of the IGF2 gene (insulin-like growth factor, a key factor in human growth and development) compared to their unexposed same-sex siblings (35). Additionally, rodent studies have shown that protein restriction during gestation induces epigenetic modification of genes involved in lipid and carbohydrate metabolism in the offspring (36, 37). We aimed to increase the mechanistic understanding of metabolic programming, in order to eventually allow for the targeted prevention of long-term health consequences of early environmental conditions. We determined in an established experimental model the effect of a low protein diet during gestation on maternal-fetal cholesterol transport and on fetal lipid synthesis in mice. To set the context of our studies, we first provide a concise overview addressing cholesterol and lipid metabolism in the fetal, neonatal and adult period.

CHOLESTEROL SYNTHESIS

In mammals, cholesterol originates from either *de novo* synthesis or from exogenous sources (i.e. dietary absorption or placental transfer). All nucleated mammalian cells can synthesize cholesterol from acetyl-CoA via the mevalonate enzymatic pathway. The liver and intestine are considered major sites of cholesterol synthesis in the adult situation (38). The first step of cholesterol biosynthesis is the formation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) from acetyl-CoA and acetoacetyl-CoA, catalyzed by the enzyme HMG-CoA synthase. HMG-CoA is then converted to mevalonate by HMG-CoA-reductase, which under physiological conditions represents the rate-controlling step. Mevalonate is converted to isopentenyl-5-pyrophosphate, condensated to squalene and further metabolized to lanosterol. Conversion of lanosterol into cholesterol takes another 19 enzymatic reactions. In adult humans, the daily synthesis of cholesterol amounts to approximately 10 mg/kg body weight. Synthesis inversely adapts to the dietary intake, i.e. synthesis increases when intake is low and vice versa. Cholesterol is transported in the circulation in lipoproteins: chylomicrons, very-low-density lipoproteins (VLDL), high-density lipoproteins (HDL) and low-density lipoproteins (LDL). The body can dispose itself from excess cholesterol via fecal excretion, either as cholesterol or after hepatic metabolism, as bile acids.

REGULATION OF CHOLESTEROL METABOLISM

Refined mechanisms exist to maintain cholesterol balance in different metabolic circumstances. The liver-X-receptors (LXRs) are key players in the regulation of cholesterol homeostasis. LXRs are ligand-activated transcription factors that belong to the nuclear receptor superfamily (39). There are two LXR isoforms; LXRα (NR1H3) and

LXRβ (NR1H2) that regulate gene expression via direct binding to DNA response elements in promoter regions of their target genes. LXRα is mainly expressed in liver, intestine and adipose tissue whereas LXRβ is more ubiquitously expressed (40). Apart from their endogenous ligands which are oxysterols (oxidized cholesterol derivatives) synthetic LXR agonists such as T0901317 have been developed (41). In the absence of ligands, LXR and its obligate heterodimer Retinoid X Receptor (RXR) are bound to LXR response elements (LXRe) in complex with corepressors in the promoter sites of their target genes. Upon ligand induced activation, the corepressor complexes are exchanges for coactivators after which gene expression of the target genes takes place (fig. 1). Both LXR isoforms bind to their obligate heterodimer Retinoid X Receptor (RXR) after which they bind to LXR response elements (LXRe) in the promoter sites of their target genes, thereby regulating gene expression. LXR serves as a cholesterol sensor: when cellular oxysterols accumulate due to increased cholesterol concentration, LXR generates the transcription of genes that induce efflux from cholesterol out of the cell. Significant LXR target genes are members of the family of ATP-binding cassette (ABC) transporters. Upon LXR activation, ABC transporters, encoding for proteins involved in cellular cholesterol efflux to bile or the intestinal lumen (ABCG5, ABCG8), to high-density lipoproteins (HDL) (ABCA1, ABCG1) are activated (see figure 2). Additionally, activation of LXR leads to induction of the conversion of cholesterol into bile acids via activation of CYP7a1 (the rate-limiting enzyme in

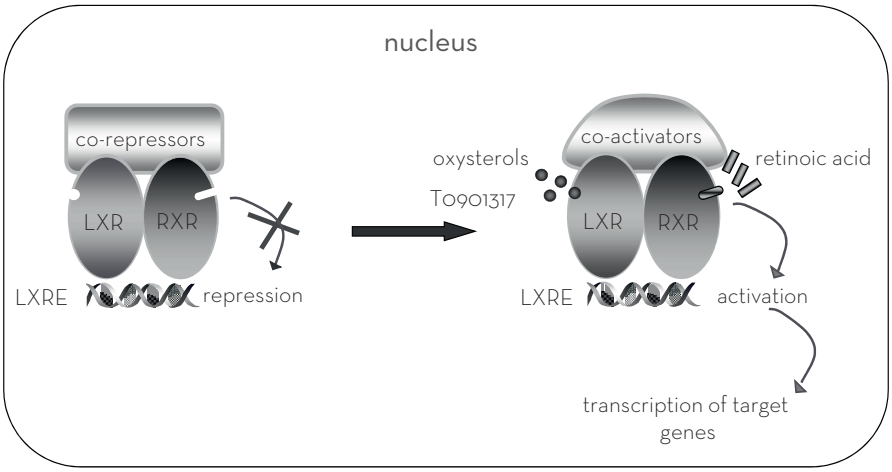

Figure 1. In the nucleus, RXR and LXR associate as heterodimers and are bound to LXREs in the promotor regions of target genes in complex with co-repressors. In the absence of ligands, they repress target gene transcripticon. After ligand activation (with oxysterols or T0901317) a conformational change in the receptor takes place after which the co-repressors are dissociated and the co-activators are recruited to initiate gene transcription.

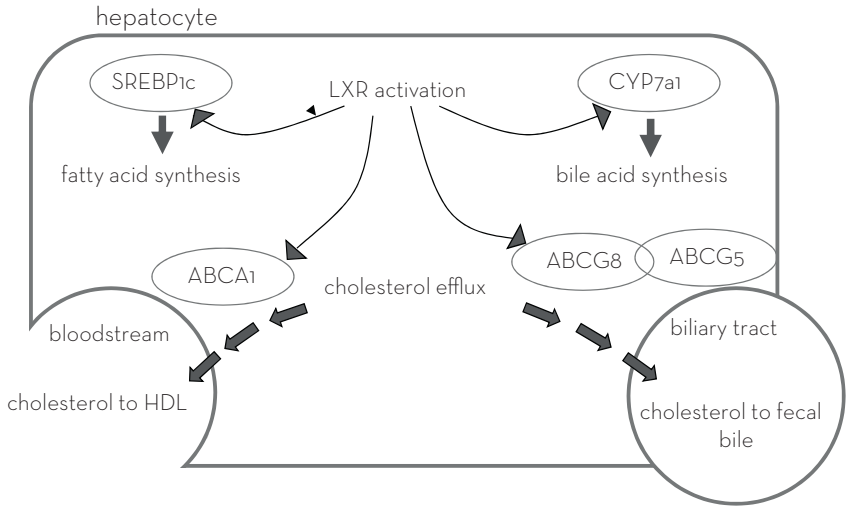

Figure 2. Upon LXR activation, LXR target genes expression is induced. LXR induces hepatic ABCA1 expression leading to the efflux of cholesterol to HDL. LXR induced the hepatic expression of both heterodimers ABCG5 and ABCG8 leading to cholesterol secretion into bile. Furthermore, LXR activation induces expression of SREBP1c resulting in fatty acid synthesis. LXR activation results in upregulation of CYP7a1, resulting in bile acid synthesis. This is not a complete list of target genes.

bile acid synthesis), subsequently generating efflux from cholesterol out of the cell. LXR activation results in an increased plasma HDL-cholesterol, decreased intestinal cholesterol absorption, increased hepatobiliary cholesterol secretion and increased fecal neutral sterol excretion; please see for review (42). In addition to cholesterol efflux, LXR activation induces expression of genes involved in *de novo* lipogenesis; sterol regulatory element binding protein 1c (SREBP1c), acetyl CoA carboxylase (ACC), fatty acid synthase (FASN) and stearoyl-Coenzyme A desaturase 1 (SCD1) (43). Both isoforms of LXR are expressed in the murine and human placenta (44), and LXR has been postulated to have a crucial role in fetal cholesterol metabolism (45).

FETAL CHOLESTEROL AND TRIGLYCERIDE METABOLISM

The significance of cholesterol for the organism is best illustrated early in life. During the rapid growth of the fetal period, significant amounts of cholesterol are required for proper growth and embryological development. Defects in cholesterol synthesis have detrimental consequences for the individual. In humans, the Smith-Lemli-Opitz syndrome, desmosterolosis and mevalonic aciduria are well described examples of inborn errors in cholesterol synthesis leading to serious malformations in various organ systems (46). Unlike the adult situation in which cholesterol metabolism is in steady state, the fetus, due to its rapid growth and development, accumulates cholesterol (47). Placental steroid hormone synthesis and the rapidly growing fetus render gestation a high cholesterol demanding state. Both placental (48), and fetal tissues are capable of *de novo* cholesterol synthesis (49). Compared to adult synthesis rates, fetal sterol synthesis rates are much higher (50). Besides *de novo* synthesis, several lines of evidence support the concept that maternal cholesterol (via placental transport) contributes to fetal cholesterol accretion. First, maternal hypercholesterolemia strongly correlates with the formation of aortic fatty streaks in the human fetus (51). Second, in the Dhcr7$^{-/-}$ mouse, a model of the Smith-Lemli-Opitz syndrome which is unable to synthesize cholesterol, the presence of cholesterol was demonstrated in the fetus indicating that exogenous cholesterol must have been transported to the fetus (52). Third, transfer of labeled cholesterol ([3,4-$^{13}C_2$]-cholesterol) from mother to fetus was described in mice, indicating that cholesterol from the maternal circulation is transported to the fetus (53). Finally, synthetic activation of LXR during gestation increased the expression of ABCA1 and ABCG1 in the murine placenta followed by a functional increase of maternal-fetal cholesterol transport measured using ^{14}C labeled cholesterol (54).

Triglycerides represent the storage and transport form of lipids and are composed of a glycerol back bone connected to three fatty acid molecules. In parallel to cholesterol, fatty acids can be taken up from the diet or can be synthesized by the body itself in the liver: *de novo* lipogenesis. Although in adults on a western-type diet, hepatic *de novo* lipogenesis is a quantitatively minor pathway as was shown by Hellerstein et al (55), biosynthesis of fatty acids might be a relevant compensatory pathway in the growth restricted fetus. In the present project, we addressed the effects of different fetal nutritional conditions on fetal lipid metabolism and on maternal-fetal lipid transport. In a mouse model, we determined the effects of synthetic activation of LXR during gestation on fetal lipid metabolism in mice. In growth restricted premature human newborns, we determined the influence of fetal growth restriction on the biosynthesis of lipids.

POSTNATAL NUTRITION

Comparable to fetal nutrition, the composition of early postnatal nutrition can exert effects on adult health. Human milk contains a substantial larger amount of cholesterol (≈ 2-4 mmol/l) than cow's milk based formulas (0.3-0.8 mmol/l) (56, 57). Breastfeeding has been associated with a decreased epidemiological risk to develop cardiovascular disease and obesity, and it is linked to a protective lipid profile when compared to formula feeding (25, 58). Higher dietary cholesterol intake is inversely related to endogenous or *de novo* cholesterol synthesis in infants. It has been hypothesized that the inverse relationship between the intake of human milk and the *de novo* cholesterol synthesis could lead to smaller central cholesterol pools thereby potentially reducing cardiovascular disease risk (14, 26, 56, 59). In addition to higher cholesterol content, human milk differs from formulas in other qualities, including the composition and concentration of oligosaccharides. Oligosaccharides have become under scientific attention, because of demonstrated and presumed beneficial prebiotic effects (60-62).

PREBIOTICS AND BILE ACID METABOLISM

Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating growth and/or activity of one or a limited number of bacteria (bifidobacteria, lactobacilli) in the colon (63). Oligosaccharides are a major constituent of human milk. Infants fed human milk have a larger proportion of bifidobacteria and lactobacilli in their intestinal bacterial flora compared with formula fed infants (64, 65) It is well known that colonic bacterial species can metabolize bile acids present in the intestinal lumen, for example by deconjugation and dehydroxylation. Microbial bile acid metabolism can thus affect bile acid metabolism. Comparable to inborn errors in cholesterol synthesis, several defects in bile acid synthesis with devastating consequences have been described, underscoring the relevance of bile acid metabolism (66). Bile acids have several important functions in addition to facilitating the absorption of dietary fat and the fat-soluble vitamins A, D, E and K. Firstly, the secretion of bile acids from hepatocytes into the biliary system is the main driving force for bile formation (67). Secondly, bile acids are essential in cholesterol homeostasis as conversion of cholesterol into bile acids and their subsequent fecal loss provides the major route for removal of excess cholesterol from the body. Interestingly, Infants fed human milk have a larger bile acid pool size and a more efficient fat absorption compared to formula fed infants (68). It has remained unclear, however, whether these observations are related to prebiotic effects of human milk constituents.

BILE ACID SYNTHESIS

Bile acids are synthesized from cholesterol, exclusively in the liver. The enzymatic conversion of cholesterol can occur via two main pathways. In the neutral or classical pathway, the steroid nucleus is modified by hydroxylation reactions prior to the side-chain shortening. Cholesterol 7α-hydroxylase (CYP7A1) catalyzes conversion of cholesterol into 7α-hydroxycholesterol. This step is considered to be rate-controlling in bile acid synthesis and is subject to negative feedback of enterohepatic cycling of bile acids. In the acidic pathway, the first step involves 27-hydroxylation of the cholesterol side-chain (to yield 27-hydroxycholesterol), catalyzed by the enzyme sterol-27-hydroxylase (CYP27A1). Finally, C27-3β-steroid dehydrogenase converts the 7α-hydroxycholesterol formed via either pathway into the two primary bile acids: cholate (CA) and chenodeoxycholate (CDCA). Under physiological conditions, bile acids are conjugated with glycine or taurine and secreted via the bile in the intestinal lumen, where they facilitate the uptake of dietary fat and other hydrophobic compounds. The majority of bile acids is reabsorbed from the ileum mediated by the apical sodium bile salt transporter (ASBT) (69) after which transport via the portal system back to the liver and uptake by hepatocytes completes the enterohepatic circulation of bile acids. A small fraction of the bile salts escapes ileal reabsorption and enters the colon, where they are deconjugated and subsequently dehydroxylated by intestinal bacteria resulting in the so-called secondary bile acids (deoxycholate and lithocholate) and tertiary bile acids (ursodeoxycholate). These unconjugated bile salts can be passively reabsorbed from the colon, which results in the presence of the subsequent secondary and tertiary bile acids in the enterohepatic circulation.

AIM AND OUTLINE OF THE THESIS

Lipids and bile acids are indispensable for various physiological functions in mammals and their adequate intake or biosynthesis is thus essential for normal development. Genetic inability to synthesize cholesterol during the fetal period leads to severe congenital malformations. The relevance of adequate bile acid and/or cholesterol synthesis is emphasized by the profound health consequences of inborn errors in bile acid and cholesterol synthesis as reviewed in **chapter 2.** Lipids are of crucial importance for all cells in human body as a structural component for cell membranes. On the other hand, excess amounts of cholesterol and/or triglycerides contribute to an increased risk for the development of cardiovascular diseases, clarifying the importance of a carefully balanced regulation of lipid metabolism. Human and animal studies indicate that maternal nutrition during pregnancy and the type and duration of nutrition after birth affect short-term and long-term (adult) lipid metabolism. The mechanisms underlying these lasting effects of pre- and early postnatal events are largely unknown. **We aimed to characterize and unravel the role of specific dietary and pharmacological interventions during prenatal and early postnatal life on physiologically relevant parameters of lipid and bile acid metabolism.** First, we determined the effect of a low protein diet during gestation on cholesterol transport from mother to fetus and on fetal cholesterol and fatty acid synthesis in mice. A low protein diet during gestation is an established model to study metabolic consequences of maternal under-nutrition during gestation. We provided mice with a protein deficient diet (9% casein instead of 18 % casein) during pregnancy and determined relevant lipid fluxes using stable isotopes **(chapter 3).** Previous research shows that fetal hepatic expression levels of genes involved in lipid metabolism are lowered after maternal protein restriction during gestation (37). We tested if this alteration in mRNA expression levels was translated into quantitative changes in physiological relevant lipid fluxes.

Infants born prematurely, may constitute a human model of the last trimester of gestation in which the impact of environmental factors can be studied, **Chapter 4** deals with the *de novo* lipogenesis and cholesterogenesis in preterm human infants in the first days after birth. Two questions were attended to. First, does fetal undernutrition, as reflected by a birth weight below the tenth percentile for gestational age influence the rate of *de novo* cholesterol synthesis? Second, does intrauterine growth restriction enhance *de novo* lipogenesis, for example, as a compensatory mechanism?

The nuclear receptor LXR, a key player in the regulation of cholesterol and in the synthesis of lipids, is suggested to be involved in maternal-fetal cholesterol transport. Theoretically, manipulation of LXR during gestation could affect fetal lipid metabolism. Pharmacological interventions in the fetal environment may induce long-term programming effects. In **chapter 5** we describe both acute and long-term effects of pharmacological LXR activation during gestation on lipid metabolism in the fetal and adult offspring in mice.

Infants fed human milk have a larger bile acid pool and a more efficient fat absorption than infants fed artificially cow's milk formula. Unlike 'classic' formula feeding,

human milk contains natural oligosaccharides, and infants fed human milk have a lager proportion of bifidobacteria and lactobacilli in their intestinal bacterial flora compared to formula fed infants. Colonic bacteria can metabolize bile acids present in the intestinal lumen. In **chapter 6** we tested if oligosaccharides comparable to those present in human milk increase the bile acid pool size. We described the effect of alteration of the colonic bacterial flora, mimicking the bacterial flora of infants fed human milk, on bile acid metabolism using a prebiotic mixture in rats.

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CHAPTER

2

INBORN ERRORS IN BILE ACID AND CHOLESTEROL BIOSYNTHESIS

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INTRODUCTION

There is a multitude of cholesterol and bile acid synthesis defects. Their frequency is relatively rare, and individual diseases frequently require different approaches. This chapter provides an overview of the most relevant defects in cholesterol and bile acid biosynthesis and their presentation, and diagnostic and therapeutic approaches.

CHOLESTEROL METABOLISM

Cholesterol is essential for eukaryotic cells as a structural component of plasma and organelle membranes. Cholesterol is also the precursor for biologically important compounds such as steroid hormones and bile acids. Additionally, cholesterol activates Sonic Hedgehog (1), a signalling protein essential for normal embryonic development. Cholesterol is derived from the diet, but can also be synthesized *de novo*. Particularly during rapid fetal growth, significant amounts of cholesterol are required, which can be derived from the mother via placental supply, or via fetal or placental synthesis. Cholesterol is synthesized by all nucleated mammalian cells. Biosynthesis occurs from acetyl-CoA via the mevalonate pathway in which several enzymatic steps are involved (Figure 1 shows a simplified scheme of the pathway). The first step is the formation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) from acetyl-CoA and acetoacetyl-CoA, catalyzed by the enzyme HMG-CoA synthase. HMG-CoA is then converted to mevalonate by HMG-CoA-reductase, which represents the rate-controlling step. Mevalonate is converted to isopentenyl-5-pyrophosphate, condensated to squalene and further metabolized to lanosterol. Conversion of lanosterol into cholesterol takes another 19 enzymatic reactions. The importance of the cholesterol synthesis pathway is evident from the profound effects of several inborn errors in cholesterol synthesis. As most inborn errors in cholesterol synthesis are very rare, evidence for the treatment of these inborn errors is limited.

Bile acid metabolism

Bile acids are synthesized from cholesterol in the liver exclusively. The enzymatic conversion of cholesterol can occur via two pathways (Fig. 2). In the neutral or classical pathway, the steroid nucleus is modified by hydroxylation reactions prior to the side-chain shortening. Cholesterol 7α-hydroxylase (CYP7A1) catalyzes conversion of cholesterol into 7α-hydroxycholesterol. This step is considered to be rate-controlling in bile acid synthesis. In the acidic pathway, the first step involves 27-hydroxylation of the cholesterol side-chain (to yield 27-hydroxycholesterol), catalyzed by the enzyme sterol-27-hydroxylase (CYP27A1). Finally, C27-3α-steroid dehydrogenase converts the 7α-hydroxycholesterol formed via either pathway into the two primary bile acids: cholic acid (CA) and chenodeoxycholic acid (CDCA). After their synthesis, primary bile acids are conjugated with glycine or taurine and secreted via the bile into the intestinal lumen, where they facilitate the uptake of dietary fat and other

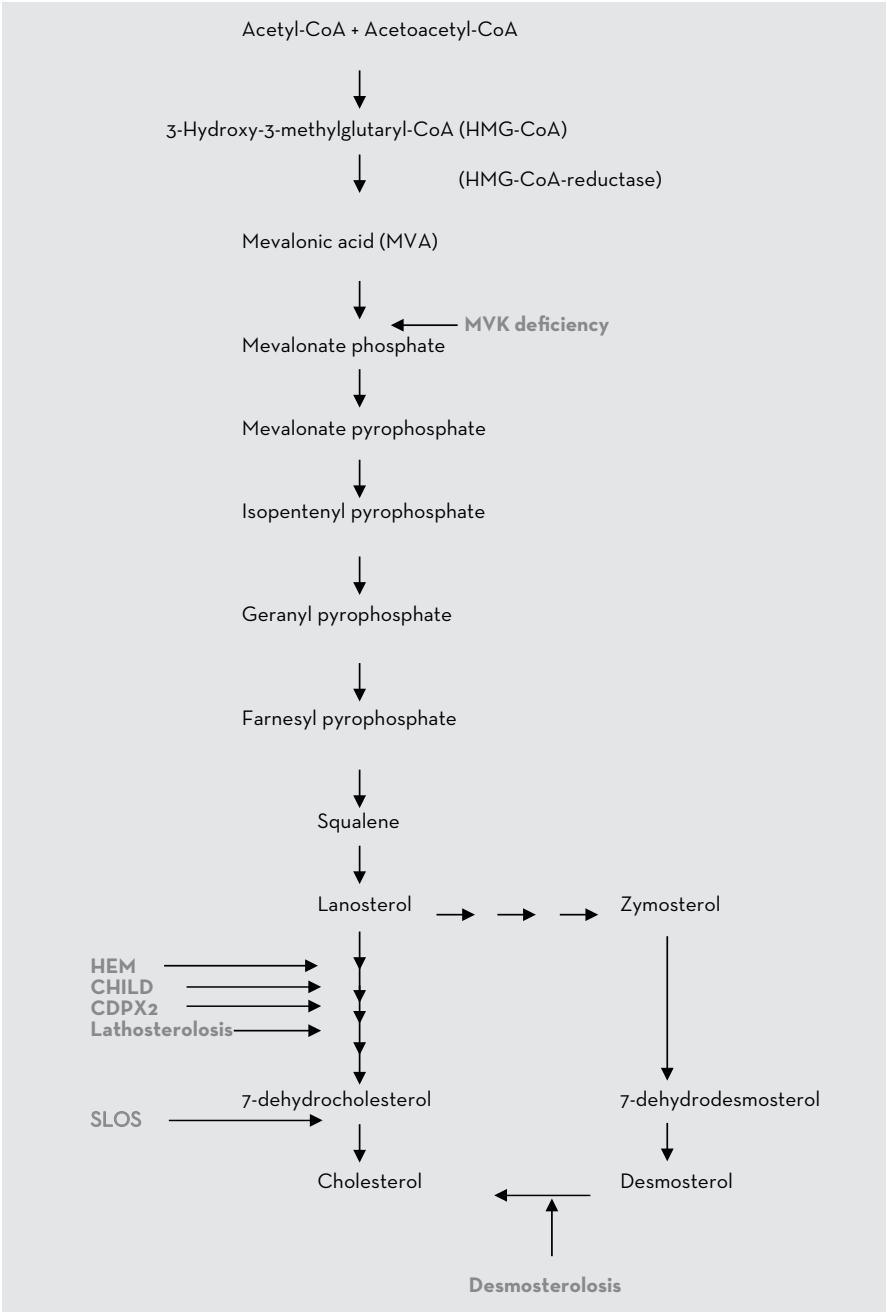

Figure 1. Simplified scheme of the cholesterol biosynthesis pathway. The described inborn errors in cholesterol biosynthesis are depicted in grey. *Adapted from Herman GE. Human Molecular Genetics. 2003;12:R75-R88*

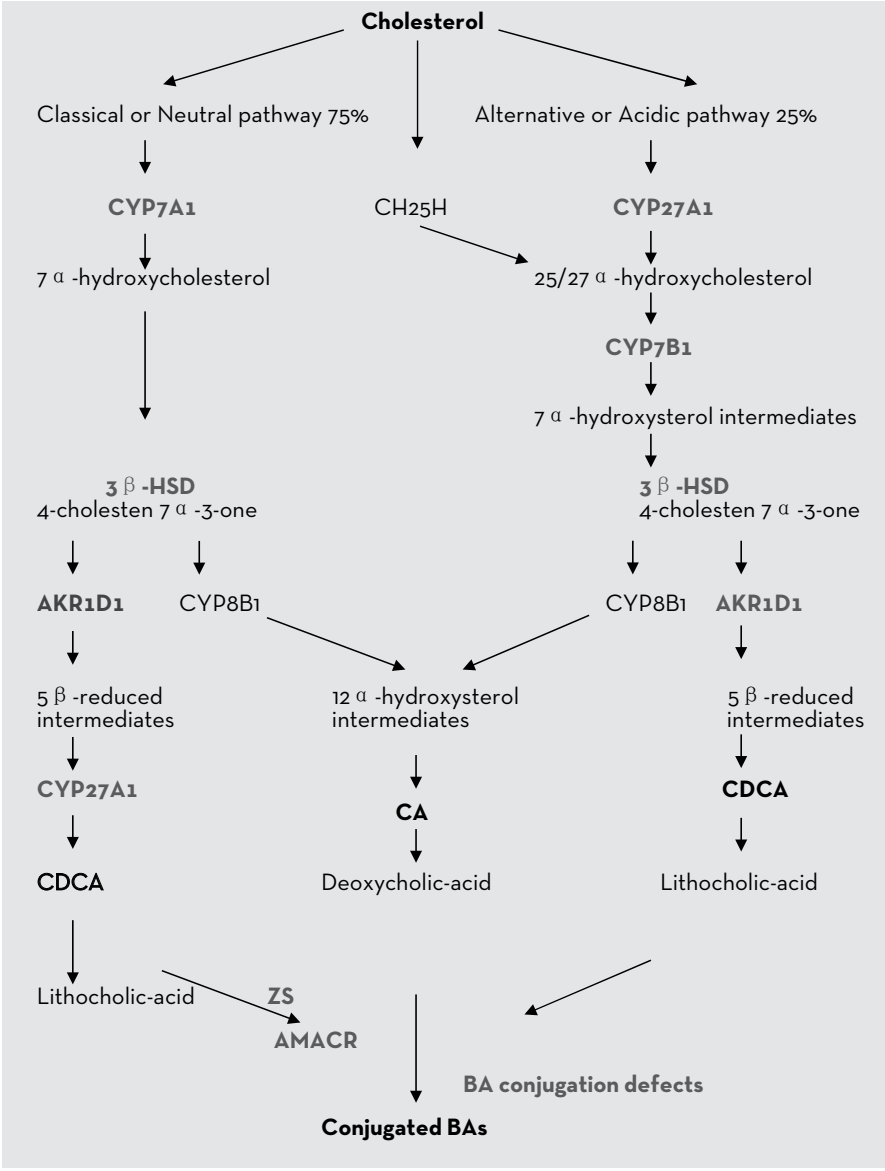

Figure 2. Pathway for the biosynthesis of the primary bile acids. Inborn errors of bile acid synthesis are depicted in bold. CYP7A1, cholesterol 7α-hydroxylase; CYP27A1, sterol 27-hydroxylase; CYP7B1, oxysterol 7α-hydroxylase; 3β-HSD, 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase; CYP8B1, Sterol 12-alpha-hydroxylase; AKR1D1, 5βRD: 3-oxo-Δ^4-steroid 5β-reductase; CH25H, cholesterol 25-hydroxylase. *Adapted from Lefebvre P et al. Role of bile acids and bile Acid receptors in metabolic regulation. Physiol Rev. 2009; 89(1):147-91, Thomas C et al.Targeting bile-acid signalling for metabolic diseases. Nat Rev Drug Discov. 2008;7(8):678-93. Liver disease in Children,3rd edition ,Edited by FJ Suchy RJ Sokol WF. Balistreri, Book chapter 31, Setchell KDR*

hydrophobic compounds. The majority of bile acids are reabsorbed in the ileum, transported via the portal system back to the liver to complete the enterohepatic circulation. A small fraction of the bile salts escapes ileal reabsorption and enters the colon, where they are deconjugated and subsequently dehydroxylated by intestinal bacteria. These unconjugated bile salts can be passively reabsorbed from the colon, which results in the presence of so-called secondary and tertiary bile acids in the enterohepatic circulation (Fig. 3). Bile acids have several important functions in addition to facilitating the absorption of dietary fat and fat-soluble vitamins. The secretion of bile acids from hepatocytes into the biliary system drives bile formation. Bile acids are essential in cholesterol homeostasis; conversion of cholesterol into bile acids and their subsequent fecal loss provides the major route for removal of excess cholesterol from the body.

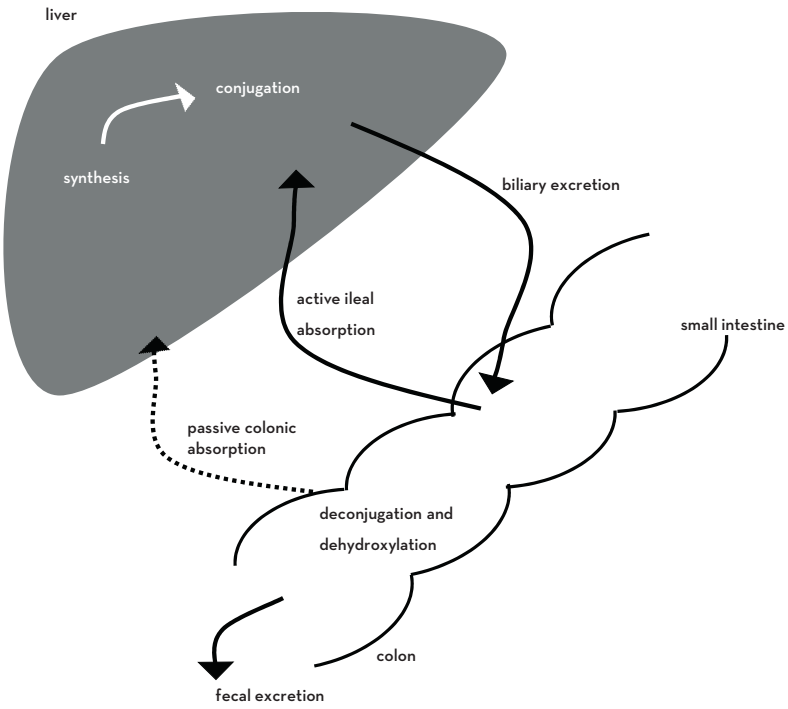

Figure 3. Enterohepatic circulation of bile acids. Bile acids are synthesized from cholesterol in the liver. After synthesis, primary bile acids are conjugated to with glycine or taurine and secreted into the intestinal lumen. The majority of bile acids is reabsorbed in the ileum and transported back to the liver via the portal system. Some bile acids enter the colon where they are deconjugated and dehydroxylated by the intestinal bacterial flora. These secondary and tertiary bile acids can be passively absorbed from the colon and are transported back to the liver via the enterohepatic circulation. The fecal excretion of bile is a route for removal of excess cholesterol from the body.

INBORN ERRORS OF CHOLESTEROL BIOSYNTHESIS

Mevalonic aciduria (MA) and hyperIgD and periodic fever syndrome (HIDS)

MA and HIDS are disorders caused by deficiency of mevalonate kinase (MVK). MA is a severe and often fatal disease presenting with a variety of symptoms (dysmorphic features, cataracts, hypotonia, developmental delay, ataxia and cerebellar atrophy, failure to thrive, recurrent febrile illnesses with enteropathy, hepatosplenomegaly, lymphadenopathy, arthralgia, oedema and rashes) (2). HIDS is a less severe condition, characterized by recurrent fever episodes associated with lymphadenopathy, arthralgia, gastrointestinal problems and rash, but without neurological symptoms or dysmorphic features (3). MA and HIDS are rare diseases with about 30 and 180 patients respectively described worldwide (4). Since identification of the affected gene in 1992 (5), several disease causing mutations have been reported. Diagnosis is based on detection of elevated levels of mevalonic acid in urine or plasma. In HIDS, however, this may not always be conclusive. MVK activity is usually below detection levels in MA patients, whereas some residual MVK activity is present in HIDS. Plasma levels of cholesterol are usually in the lower range of normal (6). In HIDS, serum IgD and IgA are frequently elevated. The diagnosis of MA and HIDS is confirmed by demonstrating mutations in the gene. The pathogenesis of both diseases is still unclear, either accumulation of mevalonic acid or deficiency of cholesterol and intermediates have been postulated to play a role.

Several attempted therapeutic strategies have not been successful in treating MA. Oral cholesterol supplementation worsened diarrhoea (6). Antioxidant treatment with ubiquinone combined with vitamin C and E to treat a postulated increased sensitivity to reactive oxygen stress, appeared to stabilize clinical symptoms in a few patients (7). However, a combination of cholesterol, ursodeoxycholic acid (UDCA), ubiquinone and vitamin E did not lead to clinical improvement (6). Treatment in 2 patients with lovastatin to block mevalonic acid production resulted in the development of severe clinical crisis (hyperthermia, vomiting, diarrhoea and marked elevation of serum creatinin kinase levels) (6). Intervention with prednisone (2 mg/kg/d) was effective during clinical crisis in 2 patients (6). One MA patient with associated nephritis was successfully treated with anakinra (an interleukin-I-receptor antagonist) (8). Remission (during a 15-month follow-up period) of febrile attacks and inflammation after allogenic bone marrow transplantation was described in a three year old patient (9).

The prognosis of HIDS is benign with complications (amyloidosis) being rare. Various pharmacological agents have been tried in the treatment of febrile episodes and associated symptoms in HIDS. Anti-inflammatory drugs (colchicine, thalidomide, cyclosporine and intravenous immunoglobulin) did not consistently suppress or shorten febrile episodes (3, 10, 11). Individual patients responded to prednisone (3, 10) or non-steroidal anti-inflammatory drugs (NSAIDs) (12). A randomized, double blind, crossover study with simvastatin in 6 patients, did not significantly affect duration or frequency of febrile illnesses (13). As plasma levels of TNFα and interleukin (IL)-1β are elevated during febrile attacks, anticytokine therapy has been attempted. Case reports described a limited effect of antitumor necrosis factor (TNF)

treatment with etanercept (14,15). Anakinra shortened frequency and duration of febrile attacks in sporadic cases (3,15). Studies have been performed in small, heterogeneous groups and sofar; there is no consensus on treatment of HIDS.
Concluding, for patients with MA, intervention with prednisone or with anakinra may be successful (III/C). The use of statins is not recommended in MA patients (III/C). In the treatment of HIDS, thalidomide (II/B), cholchicine (II/B) and statins (I/B) are not recommended whereas symptomatic treatment of fever and pain with prednisone or with NSAIDs may be successful (II/C). Anticytokine therapy with etanercept or anakinra may be successful in HIDS patients (III/C).

Smith-Lemli-Opitz syndrome (SLOS)

SLOS was first described in 1964 as an autosomal recessive malformation syndrome with microcephaly, dysmorphic facial features, genital abnormalities, limb defects and mental retardation (16). Decades later, the underlying biochemical cause was identified (17). Deficiency of 7-dehydrocholesterol-Δ^7 reductase (7-DHCR) impairs normal cholesterol synthesis leading to hypocholesterolemia and accumulation of the precursors 7-dehydrocholesterol (7-DHC) and 8-dehydrocholesterol (8-DHC). The gene DHCR7, defective in SLOS, was identified in 1998 (18) and since then over 100 different mutations have been reported. SLOS is characterized by a broad spectrum of malformations in various organ systems (reviewed in (19). Its incidence is estimated between 1:20.000 - 1:70.000 births (19) The disruption of normal development and growth is classically attributed to the deficiency of cholesterol or to the accumulation and deposition of the (potentially toxic) precursors. SLOS is diagnosed by the determination of elevated concentrations of 7DHC and 8DHC in plasma. DHC7 mutation analysis can be performed to confirm the diagnosis. Cholesterol concentrations in plasma are low or normal (19). Prenatal diagnosis can be performed by measuring elevated levels of 7-DHC concentrations in amniotic fluid or chorionic villus biopsies (20).
No pharmacological strategy has been proven effective in curing SLOS. Dietary supplementation with cholesterol seems a logical treatment because it could be expected to increase plasma and tissue cholesterol levels and to down-regulate its synthesis and the levels of, potentially toxic, precursors. Unfortunately, because brain cholesterol in mammals is dependent on *de novo* synthesis and plasma cholesterol does not efficiently cross the blood brain barrier, the effect of cholesterol supplementation on brain function is expected to be limited. Furthermore, prior developmental defects cannot be reversed by cholesterol treatment. Cholesterol supplementation (administered as natural products such as egg yolk, meat based formulas and liver or crystalline cholesterol in oil or aqueous suspension) has been evaluated in several trials. Doses of cholesterol used ranged between about 20-150 mg/kg/d (21-23). Cholesterol supplementation improved the serum ratio of cholesterol to total sterols and decreased the level of 7DHC (24). Some observational studies show improvement in growth, behaviour, gastrointestinal symptoms and photosensitivity (21-23). However, these studies were conducted in small and heterogeneous patient groups and the described improvements have not been correlated to the biochemical changes. Cholesterol treatment

Table 1. Features of inborn errors in cholesterol biosynthesis. *(a)* reviewed in Porter F *et al.* human malformation syndromes due to inborn errors of cholesterol synthesis. Curr Opin Pediatr 2003 Dec;15(6):607-13.

	MA	**HIDS**	**SLOS**	**Desmosterolosis**
Deficient enzyme	mevalonate kinase (EC 2.7.1.36)	mevalonate kinase (EC 2.7.1.36)	7-dehydrocholesterol Δ^7-reductase (EC 1.3.1.21)	3β-hydroxysterol Δ^{24}-reductase (EC 1.3.1.72)
Gene	MVK	MVK	DHCR7	DHCR24
Clinical features	Dysmorphic features Cataracts Hypotonia Developmental delay Ataxia Cerebellar atrophy Failure to thrive Recurrent febrile episodes with: -lymphadenopathy -enteropathy -hepatosplenomegaly -arthralgia -edema -rashes Often lethal	Recurrent febrile episodes with: -fever -lymphadenopathy -vomiting -diarrhoea -abdominal pain -rash -arthralgia -splenomegaly	Multiple congenital malformations in various organ systems	Multiple congenital malformations in various organ systems
Laboratory features	↑ mevalonic acid in urine ↑ mevalonic acid in plasma	During attacks: ↑ ESR and leukocytosis ↑ urinary concentration mevalonic acid Constantly present: ↑ serum IgD ↑ serum IgA	↑ 7-DHC/8-DHC Low/normal plasma cholesterol	↑ plasma desmosterol ↑ tissue desmosterol
Inheritence	Autosomal recessive	Autosomal recessive	Autosomal recessive	Suggested autosomal recessive
Prevalence	± 30 cases	± 180 cases	1:20.000-70.000	2 cases
Animal model		Mvk +/- mouse	Dhcr7 -/- mouse *(a)*	DHCR24 -/- mouse *(a)*

	Conradi-Hunerman (CDPX2)	CHILD	HEM
	3β-hydroxysteroid-Δ,^{8}Δ^{7}-isomerase (EC 5.3.3.5)	3β-hydroxysteroid dehydrogenase (EC 1.1.1.170)	3β-hydroxysterol Δ^{14}-reductase (EC 1.3.1.70)
	EBP	NSDHL	LBR gene
	At birth: -ichthyosiform erythoderma -hyperkeratotic rahs resolving after a few months Ichtyosis Patchy alopecia Chondroplasia punctata Asymmetrical rhizomelic limb shortening Scoliosis Cataracts	Unilateral ichthyosiform skin lesions Unilateral limb reduction No cataracts Alopecia	*In utero* lethality Fetal hydrops Short-limbed dwarfism Disorganisation of chondro-osseous calcification
	↑ 8-DHC ↑ 8(9)-cholestenol in plasma and tissues	↑ C4-methylated and ↑ C4-carboxy sterol intermediates in plasma and tissue	↑ cholesta-8,14-dien-3ß-ol ↑ cholesta-8,14,24-trien-33ß-ol in tissue
	X-linked dominant	X-linked dominant	Autosomal recessive
	± 100 cases	± 30 cases	± 10 cases
	Tattered (Td) mouse *(α)*	Bare patches (Bpa) and striated (Str) mouse *(α)*	Ichthyosis mouse *(α)*

does not influence the developmental progress in children and adults with SLOS (24). Bile acids including CDCA and UDCA added to cholesterol treatment in some studies did not provide benefit in treatment (22, 23). Treatment of SLOS with simvastatin, an inhibitor of cholesterol synthesis resulted in inconclusive effects. Adverse effects in two patients are reported (25), whereas simvastatin treatment in two other patients was well tolerated and lowered plasma 7DHC and 8DHC. In these patients, improvement of anthropometric measurements was suggested (26). Haas *et al* compared cholesterol monotherapy with cholesterol and simvastatine therapy in a group of 39 SLOS patients. Positive effects of simvastatin on anthropometric measurements could not be confirmed, On the other hand, a negative effect of simvastatin on physical development and behavioural problems could not be excluded (27). In summary, oral cholesterol supplementation may have a positive effect on photosensitivity, behaviour and growth in SLOS patients (II/B).

Desmosterolosis

Desmosterolosis is caused by deficiency of 3β-hydroxysterol Δ^{24}-reductase, which catalyses the reduction of the Δ^{24} double bond of sterol intermediates (such as desmosterol) in the cholesterol synthetic pathway. Two patients have been reported so far. The first was a premature infant with multiple lethal malformations and generalized accumulation of desmosterol. Malformations consisted of macrocephaly, a hypoplastic nasal bridge, cleft palate, total anomalous venous drainage, clitoromegaly, short limbs and generalized osteosclerosis (28). The second patient showed a less severe phenotype at the age of four years. His clinical presentation included microcephaly, agenesis of the corpus callosum, dysmorphic facies, submucous cleft palate, persistent patent ductus arteriosus, limb abnormalities and developmental retardation (29). Desmosterol was elevated in plasma and in cultured lymphoblasts. Parents of both cases had mildly elevated levels of plasma desmosterol suggesting autosomal recessive inheritance. Homozygous mutations in the DHCR24 gene were demonstrated in both cases (30). No treatment is currently available.

X-linked dominant chondrodysplasia punctata (Conradi-hunermann syndrome or Happle syndrome) and Congenital Hemidysplasia with Ichthyosiform nevus and Limb Defects (CHILD)

Chondrodysplasia punctata is a term used for a heterogeneous group of genetic disorders characterized by abnormal foci of calcification in the cartilaginous skeleton (epiphysial stippling). X-linked dominant chondrodysplasia punctata (CDPX2) is a rare disorder consisting of skeletal, cutanous and ocular malformations (reviewed in (31). CPDX2 almost exclusively affects females although a few affected males have been reported (31). Cutaneous manifestations contain ichtyosis, patchy alopecia and atrophoderma. Skeletal abnormalities include epiphysial stippling, asymmetric rhizomelia and scoliosis. Ocular malformations are cataracts, microphtalmus, nystagmus, glaucoma and optic nerve atrophy. Intelligence is usually normal. Other abnormalities (dysmorphic facies, and renal or cardiac manifestations) have been described. CDPX2 is caused by deficiency of 3β-hydroxysteroid-Δ8-Δ7-isomerase which cataly-

ses a step in the conversion of lanosterol to cholesterol. The enzyme defect leads to accumulation of 8-dehydrocholesterol and 8(9)-cholestenol in plasma and tissues. Mutations in the gene encoding the emopamil-binding protein, which functions as a 3β-hydroxysteroid-Δ8-Δ7-isomerase, have been identified as the underlying genetic defect and to date at least 46 different mutations have been described (32). The pathogenesis of CDPX2 remains to be investigated.

CHILD syndrome is an x-linked disorder with phenotypical similarities to CPDX2, but with a remarkable unilateral distribution of anomalies. Unilateral skin lesions, characteristically a large epidermal plaque or nevus, are usually present at birth and persist throughout life. Unilateral hypoplasia of limbs and internal organs located at the same side as the skin lesion can be present. Cataracts are not reported in CHILD syndrome and the skeletal malformations are more severe compared to those in CPDX2, reviewed in (31, 33). Approximately 30 patients have been reported amongst which 2 males. The defective gene underlying CHILD syndrome has been identified to be NSDHL, encoding the enzyme 3β-hydroxysteroid dehydrogenase (34). Treatment of CDPX2 and CHILD syndrome is symptomatic.

Hydrops-Ectopic Calcification-Moth-Eaten Skeletal Dysplasia (HEM or Greenberg dysplasia)

HEM is a rare, *in utero* lethal, skeletal dysplasia characterized by polyhydramnios, hydrops, dysmorphic facies and short-limbed dwarfism. About 10 cases have been described in the literature.

HEM is caused by a deficiency of the enzyme 3β-hydrosterol Δ^{14}-reductase caused by mutations of the LBR gene encoding the lamin B receptor (35).

Table 2. Recommendations for the treatment of inborn errors in cholesterol biosynthesis

	Recommendations	**Level of evidence**
MA	prednisone anakinra bone marrow transplant statins can be dangerous in MA	III/C 1 case described 1 case described III/C
HIDS	symptomatic treatment of fever and pain: - NSAIDs - prednison anticytokine therapy: - etanercept - anakinra thalidomide and colchicine are not recommended statins are not recommended	 II/C II/C III/C III/C II/B 1/B
SLOS	oral cholesterol supplementation (20-150 mg/kg/d) may have a positive effect on photosensitivity, behaviour and growth	II/B
Desmosterolosis	no treatment available	
Conradi-Hunerman	symptomatic treatment	
CHILD	symptomatic treatment	
HEM	no treatment available	

INBORN ERRORS IN BILE ACID SYNTHESIS

The conversion of cholesterol into the primary bile acids CA and CDCA is disturbed in a number of inborn bile acid synthesis defects (BASD). BASD are rare, with an estimated incidence of 1-2% of childhood cholestasic diseases (36). BASD can lead to cholestasis and impaired hepatic function based on accumulation of (toxic) intermediates, unusual bile acids and bile alcohols. The lack of competent bile acids in the intestine will frequently cause malabsorption of dietary fat and fat-soluble vitamins., The recognition of BASD was accelerated after the implementation of an international screening program for genetic causes of cholestatic liver disease at the Cincinnati Children's Hospital Medical Center in 2000 (37). Identification of BASD relies on fast atom bombardment ionisation-mass spectrometry (FAB-MS) or liquid secondary ion mass spectrometry (LSI-MS) of urine and serum to measure absence or reduction of the normal primary bile acids and accumulation of atypical bile acids or bile alcohols (37). Sequencing of genomic DNA has led to identification of responsible genes in most of the cases. Three clinical symptoms should lead to suspicion of a BASD in patients with cholestatic liver disease. First, most BASD are associated with normal to low serum bile acid concentrations, as determined by the conventional laboratory methodologies based on availability of substrates for 3 alpha steroid dehydrogenase, in contrast to elevated bile salt concentrations in other cholestatic liver diseases. Second, serum γ glutamyl transpeptidase (GGT) is usually normal in BASD compared to most other other cholestatic liver diseases (except for Progressive Familial Intrahepatic Cholestasis type 1 or type 2). Third, pruritis, which is common in cholestatic liver disease, is usually absent in BASD (38). The clinical picture of BSAD depends on the specific defect. In general, defects in modification of the sterol nucleus frequently present with neonatal progressive cholestatic liver disease whereas defects in modification of the side chain cause more neurological symptoms and less severe liver disease. Early recognition of BASD is essential because several of these conditions are treatable with oral bile acid supplementation. General features of inborn errors in bile acid synthesis are depicted in Table 3. Key points and recommendations in the treatment of inborn errors in bile acid synthesis are displayed in Table 4. Due to their low incidence, there is little evidence for therapeutic interventions for inborn errors in bile acid synthesis.

DEFECTS INVOLVING MODIFICATION OF STEROL NUCLEUS

Cholesterol 7α-hydroxylase deficiency (CYP7A1)

The most recently described disorder of modification of the sterol nucleus is cholesterol 7α- hydroxylase deficiency, in which bile acid synthesis via the 'classical pathway' is

Table 3. Features of inborn errors in bile acid synthesis. Adapted from Sundaram *et al* (8)

Deficient enzyme	Gene	Clinical features
Cholesterol 7α-hydroxylase (EC 1.14.13.17)	CYP7A1	Adult patients: ↑ serum LDL levels, resistant to statin therapy Hypertriglyceridemia Cholesterol gall stones
Oxysterol 7α-hydroxylase (EC 1.14.13.100)	CYP7B1	Neonatal cholestasis Rapid progression to cirrhosis
3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase (EC 1.1.1.145)	3β-HSD	Cholestasis in children at any age
3-oxo-Δ^4-steroid 5β-reductase (EC 1.3.1.3)	AKR1D1	Neonatal cholestasis Rapid progression to cirrhosis GGT can be elevated
Sterol 27-hydroxylase (EC 1.14.13.15)	CYP27A1	Adults (20-30 years) - neurological dysfunction - dementia - cerebellar ataxia - cataracts - premature atherosclerosis - xanthomas Infancy -juvenile cataracts -chronic diarrhoea -neonatal cholestasis
Alpha-Methylacyl-CoA Racemase (EC 5.1.99.4)	AMACR	Adult onset peripheral neuropathy Neonatal cholestasis
Zellweger syndrome	PEX(1-12)	Malformations in various organ systems Frequently lethal within 2 years of age
Bile acid conjugation defects (EC 2.3.1.65)	BAAT	Fat-solube vitamin deficiency (Transient) neonatal or childhood cholestasis

	BA profile (urine)	*BA profile (serum)*	*Prevalence*	*Animal model*
			< 10 cases	Cyp7a1 -/- mouse (1)
	Absent primary BA ↑ sulphate/glycosulfate conjugates of 3β-δ5-monohydroxy BA	↑↑ BA level (3β-δ5-monohydroxy BA)	2 cases	Cyp7b1 -/- mouse (2)
	↓ primary BA ↑ dihydroxy and trihydroxy-cholenoic acids	↓ or absent primary BA	± 40-50 cases	-
	↓ primary BA ↑ 3-oxo-4 bile acids ↑ allo BA	↓ primary BA ↑ 3-oxo-4 bile acids ↑ allo BA	± 5-10 cases	-
	↑ bile alcohol glucuronides	↑ bile alcohol glucuronides ↑ cholestanol/cholesterol ratio	Many cases	CYP27 -/- mouse (3)
	↓ primary BA ↑ C-27 trihydroxycholestanoic acid ↑ pristanic acid	↓ primary BA ↑ C-27 trihydroxycholestanoic acid ↑ pristanic acid	± 5 cases	AMACR -/- mouse (4)
	↓ primary BA (a) Atypical mono-,di-, and trihydroxy C-27 BA (a)	↓ primary BA (a) ↑ long-chain fatty acids (a) ↑ cholestanoic acid (a) ↑ pipecolic acid (a) ↑ C29 dicarboxylic acid (a)	Many cases	Several PEX knock-out mice (5-7)
	Unconjugated CA	Onconjugated CA and CDCA	± 5 cases	-

Tabel 4. Key points and recommendations for the treatment of inborn errors in bile acid biosynthesis

	Recommendations and key points	**Level of evidence**
General	BASD present most frequently as neonatal cholestasis Presentation as chronic liver disease in older patients occurs In contrast to other cholestatic liver diseases, patients with BASD commonly present with: - normal serum GGT - low serum bile acid levels - pruritus is usually absent Suspicion of, and early diagnosis of BASD is important, as subsequent early treatment with oral bile acid therapy can prevent progressive cholestatic liver disease.	
Oxysterol 7α-hydroxylase deficiency	OLT was performed in 1 case	-
3β-hydroxy-Δ^5-C_{27}-steroid Dehydrogenase deficiency	Oral CDCA or CA and CDCA combined UDCA may be of temporary benefit	III/B
3-oxo-Δ^4-steroid 5β-reductase deficiency	Oral CDCA combined with CA (8 mg/kg/d) Caution with the use of CDCA in patients with normal/high serum concentrations of CDCA Oral UDCA insufficient as sole therapy	III/B
CTX	children: oral CDCA 15 mg/kg/d adults: oral CDCA 250 mg/d Treatment should be started before the onset of neurological symptoms.	I/B
AMACR	Oral CA and fat-soluble vitamins	III/B
Zellweger syndrome	Treatment is supportive	
Bile acid conjugation defects	Suggested therapy is oral primary BA and fat-soluble vitamins	-

decreased. A prominent feature in adult homozygous patients is an elevated serum LDL level, resistant to statin therapy, elevated serum triglycerides, and decreased (fecal) bile acid excretion Patients may present with cholesterol gallstones, but liver disease is not observed. Heterozygous subjects presented with milder dyslipidaemia (39).

Oxysterol 7α-hydroxylase deficiency (CYP7B1)

Deficiency of the oxysterol 7α-hydroxylase, results in a defective conversion of 27-hydroxy-cholesterol to 7α, 27-dihydroxy-cholesterol. Only two cases of oxysterol 7α-hydroxylase deficiency have been described so far (40, 41). The first child presented with cholestasis, elevated transaminases and normal GGT at the age of six weeks. Hepatic failure with clotting anomalies was present. The serum concentration of total bile acids was low and GC-MS analysis revealed the presence of 3-β-hydroxy-5-choleonic and 3-β-hydroxy-5-cholestenoic acids. Serum analysis showed high levels of 27-hydroxy-cholesterol, while 7α-hydroxylated sterols were absent, suggesting a defect at the level of oxysterol 7α-hydroxylase. Genetic analysis revealed a homozygous mutation of CYP7B1. Liver biopsy findings consisted of cholestasis, bridging fibrosis, giant cell transformation and proliferation of bile ductules. Treatment with oral UDCA worsened liver function tests and the child was ineffectively treated with oral CA, after which he was subjected to orthotropic liver transplantation (OLT). The second case, a Taiwanese infant of non-consanguineous parents presented with progressive jaundice at the age of 5 months. Laboratory investigations showed elevated transaminases, a normal GGT and conjugated hyperbilirubinaemia. Mass spectrometry in urine revealed large amounts of sulfate or glycosulfate conjugates of monohydroxycholeonic acids, while "normal bile" acids were absent. In serum, over 90% of bile acids consisted of 3β-hydroxy-5-cholenoic and 3β-hydroxy-5-cholestanoic acid. Liver biopsy findings were consistent with cirrhosis and bile duct proliferation accompanied by giant cell transformation. Genetic analysis showed a homozygous mutation in CYP7B1. Treatment with oral UDCA did not improve liver function or cholestasis. CDCA and CA could not be administered because of non-availability in Taiwan. The patient was referred for OLT, but died of cholestatic liver failure before OLT could be performed.

3β-hydroxy-Δ^5-C_{27}-Steroid Dehydrogenase deficiency (3β-HSD deficiency)

The second step in the bile acid synthesis is catalyzed by 3β-hydroxy-C27-steroid dehydrogenase. Deficiency of this enzyme leads to accumulation of 7α-hydroxycholesterol in hepatocytes. As side chain oxidation and hydroxylation of 7α-hydroxycholesterol by other enzymes progresses, 3β, 7α-dihydroxy-5-choleonic acid and 3β, 7α, 12α -trihydroxy-5-choleonic acid will be formed. These non-functional bile acids undergo sulphation, and are found in high concentrations in urine and plasma (42). Diagnosis of 3β-HSD-deficiency includes demonstration of abnormal bile acids in urine or plasma using mass spectrometry. 3β-HSD expression in fibroblasts is absent in affected subjects (43). Several disease-causing mutations have been identified (44). 3β-HSD deficiency is a rare autosomal recessive disease with a broad clinical spectrum. Clinical manifestations in children may begin at any age and include cholestasis

with jaundice, fat-soluble vitamin deficiency and steatorrhea. Serum transaminases and serum bilirubin are elevated, but the serum GGT is usually normal (42;45). In 1999, Akobeng reported two patients with 3β-HSD deficiency, who presented with rickets at the ages of seven and nine months, but did not develop clinical signs of liver disease until the age of three years (45). Cholestasis has been identified in adult patients as well, the oldest case being 26 years at the time of diagnosis (46). The late onset cholestasis is usually preceded by a transient elevation of serum transaminases in the neonatal period. Histopathological findings in the liver, ranging from giant cell hepatitis to cirrhosis, depend on the age of the patient and on the rate of progression of liver disease. The therapeutic approach includes oral administration of primary bile acids. The rationale behind primary bile acid therapy is that these primary bile acids provide bile acid-dependent bile flow, counteracting cholestasis, as CYP7A1 is suppressed by cholic acid (CA) and chenodeoxycholic acid (CDCA), thereby reducing the production of the abnormal bile acids. Treatment with oral CDCA (doses ranging from 9-18 mg/kg/d) resulted in marked clinical, biochemical and histological improvement (45, 47). Kobayashi showed that combined therapy with CDCA and CA was more effective than CDCA alone in the treatment of a 23 year old woman with 3β HSD deficiency (48). Treatment with oral ursodeochycholic acid (UDCA) may be of temporary benefit (49) but its long term effect will be limited, because UDCA is not capable of suppressing bile acid synthesis (42).
Thus, oral supplementation of CDCA results in improvement of clinical, biochemical and histological features (III/B). Oral supplementation of CDCA combined with CA was more effective than CDCA treatment alone, in an adult patient (III/B).

3-oxo-Δ^4-steroid 5β-reductase deficiency (5βRD)

3-oxo-Δ^4-steroid 5β-reductase deficiency is an autosomal recessive disease caused by mutations in the gene AKR1D1 (previously known as SRD5B1). 5 βRD was first described by Setchell et al. in identical twins suffering from neonatal hepatitis and progressive cholestasis (50). Deficiency of 5βRD impairs the reduction of the double bond between C4 and C5 of the sterol nucleus, and the conversion of 3-oxo intermediates to their corresponding 3α-hydroxylated products. Urine and plasma levels of primary bile acids are low and intermediate bile acid synthesis products proximal to the enzyme defect (3-oxo-4 bile acids) accumulate, and are detectable by mass spectrometry (36). However, hyper-3-oxo-Δ^4 bile aciduria is also seen in children with cholestatic liver disease other than inborn errors in bile acid synthesis (e.g. tyrosinemia, α-1-antitrypsin deficiency), rendering it difficult to distinguish between primary 5 βRD-deficiency and secondary inhibition of the enzyme by liver damage (51). As 3-oxo-Δ4-steroid 5β-reductase is not expressed in fibroblasts or leukocytes, cell based enzyme activity studies are not available. Definite diagnosis of 3-oxo-Δ^4-steroid 5β-reductase deficiency depends on demonstrating mutations in AKR1D1 (52, 53). Approximately 10 patients with 5βRD have been described (41, 54). The clinical picture resembles that of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase deficiency; neonatal cholestasis with elevated plasma concentrations of aminotransferases and conjugated bilirubin. Yet GGT can be elevated in 5βRD. Malabsorption of the fat-soluble vitamin

D may cause rickets. Infants with 5βRD tend to have more severe liver disease at an earlier age compared to 3βHSDH. Hepatic histopathology is typical of neonatal hepatitis, with giant cell hepatitis, pseudoacinar transformation, hepatocellular and canalicular cholestasis and extramedullary haematopoiesis (36). Liver failure will progress rapidly, and without treatment the mortality rate is about 50% (36).
Oral administration of CDCA and CA (both at a dose of 8 mg/kg/d) improved liver disease, both clinically and biochemically (53, 55). Caution with the use of CDCA in patients with normal/high serum concentrations of CDCA should be urged, because CDCA is potentially hepatotoxic (55). The choleretic bile acid UDCA is frequently used in pediatric cholestatic diseases. In 5βRD, UDCA is insufficient as sole therapy, probably because UDCA does not exert a strong negative feedback on bile acid synthesis via CYP7A1 (55). Concluding, oral supplementation of CDCA combined with CA improved clinical, biochemical and histological features (III/B).

DEFECTS IN SIDE CHAIN MODIFICATION

Cerebrotendinous xanthomatosis (CTX)

CTX is a rare autosomal recessive lipid storage disease caused by the deficiency of the mitochondrial enzyme sterol 27-hydroxylase, which results in impaired side chain modification during bile acid synthesis. The intermediates 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α-diol are not hydroxylated at the c-27 position and therefore side-chain oxidation to the usual C24-primary bile acids cannot take place. These intermediates are hydroxylated at multiple sites to form bile alcohols which become glucuronidated. The production of primary bile acids, in particular CDCA, is reduced while bile alcohol glucuronides (which are diagnostic) accumulate. Some CA can still be synthesized via an alternative C-25 hydroxylation pathway. The absent negative feedback of primary bile acids on CYP7A1 leads to the accumulation of cholestanol which results in its deposition in various tissues. The molecular defect of CTX was identified to reside in the sterol 27-hydroxylase gene (CYP27A1) (56) and over 50 different mutations have been described (57).
Plasma concentrations of cholestanol are increased, whereas cholesterol concentrations are normal. Large amounts of bile alcohol glucuronides are excreted in bile, faeces and urine. Patients usually present in the second or third decade of life with various symptoms, including progressive neurological dysfunction, dementia, cerebellar ataxia, cataracts, premature atherosclerosis and the presence of xanthomas in tendons and brain (57). Chronic diarrhoea and juvenile cataracts may precede the onset of neurological symptoms, and a few paediatric cases have also been reported (58). Although liver disease is not observed in CTX, neonatal cholestasis due to mutations in the CYP27A gene has been described (59). Diagnosis is based on an increased plasma cholestanol/cholesterol ratio and the detection of increased levels of bile alcohol glucuronides in urine. Definitive conformation of the diagnosis is achieved by analysis of the CYP27A1 gene. For the treatment of CTX, several approaches have

been tried of which oral administration of CDCA was most effective (60). Berginer *et al.* showed clinical and biochemical improvement in 17 adult CTX patients, upon administration of CDCA (750 mg/d) for at least one year (61). Comparable results were found after treatment of paediatric CTX patients with CDCA (15 mg/kg). (58) Clinical improvement was most notable if CDCA treatment was initiated before the onset of significant (neurological) symptoms (62). UDCA is not effective in treatment of CTX (60, 63). Statins have been studied as a treatment regime for CTX. Even though data are limited, there is no evidence supporting statin monotherapy as treatment for CTX (63). Statins combined with CDCA treatment showed additional reduction of cholestanon in one patient (64). The addition of simvastatin (10-40 mg/d) to CDCA did lower serum levels of cholestanol in seven patients, however, additional clinical improvement was not objectified (65). In summary, oral supplementation of CDCA (15 mg/kg/d in children and 250 mg/d in adults) improved clinical and biochemical features in CTX patients (I/B). Treatment should be initiated before the onset of neurological symptoms.

Alpha-Methylacyl-CoA Racemase (AMACR) Deficiency

AMACR deficiency is an autosomal recessive defect impairing the side-chain oxidation. AMACR catalyzes the racemation of trihydroxycholestanoic acid and pristanic acid into their stereo-isomers. This conversion is required for the subsequent peroxisomal β-oxidation of the C27 bile acid side-chain. AMACR deficiency affects both bile acid and fatty acid synthesis, resulting in elevated levels of pristanic acid and trihydroxycholestanoic acid in urine and plasma. Ferdinandusse described two patients presenting with adult-onset periferal neuropathy and one with symptoms resembling Niemann-Pick type C in childhood (66). A two week old infant with fat-soluble vitamin deficiency, cholestasis and hematochezia was described. This infant had a sibling who became a liver transplant donor after death in infancy because of intracranial hemorrhage due to vitamin K deficiency. The transplant recipient was diagnosed with AMACR deficiency. Diagnosis was confirmed with absence of AMACR activity, an abnormal bile acid profile, increased plasma pristanic acid concentrations or mutations in the AMACR gene. The two week old infant and her siblings liver recipient were successfully treated with oral CA and fat-soluble vitamins (67). For the treatment of AMACR deficiency oral supplementation of CA combined with fat-soluble vitamins may improve clinical, biochemical and histological features (III/B).

Zellweger syndrome (ZS or cerebro-hepato-renal syndrome)

The final steps of bile acid synthesis take place in peroxisomes: beta-oxidation of the side-chain of specific bile acid intermediates. ZS is an autosomal recessive disorder in which peroxisomes are absent. Patients have dysmorphic facies, hypotonia, chronic liver disease, psychomotor retardation and renal cystic abnormalities. ZS is frequently lethal within two years of age. Mutations in 12 genes, PEX1 to PEX12, have been identified with PEX1 being the most common affected gene in ZS (reviewed in (68). Amongst biochemical abnormalities, hyperpipecolic acidemia and increased plasma levels of monohydroxy, di-and trihydroxy bile acids with elongated side chains

are found whereas levels of primary bile acids are reduced (68). Other, less severe peroxisomal disorders (Refsum disease and neonatal adrenoleukodystrophy) have been associated with abnormal bile acid synthesis. The treatment of these conditions is supportive.

BILE ACID CONJUGATION DEFECTS

The two enzymes catalysing the final step in bile salt synthesis (conjugation of CA and CDCA to taurine or glycine) are bile acid-CoA ligase enzyme and CoA:amino acid N-acyltransferase (encoded by the genes BAAT and BALT) (69, 70). Unconjugated bile acids are more rapidly absorbed in the intestine. Due to the decreased secretion of conjugated bile acids, less mixed micelles can be formed. The main feature in patients with bile acid conjugation defects is severe malabsorption of fat and fat -soluble vitamins in infancy or childhood. Conjugated hyperbilirubinemia, pruritis and liver failure have also been described. Mass spectrometry analysis on urine, bile and serum reveals absence of glycine- and taurine conjugated bile acids whereas unconjugated CA and glucuronide- and sulphate conjugates are present. (reviewed in Ref. 38). Mutations in the gene BAAT have been found in several Amish patients with this clinical picture (69). Supplementation with oral primary conjugated bile acids and fat soluble vitamins could provide a potential treatment.

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CHAPTER 3

THE EFFECTS OF INTRAUTERINE MALNUTRITION ON MATERNAL-FETAL CHOLESTEROL TRANSPORT AND FETAL LIPID SYNTHESIS IN MICE

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ABSTRACT

Background and aim

Intrauterine malnutrition is associated with increased susceptibility to chronic diseases in adulthood. Growth-restricted infants display a less favorable lipid profile already shortly postnatal. Maternal low protein diet (LPD) during gestation is a well-defined model of fetal programming in rodents and affects lipid metabolism of the offspring. Effects of LPD throughout gestation on physiological relevant parameters of lipid metabolism are unclear. We aimed to determine effects of gestational LPD on maternal-fetal cholesterol fluxes and fetal lipid synthesis in mice.

Methods

Pregnant mice (dams) were fed a control (18% casein) or a low protein diet (9% casein) from E0.5 onward. We quantified maternal-fetal cholesterol transport and maternal cholesterol absorption at E19.5 using stable isotopes. We determined fetal lipid biosynthesis at E19.5, after administration of [1-^{13}C]-acetate from E17.5 onward.

Results

LPD did not change fetal and maternal plasma and hepatic concentrations of cholesterol and triglycerides. LPD affected neither the magnitudes of maternal-fetal cholesterol flux, maternal cholesterol absorption, nor fetal synthesis of cholesterol and palmitate (both groups, ~14% and ~13%, respectively).

Conclusion

We conclude that LPD throughout gestation in mice does not affect maternal-fetal cholesterol transport, fetal cholesterol or fatty acid synthesis, indicating that programming effects of LPD are not mediated by short-term changes in maternal-fetal lipid metabolism.

INTRODUCTION

Epidemiological studies indicate that inadequate intrauterine nutrition is associated with increased susceptibility to develop chronic diseases in adulthood (e.g. diabetes, hypertension and cardiovascular disease; 'metabolic programming') (1). Comparably, associations between small body size at birth and a less favourable lipid profile in adulthood have been described (2-4). Already in the first postnatal days, growth restricted human infants display a more atherogenic lipid profile (increased plasma concentrations of triglycerides, total cholesterol and LDL-cholesterol) compared with infants with a birth weight appropriate for gestational age (5).

Elevated plasma cholesterol is an established risk factor for the development of cardiovascular diseases later in life, however, insufficient cholesterol supply to the fetus impairs its development (6). It was demonstrated that the fetus is capable of synthesizing a large fraction of the required cholesterol *de novo* (7-9). As an independent source of cholesterol, maternal cholesterol can be transported to the fetal circulation (10). Recently it was shown that maternal-fetal cholesterol transport is influenced by maternal plasma cholesterol levels (11). Interestingly, high maternal cholesterol levels increase the development of fetal aortic fatty streaks, as shown in human and animal studies (12, 13). Hence, the metabolic condition of the mother during pregnancy can affect lipid metabolism in the offspring.

In humans, exposure to the Dutch Hunger Winter during late gestation is associated with increased adult obesity, glucose intolerance, and hypertension (14). Several animal models were developed to mimic the caloric restriction during gestation. An established animal model to investigate metabolic consequences of gestational under nutrition in the offspring, is a low protein diet during gestation (15).

Studies in rats have indicated that maternal protein restriction during gestation leads to features of the metabolic syndrome in adulthood, e.g., increased blood pressure (16), decreased insulin sensitivity and alterations in lipid profile (17, 18). In mice, maternal protein restriction during pregnancy impaired glucose clearance in the adult offspring (19). Furthermore, experiments in mice showed that an LPD affected the renin-angiotensin system (important in the regulation of blood pressure) in the fetus already (20).

Recently, we demonstrated in mice that maternal gestational protein restriction reduced the expression of genes involved in lipid synthesis and cholesterol metabolism in the fetal liver (21). Because the liver is a key player in regulating cholesterol and fatty acid homeostasis, we hypothesized that changes in fetal hepatic gene expression could translate into short term ("acute") effects on cholesterol and fatty acid metabolism (such as maternal-fetal cholesterol flux and/or fetal cholesterol and lipid synthesis). If true, short-term effects on fetal lipid metabolism could be involved in the mechanism by which metabolic programming takes place. We aimed to determine the acute effects of maternal protein restriction during gestation on maternal-fetal cholesterol flux and on *de novo* fetal cholesterol synthesis and on fatty acid synthesis in mice.

MATERIALS AND METHODS

Animals

C57BL/6J mice (age 20 ± 1 wk) were obtained from Harlan (Horst, The Netherlands). Animals were housed in temperature-controlled rooms (23 °C) with 12-h light cycling, and free access to standard RMH-B mouse chow before the experiments (Arie Blok BV, Woerden, The Netherlands) and water *ad libitum*. Experimental procedures were approved by the local Ethical Committee for Animal Experiments of the University of Groningen.

Experimental procedures

All female mice received the control diet containing 180 g casein/kg (purified diet, 4400.18, Arie Blok BV, Woerden, The Netherlands) 2 weeks before mating. Virgin females were time mated using vaginal smears to assess their stage of estrous before introducing the male. After confirmation of mating by detection of a vaginal plug, the females were allocated to either the control diet or the isocaloric low protein diet (90g casein/kg, purified diet 4400.17, Arie Blok BV, Woerden, The Netherlands) during the gestational period. The experimental diets are identical to the diets used by Langley-Evans *et al*. (22) and are described in detail in Table 1. We originally planned an independent intervention study with a fourth experimental group (n=5) and own controls (n=5). However, the intervention was not executed, in contrast to the control treatment in the second group of five (control) mice. Because the design on the control group was identical, we considered it scientifically appropriate to include the control mice into this study, although it caused unequal distribution (n = 10 for controls and n = 5 for treated dams).

Animals were weighed every two d. On embryonic day 14.5 (E14.5) pregnant mice received an i.v. dose of 0.52 mg (1.265 μmol) cholesterol-D_7 dissolved in Intralipid®

Table 1. Composition of the diets provided

Component (g per 100g diet)	Control Diet (18% casein)	Low Protein Diet (9% casein)
Casein	18.00	9.00
Sucrose	21.30	24.30
Corn Starch	42.5	48.50
Cellulose Fibre	5.00	5.00
Vitamin mix AIN-76	0.12	0.12
Mineral mix AIN-76	0.12	0.12
Corn oil	10.00	10.00
Choline choride	0.20	0.20
Methionine	0.50	0.50

(20%, Fresenius Kabi, Den Bosch, The Netherlands) and an oral dose of 0.97 mg (2.30 μmol) cholesterol-D_5 dissolved in medium-chain triglyceride oil. Weights of the dams ranged from 27.3 to 33.1 g at E14.5 of gestation and were not significantly different between the two dietary groups. From E17.5 onward both diets were supplemented with 10 mg/g [1-^{13}C]-acetate (Isotec, Miamisburg, OH, USA). Blood spots were collected from the tail on filter paper before administration of labeled cholesterol and acetate and daily from E14.5 onward until E19.5 of gestation. At E19.5 females were anaesthetized with isoflurane and terminated by cardiac puncture. Figure 1 shows a schematic overview of the experimental set-up

Blood was collected in EDTA tubes. Liver and brain were snap frozen in liquid nitrogen. Fetuses were removed from *uteri*, weight and length were measured, and fetuses were killed and dissected. Blood samples were taken by exsanguination. Liver and brain of fetuses were collected, immediately snap frozen in liquid nitrogen and stored at -80 °C. Sex of the fetuses was determined by PCR as previously described (23, 24)

Analytical procedures

Cholesterol was extracted from blood spots and plasma according to Neese *et al* (25). Hepatic and brain lipids from mothers and pups and lipids from whole (complete) pups were extracted according to Bligh and Dyer (26). Unesterified cholesterol from blood spots, plasma and tissues were analyzed by gas chromatography quadrupole mass spectrometry (27). Commercially available kits were used for the determination of total cholesterol in brain, liver and plasma samples (Roche, Mannheim, Germany). Hepatic fatty acids from livers of mothers and fetuses were analyzed by gas chromatography quadrupole mass spectrometry according to Oosterveer *et al* (28). In short, lipids were extracted from livers, and were hydrolyzed using HCl and acetonitril. Fatty acids were extracted with hexane, after which the samples were derivatized using pentafluorobenzyl:triethanolamine:acetonitril solution. Enrichments of the PFB-derivatives of palmitate were measured by gas chromatograpy-mass spectrometry. The ion monitored were m/z 255-259 corresponding to the m0-m4 mass isotopomers for C16:0.

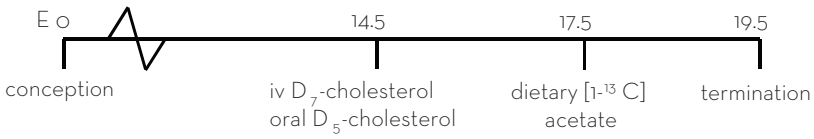

Figure 1. Experimental set-up.

Fractional cholesterol absorption measurement

Fractional cholesterol absorption was measured using an adapted plasma dual isotope ratio method (29) using blood spots obtained at 120 h after i.v. and oral administration of stable-isotopically-labeled cholesterol. For calculation of the fractional cholesterol absorption, the ratio of D_5-cholesterol and D_7-cholesterol in blood spots was divided by the ratio of D_5-cholesterol and D_7-cholesterol in the administered dose. The resulting value represents the fractional cholesterol absorption (27).

Mass isotopomer distribution analysis (MIDA)

To determine *de novo* cholesterol and palmitate synthesis, the MIDA approach was used (25, 30). MIDA allows determination of the enrichment of the pool of acetyl-CoA precursor units that has entered newly synthesized cholesterol or palmitate molecules during the course of [1-^{13}C]-acetate administration. Analysis of the isotopomer pattern of these molecules allows for determination of the fraction (f) of newly synthesized cholesterol or palmitate, respectively in plasma or tissue. All normalized mass isotopomer distributions measured by GC-MS (m_0- m_{0x}) were corrected for the natural abundance of ^{13}C by multiple linear regression as described by Lee (31) et al. to obtain the excess fractional distribution of mass isotopomers (m_0- m_{0x}) due to incorporation of the infused [1-^{13}C]-acetate as described in Ref 28. For determination of the absolute amount of newly synthesized hepatic palmitate, we multiplied f by the total amount of hepatic palmitate at the end of the experiment.

Statistics

We regarded the whole litter as one experimental unit, according to Festing (32).
All data are presented as means ± SD. Statistical analyses were performed using SPSS 14.0 for Windows software (SPSS Inc., Chicago). All data were analyzed using the Mann-Whitney-U-test with a $p < 0.05$ considered significant.

RESULTS

Parameters in the control group

As stated above, the group size of the control mice was twice that of the experimental (low protein) intervention, based on the original design of two control groups of each n=5, undergoing identical (control) treatment. We analyzed whether results between the two n=5 control mice differed, but the parameters measured were virtually identical. Based on this observation, we pooled the data of the n=10 control mice.

LPD affects body weight in dams but not in fetuses

At E0.5, there was no difference in body weight of dams between both groups. At E19.5, dams on a LPD had lower body weights than control dams (Table 2. Food intake during gestation was not different until E17.5 (data not shown), but was lower

Table 2. Parameters of dams and fetuses at E19.5 of gestation

Parameter	Control diet	Low protein diet
weight dam (g)	35.6 ± 2.0	32.1 ± 1.0*
food intake dam (g/day)	6.3 ± 1.2	4.2 ± 0.7*
liver weight to body weight dam (%)	4.5 ± 0.4	5.0 ± 0.2
litter size (n)	7.3 ± 1.7	6.2 ± 2.1
weight fetus (g)	1.07 ± 0.11	0.99 ± 0.15
length fetus (mm)	20.4 ± 1.4	20.5 ± 1.5
liver weight to body weight fetus (%)	2.8 ± 0.8	2.9 ± 1.1

Parameters of C57BL/6J dams and fetuses at E19.5 of gestation receiving control diet or low protein diet during gestation. Data are mean ffl SD. N= 5 (low protein) and 10 (control) dams per group; *, p<0.05 LPD vs control diet

in dams on a LPD between E17.5 and E19.5. There was no significant difference in the other parameters analyzed, including liver weight to body weight ratio of dams, litter size, body weight, body length or liver weight to body weight ratio in fetuses (Table 2).

LPD does not affect total cholesterol concentrations in tissues

To investigate whether protein restriction diet during gestation influenced total cholesterol concentrations in dams and fetuses, we measured total cholesterol in tissues on E19.5. Figure 2A shows that total cholesterol levels in liver or brain in dams did not significantly differ between the two groups. In fetuses, protein restriction did not significantly affect total cholesterol levels in liver, brain, or whole body (the complete fetus; Fig. 2B).

LPD does not affect maternal fractional absorption of cholesterol

We investigated whether the LPD quantitatively affected cholesterol absorption in the dam and cholesterol transport to the fetus during the last stage of gestation. Cholesterol absorption in dams was found to be 64 ± 8% in control dams and 67 ± 8% in low protein (NS).

LPD does not quantitatively affect maternal-fetal cholesterol transport

Maternal-fetal transport of cholesterol was determined by measurement of the fraction of labeled cholesterol in several tissues of dams and fetuses at E19.5. To determine

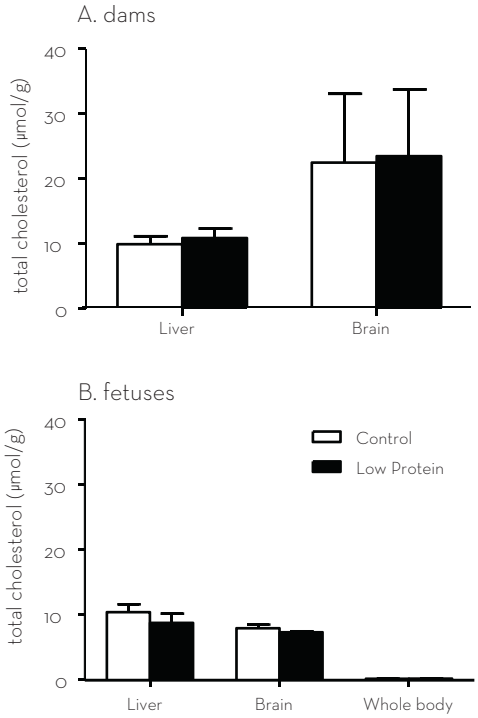

Figure 2. Total cholesterol levels in tissues of (**A**) dams and (**B**) fetuses. Whole body cholesterol in fetuses represents the amount of total cholesterol in the complete fetal body. Open bars, control mice; closed bars, mice receiving an LPD during gestation. Values represent the mean ± SD. n = 5 for low-protein dams and 10 for control dams.

whether the administration route to the dam influenced on cholesterol transport to the fetus, fractions of both D_5 and D_7-labeled cholesterol in tissue and plasma were calculated. The rate of elimination of D_5 and D_7 cholesterol was measured every 24 h after administration (E14.5) until termination (E19.5) and did not significantly differ between dams on LPD or on control diet (data not shown).

The fraction of orally administered D_5 cholesterol constituted about 0.5% of total cholesterol in livers and plasma of both groups. The fraction of orally administered D_5 cholesterol in brain was near detection level in dams of both groups (Fig. 3A). In fetuses at E19.5, the fraction of D_5 cholesterol was measurable in liver, plasma, brain and whole body, albeit very low in both groups (Figure 3B).

The fraction of i.v.-administered D_7 cholesterol in plasma was higher in low protein dams compared with control dams (0.16 ± 0.04% in control dams and 0.36 ± 0.06% in low protein dams ($p=0.028$). In livers, this fraction was 2.2 ± 0.2% in control dams and 2.8 ± 0.2% in low protein dams. In brains of dams, the fraction of i.v.-

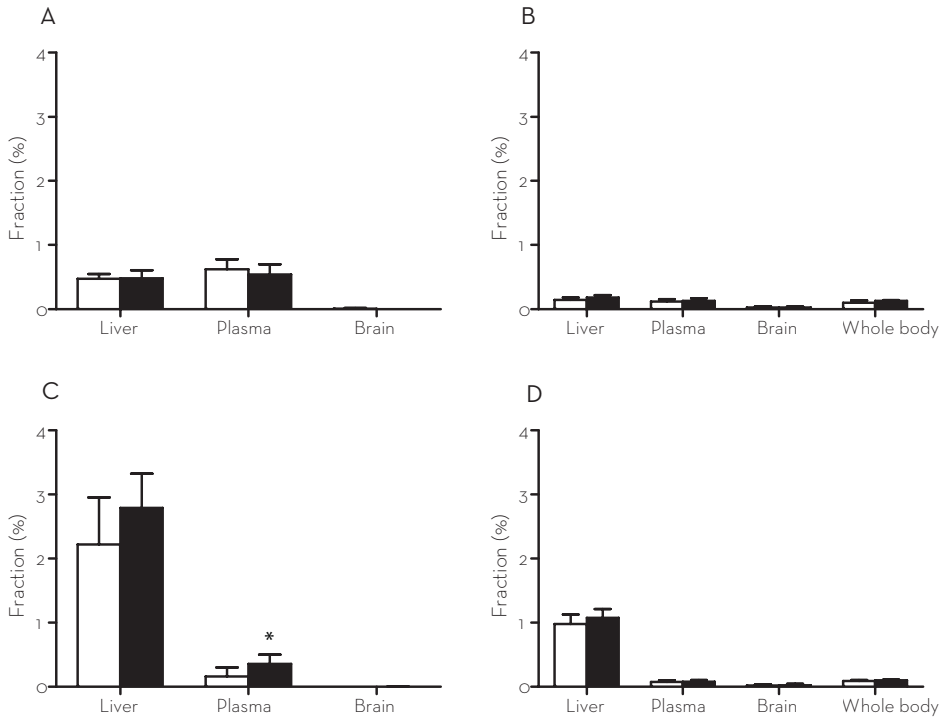

Figure 3. Fractional enrichments of orally administrated D_5-cholesterol in tissues of (**A**) dams and (**B**) fetuses and of i.v.-administrated D_7-cholesterol in tissues of (**C**) dams and (**D**) fetuses at day 19.5 of gestation. Open bars, control mice; closed bars, mice receiving an LPD during gestation. Values represent the mean ± SD. n = 5 for low-protein dams and 10 for control dams.

administrated D_7 was below detection at E19.5 of gestation for both groups (Fig. 3C). In fetuses at E19.5, the fraction of D_7 cholesterol was 1.0 ± 0.1% in liver of control fetuses and 1.1 ± 0.1% in liver of low protein fetuses. Fractions of D_7 cholesterol in plasma brain and whole body of fetuses were detectable, but below 0.5% in both groups (Fig. 3D). No significant differences were found in the fractional enrichments of D_5 or D_7 cholesterol between control or low protein dams or fetuses.

LPD does not quantitatively affect maternal or fetal lipid synthesis

All dams received [1-^{13}C]-acetate in the diet from E17.5 onward to investigate if receiving an LPD during gestation would influence cholesterol or palmitate synthesis in dams and fetuses in the last stage of gestation. Precursor pool enrichments were ~9% for control dams and low protein dams at 24 and 48h (Fig. 4A), indicating steady-state and comparable levels of precursor pool enrichment in both groups. Fractional contribution of newly synthesized cholesterol was 11.9 ± 0.4% for control dams and

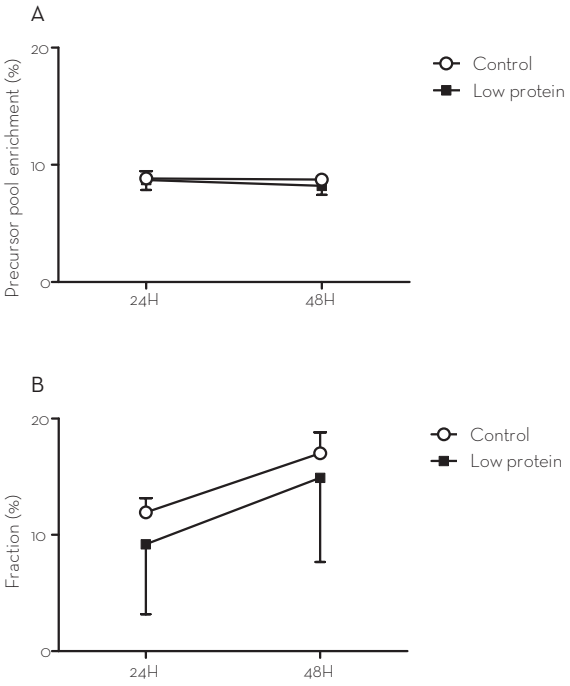

Figure 4. Fractional precursor pool enrichment (**A**) and fractional contribution of newly synthesized cholesterol (**B**) in dams on a control diet (open circles) or an LPD (closed squares) during gestation. Fractions were measured making use of blood spots collected on filter paper (n = 5 for low-protein dams and 10 for control dams). Dams received ^{13}C-labeled acetate in the diet from day 17.5 of gestation onward. Blood spots obtained 24 and 48 hours after administration were used for the calculations of the precursor pool enrichment and fractional contribution of newly synthesized cholesterol. Values represent the mean ± SD.

9.2 ± 2.7% for low protein dams at 24h (NS) and 17.0 ± 0.6% for control dams and 14.9 ± 3.2% for low protein dams at 48h (NS, Fig. 4B).

We determined the incorporation of newly synthesized cholesterol in the fetus by calculating fractional newly synthesized cholesterol at E19.5 in different tissues of dams and fetuses receiving the control diet or the LPD using MIDA. Fractional synthesis in dams was 17.4 ± 0.6% in liver for control dams and 15.0 ± 3.0% in livers of low protein dams, 16.7 ± 1.5% in plasma of control dams and 14.4 ± 6.9% in plasma of low protein dams, 1.4 ± 0.4% in brain of control dams and 1.5 ± 0.4% in brain of low protein dams (all NS, Fig. 5A).

Fractional synthesis in fetuses was 12.6 ± 0.5% in liver of control fetuses and 14.8 ± 2.1% in liver of low protein fetuses, 13.1 ± 2.1% in plasma of controls and 15.4 ± 5.3% in plasma of low protein fetuses, 3.3 ± 0.02% in brain of control fetuses and 4.0 ± 0.4% in brain of low protein fetuses, 9.7 ± 1.4% in whole body of control fetuses and 10.7 ± 1.2% in whole body of low protein fetuses (all NS, Fig. 5B).

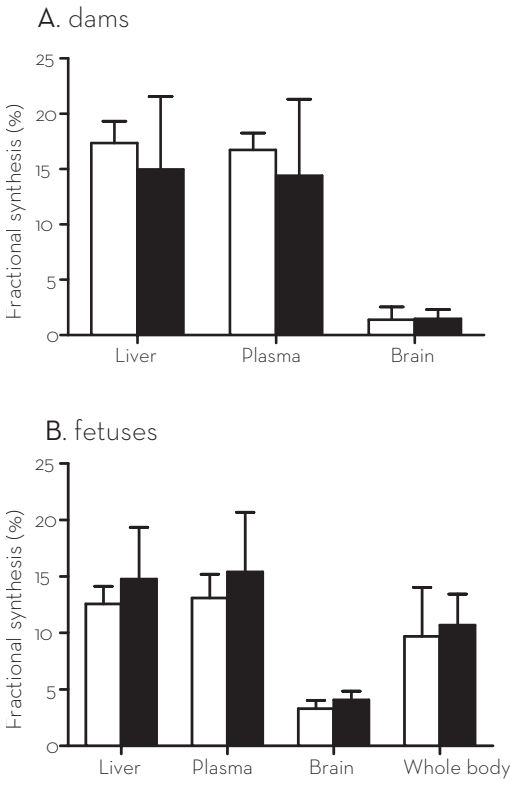

Figure 5. De novo fractional cholesterol synthesis in tissues of (**A**) dams and (**B**) fetuses. Open bars, control mice; closed bars, mice receiving an LPD during gestation. ^{13}C-acetate was administrated in the diet and fractional synthesis was calculated using MIDA. n = 5 for low-protein dams and 10 for control dams. Values represent the mean ± SD.

No significant differences were found in fractional cholesterol synthesis between dams and fetuses on a control diet compared with dams and fetuses on an LPD.
Fractional and absolute *de novo* synthesis of palmitate (C16:0) in livers of dams and fetuses was calculated using MIDA. Fractional *de novo* synthesized palmitate was 23 ± 2% in control dams and 23 ± 5% in low protein dams. Fractional *de novo* synthesized palmitate was 12.3 ± 0.4% in control fetuses and 14.8 ± 1.0% in low protein fetuses (Fig. 6A). Absolute *de novo* synthesis of palmitate was 7.3 ± 1.4 μmol/gram liver in control dams and 4.7 ± 0.9 μmol/gram liver in low protein dams (p 0.2). Absolute *de novo* synthesis of palmitate was 18.7 ± 1.4 μmol/gram liver in control fetuses and 25.0 ± 2.8 μmol/gram liver in low protein fetuses (Fig. 6B). No significant differences considering fractional or absolute *de novo* synthesis of palmitate between the two groups of dams or fetuses were found.

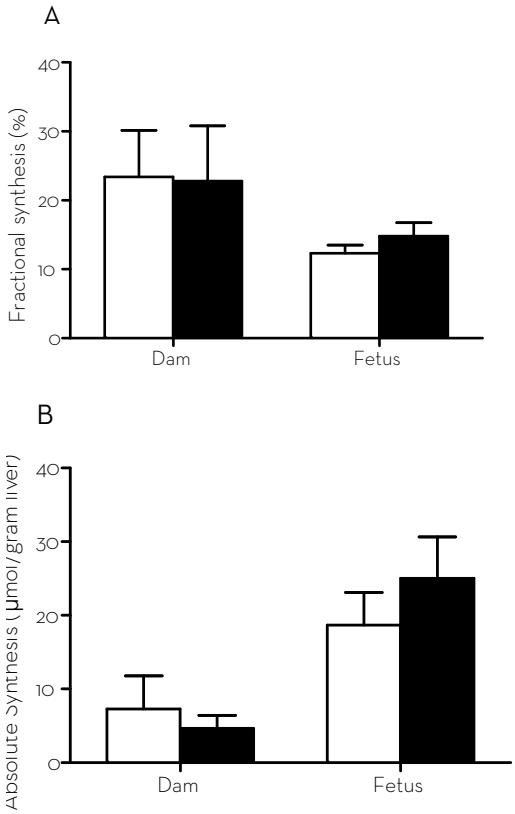

Figure 6. De novo (**A**) fractional and (**B**) absolute palmitate (C16:0) synthesis in livers of dams and fetuses. Open bars, control mice; closed bars, mice receiving an LPD during gestation. ^{13}C-acetate was administrated in the diet and fractional and absolute synthesis were calculated using MIDA. n = 5 for low-protein dams and 10 for control dams. Values represent the mean ± SD.

DISCUSSION

A maternal LPD during the gestational period is a widely used animal model for intrauterine malnutrition to study the mechanisms underlying 'metabolic programming'. The purpose of using this diet during gestation is to mimic intrauterine malnutrition and its possible metabolic consequences. Most frequently, the LPD has been applied in rats in which gestational protein restriction resulted in impaired nephrogenesis and hypertension (33), disturbed glucose tolerance, obesity and alterations in lipid metabolism (17) in the adult offspring. Several studies in mice demonstrate that maternal low protein during gestation leads to features of the metabolic syndrome in offspring. Male offspring from protein-restricted dams developed increased adiposity and glucose intolerance at adult age (34). When fed a high-fat diet, male offspring from protein-restricted dams show a higher increase in body weight, relative fat mass, hyperglycemia, hypercholesterolemia and hyperleptinemia, compared to high-fed diet offspring from control dams (35). Studying the metabolic effects of protein restriction during gestation in mice allows for extrapolation to studies on programming effects in different knock out mice. In the ApoE*3-Leiden mouse, a model for development of atherosclerosis, maternal protein restriction "aggravated" the dyslipidaemia and induced more severe atherosclerotic lesions in female adult offspring fed an atherogenic diet (36).

Some human and animal studies showed that metabolic programming has differential effects in adult male and female offspring, albeit inconsistent. In the present study, no differences between male and female pups in the studied parameters were observed.

We used a unique stable isotope method to determine maternal-fetal cholesterol fluxes and fetal lipid synthesis in mice. Administration of D_5 and D_7-labeled cholesterol to the dam enabled us to distinguish between cholesterol of different maternal sources transported from dam to fetus. The simultaneous administration of ^{13}C-acetate to the dam allows determination of cholesterol and fatty acid synthesis in maternal and fetal organs.

In this study, the LPD slightly decreased maternal food intake and maternal weight, when compared to control diet. However, no differences in fetal weight or length were seen in protein-restricted fetuses compared with controls. The observation on fetal weight corresponds with several other studies using the same diet in rats (37, 38).

In previous experiments in mice, we showed that protein restriction during gestation decreased mRNA expression of genes important in cholesterol transport and fatty acid synthesis in fetal livers (21). These data supported the hypothesis that acute effects on lipid homeostasis could be related to the induction of programming. The present study, however, unequivocally demonstrates that maternal protein restriction during gestation does not induce acute, major quantitative changes in maternal-fetal cholesterol transport, cholesterol biosynthesis or fatty acid biosynthesis in the last stage of pregnancy in mice. Discrepancy between mRNA data and metabolic fluxes has been reported before, underscoring the notion that the 'proof of the pudding' of reporting changes in mRNA and even protein activities is in measuring the relevant metabolic fluxes *in vivo*.

The fraction of i.v. administered cholesterol was significantly higher in plasma of the low protein dams compared with controls. This effect, however, was not reflected in the total plasma cholesterol levels of dams, or in the fractions of D_7-labeled cholesterol in plasma or other tissues of pups. We therefore assume that the effect on i.v. fractions is of limited physiological relevance.

We do realize that a theoretical limitation of the present study is the rapidly changing metabolism of dam and fetus during gestation, which constitutes metabolically a "non-steady state" condition per definition. However, we feel that this limitation most likely applies equally for both the control and the low protein group, and does not invalidate our main conclusions. Another realization involves the timing of our experiment to the late stage of gestation. Theoretically, the protein restriction diet could have influenced cholesterol flux to the fetus in the first stage of gestation. We do not have indications for this possibility as we did not find any differences in total cholesterol content in fetal organs in the last stage of gestation. Based on the results, it seems reasonable to assume that the effects of the maternal diet on cholesterol fluxes and/or on fetal hepatic lipid synthesis during the first stage of gestation are negligible. The presently used model does allow determination of effects of the maternal environment on maternal-fetal transport and on fetal cholesterol and fatty acid synthesis. The method could be applicable to studies investigating the influence of various maternal conditions such as maternal overnutrition or maternal diabetes on fetal lipid metabolism. In conclusion, programming effects of maternal protein restriction during gestation are not accompanied by immediate changes in fetal-maternal cholesterol transport or lipid synthesis in mice in the last stage of pregnancy. Apparently, programming effects of maternal LPD on lipid metabolism are more mediated by more complex pathways than these acute effects.

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CHAPTER

4

INTRAUTERINE GROWTH RESTRICTION AND *DE NOVO* FATTY ACID AND CHOLESTEROL SYNTHESIS IN PRETERM NEONATES

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ABSTRACT

Background

IUGR is linked to increased development of features of the metabolic syndrome in adulthood. IUGR results in small-for-gestational-age (SGA) neonates and is associated with elevated plasma cholesterol and triglyceride concentrations in neonatal and adult life. It is unclear whether these associations are based on increased cholesterol and fatty acid synthesis.

Aim

We compared the rates of cholesterogenesis and *de novo* lipogenesis (DNL) in six SGA and eight appropriate-for-gestational-age (AGA) preterm infants.

Methods and results

[1-^{13}C]-Acetate was administered intravenously for 24 hours in the first two postnatal days. We quantified DNL and cholesterogenesis in blood by mass isotopomer distribution analysis. During the study period, patients did not yet receive intravenous lipid emulsion and enteral intake was limited. Plasma cholesterol and triglycerides were comparable in SGA and AGA infants (cholesterol ~2 mM; triglycerides ~0.5 mM). The fraction newly synthesized cholesterol in plasma free cholesterol (~13%) and the absolute cholesterol synthesis rate (~23 mg/kg/day) were similar in both groups. DNL was ~50% higher in SGA than in AGA infants: 9.3% vs. 6.1%, respectively; $p<0.05$.

Conclusion

We conclude that IUGR does not affect cholesterogenesis and slightly increases DNL. Our results also indicate that DNL does not strongly affect neonatal plasma triglyceride concentrations.

INTRODUCTION

Epidemiological studies have linked IUGR to chronic diseases such as hypertension, coronary heart disease and type II diabetes in adulthood (8, 31). Additionally, IUGR is related to a more atherogenic lipid profile in later life (2, 12, 13, 16, 24, 29). Interestingly, IUGR influences lipid profiles already in fetuses (14, 19) and in newborn infants. It has been shown that SGA neonates had higher plasma triglycerides (TG), total cholesterol (TC) and LDL (C) concentrations compared to AGA neonates within 72 hours after birth (30). Moreover, aortic wall thickening, an early marker of atherosclerosis in children, is increased in SGA compared to AGA neonates (16, 27). The underlying mechanism linking IUGR to these short-term changes in lipid metabolism has remained unclear.

In contrast, indispensible functions of cholesterol have been unraveled to a large extent. Cholesterol serves as a structural membrane component and a precursor for steroid hormones and bile acids (18). During the embryonic and fetal period, cholesterol is required to activate Sonic Hedgehog, a signaling protein which is essential for normal embryonic development (25). The rapid fetal growth and development renders the fetal period a high cholesterol demanding state. Cholesterol can be acquired via external supply (i.e. dietary intake or placental transport) or via *de novo* synthesis from acetyl-CoA which takes place mainly in the liver. Four-month old infants fed human milk (containing ~0.3 mM cholesterol) had significantly lower cholesterol synthesis rates compared to infants fed formula (containing ~0.1 mM cholesterol) (32). This observation underscores the notion that the *de novo* cholesterol synthesis rate is inversely related to the amount of exogenous supplied cholesterol. It has been postulated that alterations in cholesterol synthesis rates due to early nutrition may sustain for a long period (metabolic imprinting) affecting the future plasma cholesterol profile (20). Similar to effect of early postnatal nutrition on cholesterol synthesis, is seems reasonable to assume that differences in the prenatal (i.e. placental) cholesterol supply may affect the rate of *de novo* cholesterol synthesis. It has been shown previously that synthesis rate of cholesterol is relatively high in premature infants compared to adults (26). To date, the influence of intrauterine growth restriction on cholesterol synthesis rates is unclear. Analogous to cholesterol, fatty acids can be derived from the diet or can be *de novo* synthesized in the body. In adults on a Western type diet, *de novo* lipogenesis (DNL) is quantitatively insignificant to the total fatty acid accretion (11). Preterm neonates, however, are born during the physiological third term of gestation, i.e., when body fat accretion is highest. Preterm-born human infants may represent a human model of the third trimester of gestation. It is unknown whether intrauterine growth restriction is compensated by enhanced DNL.

Since IUGR is linked to health consequences in early and adult life, we aimed to elucidate the consequences of IUGR on cholesterol and fatty acid synthesis rates. We also hypothesized that increased *de novo* synthesis of lipids contributes to the elevated plasma concentrations of TG and cholesterol in SGA neonates.

MATERIALS AND METHODS

Patients

We performed our studies in 6 SGA and 8 AGA premature neonates admitted to the NICU of the Beatrix Children's Hospital in Groningen. Infants were considered SGA if their birth weight was at or below the 10th percentile for GA, using the Dutch intrauterine growth curves (15). Parenteral nutrient administration (glucose and amino acids) was initiated after birth according to protocol, resulting in a glucose intake of 3-5 mg/kg/min during the time of the experiment. To exclude possible influences of postnatal dietary lipid or nutrient supply on the analyses, we chose to determine DNL and *de novo* cholesterol synthesis in the first postnatal days. Before and during the experiment parenteral lipid emulsion was not administered and enteral feeding was limited (less than 20 ml/kg/day). All mothers of patients had received antenatal steroids, except for one.

Experimental design

The study protocol was approved by the University Medical Center Groningen Ethical Committee. Written informed consent was obtained from the parents of the infants. The experimental protocol did not intervene with the standard treatment for this group of patients. Exclusion criteria were metabolic or chromosomal disorders, clinical suspicion of sepsis or meningitis and maternal endocrine disorders. Infants that were born large for gestational age (a birth weight exceeding the 90th percentile of the intrauterine growth curves) were excluded. The experimental set-up is depicted in figure 1. All infants received an intravenous infusion of [1-^{13}C]-acetate (Isotec, Miamisburg, OH) at a constant rate of 0.14 mmol/kg/h during 24 hours. The start of the experiment (t=0) was defined by the start of the [1-^{13}C]-acetate infusion. A blood sample of 1.0 ml was obtained before the start (t=0), and at the end (t=24) of the experiment. Blood samples of 0.4 ml were obtained at 8, 12, 16 and 20 hours after the start of the [1-^{13}C]-acetate infusion. Blood samples were obtained via an intra-arterial line which the infants had received for their standard treatment.

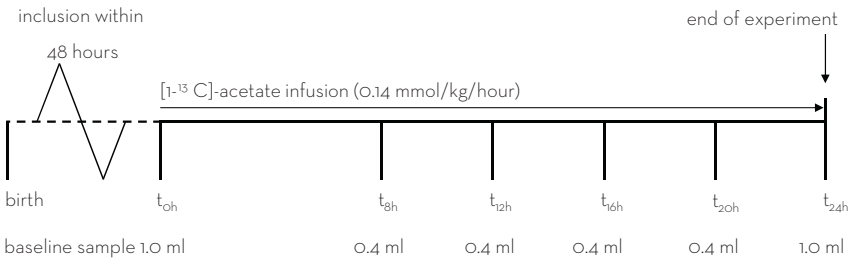

Figure 1. Design of the experiment

Analytical procedures

Blood samples were collected in EDTA-containing tubes and were directly centrifuged for 10 min at 4000 rpm at 4°C, after which the plasma was stored at -80 °C until analysis. Commercially available kits were used for the determination of total cholesterol and triglycerides in plasma (Roche, Mannheim, Germany). Cholesterol was extracted from plasma according to Neese *et al* (22). Unesterified cholesterol was derivatized as described (26). Samples were dried under nitrogen and dissolved in hexane, followed by Mass Isotopomer Distribution Analysis (MIDA, described below) on plasma unesterified cholesterol. All analysis were performed on a Trace MS plus GC/MS (Interscience, Breda, the Netherlands). Derivatives were separated on a DB-17MS 20m x 0.18mm ID (0.18µm film thickness) capillary column (Alltech, Breda, the Netherlands). Ions monitored were m/z 458-462 corresponding to the m_0-m_4 mass isotopomers. Plasma VLDL was isolated by density gradient ultracentrifugation as previously described (9). Fatty acids were extracted and derivatized as described earlier (23) followed by MIDA on palmitate (16:0) as the marker of fatty acid synthesis. Fatty acid derivatives were analyzed by GC-MS, 5975C MSD, (Agilent Technologies, Amstelveen, The Netherlands). Fatty acids were separated on a Zebron ZB-1 30m x 0.25 mm ID capillary column (Phenomenex, Torrance, USA). Ions monitored were m/z 255-259 corresponding to the m0-m4 mass isotopomers for palmitate (C16:0). The normalized isotopomer distributions measured by GC/MS (m_0-m_4) were corrected for natural abundance of ^{13}C by multiple linear regression (17) to obtain the excess fractional distribution of mass isotopomers (M_0-M_4) due to incorporation of [1-^{13}C]-acetate. This distribution was used in MIDA algorithms.

Determination of cholesterol and fatty acid synthesis using Mass Isotopomer Distribution Analysis (MIDA)

De novo synthesis of cholesterol and fatty acids was calculated by MIDA, as described in detail elsewhere (10, 22). In short, MIDA allows the determination of the fraction newly synthesized polymers like cholesterol and palmitate. In this experiment we used these fractions as a marker of *de novo* lipogenesis. Fractional cholesterol synthesis was calculated on regular time points (f_t) during [1-^{13}C] acetate infusion. We assumed that equation $f_t = f_\infty (1-e^{-kt})$ is valid to describe the fractional synthesis during the infusion. Using the SAAM II program (SAAM Institute, Seattle, WA, U.S.A.) we estimated the rate constant; k (h^{-1}) and the fractional enrichment at infinite time; f_∞. To calculate absolute synthesis rates of cholesterol, we used the estimated total body pool size of free cholesterol (A) of 130 mg/kg body weight, as postulated by Neese *et al.* (22). The absolute synthesis rate of cholesterol (K) is calculated as $K = k * f_\infty * A$. The fraction of newly synthesized lipoprotein palmitate at t=24 h, after cessation of the [1-^{13}C]-acetate infusion was calculated as described elsewhere (23).

Statistics

All data are presented as medians and range. Statistical analyses were performed using SPSS 16.0 for Windows software (SPSS Inc., Chicago, OH), using the Mann-Whitney-U-test. A p-value < 0.05 was considered significant.

Table 1. Characteristics of preterm SGA and AGA newborns

	SGA	AGA
Gestational age (wk)	29.0 (27.6-30.4)	29.5 (28.1-32.6)
Birth weight (g)	903 (700-1062)*	1333 (1054-2413)
Age at the start of the study (h)	24 (15-44)	22 (5-43)
Plasma cholesterol (mmol/l)	1.8 (1.2-2.1)	2.1 (1.1-3.0)
Plasma triglycerides (mmol/l)	0.4 (0.1-0.6)	0.5 (0.1-1.2)
Female/male	4/2	4/4

Parameters of premature SGA and AGA newborns at the start of the experiment. Data are medians (range). n= 6 (SGA) and 8 (AGA) neonates per group.
* $p<0.05$ SGA vs AGA.

RESULTS

Clinical Characteristics

Table 1 shows the main characteristics of the SGA and AGA preterm neonates. Gestational age and postnatal age at the start of the study were similar. The median birth weight of the SGA neonates was 40% lower than that of the AGA neonates ($p<0.05$). The distribution of sex in both groups was not significantly different. Plasma concentrations of total cholesterol and of triglycerides were not significantly different between the SGA and AGA preterm infants.

Intrauterine growth restriction does not affect de novo synthesis of cholesterol

In order to quantify the effect of IUGR on *de novo* cholesterol synthesis, we calculated the enrichment of the precursor pool acetyl-CoA for cholesterol and subsequently, the fractional synthesis of plasma cholesterol at subsequent time points during the experiment. The precursorpool enrichment remained unchanged during the experiment (Figure 2A) and was similar in SGA and AGA infants (Figure 3A). The fractional contribution of newly synthesized cholesterol rose steadily over time in both groups (Figure 2B) but at infinite time ($f\infty$) there were no differences between SGA and AGA infants (Figure 3B). Using these curves of fractional synthesis rates, the rate constant of appearance of newly synthesized cholesterol k (h^{-1}) could be determined. The median k-value in the SGA group [0.07 (0.05-0.10) h^{-1}] was similar to that in the AGA group [0.06 (0.03-0.10] h^{-1}, Figure 3C]. The absolute rate of cholesterol synthesis was calculated using the following equation: $A = k * f_{\infty} *$ pool

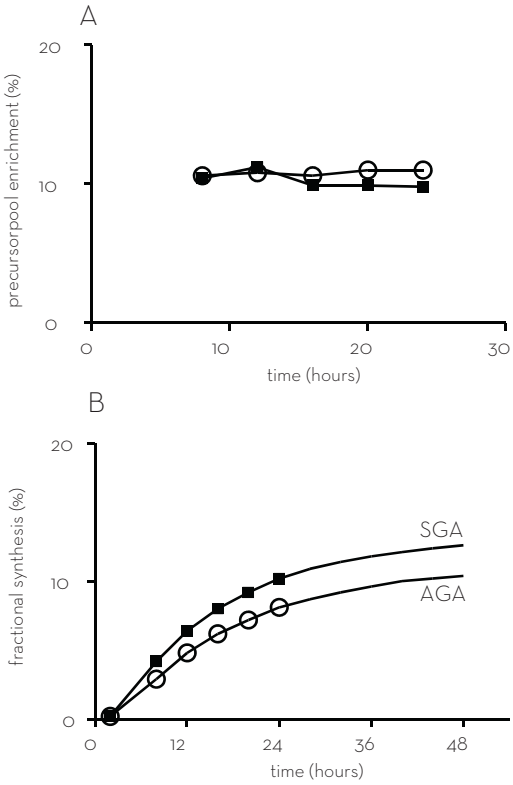

Figure 2. The enrichment of the precursorpool acetyl-CoA remained in steady state from eight hours after infusion onward in both groups (**A**). (**B**) depicts the fractional synthesis of plasma unesterified cholesterol in preterm SGA (n=6; closed squares) and AGA (n=8; open circles) neonates. Values were calculated at the indicated time points using the blood samples obtained during the 24 h infusion of [1-^{13}C]-acetate. Curves of fractional synthesis rates were fitted using SAAM2 (for experimental details, please see Materials and Methods), and showed a plateau phase depicting fractional synthesis at infinite time (f_{∞}) after about 50 hours. Values represent the means.

size. The calculated absolute synthesis rate of cholesterol was 26 (22-46) mg/kg/day in the SGA infants, similar to 20 (4-43) mg/kg/day in the AGA group (Figure 3D).

Fractional de novo lipogenesis is increased in SGA compared to AGA premature newborns

To compare *de novo* lipid synthesis rates between SGA and AGA premature neonates, we performed Mass Isotopomere Distribution Analysis (MIDA) on VLDL-palmitate (16:0) in the obtained plasma samples. Figure 4 shows the fractional contribution of *de novo* synthesized palmitate to VLDL-palmitate after 24 hours of [1-^{13}C] acetate infusion. The fraction of newly synthesized VLDL-palmitate after 24 hours of infusion ($f\text{-}_{24}$) was 52% higher in SGA than in AGA preterm neonates ($p < 0.05$).

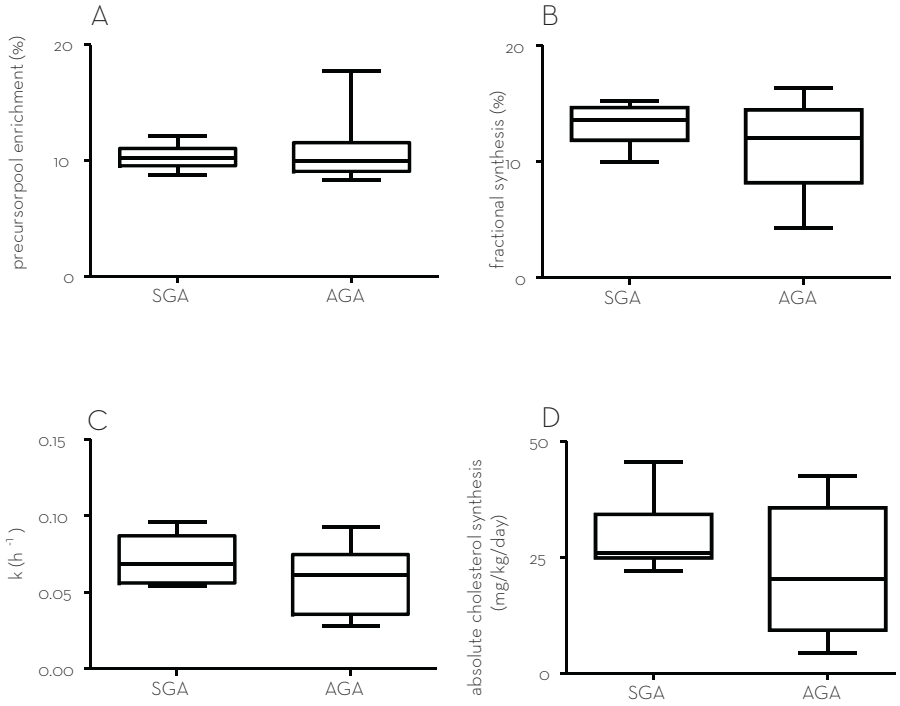

Figure 3. Parameters of the de novo cholesterol synthesis
Enrichment of the precursorpool acetyl-CoA for cholesterol after 24 hours of [1-^{13}C]-acetate infusion in SGA and AGA pre-terms (**A**). The fraction of newly synthesized unesterified cholesterol at infinite time, (fffi) in plasma in SGA and AGA neonates (**B**). Using the curves of fractional synthesis rates (Figure 2B), the rate constant of appearance of newly synthesized cholesterol k (h^{-1}) could be determined (**C**). The absolute rate of cholesterol synthesis was calculated using the following equation: $K = k * f_{ffi} * A$ (D). Values represent the median and range. n= 6 (SGA) and 8 (AGA) per group.

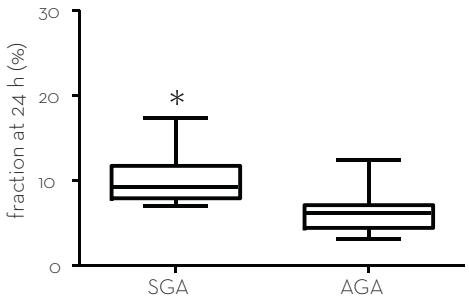

Figure 4. Parameters of DNL.
The fraction of newly synthesized lipoprotein palmitate after 24 hours of infusion (f_{-24}) in SGA and AGA preterm infants. Values represent the median and range. N= 6 (SGA) and 8 (AGA) per group. *p<0.05 SGA vs. AGA preterm infants.

DISCUSSION

Exposure to an adverse fetal environment may permanently alter the structure, function or metabolism of major tissues or organs, herewith increasing the susceptibility to diseases later in life (1). Metabolic consequences of IUGR have been recognized and immediate and long-term effects of IUGR, resulting in SGA, on plasma lipid levels are reported (30). We determined the influence of IUGR on *de novo* synthesis rates of cholesterol and fatty acids in SGA and AGA preterm neonates as an indicator of intrauterine lipogenesis. We demonstrated similar synthesis rates of cholesterol whereas fractional hepatic palmitate synthesis was increased in SGA compared to AGA preterm infants.

DNL, as reflected by the fraction of VLDL-palmitate that was newly synthesized after 24 h of [1-^{13}C]-acetate infusion, was ~50% higher in SGA than in AGA infants. Although this difference is substantial the clinical relevance of this change in DNL regarding plasma TG levels needs to be established. We speculate that this relevance may be limited considering the relatively low fractions of hepatic DNL in VLDL-palmitate in SGA and AGA neonates. Moreover plasma concentrations of triglycerides were comparably low in both groups, excluding any substantial effect of enhanced DNL. We cannot exclude, however, a relevant effect of the mildly increased DNL to the longer term fat accretion in SGA neonates. We did not confirm the higher levels of triglycerides in the SGA infant as reported by others (30). Wang *et al* demonstrated significantly higher plasma concentrations of TG, TC and LDL-c in SGA neonates compared to AGA neonates, either when born preterm or term, in the first days after birth. The plasma TG concentrations determined in our study were generally lower (~0.5 mM) compared to TG concentrations reported by Wang *et al* (~ 2.3 and 1.5 mM in respectively SGA and AGA neonates) whereas TC levels between the two studies were within the same range (~2 mM). A possible explanation for the lower plasma triglyceride levels in the present study could be related to differences in the use of intravenous lipid emulsions and enteral nutrition. In our present study, the neonates did not receive parenteral lipids and only limited amounts of enteral feeding (< 20 ml/kg/day). In support of this possibility, plasma triglyceride concentrations measured in the present study are in line with concentrations found in cord blood samples of preterm and term infants (7), and with serum triglyceride concentrations in premature infants within the first day after birth (6). In the present study, hepatic *de novo* lipogenesis was increased in the SGA group whereas plasma TG concentrations were similar in both groups. Apparently, plasma triglyceride levels are not strongly correlated to DNL.

Since cholesterol is indispensible for normal fetal development, adequate supply to the fetus needs to be warranted during gestation. Both placental and fetal human tissues are capable of *de novo* cholesterol synthesis (4). Several observations support the notion that cholesterol is transported from mother to fetus (28, 33, 21). Fetal growth depends on adequate maternal-fetal transport of nutrients. Factors that impair fetal nutrional supply, such as maternal undernutrition or placenta insufficiency are associated to perturbed cholesterol metabolism in adulthood. We hypothesized that

IUGR in humans could influence fetal cholesterol synthesis rates, thereby altering the 'metabolic set point' and influence future response to dietary cholesterol. The present study in preterm neonates, using undisputed stable isotope methodologies, shows that IUGR does not generate effects on endogenous synthesis rates of cholesterol or on plasma cholesterol concentrations.

Our results on cholesterol synthesis rates are in the same range with results by others using MIDA in preterm neonates (26) and deuterium incorporation techniques in older infants (3, 5, 32).

An advantage of the presently used MIDA method is that it allows for the use of small blood samples. The alternative method, based on deuterium incorporation into newly synthesized lipids, required substantially larger blood samples of 8 ml (3, 32). Previously, the deuterium method was used in infants of four to five months of age, but this scale of blood sampling is definitively not applicable to the currently studied population.

In summary, we conclude that intrauterine growth restriction does not influence the synthesis rate of cholesterol whereas it increased the *de novo* fatty acid synthesis. Our results indicate that the neonatal plasma concentration of triglycerides does not strongly depend on the *de novo* fatty acid synthesis rate. The present results do not support the notion that IUGR exerts major effects on lipid metabolism within the time frame studied.

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CHAPTER

5

FETAL LIVER X RECEPTOR ACTIVATION ACUTELY INDUCES LIPOGENESIS, BUT DOES NOT AFFECT PLASMA LIPID RESPONSE TO A HIGH-FAT DIET IN ADULT MICE

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ABSTRACT

Background and aim

There is increasing evidence that the metabolic state of the mother during pregnancy affects long-term glucose and lipid metabolism of the offspring. The Liver X Receptors (LXR) α and β are key regulators of cholesterol, fatty acid and glucose metabolism. LXRs are activated by oxysterols and expressed in fetal mouse liver from day 10 of gestation onwards. In the present study, we aimed to elucidate whether *in utero* pharmacological activation of LXR would influence fetal fatty acid and glucose metabolism and whether this would affect lipid homeostasis at adult age.

Methods and results

Exposure of pregnant mice to the synthetic LXR agonist T0901317 increased hepatic mRNA expression levels of Lxr target genes and hepatic and plasma triglyceride levels in fetuses and dams. T0901317 treatment increased absolute *de novo* synthesis and chain elongation of hepatic oleic acid in dams and fetuses. T0901317 exposure *in utero* influenced lipid metabolism in adulthood in a gender specific manner: hepatic triglyceride content was increased (+45%) in male offspring and decreased in female offspring (-42%) when fed a regular chow diet, compared with untreated gender controls. Plasma and hepatic lipid contents and hepatic gene expression patterns in adult male or female mice fed a high fat diet were not affected by T0901317 pretreatment.

Conclusion

We conclude that LXR treatment of pregnant mice induces immediate effects on lipid metabolism in dams and fetuses. Despite the profound changes during fetal life, long-term effects appeared to be rather mild and gender selective without modulating the lipid response to a high-fat diet.

INTRODUCTION

In humans, the nutritional condition during pregnancy has been shown to have a persistent effect on aspects of lipid and carbohydrate metabolism of the developing fetus. Maternal overnutrition leads to a higher incidence of insulin resistance, obesity, hypertension and cardiovascular diseases in adult offspring (6). Effects of a high-fat maternal diet during pregnancy on long-term health status of the offspring are relatively well-studied in animal models (3). However, relatively little is known about long-term consequences of targeted alterations in fetal lipid metabolism *in utero*.

The Liver X Receptors have been identified as key players in the regulation of cholesterol, fatty acid and carbohydrate metabolism in adult mammals. Liver X Receptors LXRα(NR1H3) and LXRβ (NR1H2) are members of the nuclear receptor superfamily of ligand-activated receptors. Both LXR isoforms are activated by oxidized cholesterol metabolites (oxysterols) with no LXRα/β specificity documented (20). Expression of LXRα is mainly restricted to liver, intestine, adipose tissue and macrophages, while LXRβ is broadly expressed (23). Activated LXRs heterodimerize with ligand-activated Retinoid X receptor (RXR) at LXR response elements present in the promotors of target genes to induce their transcription. Activation of LXRs induces expression of the sterol regulatory element binding protein 1c (*SREBP1c*) that subsequently activates the lipogenic genes acetyl-CoA carboxylase (*ACC*), fatty acid synthase (*FASN*) and stearoyl-Coenzyme A desaturase 1 (*SCD1*) (29). It has been postulated that activation of LXR could induce carbohydrate response element-binding protein (*ChREBP*) and in this way indirectly influence glycolysis via influencing expression of liver-specific pyruvate kinase (PKLR) (7).

Expression of *Lxra* and *Lxrb* has been demonstrated in mouse fetuses from day 11.5 *post coitum* onwards (2), however, the functional role of LXRs during fetal development and in the newborn has not been defined. LXR does not appear to be essential for normal fetal development as mice with targeted inactivation of *Lxra*-, *Lxrb*-, or *Lxra/b* (1, 17, 21) show normal fetal development.

Mechanisms by which fetal and early postnatal environment influence lipid and glucose homeostasis in adult life are poorly defined. We previously showed that treatment of pregnant mice with the widely used LXR agonist T0901317 affects cholesterol metabolism in the fetus. T0901317 treatment of pregnant mice activated LXR in the fetuses, resulting in induction of genes involved in fetal cholesterol metabolism. Interestingly, the treatment did not profoundly influence cholesterol metabolism at adult age (27, 27). To delineate the functional importance of LXRs in control of fetal fatty acid and carbohydrate metabolism, we supplemented the diet of pregnant mice with T0901317. In the present study we aimed 1. to determine whether pharmacological activation of Lxr in fetuses through maternal treatment with T0901317 activated Lxra or Lxrb in the fetal liver and influenced fetal fatty acid and glucose metabolism; and 2. to assess whether Lxr activation during fetal development changed lipid homeostasis into adulthood, when weaned onto standard chow or on a high-fat diet. Our results indicate that maternal T0901317 treatment activates Lxrs and strongly affects lipid metabolism in the fetus. The prenatal treatment only had relatively minor, gender-

selective effects on lipid homeostasis in (young) adulthood and did not influence the response to a short-term high-fat diet.

MATERIALS AND METHODS

Animals

Animals were housed in temperature-controlled rooms (23 °C) with 12 hours light cycling and received standard RMH-B mouse chow (Arie Blok BV, Woerden, The Netherlands) and water *ad libitum*. Pregnant C57BL/6J mice were obtained from Harlan (Horst, The Netherlands) at 2 days post coitum (dpc). *Lxrα*$^{+/-}$ female mice (25) on a C57BL/6J background were crossed with *Lxrα*$^{+/-}$ male mice on the same background in our laboratory to obtain offspring with *Lxrα*$^{+/+}$, *Lxrα*$^{+/-}$ and *Lxrα*$^{-/-}$ genotypes as previously described (27). All experimental procedures were approved by the local Ethical Committee for Animal Experiments of the University of Groningen.

Experimental procedures

From day 10 *post coitum* until day one after delivery, C57BL/6J wild-type females received standard chow only or chow supplemented with 0.015% w/w T0901317 (Cayman Chemicals, Ann Arbor, Michigan), as described in (26). At days 13.5, 15.5, 17.5 and 19.5 *post coitum* pregnant C57BL/6J mice were anaesthetized with isoflurane and terminated by heart puncture. *Lxrα*$^{+/-}$ females received the T0901317 diet from day 10 *pc* till day 19.5 *pc* and were terminated at day 19.5 *pc*. Blood was collected in EDTA containing tubes. Liver samples of the dams were snap-frozen in liquid nitrogen. Fetuses were removed from *uteri*, their weight and length were measured, and they were terminated by decapitation and dissected. Pups were sacrificed at day 1 *post partum*. Blood samples were taken by exsanguisation. Livers and intestines of fetuses and pups were collected, immediately snap-frozen in liquid nitrogen and stored at -80 °C until mRNA isolation or biochemical analysis. Samples for microscopic evaluation were snap-frozen in liquid nitrogen for Oil-Red-O staining. Pups were genotyped by PCR using allele-specific primers (wild-type: sense 5'-CACCCATTCTCCCGTGCTTCTCTT G-3'; knockout: sense, 5'-GGGCCAGCTCATTCCTCCCACTCAT-3'; antisense for both, 5'-GTTTCTCTCCCCTATCTAGGGAGAC-3').

To investigate lipogenesis and chain elongation of several fatty acids in the liver, a separate group of pregnant females on chow or on chow with T0901317 received these diets with 10 mg/g [1-^{13}C] acetate (Isotec, Miamisburg, OH, USA) added from day 17.5 of gestation till day 19.5 *pc* and were terminated at 19.5 *pc*.

To investigate the influence of receiving T0901317 during fetal development on coping with a high fat/high cholesterol diet at adult age, pregnant C57BL/6J females received chow only or chow supplemented with 0.015% w/w T0901317 from day 10 of gestation until day 1 after delivery. All dams received chow till the pups were weaned. Offspring received chow until 6 weeks of age and received either chow or a semi-synthetic Western-type diet (HFHC) containing 15% (w/w) cacao butter and

0.25% (w/w) cholesterol (Diet W; Special Diet Services, Witham, UK) for two weeks. Offspring was terminated at 8 weeks of age.

Analytical procedures

Liver homogenates were made by homogenization of the complete fetal liver or (approximately 20-80 mg, depending on age) in 200 µL ice-cold water. Hepatic lipids were extracted using the Bligh and Dyer method (4). Pooled plasma samples from all animals of one group were used for lipoprotein separation by fast protein liquid chromatography (FPLC) as described previously (28). Commercially available kits were used for the determination of triglycerides in liver extracts, plasma samples and FPLC fractions (Roche, Mannheim, Germany).

Determination of fatty acid synthesis using MIDA

MIDA allows quantitation of the biosynthesis of polymers *in vivo* and is described in detail elsewhere (13). Hepatic lipids from mothers and their fetuses receiving [1-^{13}C] acetate were extracted and hydrolyzed by adding 0.5 ml 0.5M HCl in acetonitril and heating for 45 minutes at 100° C. As an internal standard, 100 ul or 5 ul of C17:0 (0.5 mg/ mL) in chloroform was added to the mother and fetal liver extracts, respectively. Fatty acids were extracted by adding 1,5 ml hexane, 5 minutes shaking and centrifugation for 5 minutes at 2500 rpm. The organic phase was transferred to a clean tube, and samples were dried down at 40° C under N_2. Samples were derivatized for 15 minutes at RT using 50 ul of 1: 2: 6 pentafluorobenzyl: triethanolamine: acetonitril solution. 0.5 ml 0.1 M HCl was added and the derivatives were extracted using 1 ml hexane, 5 minutes shaking and 5 minutes centrifugation at 2500 rpm after which the organic phase was transferred to a GC-MS vial. Enrichments of the PFB-derivatives of hepatic fatty acids were measured by gas chromatography-mass spectrometry. Derivatives were separated on a Zebron ZB-1 30 m x 0.25 mm ID (0.25 µm film thickness) capillary column (Phenomenex, Torrance, USA). The oven temperature started at 100° C for 1 min, increased to 200° C at a rate of 50° C/min, then increased to 270° C at a rate of 5° C/min, and finally increased to 300° C at a rate of 50° C/min, and remained at 300° C for 4 minutes. Mass spectrometry analysis was performed by electron capture negative ionization (ECNI) with methane as a moderating gas. The ion monitored were m/z 255-259 corresponding to the m0-m4 mass isotopomers for C16:0, m/z 269-273 for C17:0, m/z 283-287 for C18:0 and m/z 281-285 for C18:1ω9. Calculations on the newly synthesized polymers, the isotope enrichments of their monomer precursor (acetyl-CoA), and synthesis and chain-elongation of stearic acid and oleic acid are described elsewhere (19, 22).

RNA isolation and PCR procedures

Total RNA was extracted from frozen tissues with TriReagent (Sigma, St. Louis, MO, USA) and quantified using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). cDNA synthesis and real-time quantitative PCR were performed as described by Plösch et al (21). Primer and probe sequences for *18S*, *Srebp1c*, *Acc1*, *Fasn*, *Gk*, *G6p*, *Pck1*, *Pk* (12), *Scd1* (25), *Fxr*, *Ppara* (15), *Lxra*, *Lxrb*

(27), have been published, with the exception of *Rxra*, sense 5'-GGCAAACATGGGGC-TGAAC -3'; antisense 5'- GCTTGTCTGCTGCTTGACAGAT -3'; and probe 5'- CCA-GCTCACCAAATGACCCTGTTACCAAC -3' (accession number NM_011305), *Insr*, sense 5'- TGAGTCAGCCAGTCTTCGAGAA -3'; antisense 5'-ACTACCAGCATTGGCT-GTCCTT- 3'; and probe 5'- CTGCCATCATGTGGTCCGCCTTCT -3' (accession number NM_010568). Expression levels were normalized to those of 18S ribosomal RNA. 18S rRNA was analyzed in separate runs and not found significantly different between the experimental groups.

Histology

Liver histology was examined on frozen liver sections after Oil-Red-O staining for neutral lipids by standard procedures.

Statistics

Statistical analyses were performed using SPSS 14.0 for Windows (SPSS Inc., Chicago, USA). Differences between the groups were analyzed by Kruskal Wallis test followed by Mann-Whitney-U-test. Data presented are means ± SD. A p-value smaller than 0.05 was considered to be statistically significant.

RESULTS

T0901317 treatment to dams strongly induces genes involved in lipogenesis in dams and fetuses and acts mainly via Lxra

As described previously, dams receiving T0901317 from 10.5 dpc to 19.5 dpc had slightly lower body weights on day 19.5 of gestation compared to control dams (26). Liver weights of the treated dams, expressed as a percentage of total body weight, were increased by ~50% during the entire treatment period. Body weight of the fetuses on day 13.5, 15.5, and 17.5 pc was not influenced by T0901317 administration; only on day 19.5 it was slightly reduced in the treated animals (26). T0901317 treatment had no influence on the number of offspring per dam or on their body weights (1.26 ± 0.19 vs. 1.38 ± 0.13 g in treated and untreated animals, respectively, at day 1 after birth) (26).

Our first aim was to establish whether T0901317 treatment of the wild-type dams affects gene expression levels of lipogenic Lxr target genes in dams and fetuses. In dams (Suppl. Figure 1a to c) and in fetuses (Figure 1a to d), administration of T0901317 strongly increased hepatic mRNA levels of *Srebp1c*, *Fasn* and *Scd1* compared to controls. *Acc1* induction in the fetus was less pronounced by T0901317 treatment. Administration of T0901317 to pregnant mice did not significantly affect expression levels of nuclear receptors *Rxra*, *Ppara* or *Fxr* in the fetal liver (Suppl. Figure 2a to c).

To ascertain to what extent T0901317-induced effects on lipid metabolism were Lxrα or -β mediated, *Lxra*$^{+/-}$ females were crossed with *Lxra*$^{+/-}$ males. T0901317 treatment to the heterozygous dams resulted in significantly lower expression levels of lipogenic

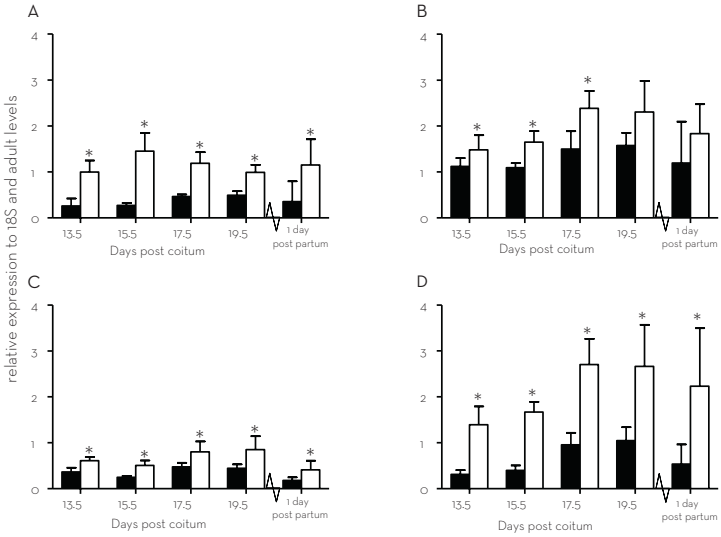

Figure 1. Changes in relative hepatic gene expression of fetuses on several days of gestation upon treatment of the dam with T0901317. Expression of fetal (**A**) *Srebp1c*, (**B**) *Acc1*, (**C**) *Fasn*, and (**D**) *Scd1*. Results were normalized to *18s* mRNA levels. Adult expression levels are arbitrarily defined as 1. Black bars, control fetuses; open bars, fetuses from dams receiving 0.015% T0901317. Values represent the mean ± SD. n=6 dams per group; *, $p < 0.05$ for treated vs control.

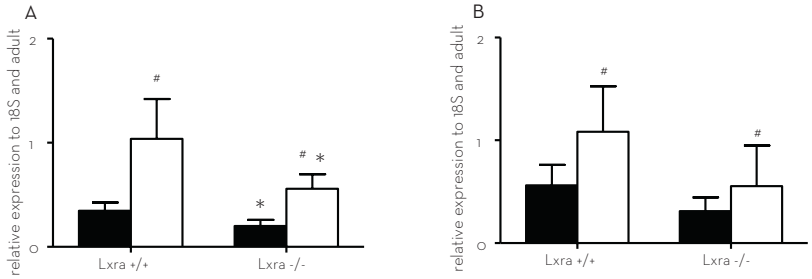

Figure 2. Changes in relative hepatic gene expression in wild type and Lxrα-knockout fetuses at day 19.5 of gestation upon treatment of the heterozygote dam with T0901317. Hepatic expression levels of fetal (**A**) *Srebp1c* and
(**B**) *Fasn* are displayed. Results were normalized to *18s* mRNA levels. Adult expression levels are arbitrarily defined as 1. Values represent the mean ± SD. n=6 dams per group, with an average of 5 fetuses per dam; #, $p < 0.05$ for treated vs control, *, $p < 0.05$ for knockout vs wild type.

LXR target genes (*Srepb1c*, *Fasn*) in the liver of *Lxra* knockout fetuses than in wild-type fetuses (Figure 2a and 2b), indicating that the observed effects are, at least partly, mediated via an Lxr alpha-specific effect.

T0901317 administration induces transient hepatic steatosis in pregnant mice and their fetuses

T0901317 treatment increased hepatic triglyceride content ~ 4-fold in pregnant females after three days of treatment (Figure 3a). This effect gradually declined during gestation, but was still apparent at the time of delivery. T0901317 treatment decreased plasma triglyceride concentrations in dams, but only during the pregnancy (Figure 3b). FPLC analysis of maternal plasma at day 19 of gestation revealed that the reduction in triglycerides in the T0901317-treated dams was mainly in VLDL-sized fractions (Figure 3c).

Maternal T0901317 treatment increased hepatic triglyceride concentrations in the fetuses (Figure 4a). At day one after delivery, however, triglyceride concentrations in livers of T091317-exposed pups remained at (induced) fetal level, whereas concentrations in control pups surged. Fetal plasma triglyceride concentrations, measurable at 19.5 dpc and at day 1 after delivery, were significantly elevated upon LXR activation (Figure 4b). Both in the control and in the T0901317-exposed pups, plasma lipid levels strongly increased after delivery. FPLC analysis showed a profile typical for fetal

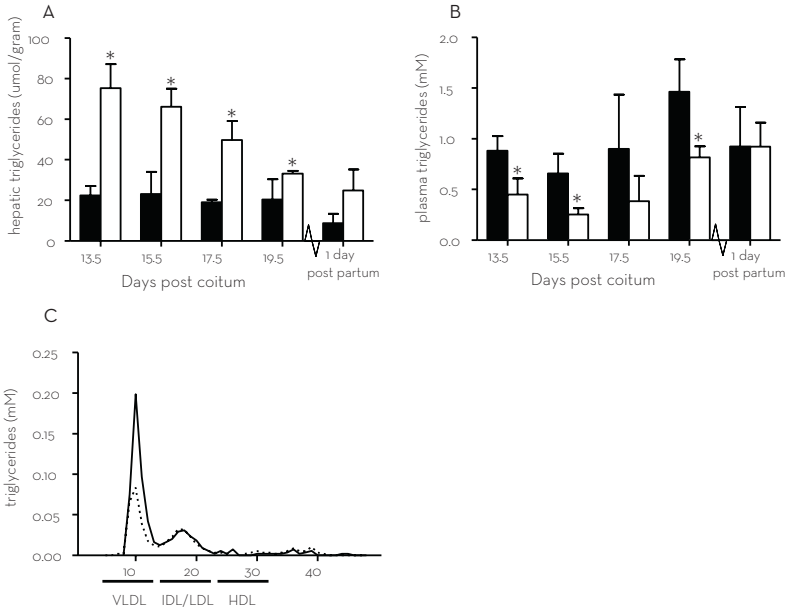

Figure 3. (**A**) Hepatic and (**B**) plasma triglyceride levels on several days of the gestation of dams, and (**C**) triglyceride levels in FPLC samples of pooled plasma (n=6 per group) of dams at day 19 of gestation. Black bars or black line, control mice; open bars or dotted line, mice receiving 0.015% T0901317 in the diet. Values represent the mean ± SD. n=6 dams per group; *, $p < 0.05$ for treated vs control.

murine plasma and revealed that the increase in plasma TG reflects elevated IDL/LDL levels (Figure 4c). Consistent with gene expression data, in Lxra knockout fetuses T0901317 administration increased hepatic triglyceride concentrations to a lower extent compared to wild type fetuses (~70% vs. ~150%, respectively; each $p < 0.01$; Figure 4d). Oil-Red-O staining for neutral lipids confirmed lipid accumulation in treated wild-type pups at day 13.5, 15.5, 17.5 and 19.5 of gestation and at day one after delivery. Livers of control and T0901317-treated wild-type fetuses on day 17.5 and day one after delivery are shown in Figure 5a to d).

T0901317 treatment increases absolute synthesis and total pool of oleate in pregnant dams and fetuses on E19.5

Using a ^{13}C-acetate method, we quantified to what extent T0901317 treatment affected the *de novo* hepatic synthesis of palmitate, stearate and oleate and the hepatic pool size of these fatty acids in dams and fetuses. T0901317 treatment increased total *de novo* synthesis and chain elongation of oleate (C18:1) in dams and fetuses at day 19.5 of gestation (Figure 6a and b), but did not significantly altered palmitate (C16:0) or

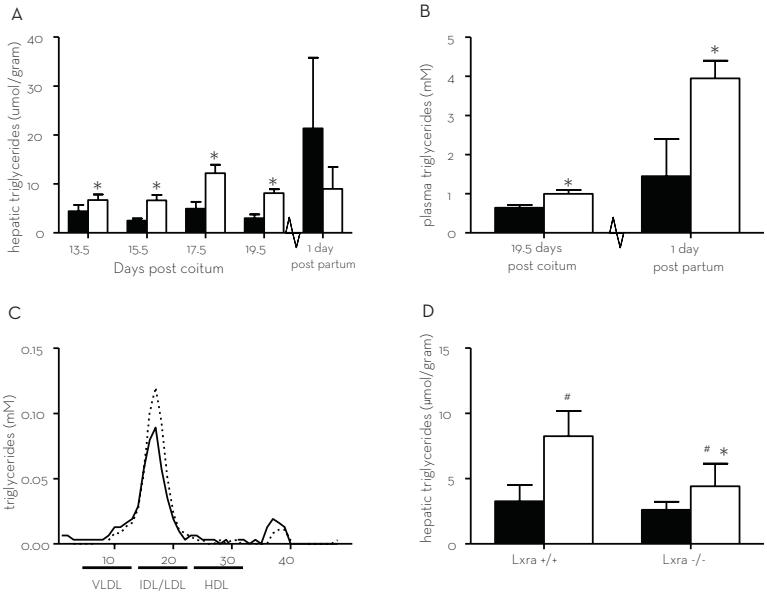

Figure 4. (**A**) Hepatic and (**B**) plasma and triglyceride levels on several days of the gestation of fetuses. (**C**) Triglyceride levels in FPLC samples of pooled plasma (n=20 per group) of 19 dpc fetuses. (**D**) Hepatic triglyceride concentrations in wild type and *Lxr* knockout fetuses on day 19 of gestation upon treatment of the heterozygote mother with T0901317. Black bars or black line, control mice; open bars or dotted line, mice receiving 0.015% T0901317 in the diet. Values represent the mean ± SD. n=6 dams per group; *, $p < 0.05$ for treated vs control.

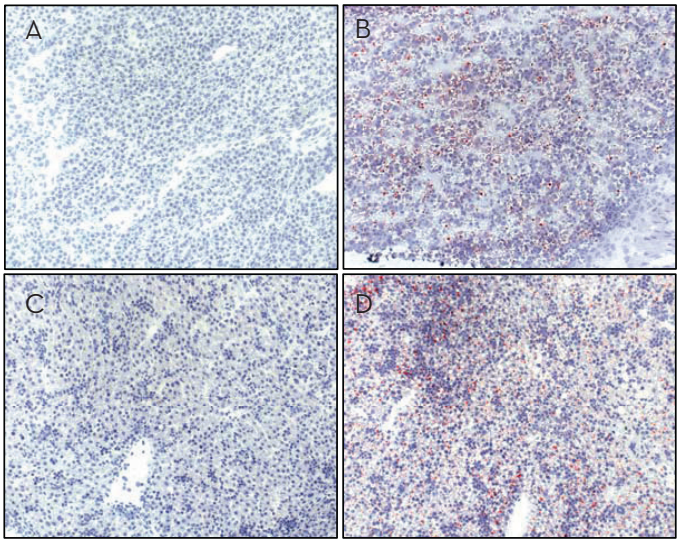

Figure 5. Oil-Red-O (ORO) staining for neutral fat in developing fetal liver on several days of gestation upon treatment of the mother with T0901317. No staining was observed in liver sections from fetuses from dams receiving the control diet (**A**, 17.5dpc and **C**, 1dpp, respectively). Oil-red-O staining was found in liver sections from fetuses from dams treated with T0901317 (**B**, 17.5 dpc and **D**, 1 day post partum, respectively). Original magnification 20x.

stearate (C18:0) synthesis. Chain elongation of stearate was reduced in treated fetuses. In dams, T0901317 administration increased the total hepatic pool of oleate and it increased both the oleate and the palmitate pool in fetuses (Figure 6c and d).

In utero Lxr activation does not influence basal insulin signaling

To test the hypothesis that Lxr activation influences carbohydrate metabolism in the wild-type mouse, via induction of gene expression of *Srebp1c* and *Chrebp* and their subsequent target genes, we measured hepatic expression levels of several genes involved in carbohydrate metabolism in fetuses from untreated and treated wild-type dams. Treatment of dams with T0901317 had no influence on hepatic gene expression levels of *G6pc*, *Pck1*, *Gck*, *Chrebp*, *Pklr* or *Insr* in the fetuses (Suppl. Fig 3a to f). Since gene expression levels do not necessarily reflect physiological changes, we examined whether T0901317 treatment influenced insulin signaling in offspring. We performed an adapted insulin tolerance test in 1-day old pups of treated and untreated dams. Intraperitoneal injection of 1-day old pups with 0.75U/kg insulin lowered blood glucose levels by 50% compared to pups injected with saline. Prenatal T0901317 treatment of the dams did not influence this insulin response, compared with controls.

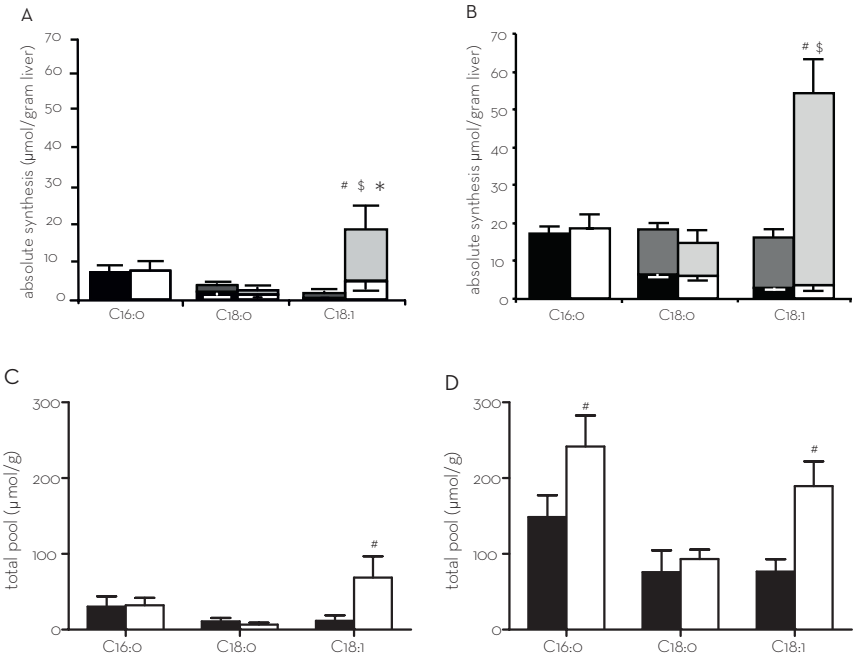

Figure 6. Absolute de novo lipogenesis and chain elongation, and total pool size of several fatty acids in livers of dams and fetuses at day 19 of gestation. (**A**) Absolute synthesis in dams; (**B**) absolute synthesis in fetuses; (**C**) total pool of fatty acids in dams; (**D**) total pool of fatty acids in fetuses. C16:0 (palmitate), C18:0 (stearate) and C18:1 (oleate) fractions were analyzed and calculated as described in the Materials and Methods section. Black bars: de novo lipogenesis in control animals; Open bars: de novo lipogenesis in T0901317 treated animals; dark grey bars: chain elongation in control animals; light grey bars: chain elongation in T0901317-treated animals. Values represent the mean ± SD. n=4 fetuses from 3 dams in the T0901317-treated group; n=4 fetuses from 8 dams in the control group; #, $p < 0.05$ for total new synthesis (A and B) or total pool (C and D) in treated animals versus controls; *, $p < 0.05$ for de novo lipogenesis in treated animals versus controls; $, $p < 0.05$ for chain elongation in treated animals versus controls.

Long-term effects of prenatal Lxr activation in offspring into adulthood

We determined whether T0901317 treatment of the dam had long-lasting effects in the fetuses, i.e., into adulthood. Previous studies have indicated that a perinatal programming phenotype may only become apparent in adulthood upon exposure to a metabolic challenge. We administered T0901317 from day 10 of gestation until day 1 after delivery to C57BL/6J females via the diet (0.015% w/w in chow). Nest sizes were comparable in the two treatment groups, with an average of 6 pups per nest. After weaning, offspring received chow until 6 weeks of age; subsequently, offspring received either chow or a semi-synthetic Western-type diet (HFHC) containing 15% (w/w) cacao butter and 0.25% (w/w) cholesterol for two weeks.

Body weight, liver weight and liver weight to body weight was previously described

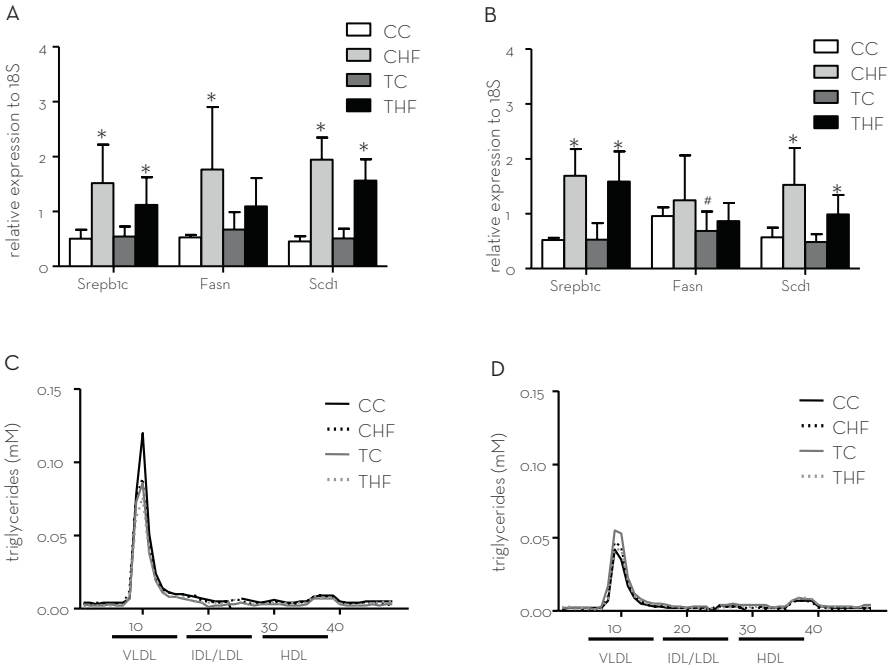

Figure 7. Changes in relative hepatic gene expression in offspring of mice fed chow containing 0.015% T0901317 or control chow during gestation, and FPLC profiles of this offspring. The offspring received either chow or a high fat/high cholesterol diet from 6 till 8 weeks of age. (**A**) Gene expression in male offspring, (**B**) gene expression in female offspring. (**C**) FPLC profile of male offspring, (**D**) FPLC profile of female offspring. Open bars and black lines: control offspring of control mice, light shaded bars and black dotted line: high fat/high cholesterol offspring of control mice; dark shaded bars and grey lines: control offspring of T0901317-fed mice; black bars and grey dotted lines: high fat/high cholesterol offspring of T0901317-fed mice. Values represent the mean ± SD. n=6; * $p < 0.05$ for high fat vs control, #, $p < 0.05$ for T0901317 vs control during gestation.

(27) and was similar in all male and in all female offspring. In chow-fed offspring, gene expression levels of Srebp1c, Fasn and Scd1 were not different between pre-treated and untreated male offspring on control diet (Figure 7a). Control-fed females from T0901317-treated dams however, showed lower Fasn expression levels (-30%) compared to untreated females (Figure 7b). Male offspring on control diet from treated dams had ~42% higher hepatic triglyceride levels than male offspring on control diet from untreated dams, while female offspring on control diet from treated dams had ~42% *lower* hepatic triglyceride levels than female offspring on control diet from untreated dams (Table 1). Plasma triglycerides in male and females were not influenced by T0901317 pretreatment (Table 1), although FPLC analysis showed that control male offspring receiving CON diet had slightly higher VLDL levels than other male offspring groups (Figure 7c). No change in triglyceride FPLC profiles was seen between the different female offspring groups (Figure 7d).

Table 1. Parameters of adult offspring

	Hepatic TG (umol/g)		Plasma TG (mM)	
	Control	**T0901317**	**Control**	**T0901317**
M CON	2.4 ± 0.6	3.4 ± 1.0 #	0.43 ± 0.08	0.34 ± 0.17
M HFHC	6.6 ± 1.2 *	6.7 ± 2.1 *	0.35 ± 0.15	0.33 ± 0.13
F CON	11.1 ± 2.3	6.4 ± 2.9 #	0.21 ± 0.05	0.25 ± 0.08
F HFHC	11.6 ± 4.5	9.5 ± 2.0	0.26 ± 0.11	0.19 ± 0.05

Parameters of C57BL/6J OlaHsd offspring of mothers that received either chow or chow supplemented with 0.015% T0901317. The offspring received either chow or a high fat/high cholesterol diet from 6 till 8 weeks of age. Data are mean ± SD. N=6; *, p<0.05 HF/HC vs CON #, p<0.05 T09 vs CON.

The high-fat diet increased hepatic gene expression levels of the lipogenic LXR target genes Srebp1c and Scd1 in all male and female offspring, regardless of exposure to T0901317 during gestation (Figure 7a and b). Interestingly, a gender specificity was observed concerning *Fasn* expression. *Fasn* expression levels were significantly *higher* in high-fat male offspring from untreated dams compared to control-fed male offspring from untreated dams (+200%, p=0.017), while *Fasn* expression levels were *not upregulated* by the high fat diet in pretreated male offspring compared to pretreated control males (p=0.247). Similarly, females pretreated with T0901317 showed *no upregulation* of *Fasn* upon receiving the high fat diet. In all male offspring, hepatic triglyceride levels were increased in animals that received the high fat diet, independent from pretreatment with T0901317 (Table 1). The high fat diet did not lead to higher hepatic triglyceride levels in female offspring. Feeding the high fat diet did not significantly change plasma triglyceride levels or FPLC profiles in either males or females from chow-fed or T0901317-fed dams (Table 1, Figure 7b and c).

DISCUSSION

We previously showed that targeted treatment of pregnant mice with the synthetic LXR agonist T0901317 activates Lxr in the fetuses. In the present paper, we investigated whether *in utero* Lxr activation by T0901317 changes fatty acid and glucose metabolism in the fetus and in adult offspring. Our data show that this treatment results in acute effects on lipid homeostasis in dams and fetuses, but not on glucose homeostasis in fetuses, and in relatively minor but persistent consequences on fatty acid metabolism during adulthood.

Supplementation of the diet of the dam with T0901317 induced LXR target genes *Srebp1c*, *Acc1*, *Fasn*, and *Scd1* in the fetal liver, suggesting that T0901317 is transported across the placenta activates Lxr. We previously showed that maternal T0901317 administration did not influence fetal hepatic gene expression levels of *Lxra* or *b* (27). Our present results in *Lxra* knockout fetuses show that hepatic Lxr effects are mainly caused by *Lxra* and that Lxrb compensates about 50% of *Lxr* action in the absence of Lxra.

Lxr-specific effects on lipid metabolism in the mother and in the fetus are comparable to the effects seen in non-pregnant adult mice (21, 24). T0901317 administration increased hepatic triglyceride levels in dams, which was similar to studies in adult male mice (12, 14). Hepatic triglyceride accumulation decreased during gestation in treated dams, possibly by increased transport from the liver, either to other tissues or to the rapidly growing fetus. The hepatic lipid concentrations in one-day old pups varied considerably within one group. Possibly, this is caused by the variation in feeding time of the pups. Prolonged fasting leads to hepatic lipid accumulation (26). Because we could not control the breast feeding times by the mother, some pups could have been fasted for a longer time than other pups, leading to higher liver triglyceride concentrations compared to pups that were fed prior to termination.

Grefhorst *et al*. found no difference in plasma triglycerides in wild-type male mice treated with T0901317 compared to controls (11). T0901317 treated dams showed a lowering of plasma triglycerides compared to controls, representing a decrease in VLDL levels. Since plasma triglycerides in fetuses and pups from treated dams were increased, this seems to imply that treated dams transfer more lipid to the fetus compared to controls. Further studies have to elucidate how LXR activation influences lipid transport to the fetus.

Treated dams and fetuses showed an increase in absolute hepatic synthesis of oleic acid. Based on the ^{13}C-acetate method used and the absence of increased palmitate and stearate synthesis, we interprete that this observation is due to increased palmitate elongation and subsequent desaturation by stearoyl-coenzyme A desaturase 1 (*Scd1*). Scd1 catalyzes the synthesis of monounsaturated fatty acids from saturated fatty acids (10, 10)). Scd1 is a target gene of Lxr, and its expression was increased in maternal and fetal liver upon T0901317 exposure.

Apart from the well-characterized role of Lxr in cholesterol metabolism, a potential role controlling glucose homeostasis via activation of Srebp1c and Chrebp has been suggested (9, 16), although this role remains controversial (8, 18). Using an adapted

insulin tolerance test in 1-day old pups, no effect of the T0901317 pre-treatment was found on glucose levels. Our results show that Lxr activation did not quantitatively influence the hepatic expression of any of the tested genes related to glucose metabolism in the fetus.

We next investigated whether Lxr activation by T0901317, which induced profound changes in lipid metabolism in perinatal life, also had long-lasting effects in lipid homeostasis, i.e. into adulthood. Female chow-fed offspring from T0901317-treated dams showed lower *Fasn* expression levels and lower hepatic triglyceride levels compared to untreated chow-fed female offspring. Strikingly, although no changes were seen on gene expression levels, in the male chow-fed offspring hepatic triglyceride levels were higher than in untreated chow-fed male offspring. Apparently, treatment with T0901317 *in utero* reduces hepatic storage of triglycerides in female offspring while it induces hepatic storage of triglycerides in male offspring.

The subtle changes in adult lipid metabolism caused by *in utero* Lxr activation could be enlarged by receiving a high-fat diet at adult age. As expected (5), short term (two weeks) high fat feeding led to higher expression levels of lipogenic genes in all offspring and higher hepatic triglyceride levels in male offspring. However, these effects were regardless of *in utero* treatment with T0901317. Apparently, receiving a high-fat diet for two weeks only eliminates rather than increases the gender-specific effects caused by *in utero* Lxr activation. Based on this observation it is tempting to speculate that long-term high-fat feeding would possibly override the relatively small effects of the prenatal treatment.

We conclude that Lxr activation by T0901317 treatment of pregnant mice induces immediate effects on lipid metabolism in dams and fetuses, including increases in fatty acid synthesis and triglyceride levels. Lxr activation did not significantly influence expression of carbohydrate metabolism related genes, nor the glucose response to insulin administration. Despite profound changes in lipid metabolism during fetal life, long-term effects on lipid metabolism appear to be rather mild, and not related to the lipid response to a high-fat diet.

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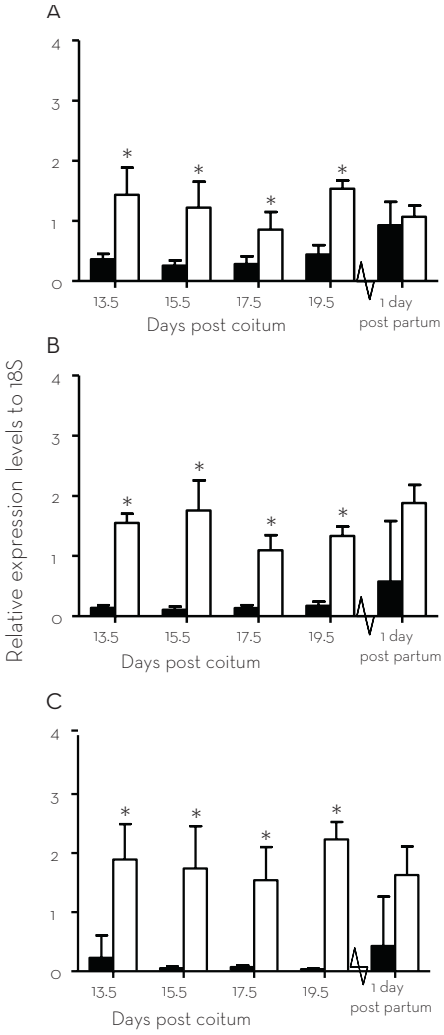
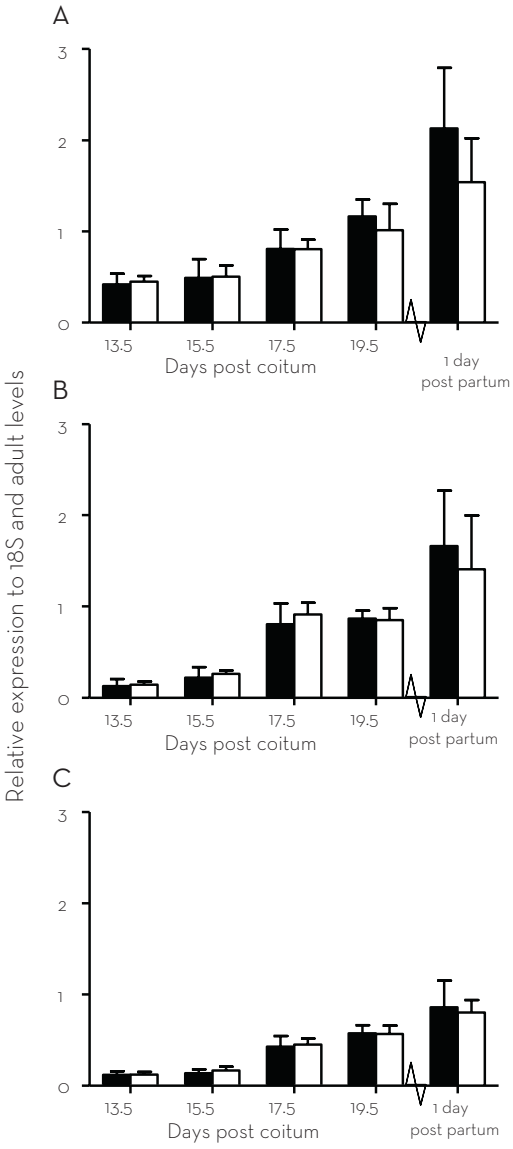
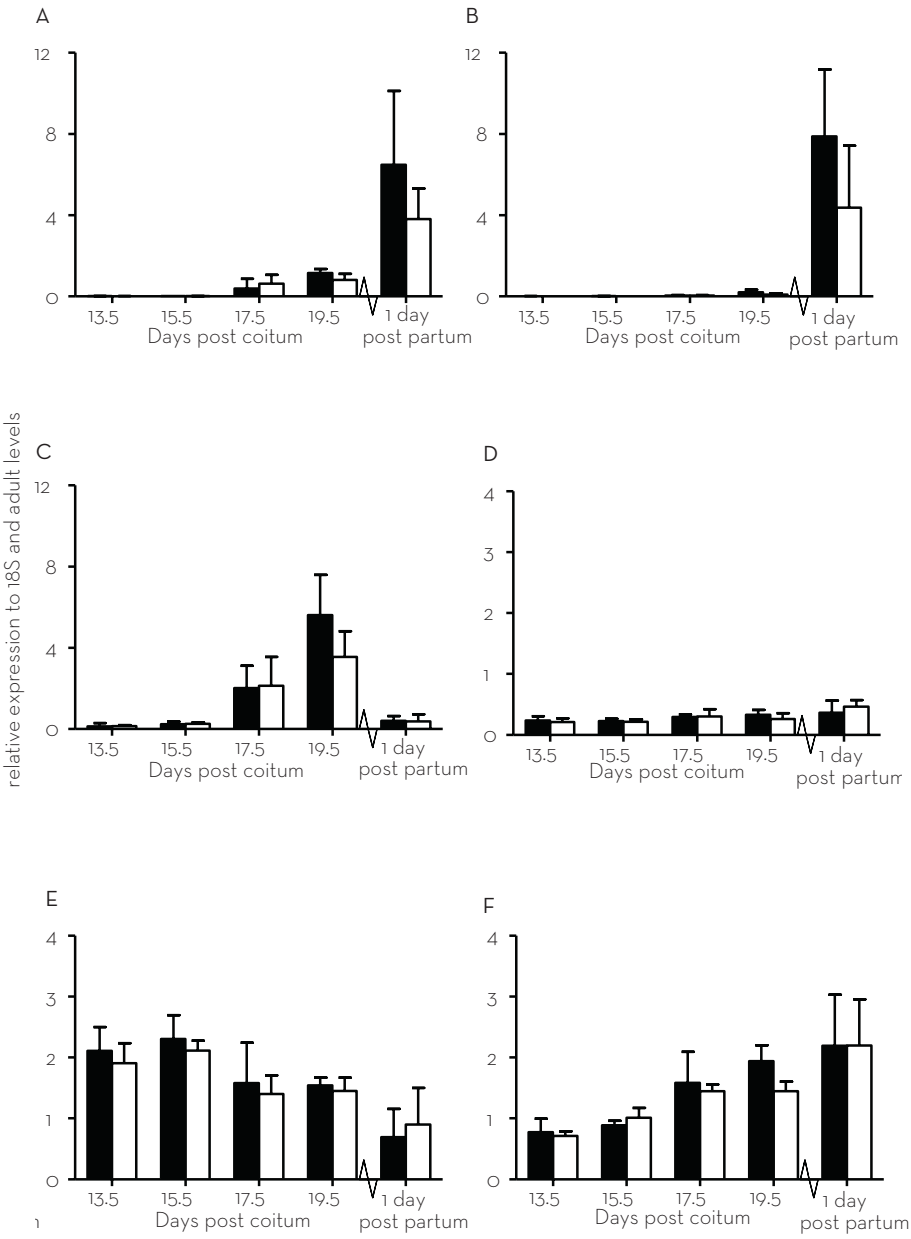
SUPPLEMENTAL FIGURES



Supplemental Figure 1. Changes in relative hepatic gene expression in genes encoding proteins involved in de novo lipogenesis in C57BL/6 dams on several days of gestation upon treatment of the dam with T0901317. Expression of maternal hepatic (**A**) *Srebp1c*, (**B**) *Fasn*, and (**C**) *Scd1*. Results were normalized to 18s mRNA levels. Black bars, control dams; open bars, dams receiving 0.015% T0901317. Values represent the mean ± SD. n=6; *, $p < 0.05$ for treated vs control.



Supplemental Figure 2. Changes in relative hepatic gene expression in C57BL/6 fetuses on several days of gestation upon treatment of the dam with T0901317. Hepatic expression levels of fetal (**A**) *Rxra*, (**B**) *PPARa* and (**C**) *Fxr* are displayed. Results were normalized to *18s* mRNA levels. Adult expression levels are arbitrarily defined as 1. Black bars, control fetuses; open bars, fetuses from dams receiving 0.015% T0901317. Values represent the mean ± SD. n=6; *, $p < 0.05$ for treated vs control.



Supplemental Figure 3. Hepatic gene expression of proteins involved in glucose metabolism in C57BL/6 fetuses on several days of gestation upon treatment of the dam with T0901317. Hepatic expression levels of fetal (**A**) *G6pc*, (**B**) *Pck1*, (**C**) Gck, (**D**) *Chrebp*, (**E**) *Pklr* and (**F**) *Insr* are displayed. Adult expression levels are arbitrarily defined as 1. Black bars, control fetuses; open bars, fetuses from dams receiving 0.015% T0901317. Values represent the mean ± SD. n=6; *, $p < 0.05$ for treated vs control.

CHAPTER

6

PREBIOTIC OLIGOSACCHARIDES AND THE ENTEROHEPATIC CIRCULATION OF BILE SALTS IN RATS

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ABSTRACT

Background and aim

Human milk contains prebiotic oligosaccharides which stimulate the growth of intestinal bifidobacteria and lactobacilli. It is unclear whether the prebiotic capacity of human milk contributes to the larger bile salt pool size and the more efficient fat absorption in infants fed human milk compared to formula. We determined the effect of prebiotic oligosaccharides on bile salt metabolism in rats.

Methods and results

Rats were fed a control diet, or an isocaloric diet containing a mixture of galactooligosaccharides (GOS), long chain fructooligosaccharides (lcFOS) and acidified oligosaccharides (AOS) for 3 wk. We determined synthesis rate, pool size and fractional turnover rate (FTR) of the primary bile salt cholate using stable isotope dilution methodology. We quantified bile flow and biliary bile salt secretion rates through bile canulation. Prebiotic intervention resulted in significant changes in fecal and colonic flora: the proportion of lactobacilli increased with 344% ($p<0.01$) in colon content and with 139% ($p<0.01$) in feces compared to the control group. The number of bifidobacteria also increased with 366% ($p<0.01$) in colon content and with 282% in feces after the prebiotic treatment. Furthermore, pH in both colon and feces decreased significantly with 1.0 and 0.5 pH point, respectively. However, despite this alteration of intestinal bacterial flora, no significant effect on relevant parameters of bile salt metabolism and cholate kinetics was found.

Conclusion

The present data in rats do not support the hypothesis that prebiotics naturally present in human milk contribute to a larger bile salt pool size or altered bile salt pool kinetics.

INTRODUCTION

The enterohepatic circulation of bile salts, the major constituents of bile, serves two important functions in the human body. Bile salts enhance the absorption of long chain saturated fatty acids and fat-soluble vitamins from the intestine. Furthermore, the enterohepatic circulation of bile salts promotes the excretion of lipophilic molecules via the bile into the feces (e.g. cholesterol and bilirubin) and is critically important for cholesterol homeostasis in the body. The dietary fat intake in infants is relatively high compared to the dietary fat intake in adults, whereas their bile salt pool size is lower (33). Efficient absorption of dietary fat is essential for optimal growth and development during infancy.

Human milk is the gold standard in infant feeding. One of the beneficial qualities of human milk involves the more efficient absorption of dietary fat compared to that from formula (34). Interestingly, the bile salt pool is larger in premature infants fed human milk compared to formula, but it has not been clarified whether this contributes to the mechanism underlying the more efficient fat absorption (34). Previously, we reported that the maturation of fat absorption in human neonates is functionally related to an increased capacity to absorb long chain fatty acids from the intestine, possibly due to developmental changes in bile salt composition and bile salt pool (28).

Prebiotics are non-digestible food ingredients that stimulate the growth and activity of specific bacteria in the colon (i.e. bifidibacteria and lactobacilli) (14). Oligosaccharides are a major constituent of human milk and have been demonstrated to increase the proportion of bifidobacteria and lactobacilli in the infant's colon (4, 9). The prebiotic galactooligosaccharides (GOS) and fructooligosaccharides (FOS) are known to increase the number of bifidobacteria and lactobacilli in the gut in both human and animal studies (3, 26, 32). Previous studies in rats, using the prebiotic substrate FOS, have shown an increase in the amount of intestinal bifidobacteria and lactobacillus (25, 31).

It is not known whether oligosaccharides, the prebiotic constituent of human milk, influence the bile salt metabolism and thereby play a role in the more efficient fat absorption of breast milk. Interactions between intestinal flora and bile salts are well known. The bacterial metabolism of bile salts in the intestinal lumen can involve deconjugation and dehydroxylation, converting primary bile salts such as cholate and chenodeoxycholate, into secondary bile salts (i.e. deoxycholate and lithocholate). Bacterial metabolism of bile salts is partly responsible for the fractional turnover rate of primary bile salts i.e. the portion of the pool that is newly synthesized per day. Bacterial metabolism may also influence the physiological activity of bile salts, since secondary bile salts are more hydrophobic than primary bile salts and therefore have a greater capacity to interact with dietary fat.

Recently we developed a method to quantify cholate fluxes in the enterohepatic circulation of experimental animals (19). This method is based on a stable isotope dilution methodology used in humans that was successfully downscaled to allow measurement of cholate fluxes in experimental animals (19). The enterohepatic circulation of

bile salts can be quantitatively characterized by specific kinetic parameters: the pool size (the amount of bile salts in the body), the FTR (the fraction of the pool that is newly synthesized each day), the synthesis rate, and, finally, the cycling time (the time it takes the cholate pool to undergo one full cycle in the enterohepatic circulation). In the present study, we determined the effect of dietary prebiotic oligosaccharides on the enterohepatic circulation of cholate, the major primary bile salt in rats.

MATERIALS AND METHODS

Animals and materials

Six week old male rats (Harlan Laboratories, Zeist, The Netherlands) were housed in a light- and temperature- controlled facility, with free access to tap water and to either the prebiotic diet or the control diet. Experimental protocols were approved by the Ethical Committee for Animal Experiments, Faculty of Medical Sciences (University Medical Center Groningen, The Netherlands). 2, 2, 4, 4 -Tetradeuterated cholic acid ([$^2H_4$]-CA; isotopic purity, 98%) was obtained from Isotec (Miamisburg, OH). All other chemicals and solvents used were of the highest purity commercially available. Animals received semi-purified AIN-93-based diets pressed into pellets (Research Diet Services, Wijk bij Duurstede, The Netherlands). In the prebiotic diet, the supplemented oligosaccharides were exchanged for the same amount of carbohydrates. The experimental diet was supplemented with GOS, lcFOS and AOS, in a dose of 7.65, 0.85 and 0.90 wt%, respectively (Table 1).

Table 1. Composition of the prebiotic diet and the control diet

Ingredients (g/kg)	Control diet (AIN-93G)	Prebiotic diet (GOS/lcFOS/AOS supplemented)
Cornstarch	397.5	397.5
Dextrinized cornstarch	132.0	65.9
Sucrose	100.0	72.3
Cellulose	50.0	50.0
Pure carbohydrate mix Numico*	-	93.8
Soybean oil	70.0	70.0

*85 g galactooligosaccharides (GOS), long-chain fructooligosacharides (lcFOS) (9:1), and 8.8 g acidified oligosaccharides (AOS).

Experimental procedures

Male Wistar rats (body weight 253 ± 11 g) were fed the control diet for three weeks as a run in, after which they were randomly assigned to the prebiotic or the control diet for another 3 wk (n=8 per group). Body weight was measured weekly; food intake and fecal production were measured during 72 h in the third week after randomisation. Three weeks after randomisation, relevant parameters of synthesis and enterohepatic circulation of cholate were determined using the previously mentioned stable isotope dilution technique (19). In short, 3.0 mg of $^{2}H_{4}$-CA in a solution of 0.5% $NaHCO_3$ in phosphate-buffered saline was slowly injected via the penile vein during isoflurane anesthesia. Blood samples were taken before injection and at 12, 24, 36, 48, 60, and 72 h after injection. Blood samples (300 µl) were collected by tailbleeding under isoflurane anesthesia. Blood was collected in EDTA tubes and centrifuged to obtain plasma. After centrifugion (3,000 rpm for 10 min at 4°C), plasma was stored at -20°C until analysis. At the last day of the experiment, rats were equipped with a catheter in the bile duct, and bile was collected for 30 min. Animals were euthanized by heart puncture, after which the liver and colon contents were collected and stored at -80°C until analysis.

Analytical procedures

Plasma alanine transaminase, aspartate transaminase, alkaline phosphatase, cholesterol and triglyceride concentrations were determined by routine laboratory techniques. Concentrations of biliary cholesterol and phospholipids were measured as described (13, 16), as were bile salt concentrations in feces and in bile (24). Fecal bile salt composition and fecal neutral sterols were analyzed as follows: 50 mg of dried feces was boiled in 1 ml alkaline methanol (1M NaOH / Methanol, 1:3 vol/vol) at 80°C for 2 h after addition of 50 nmol 5α-Cholestane and 14 nmol 7α,12α-dihydroxy-5β-cholanic acid as internal standard for neutral sterols and bile salts, respectively. After cooling down to room temperature, neutral sterols were extracted by using 3 x 3 ml of petroleum ether, boiling range 60-80 °C. The residual sample was diluted 1: 9 with distilled water. A sample (100 µl) of the solution was subjected to an enzymatic total bile salt measurement (24). The residual sample was used for bile salt isolation by reversed phase solid phase (C18) extraction (24, 30). The eluate was evaporated to dryness, and bile salts were derivatized to the methyl ester–trimethylsilyl derivatives for gas chromatography analysis. The extracted neutral sterols were derivatized to the trimethylsilyl derivatives applying the same procedure that was used for bile salts. Bile salt composition of prepared bile samples, fecal samples and neutral sterol composition of prepared feces samples were determined by capillary gas chromatography on an Agilent gas chromatograph (HP 6890) equipped with a 25 m x 0.25 mm CP-Sil-19 fused silica column (Varian, Middelburg, The Netherlands) and a flame ionization detector. The conditions were as follows: Injector temperature 280 °C; pressure 16.0 psi; column flow constant at 0.8 ml/min; oven temperature program: 240 °C (4 min), 10 °C/ min to 280 °C (27 min); detector temperature 300 °C. Hepatic concentrations of triglycerides and cholesterol were measured using commercial kits (Wako Chemicals, Neuss, Germany and Roche Diagnostics, Mannheim, Germany)

after livers were homogenized. Pooled plasma samples were used for lipoprotein separation by fast-protein liquid chromatography (FPLC) on a Superose 6B 10/30 column (Amersham Biosciences). The concentration of triglycerides in the various fractions was determined using a commercial kit (Wako Chemicals).

Gas-liquid chromatography electron capture negative chemical ionisation mass spectrometry and calculations

Plasma samples were prepared for isotopic analysis of bile salts by gas chromatography mass spectrometry (GC-MS) as described (19; 27). Analyses were performed at the pentafluoro-TMS derivative using a Finnigan SSQ7000 Quadrupole GC-MS (Finnigan MAT, San José, CA). GC separation was performed on a 15 m x 0.25 mm column, 0.25 µm film thickness (AT-5MS, Alltech Associates, Deerfield, IL). The area ratio M4/M0 is calculated after computerized integration of peak areas of M4 CA and M0 CA in the mass chromatograms for m/z 627.3 and 623.3, by using LCQuan software (Finnigan MAT). Enrichment is defined as the increase of M4/M0 after administration of [$^2H_4$]-CA and expressed as the natural logarithm of the atom% excess (ln APE) value. The decay of ln APE over time was described by linear regression analysis. From this linear decay curve the FTR and pool size of CA were calculated. The FTR (day^{-1}) equals the slope of the regression line. The pool size (µmol/100 g body wt) is determined according to the formula: Pool size = (D x b x 100/e^a) –D, where D is the administered amount of label, b is the isotopic purity, and a is the intercept on the y axis of the ln APE-vs.-time curve. The CA synthesis rate (µmol·100 g body wt^{-1}·day^{-1}) is determined by multiplying pool size and FTR.

Enterohepatic cycling time

The cycling time of the enterohepatic circulation is the time it takes for a bile salt to undergo one full cycle. The cycling time for cholate can be estimated by dividing the cholate pool size by the biliary secretion rate of cholate (which was calculated using bile salt composition in bile and bile flow rates after bile canulation). The fraction of cholate lost per cycle was calculated by dividing fractional cholate synthesis rate by cholate cycling frequency.

Fecal flora composition

Frozen fecal or colon samples were thawed by adding 1 ml of MilliQ water and heating at 90 °C. The suspensions were subsequently homogenized and frozen overnight at −20 °C. The homogenized samples were thawed at room temperature, followed by DNA isolation using the NucliSense Isolation Extraction Kit (BioMerieux, Boxtel, The Netherlands). For the relative quantification of the genera *Bifidobacterium* and *Lactobacillus* in relation to the total bacterial load, a duplex 5′ nuclease quantitative real-time PCR assay was used (15).

Briefly, different primers and probes for the genus *Bifidobacterium* or *Lactobacillus* in combination with primers and probes for total bacterial were used in a temperature profile consisting of 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 60 °C for 1 min, run on ABI Prism 7700 PCR equipment (Applied

Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The relative amounts of the genus *Bifidobacterium* or *Lactobacillus* in the samples were calculated with respect to the total bacterial load according to Liu *et al.* (23), and expressed in percentages. All samples were analyzed in triplicate.

Statistics

Values represent means ± SD for the indicated number of animals per group. Differences between the two groups were determined by Student's t-test for normally distributed values, and Mann-Whitney exact 2-tailed U-test was used for nonnormally distributed data. $P < 0.05$ was considered significant. Analysis was performed using SPSS 12.0 for Windows software (SPSS, Chicago, IL).

RESULTS

Animal characteristics

Animals fed the prebiotic diet or the control diet, were comparable in body weight, growth, fecal production and food intake (Table 2). The prebiotic diet significantly elevated serum lathosterol, an intermediate in the cholesterol synthesis pathway, whereas plasma concentrations of cholesterol, triglycerides, alanine transaminase, aspartate transaminase and alkaline phosphatase were not altered upon treatment (Table 2).

Table 2. Animal characteristics, plasma and hepatic parameters of lipid metabolism and liver function after feeding rats a prebiotic or a control diet

	Control diet	Prebiotic diet
Animal characteristics		
Body weight (g)	252 ± 12	254 ± 11
Body weight at termination (g)	456 ± 35	476 ± 28
Feces (wet) (g/day/100 g body weight)	0.4 ± 0.1	0.4 ± 0.1
Food intake (g/24 h)	27.9 ± 1.7	26.7 ± 1.6
Hepatic parameters		
Liver weight (g)		
- absolute	13.8 ± 1.4	14.8 ± 1.7
- relative (% of bodyweight)	3.0 ± 0.2	3.1 ± 0.2
Triglycerides (nmol/mg liver)	14.3 ± 5.4	14.7 ± 5.6
Cholesterol (nmol/mg liver)	5.4 ± 0.7	5.7 ± 1.7
Plasma parameters		
Alanine transaminase (units/l)	46 ± 10	45 ± 7
Aspartate transaminase (units/l)	153 ± 75	155 ± 49
Alkaline phosphatase (units/l)	6 ± 7	2 ± 1
Cholesterol (mmol/l)	1.8 ± 0.3	1.9 ± 0.5
Triglycerides (mmol/l)	1.4 ± 0.5	1.9 ± 1.0
Lathosterol (·mol/l)	0.7 ± 0.1	1.3 ± 0.5*

All values are expressed as means ffl standard deviation. * p < 0.05; n = 8 per group.

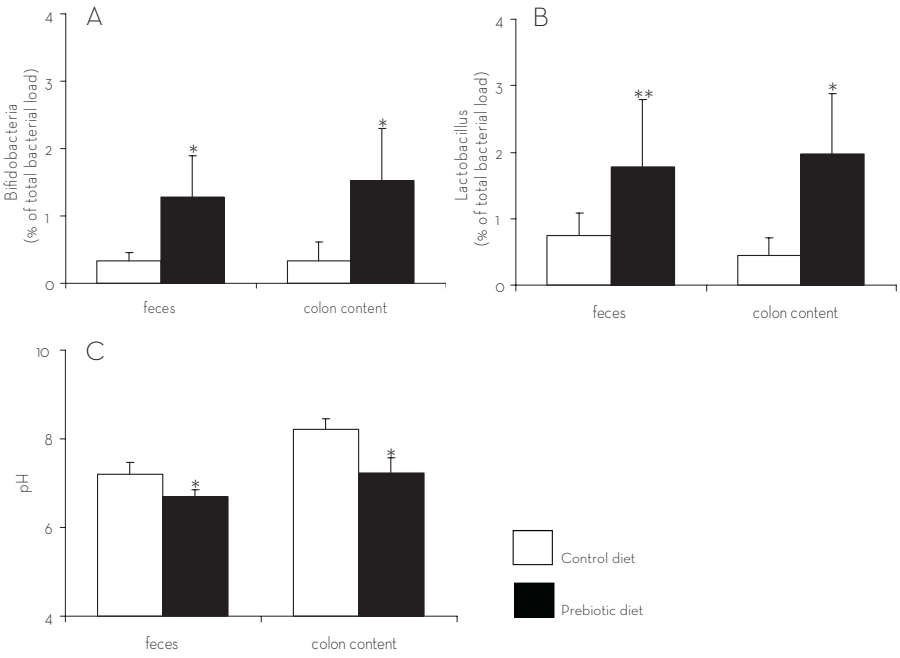

Figure 1. Effect of prebiotic treatment (solid bars) on percentage of total bacterial load of bifidobacteria (**A**) and lactobacilli (**B**) in feces and colon content compared with control rats (open bars). Effect of prebiotic treatment on pH in feces and colon content (**C**). Prebiotic-treated rats are significantly different from controls. Data are expressed in means ± SD of n = 8 per group; * $P < 0.05$; ** $P < 0.01$.

Effect of prebiotic treatment on the composition of intestinal flora

Figure 1 shows that the prebiotic diet had a prominent bifidogenic effect. After 3 wk the relative numbers of bifidobacteria and of lactobacilli were significantly higher in colon content and in feces in rats fed the prebiotic diet compared with controls. The proportion of lactobacilli increased 344% ($P < 0.01$) in colon content and 139% ($P < 0.05$) in feces compared with rats on control diet (Fig. 1).
Prebiotic treatment also increased the contribution of bifidobacteria 366% ($P < 0.01$) in colon content and 282% ($P < 0.01$) in feces compared with the control group (Fig. 1). The prebiotic diet significantly lowered pH in both colon content and feces with 1.0 and 0.5 pH point, respectively (Fig.1).

Effect of prebiotic intervention on parameters of the enterohepatic circulation of bile salts

Bile flow and biliary secretion rates of bile salts, phospholipids and cholesterol were similar between the groups (Fig. 2), as were biliary bile salt composition (Fig. 3), fecal neutral sterol composition and fecal bile salt excretion (Fig. 4). Prebiotic treatment

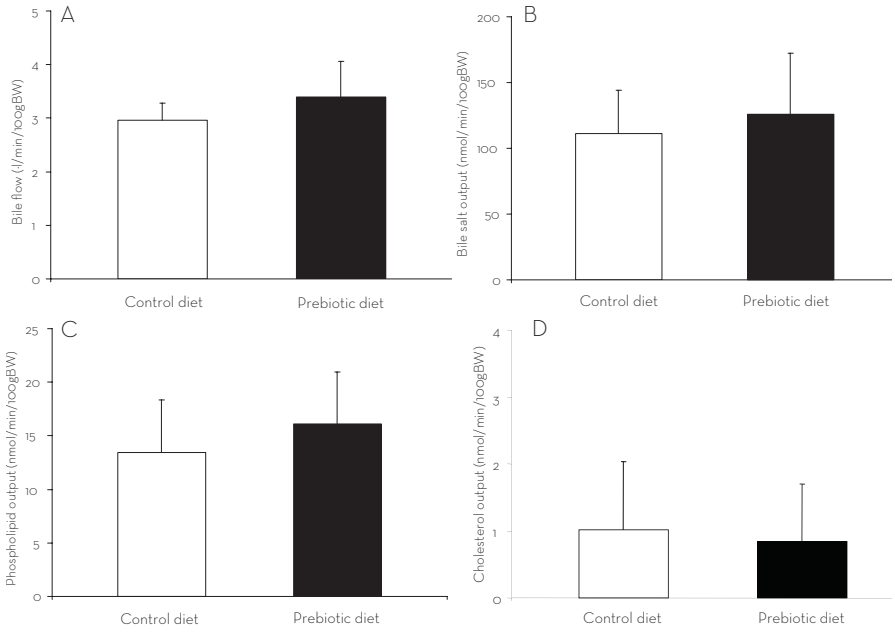

Figure 2. Effect of prebiotic diet (solid bars) on bile flow (**A**), total bile salts (**B**), phospholipids (**C**), and cholesterol (**D**) in bile compared with the control rats (open bars). Data are expressed in means ± SD of n = 7 or 8 per group. No significant differences between prebiotic-treated rats compared to controls in any of the parameters. BW, body weight.

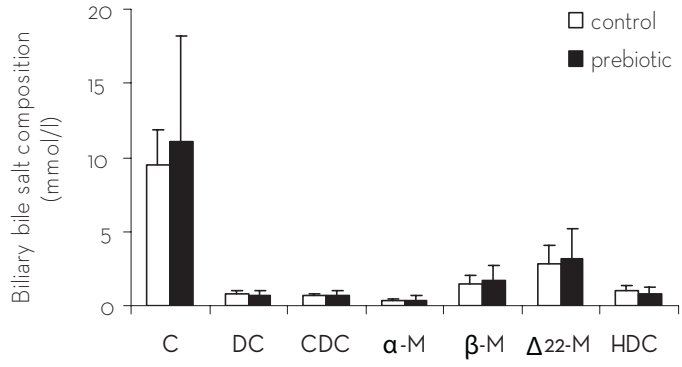

Figure 3. Effect of prebiotic treatment (solid bars) on biliary bile salt composition compared with control rats (open bars). Data are expressed in means ± SD of n = 7 or 8 per group. No significant differences between prebiotic-treated rats compared with controls. C, cholate; DC, deoxycholate; CDC, chenodeoxycholate; α-M, α-muricholate; β-M, β-muricholate; Δ22-M, Δ22-muricholate; HDC, hyodeoxycholate.

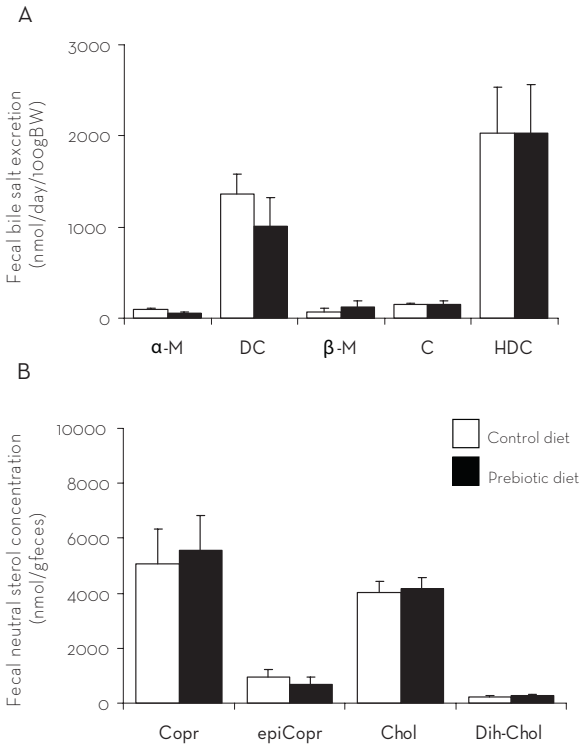

Figure 4. Effect of prebiotic treatment on fecal bile salt excretion (**A**) and fecal neutral sterol excretion (**B**). Prebiotic diet (solid bars), control diet (open bars). Values are expressed in means ± SD, and n = 8 per group. No significant differences between prebiotic-treated animals compared with controls in any of the parameters. α-M, α-muricholate; DC, deoxycholate; β-M, β-muricholate; C, cholate; HDC, hyodeoxycholate. Copr, coprostanol; epiCopr, epicoprostonol; Chol, cholesterol; Dih-Chol, dihydrocholesterol.

Table 3. Pool size, fractional turnover rate and synthesis rates of cholate

	Prebiotic diet	Control diet
Fractional turnover rate (day $^{-1}$)	0.28 ± 0.04	0.26 ± 0.07
Pool size (μmol/100 g body wt)	8.11 ± 1.82	8.80 ± 2.57
Synthesis rate (μmol/100 g /day)	2.25 ± 0.55	2.23 ± 0.72

Values are obtained by [$^2H_4$]-CA isotope enrichment measurements in plasma of rats fed the prebiotic diet or the control diet, as detailed in Materials and Methods. Values are expressed in means ± SD (n = 7-8 rats per group). No significant differences between prebiotic treated rats compared to controls in any of the parameters.

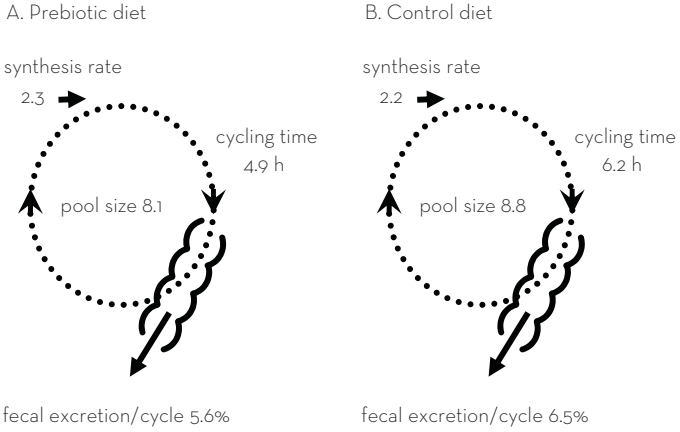

Figure 5. Cholate synthesis rate, pool size and fecal excretion in rats fed a prebiotic diet (**A**) or a control diet (**B**); (n=7 or 8 per group). The cycling time of the enterohepatic circulation is the time it takes for a bile salt to undergo one full cycle. The cycling time for cholate can be estimated by dividing the cholate pool size by the biliary secretion rate of cholate. The biliary secretion rate of cholate was similar in both groups (data not shown). The fraction of cholate lost per cycle was calculated by dividing fractional cholate synthesis rate by cholate cycling frequency. No significant differences between prebiotic treated rats compared to controls in any of the parameters.

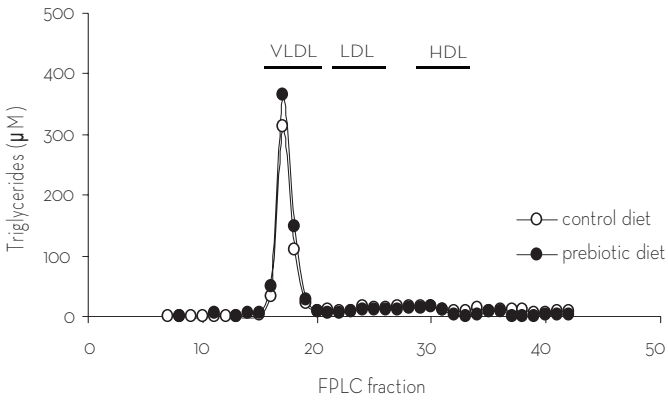

Figure 6. Distribution of triglycerides in plasma lipoprotein fractions in prebiotic treated rats (closed symbols) and controls (open symbols). Lipoproteins were separated by using fast protein liquid chromatography (FPLC). Plasma from all individual rats per group (n = 8 per group) was pooled and subjected to gel filtration using Superose 6 columns. Triglyceride concentration in each fraction was measured as described in *Analytical procedures*. The amount of triglycerides in the separated fractions is comparable in the prebiotic treated rats and controls.

did not affect the enterohepatic circulation of bile salts. Kinetic parameters of cholate, the main bile salt in rodents, were not significantly altered by the prebiotic diet. Cholate synthesis rate, pool size and FTR, as well as fecal excretion, were similar in both groups (Table 3 and Fig. 5).

Effect of prebiotic treatment on hepatic and plasma lipids

The prebiotic treatment did not affect hepatic and plasma concentrations of cholesterol and triglycerides (Table 2). Upon FPLC separation of plasma lipoproteins, the distribution of triglycerides in the different fractions was comparable in prebiotic-treated rats and controls (Fig. 6).

DISCUSSION

We investigated whether prebiotic treatment and subsequent alteration of the intestinal bacterial flora affects the enterohepatic circulation and composition of bile salts in rats. The results show that prebiotic treatment significantly increased the relative contributions of both lactobacilli and bifidobacteria in the colon and the feces of rats. Furthermore, pH in both colon and feces significantly decreased after feeding rats a prebiotic diet. This prebiotic effect, however, did not significantly affect bile flow or bile salt composition, or the synthesis and enterohepatic circulation of cholate.

The liver parenchymal cells synthesize the primary bile salts cholate and chenodeoxycholate. Primary bile salts are conjugated before their secretion into bile (in rats predominantly to taurine). Upon entering the proximal small intestine, conjugated bile salts stimulate the emulsification and absorption of dietary fat. Conjugated bile salts are efficiently reabsorbed in the terminal ileum, mediated by the apical sodium-dependent bile salt transporter (Asbt) and the basolateral transporter Ost α/β. Primary bile salts that escape absorption in the terminal ileum may be deconjugated by the bacterial flora in the colon and then undergo (7-alpha-) dehydroxylation resulting in the formation of secondary bile salts such as deoxycholate and lithocholate. Various intestinal bacterial species, including lactobacilli and bifidobacteria, have the capacity to metabolize bile salts (10). The secondary bile salts are partly reabsorbed by passive diffusion or are lost via the feces. Intestinal reabsorption of secondary bile salts, dependent on passive diffusion, is less efficient than the Asbt-mediated transport of primary bile salts.

Under steady-state conditions, fecal bile salt loss is compensated by synthesis of primary bile salts in the liver. In theory, modification of intestinal bacterial flora and subsequent altered distribution of bile salts between primary and secondary type could influence intestinal absorption and fecal excretion of bile salts and therefore affect kinetic parameters of bile salt homeostasis. In preterm infants fed human milk, the bile salt pool size is larger compared with formula-fed infants, indicating that dietary factors affect the pool size (34). Also, fecal bile salt concentrations are higher in infants fed human milk and contain a smaller fraction of secondary bile salts, com-

pared with those fed formula (16). Breast-fed infants have an intestinal bacterial flora which is characterized by relatively high amounts of bifidobacteria and lactobacilli (4, 17, 20, 29). Because of the fermenting ability of these bacteria, a lower stool pH is found in infants fed human milk compared with formula-fed infants. The prebiotic capacity of human milk induces a different intestinal environment, which could, in theory, alter the enterohepatic circulation of bile salts. Our present data show that a prebiotic treatment altered the intestinal bacterial flora in adult rats, indicated by a significant induction of colonic and fecal bifidobacteria and lactobacilli, and a significant decrease in fecal pH. Interestingly, however, the prebiotic diet did not affect either the bile salt composition or the kinetic parameters of the enterohepatic bile salt circulation (Fig. 5).

Theoretically, these observations could be due to a different response to a prebiotic diet in rats compared with other species. The present prebiotic treatment resulted in lactobacilli, accounting for 2.0% and 1.8% of total bacterial load in colon and feces, respectively. The same diet (AIN-93G) supplemented with 10 wt% GOS/lcFOS (ratio 9:1) resulted in 7.5 % lactobacilli in feces of mice (32). Haarman *et al.* (15) found fecal flora to contain 4.4 % lactobacilli in infants receiving a formula supplemented with 0.8 g/100 ml GOS/lcFOS (ratio 9:1) for 6 wk, similar to values observed in breast-fed infants. Despite the still limited absolute contribution of lactobacilli, the deceased colonic and fecal pH indicates that a physiological respons is achieved.

However, the bacterial flora of infants fed human milk contains > 60 % bifidobacteria within 1 wk after birth (17). The addition of a GOS/FOS mixture to infant formula resulted in an increase of fecal bifidobacteria of almost 60 % in healthy infants (20). Vos *et al.* (33) used an identical prebiotic diet (10 wt% GOS/lc-FOS; ratio 9:1) in a murine model, resulting in 40% bifidobacteria of total bacterial load in fecal samples. Surprisingly, the present study showed a contribution of bifidobacteria of 1.3% and 1.5% in feces and colon content, respectively, after prebiotic treatment. Thus, although the presently used diet resulted in an increase of lactobacillus in the same range as mice and human, and a significant decrease in fecal and colonic pH, the less pronounced bifidogenic response could theoretically contribute to the observed lack of effect on the enterohepatic circulation of bile salts.

It should be realized that the situation studied in the present rat model differs in several aspects from the physiology in human infants. Besides the less pronounced prebiotic respons, it cannot be excluded that the absence of a gallbladder in rats influences the dynamics of the enterohepatic circulation of bile salts. Furthermore, immaturity of intestinal absorption of nutrients (28) and bile salts (6) intervene with the enterohepatic circulation of bile salts, which may render the dynamics of bile salt metabolism in human infants not completely comparable to the presently studied rat model. Finally, only in rats, the primary bile salt chenodeoxycholate in rats is further metabolized to α-muricholic acid and β-muricholic acid, rendering the bile salt pool more hydrophilic compared with the human situation. In the present experiment, however, the amount of muricholic bile salts measured in bile did not exceed 25%. Nevertheless we are aware of the possibility that one or more of the aforementioned differences may limit the extrapolation of the present results to the human situation.

Besides the addressed differences in bile salt physiology between human infants and the presently used rat model, human milk contains high concentrations of cholesterol (2.6-3.9 mmol/L) compared with formulas (0.3-0.9 mmol/L) (5). The colonic flora of human infants develops its dehydroxylating capacity over the first months of life (2), whereas in the adult rat the dehydroxylating bacteria are well established. It is tempting to speculate that the larger bile salt pool size found in breast-fed infants could be related to the substantially higher dietary intake of cholesterol and not, based on our present observations, on differences in dietary oligosaccharides.

Enhanced conjugation of bile salts by bile hydrolase-producing bacteria such as lactobacilli could, in theory, be expected to increase the amount of deconjugated bile salts and the subsequent fecal excretion hereof. In the present study, both amount and composition of fecal bile salts were unaffected upon prebiotic treatment. We hypothesize that this can be due to the relative low contribution of lactobacillus and bifidobacteria to the total intestinal deconjugating capacity. Bile salt hydrolase is apart from lactobacillus and bifidobacteria, also detected in other bacterial species (12). Furthermore, efficient passive absorption of deconjugated bile salts may have masked a deconjugating enhancing effect, of prebiotic treatment.

The presently applied methodology allowed determination of bile salt kinetic parameters in a physiologically uncompromised animal model. The parameters of cholate kinetics determined in this experiment were in line with previous experiments performed in rats using the same method (19). Under steady-state conditions, the bile salt pool size is regulated by hepatic de novo synthesis of bile salts and by the efficiency by which bile salts are reabsorbed in the intestine. Under steady-state conditions, the amount lost via the feces is equal to the amount of bile salts newly synthesized by the liver. A difference neither in cholate synthesis rate nor in fecal bile salt excretion was observed, indicating that prebiotic treatment does not affect the two important rate-limiting parameters of the enterohepatic circulation. We also observed that the fecal excretion of chenodeoxycholate-derived metabolites (muricholate, hyodeoxycholate) was not altered either, suggesting no effect of prebiotic treatment on the synthesis of the other primary bile salt, chenodeoxycholate.

Apart from an important role in absorption of dietary lipids, bile salts are critically involved in cholesterol homeostasis in the body. Prebiotic substances are suggested to have a plasma lipid-lowering effect in both animal and human studies. Serum cholesterol concentrations in bottle-fed infants decreased as the number of lactobacilli in their stools increased (18). Possible mechanisms could be modulation of the intestinal bacterial flora with production of short-chain fatty acids inhibiting hepatic fatty acid or cholesterol synthesis or alteration of the bacterial bile acid deconjugating capacity and a subsequent altered fecal bile salt excretion (8). Studies in rats have consistently shown a decrease in plasma triglycerides after a diet supplemented with inulin and oligofructose (reviewed in Ref. 7). Data on plasma cholesterol, however, are less straightforward. A 10 wt% oligofructose supplemented diet decreased serum cholesterol concentrations in rats in one study (11), but the addition of oligofructose to the diet of lean rats did not induce a decrease in plasma cholesterol levels in another study (21). Human studies investigating the effect of oligofructans on lipid me-

tabolism have shown variable results (reviewed in Ref. 8). Our prebiotic diet did not significantly affect plasma concentrations of cholesterol in rats, whereas it increased the serum lathosterol concentration. Lathosterol is an intermediate in the cholesterol synthesis pathway, which could suggest that cholesterol synthesis was enhanced during prebiotic treatment. However, cholesterol synthesis was not determined in this experiment, and no other indications of increased cholesterol synthesis were obtained. For example, the hepatic mRNA expression of HMG-CoA reductase, encoding for the rate limiting enzyme of cholesterol synthesis, was not upregulated by prebiotic treatment (data not shown). The presently used diet did not affect serum triglycerides or hepatic triglycerides. Also, the amount of triglycerides in the VLDL fraction of lipoproteins was comparable in prebiotic-treated and control rats, indicating that the present treatment did not affect hepatic VLDL composition or triglyceride distribution over the various plasma lipoproteins (Figure 6). Rat studies on lipid metabolism or bile salt metabolism using diets supplemented with the presently used GOS/lcFOS, have, to the best of our knowledge, not been performed. In accordance with the present results, a study recently performed in human infants showed unaffected plasma levels of cholesterol and of triglycerides in infants fed a formula supplemented with identical prebiotic substances in the same ratio used in the present study (1).
In summary, we conclude that feeding rats a prebiotic diet induces modification of the intestinal flora, and decreases the intestinal pH in the colon and the feces. However, the prebiotic diet does not influence the metabolism of bile salts in rats. The present data in rats do not support the hypothesis that prebiotics naturally present in human milk contribute to a larger bile salt pool size or altered pool kinetics.

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CHAPTER 7 DISCUSSION

OVERVIEW

Epidemiological studies have indicated that nutrition during the fetal and/or the neonatal period affects adult health. Over the last decades, the different concepts regarding the relationship between early events and health in adulthood have emerged into the predictive adaptive response hypothesis as postulated by Gluckman et al (1). This hypothesis defines that, based on the prenatal environment, the fetus reacts to its expected postnatal environment i.e. if the prenatal environment of the fetus "predicts" a nutritionally poor postnatal environment, the fetus adapts to a developmental path appropriate for a lower postnatal nutritional supply (2). These adaptations are appropriate if the expected postnatal environment is in accordance with the actual one. Mismatch between the expected and the actual environment, however, is suggested to be a major determinant in the development of (features of) the metabolic syndrome. The metabolic syndrome is a cluster of metabolic and cardiovascular risk factors including insulin resistance, diabetes, hypertension, obesity, and dyslipidemia (3). Dyslipidemia (elevated triglycerides and LDL-cholesterol) contributes to the development of diabetes and of non-alcoholic fatty liver disease (NAFLD) and is, both direct and indirect, a risk factor for atherosclerosis. Epidemiological studies in humans and studies in several animal models have shown effects of early nutritional conditions on lipid metabolism in adulthood.

The mechanism behind the relation between fetal events and adult disease has remained unclear to a large extend. Insight in the mechanism behind metabolic programming represents a promising area in which preventive (nutritional) strategies could be developed to maintain or improve future health. In this thesis we investigated the effects of different fetal and neonatal nutritional conditions on lipid and bile acid metabolism in rodents and in human infants using stable isotope techniques.

Protein restriction during gestation in mice

Intrauterine growth restriction has been associated with an increased risk to develop features of the metabolic syndrome in adulthood (1, 4-13); a phenomenon termed "metabolic programming". To clarify mechanisms behind metabolic programming, different animal models have been developed (14, 15). A well-studied rodent model to investigate programming effects of intrauterine growth restriction is maternal dietary protein restriction during gestation (or fetal protein restriction). The purpose of this model is to mimic intrauterine malnutrition. Maternal protein restriction during gestation is most frequently studied in rats in which the model induced disordered regulation of lipid metabolism (16), high blood pressure (17, 18) and obesity and insulin resistance (19) in the adult offspring. Fewer protein restricted dietary studies have been performed in mice. Establishing the same model in mice, however, expands the possibilities of the model by allowing investigating the metabolic effects of intrauterine growth restriction in different genetically modified mice (for example, knock-out or transgenic).

A proposed mechanism behind metabolic programming is that of epigenetic modification which implies the alteration of gene expression levels without alterations in

the underlying DNA sequence. A widely studied example of epigenetic modification is that of DNA methylation which causes suppression of gene expression without altering the sequence of the silenced genes. Previous studies in mice performed in our laboratory showed that maternal gestational protein restriction increased the hepatic DNA methylation status of LXR (a key regulator of cholesterol and fatty acid metabolism) in the fetal offspring. Subsequently, the mRNA expression of LXR and its target genes involved in fatty acid and cholesterol metabolism was lowered (20). In this thesis, we tested if these epigenetic changes with corresponding alterations of mRNA expression of LXR and its target genes would translate into effect on actual fluxes in lipid metabolism. Our data as described in **chapter 3** clearly showed that the previously described effect of fetal protein restriction on fetal mRNA expression of genes involved in lipid metabolism was not translated into alterations in fetal fatty acid synthesis or in maternal-fetal cholesterol transport. This underscores the notion that effects on mRNA expression levels are not always translated into effects on relevant physiological fluxes.

In our studies, the protein restricted diet was isocaloric to the control diet, through the exchange of protein for glucose (+3 % w/w) and starch (low protein diet: + 6% w/w) compared to the control diet. We realize that a role of excess maternal carbohydrate intake during gestation cannot be excluded in effects of the protein restriction model. In our studies in mice, fetal protein restriction did not actually induce fetal growth restriction. Studies by others have shown that protein restriction during gestation affected fetal growth in rats in an inconsistent manner, varying from a decrease (21, 22), to no effect on fetal birth weight (20, 23). A study by Langley-Evans even described increased fetal birth weight after fetal protein restriction in rats (24).

Previous studies in mice performed in our laboratory using the same model i.e., gestational dietary protein restriction without inducing fetal growth restriction, showed that fetal protein restriction altered DNA methylation of LXR in fetal livers and lowered insulin sensitivity in the adult offspring (20). We therefore consider the model to be relevant to study metabolic programming despite the absence of fetal growth restriction.

Studies by others in mice showed that forced catch-up growth (generated by the reduction of littersize) after fetal protein restriction did induce features of the metabolic syndrome in adulthood (21). Fetal protein restriction did not exert short term effects on lipid metabolism in our study as described in **chapter 3.** We cannot exclude, however, that the earlier described effects of gestational protein restriction on fetal LXR methylation and gene expression, can modify lipid metabolism in an older animal or, according to the predictive adaptive response theory, after a second hit such as aggravated catch-up growth. The effect of forced catch-up growth of the offspring was not studied in the present thesis. It would be interesting to study the effect of an early postnatal overfeeding model (e.g. by reducing litter size during lactation) in the offspring of protein restricted mice on physiological relevant lipid fluxes such as cholesterol and fatty acid synthesis.

Intrauterine growth restriction in human infants

Intrauterine growth restriction has been related to adult dyslipidemia in humans (2, 7, 8, 25). Already in the neonatal period, infants that are small-for-gestational age (SGA) have higher plasma concentrations of total cholesterol, low-density cholesterol and triglycerides compared to normal weight infants (26).

It is known that infants fed human milk (containing a significantly higher amount of cholesterol compared to formula milk) downregulate their endogenous cholesterol synthesis (27). Breastfeeding is associated with lower mean total cholesterol and low-density cholesterol levels in adulthood, suggesting that breastfeeding may have long-term benefits for cardiovascular health, possibly by altering the metabolic set point for cholesterol synthesis (28). A study in which premature infants were randomly assigned to either human milk or formula feeding in their early postnatal life, showed a more favorable lipid profile in adolescence in the infants that had received human milk compared to the control group assigned to formula milk (29). According to the predictive adaptive response hypothesis we tested the hypothesis that a fetus subjected to a decreased placental nutrient supply would increase its cholesterol and / or triglyceride synthesis rate and thereby alter its metabolic set point. **In chapter 4**, we investigated the influence of intrauterine growth restriction on the *de novo* synthesis of cholesterol and on *de novo* lipogenesis in preterm born SGA and AGA infants as model for the third trimester of gestation. We showed, using stable isotope methodologies, that intrauterine growth restriction did not influence cholesterol synthesis rates. Hepatic de novo lipogenesis (DNL) is the process of conversion of acetyl-CoA into fatty acids. After subsequent esterification of fatty acids to glycerol, triglycerides can be released from the liver in the form of very-low-density lipoproteins (VLDL). Hepatic DNL is a quantitively minor pathway of total fat accretion in adults on a Western type diet. During gestation, the human fetus accumulates the majority of its fat during the third trimester of gestation. Thus, it would seem reasonable to assume that DNL is increased in premature neonates, to compensate for the loss of placental transfer of fatty acids particularly if preterm infants are born SGA. We hypothesized that in the SGA fetus, the hepatic DNL would be enhanced compared with the appropriate-for-gestational-age fetus (AGA) to compensate for the reduced fat accretion. Our study indeed showed that DNL was increased in the growth restricted infants compared to the AGA infants, but the magnitude of the effect was relatively mild. Yet, we cannot exclude a cumulative long term effect of a mildly increased DNL shortly after birth. It has been shown that infants born SGA who experience rapid early catch-up growth have an increased risk to develop obesity (30). Future research should thus focus on the effect of enteral nutrition and/or catch-up growth on the *de novo* synthesis of lipids.

Pharmacological LXR activation during gestation in mice

The nuclear receptor LXR is a key player in the regulation of cholesterol and in the synthesis of lipids. LXR is proposed to be involved in maternal-fetal cholesterol transport. In theory, manipulation of LXR during gestation could thus affect fetal lipid metabolism and even induce long term programming effects. **In chapter 5** we

determined if synthetic activation of LXR in utero would affect both fetal and adult lipid metabolism. Administration of the well studied synthetic LXR agonists T0901317 to pregnant females (dams) increased the expression of LXR target genes in both maternal and fetal liver. Short-term effects on fetal lipid metabolism were present in both dams and fetuses including increases in fatty acid synthesis and triglyceride levels. Despite these profound changes in lipid metabolism during fetal life, only mild effects persisted into young adulthood. A high-fat diet challenge did not induce a different lipid response in the young adult offspring of LXR agonist treated dams. It is not excluded however, that programming effects of the pharmaceutical intervention would appear at a later age.

Prebiotic oligosaccharides and the enterohepatic circulation of bile salts in rats

Human milk, the gold standard in infant nutrition, contains prebiotic oligosaccharides which are non-digestible food ingredients that stimulate the growth of health improving colonic bacteria (bifidobacteria and lactobacillus). In the last decade, infant formulas are increasingly supplemented with prebiotics in order to mimic the physiological effects of human milk in neonates.

Short-term effects of human milk on lipid and bile acid metabolism have been described: infants fed human milk have a larger bile acid pool size and a more efficient fat absorption (31, 32). We were interested if the enhanced fat absorption as found in breastfed infants could be attributed to an effect of prebiotic oligosaccharides on bile acid metabolism. In **chapter 6** we showed in (adult) rats that dietary supplementation with prebiotic oligosaccharides increased the presence of colonic lactobacillus and bifodobacteria and decreased the colonic pH. This prebiotic effect, however, did not affect kinetic parameters of bile acid metabolism, such as pool size, biliary bile salt secretion rate and cycling time. The lack of any effect of the prebiotic effect on bile acid metabolism, although studied in adult animals, does not support the hypothesis that supplementation of formula with prebiotics positively affects bile acid metabolism in infants. We do realize that the results in the presently used model of adult rats may not be the same in the situation of human infants, but an isolated effect of the prebiotic oligosaccharides is nevertheless unlikely.

CONCLUSION

The aim of the studies described in this thesis was to elucidate effects of fetal growth restriction and of a farmacological manipulation during gestation on lipid metabolism. Administration of a synthetic LXR agonist did exert short-term changes in fetal lipid metabolism without significantly affecting lipid metabolism in young adulthood. We showed in mice that previously described epigenetic changes using the identical fetal protein restriction model are not translated into short-term effects on lipid metabolism. In preterm infants, intrauterine growth restriction did not exert major effects on lipid synthesis. These studies indicate that fetal undernutrition does

not exert overt fetal adaptations in lipid metabolism that can be discriminated with the currently used techniques. We speculate that the manifestations of programming may either be subtle, visible in different body compartments, and/or may become (more) prominent after other forms of dietary/environmental challenges at adult life.

Stable isotope methodologies have been proven sensitive enough to delineate the consequences of the early environment in both rodents and in human infants. Studies investigating metabolic nutritional programming focused to a large extent on the health impact of under-nutrition during gestation. Under-nutrition during gestation and IUGR are still present in developed countries, and are more common in developing countries. Considering the increasing incidence of obesity in Western societies however, it would be logical to extend the focus of research to the metabolic consequences of maternal over-nutrition for the offspring.

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APPENDICES

ENGLISH SUMMARY

Introduction

A large volume of epidemiological data indicates that determinants of health and disease in adult life partly originate from environmental conditions in the fetal and early postnatal period. The maternal condition during gestation, the birth weight and the nutritional supply in the first postnatal year, can affect health in adulthood. Impaired nutrition of the mother during pregnancy and/or being born with a low birth weight is associated with an increased risk of developing chronic diseases at a later age. These chronic, predominantly "adult" diseases are features of the "metabolic syndrome" such as diabetes, hypertension, obesity and dyslipidemia. The scenario by which early adaptations to a (nutritional) stimulus permanently change the physiology and the metabolism of the organism, and continue to be expressed even in the absence of the stimulus that initiated them, is termed "metabolic programming". The mechanisms underlying this phenomenon are largely unknown. Insight in the pathophysiological mechanisms underlying metabolic programming could eventually allow for targeted, preventive, e.g. nutritional manipulations in the early environment, thereby improving long-term health. In this thesis we set out to characterize and unravel the consequences of and the mechanisms underlying specific dietary and pharmacological interventions during prenatal and early postnatal life on physiologically relevant parameters of lipid and bile acid metabolism. To position the context of our studies we first addressed to lipid and bile acid metabolism.

Lipid and bile acid metabolism

It is well known that elevated plasma lipid levels (low-density cholesterol and triglycerides) constitute a risk factor for the development of cardiovascular disease. Less familiar is the fact that lipids such as fatty acids and cholesterol are also indispensible for normal growth and development, especially in the fetal period. Lipids are water-insoluble molecules with several essential functions in mammals. Cholesterol is a component of cell membranes and a precursor for steroid hormones and bile acids. In addition, cholesterol is needed to activate Sonic Hedgehog, a signaling protein which is essential in embryonic development. Cholesterol can enter the body via external supply (diet, or during fetal life placental supply) or via endogenous production; *de novo* synthesis from acetyl-CoA with the liver being the major site of synthesis.

Bile acids, which are synthesized from cholesterol in the liver, are crucial for several physiological relevant functions of bile. Hepatobiliary bile acid secretion is the main driving force for generation of bile flow. As biological detergents, bile acids can solubilize lipids and facilitate the uptake of dietary fat and fat soluble lipids, thereby providing energy and structural components needed for normal growth. Bile acids are also essential for the disposal of excess cholesterol from the body via fecal secretion. Bile acids are conserved via the enterohepatic circulation implying that after secretion, the majority of bile acids is reabsorbed from the intestine and transported back to the liver via the portal circulation. The physiological relevance

of bile acids and lipids, such as cholesterol, is clarified by the profound health consequences of inborn errors in cholesterol synthesis and bile acid synthesis as reviewed in **chapter 2.**

The effects of intrauterine malnutrition on materal-fetal cholesterol transport and fetal lipid synthesis in mice

As stated previously, the nutritional conditions of the mother during gestation can affect the lipid metabolism of the offspring. Several animal models have been developed to determine the metabolic consequences of gestational under- or over-nutrition. Maternal low protein diet (LPD) during gestation is a well-defined model in rodents to study the metabolic effects of fetal under-nutrition. We used this model in mice to asses the effects of impaired nutrition on lipid metabolism. Previous research by others had shown that maternal protein restriction during gestation induced decreased fetal hepatic mRNA expression levels of genes involved in lipid metabolism. In **chapter 3** we described the effects of LPD on maternal-fetal cholesterol transport and on fetal lipid synthesis in mice. Pregnant mice received protein restriction or a control diet throughout gestation. We used stable isotope techniques to determine if the previously shown changes in fetal hepatic gene expression levels were translated into short-term (acute) effects on physiological relevant lipid fluxes. We demonstrated that gestational protein restriction did neither exert immediate effects on cholesterol transport from dam to fetus, nor on the *de novo* synthesis of cholesterol or fatty acids in the last stage of gestation. This conclusion strongly indicated that the earlier described programming effects of maternal protein restriction are not mediated by short-term changes in maternal-fetal lipid fluxes in mice.

Intrauterine growth restriction and de novo fatty acid and cholesterol synthesis in preterm neonates

Since the ultimate relevance for human physiology requires studies in infants, we than set out to investigate the effect of intrauterine malnutrition on lipid metabolism in humans. Infants born prematurely can constitute a model of the last trimester of gestation in which the impact of environmental factors can be studied. In **chapter 4** we describe the influence of intrauterine growth restriction (as a marker for impaired fetal nutrition) on lipid synthesis. We used stable isotope techniques to determine synthesis rates of cholesterol and of fatty acids. We conclude that IUGR did not quantitatively affect cholesterol synthesis whereas it only relatively mildly increased DNL. This conclusion indicates (consistent with our results in mice) that the association between impaired maternal nutrition during gestation and adult dyslipidemia is not explained by short-term intrauterine effects on lipid synthesis.

Fetal liver receptor activation acutely induces lipogenesis but does not affect plasma lipid respons to a high fat diet in mice

Not only a surplus, but also a shortage of lipids can disrupt health, especially during the fetal period. Cholesterol and lipid metabolism is under tight control of refined regulatory mechanisms in which the Liver X Receptor (LXR) is a key player.

Summarized, LXR is activated by oxidized cholesterol derivatives (oxysterols) after which LXR binds to its obligatory heterodimer, the Retinoid X-receptor (RXR) and transcription of target genes is initiated. Important LXR target genes are the ATP-binding cassette (ABC) transporters that are responsible for the efflux of cholesterol into bile, or into high-density-lipoproteins in the bloodstream. Besides regulating cholesterol efflux from the cell, LXR activation induces activation of genes involved in lipogenesis. In the murine placenta LXR's are expressed from day 10 of gestation (the second half of gestation) onwards. Furthermore, LXR is active in the fetal liver and can be stimulated via the dietary administration to the pregnant mice of the synthetic LXR agonist T0901317. We aimed to elucidate the effect of pharmacological activation of LXR in utero on fetal lipid metabolism in a mouse model. Additionally, we were interested if a short-term effect on lipid metabolism would be "programmed" into adulthood. In **Chapter 5** we showed that the administration of a pharmalogical LXR agonist (T0901317) to pregnant mice induced immediate effects on fetal lipid metabolism. Fetal hepatic LXR target genes involved in lipid synthesis were upregulated with subsequent increase in hepatic and plasma triglyceride levels in fetuses and dams. Lipogenesis, determined using a stable isotope technique, was increased in dams and fetuses after T0901317 treatment. Despite these profound immediate (fetal) effects, long term effects appeared to be rather mild.

Prebiotic oligosaccharides and the enterohepatic circulation of bile acids

Comparable to fetal nutrition, early postnatal nutrition may affect health in a later stage. One of the short-term benefits of human milk compared to formula is the more efficient absorption of fat. Bile acids are relevant for the uptake of fat and fat-soluble vitamins from the gastrointestinal tract, thereby facilitating normal growth. Interestingly, the bile acid pool size is larger in premature infants when they are fed human milk compared to formula. Extensive efforts have been made to improve the composition of infant formulas by adding ingredients that are specific for human milk such as prebiotic oligosaccharides. Prebiotic oligosaccharides are nondigestible food ingredients that stimulate the growth and activity of specific bacteria with supposed beneficial properties in the colon, e.g. bifidobacteria and lactobacilli. In **chapter 6** we evaluated the effect of a prebiotic diet on relevant parameters of bile acid metabolism. We hypothesized that alteration of the colonic bacterial flora due to the prebiotic diet would affect the bile acid metabolism via bacterial deconjugation and/or dehydroxylation. Administration of dietary oligosaccharides to rats induced a significant increase in the proportion of colonic and fecal lactobacilli and bifidobacteria. Furthermore, the colonic and fecal pH, as a marker for the fermenting capacity of bifidobacteria and lactobacilli, decreased significantly. Despite the prebiotic effect, no effect on bile acid kinetics (pool size, synthesis rate and cycling time) was observed. The present study indicates that the larger pool size observed in infants fed human milk is not related to altered bile acid kinetics due to prebiotics naturally occurring in human milk.

CONCLUSION

The aim of the research described in this thesis was to characterize and unravel the role of specific dietary and pharmacological interventions during prenatal and (early) postnatal life on physiological relevant parameters of lipid and bile acid metabolism. Mimicking fetal malnutrition using protein restriction during gestation in mice did not affect lipid metabolism in the fetal period. Previously induced epigenetic and mRNA changes after using the same model were not translated into short-term (fetal) effects on lipid metabolism. In contrast, the administration of a synthetic LXR agonist to pregnant mice did affect the lipid metabolism in the fetus. However, these effects only mildly persisted into a later age. Intrauterine growth restriction in human infants did not substantially affect lipid metabolism as was demonstrated in premature newborns. These studies indicate that fetal malnutrition does not lead to profound fetal adaptations in lipid metabolism as measured with the present techniques. Our studies also indicate that the applied stable isotope techniques are sensitive enough to investigate the consequences of the early (nutritional) environment on lipid metabolism in humans and in a mouse model. Considering the increasing incidence of obesity in the Western world, it would be interesting to extend the focus of research in this field to the metabolic consequences of maternal overnutrition during gestation.

NEDERLANDSE SAMENVATTING

Introductie

Een groot aantal epidemiologische studies laat zien dat het risico op het krijgen van chronische ziekten op latere leeftijd deels al vroeg in het leven bepaald wordt. De voedingstoestand van de moeder tijdens de zwangerschap, het geboorte gewicht en het voedingsaanbod in het eerste levensjaar kunnen de gezondheid op latere leeftijd beïnvloeden. Ondervoeding van de moeder tijdens de zwangerschap is geassocieerd met een verhoogde gevoeligheid voor het ontwikkelen van chronische aandoeningen die passen in het zogenoemde metabool syndroom. Dit is een cluster van chronische ziektes zoals diabetes, obesitas, hart-en vaatziekten, hypertensie en dyslipidemie (een verstoorde vetbalans). Het verband tussen prenatale voeding en de ontwikkeling van ziekten op latere leeftijd wordt "metabolic programming" genoemd. De oorzakelijke mechanismen die aan dit fenomeen ten grondslag liggen zijn grotendeels onbekend. Inzicht in die mechanismen zou uiteindelijk kunnen leiden tot het ontwikkelen van specifieke preventieve (voedings)interventies in het vroege leven, om de gezondheid op langere termijn gunstig te beïnvloeden.

Het doel van het in dit proefschrift beschreven onderzoek was om de fysiologische consequenties van verschillende voedings- en farmacologische interventies in de foetale en (vroege) postnatale periode op het galzouten- en vetmetabolisme te onderzoeken. Om de beschreven studies in context te plaatsen worden eerst het lipidenmetabolisme (aan de hand van een belangrijk lipide, namelijk cholesterol) en het metabolisme van galzouten behandeld.

Lipiden- en galzout metabolisme

Het is algemeen bekend dat dyslipidemie (verhoogde plasma concentraties van low-density-cholesterol en triglyceriden) een risico factor is voor het ontwikkelen van hart-en vaatziekten. Minder bekend is het feit dat lipiden zoals vetzuren en cholesterol juist essentieel zijn voor normale groei en ontwikkeling, vooral in de foetale periode. Lipiden zijn niet-wateroplosbare moleculen met een aantal essentiële functies. Cholesterol is een bestanddeel van het celmembraan en een voorloper van steroïdhormonen en van galzouten. In de foetale periode is cholesterol nodig voor de normale embryonale ontwikkeling. Het lichaam kan cholesterol verkrijgen via de externe route, uit het dieet, of in de foetale periode via de placenta. Hiernaast kan het lichaam zelf cholesterol aanmaken via het proces dat *de novo* synthese van cholesterol wordt genoemd. Hierbij wordt cholesterol gesynthetiseerd uit acetyl-CoA, wat voornamelijk plaatsvindt in de lever.

Galzouten, die in de lever uit cholesterol gesynthetiseerd worden zijn essentieel voor een aantal fysiologisch belangrijke functies van gal. Hepatobiliare uitscheiding van galzouten is de voornaamste drijfveer voor het ontstaan van de galstroom vanuit de lever. Door hun zeepachtige structuur kunnen galzouten vetten emulgeren waarmee ze de opname van vet en vetoplosbare vitamines uit de voeding mogelijk maken. Galzouten zijn daarom onmisbaar voor normale groei en ontwikkeling. Het galzout metabolisme speelt ook een belangrijke rol bij de uitscheiding van overtollig choles-

terol via de feces. Galzouten worden in het lichaam "gerecycled" door middel van de enterohepatische kringloop. Dit houdt in dat galzouten, nadat ze hun werk in de darm hebben gedaan, voor het grootste deel worden gereabsorbeerd en via het portale bloed terug worden getransporteerd naar de lever.
De belangrijke rol van lipiden en galzouten wordt duidelijk door de ernstige gevolgen van aangeboren afwijkingen in de aanmaak van cholesterol en van galzouten. In **hoofdstuk 2** worden galzout- en cholesterol metabolisme beschreven, evenals verschillende aangeboren aandoeningen in de aanmaak van cholesterol en galzouten en hun klinische consequentie.

De gevolgen van intra-uteriene ondervoeding op maternaal-foetaal cholesteroltransport en op de foetale vetsynthese in muizen

Zoals eerder vermeld kan de voedingstoestand van de moeder tijdens de zwangerschap van invloed zijn op de vetstofwisseling van haar nageslacht op latere leeftijd. Er zijn verschillende diermodellen ontworpen om de metabole consequenties van onder- en overvoeding tijdens de zwangerschap te bestuderen. Een welbeproefd model in knaagdieren om de gevolgen van maternale ondervoeding tijdens de zwangerschap te onderzoeken, is het geven van eiwitbeperking tijdens de zwangerschap. We gebruikten dit model in muizen om de effecten van maternale ondervoeding op de vetstofwisseling in de foetus te bestuderen. Eerder onderzoek waarin hetzelfde model gebruikt werd, toonde aan dat maternale eiwitbeperking tijdens de zwangerschap leidde tot verminderde expressie van mRNA van genen die betrokken zijn bij het lipidenmetabolisme in foetale levers. In **hoofdstuk 3** onderzochten we het effect van deze veranderingen in genexpressie op het maternaal-foetale cholesterol transport en op de foetale aanmaak van cholesterol en vetzuren. Zwangere muizen werden hiertoe gevoed met een controle dieet of met een eiwitarm dieet tijdens de gehele zwangerschap. We gebruikten stabiele isotopen om maternaal-foetaal cholesterol transport en foetale cholesterol en vetzuursynthese te kwantificeren. Deze studie laat zien dat maternale eiwitbeperking tijdens de zwangerschap in muizen geen kwantitatieve korte termijn veranderingen teweegbracht in maternaal-foetaal cholesterol transport of in de foetale lipiden synthese in de laatste fase van de zwangerschap.

Intra-uteriene groeivertraging en de synthese van vetzuren en cholesterol in premature neonaten

Het doel van dit onderzoek was om het effect van intra-uteriene groeiretardatie (IUGR) op de vetstofwisseling te onderzoeken. Een te vroeg geboren kind kan gezien worden als een model voor het laatste trimester van de zwangerschap waarin de gevolgen van omgevingsfactoren onderzocht kunnen worden. In **hoofdstuk 4** beschreven we de invloed van IUGR (als een maat voor verminderde foetale voeding) op de synthese van cholesterol en van vetzuren. We gebruikten stabiele isotopen om de biosynthese van cholesterol en vetzuren te meten. We concluderen dat IUGR niet tot kwantitatieve veranderingen in de synthese van cholesterol leidt, terwijl de biosynthese van vetzuren wel, maar slechts matig verhoogd was. De uitkomsten van deze studie geven weer dat de associatie tussen IUGR en dyslipidemie op de volwassen

leeftijd niet kan worden verklaard door significante korte termijn effecten van IUGR op de biosynthese van vetzuren en cholesterol.

Foetale Liver X Receptor activatie stimuleert de foetale vetzuursynthese, maar heeft geen effect op de plasma lipiderespons op een vetrijk dieet in muizen

Zowel een teveel als een tekort aan lipiden beïnvloedt de gezondheid negatief. Het cholesterol-en vetzuur metabolisme wordt dan ook nauwgezet gereguleerd. Een belangrijke regulator in het cholesterol- en vetzuurmetabolisme is de liver X Receptor (LXR). LXR is een transcriptiefactor; een eiwit dat de transcriptie van genen reguleert. LXR wordt geactiveerd door oxysterolen, metabolieten van cholesterol. LXR bindt daarna aan zijn heterodimeer retinoid X-receptor (RXR) waarna de transcriptie van verschillende genen wordt aangezet. Belangrijke targetgenen van LXR zijn ATP-binding cassette (ABC) transporters, die zorgen voor transport van cholesterol vanuit de cel naar gal, of naar high-density-lipoproteinen in de bloedstroom. Andere target genen van LXR zorgen voor het ingang zetten van de biosynthese van vetzuren. LXR is aantoonbaar in de placenta van de muis vanaf dag 10, halverwege de zwangerschap. LXR is actief in de foetale lever en kan gestimuleerd door toedienen van een synthetische agonist (T0901317) aan het dieet van zwangere muizen. De experimenten beschreven **in hoofdstuk 5** hadden als doel het effect van intra-uteriene farmacologische activering van LXR op de foetale vetstofwisseling in een muismodel te onderzoeken. We waren daarbij zowel geïnteresseerd in effecten op de korte termijn, de foetale periode, als op de lange termijn, de volwassen leeftijd. In hoofdstuk 5 laten we zien dat de toediening van een farmacologische LXR activator (T0901317) aan zwangere muizen directe effecten op de foetale vetstofwisseling induceert. Foetale LXR targetgenen in de lever werden geactiveerd en er werd een toename gezien van de lipidensynthese. De directe (foetale) effecten persisteerden echter maar in geringe mate op de volwassen leeftijd.

Prebiotische oligosacchariden en de enterohepatische circulatie van galzouten in ratten

Analoog aan voeding tijdens de foetale periode, kan vroege postnatale voeding de gezondheid op lange termijn beïnvloeden. Een van de voordelen van moedermelk, de gouden standaard in babyvoeding, is de meer efficiënte vetabsorptie in vergelijking met kunstvoeding. Ook is de hoeveelheid galzouten aanwezig in het lichaam groter bij prematuren die met moedermelk gevoed worden in vergelijking met prematuren die kunstvoeding krijgen. Galzouten zijn essentieel in de opname van vet en vetoplosbare vitamines uit het dieet en daardoor belangrijk voor goede groei. In een poging om de voordelen van moedermelk na te bootsen in kunstvoeding, verrijkt men kunstvoeding vaak met componenten die in moedermelk aanwezig zijn, bijvoorbeeld met prebiotische oligosacchariden. Prebiotische oligosacchariden zijn onverteerbare voedingstoffen die de groei en activiteit van specifieke bacteriën zoals bifidobacteriën en lactobacillen in de darm stimuleren en op die manier de gezondheid gunstig beinvloeden. **In hoofdstuk 6** evalueren we het effect van een prebiotisch dieet op het

galzout metabolisme in ratten. Onze hypothese was dat verandering van de intestinale bacteriële flora als gevolg van het toevoegen van prebiotica, het galzout metabolisme via bacteriële deconjugatie en/of dehydroxylatie zou beïnvloeden. We verwachtten dat belangrijke parameters van het galzoutmetabolisme meer zouden lijken op die van borstgevoede kinderen. Toediening van oligosacchariden in het dieet van ratten induceerde stijging van de hoeveelheid lactobaccillen en bifidobacterien in de darm. Tevens was de pH (als maat voor de fermentatie capaciteit van lactobaccillen en bifidobacteriën) significant lager na het toedienen van prebiotica. Ondanks dit prebiotisch effect, was er geen effect op het galzoutmetabolisme. Deze studie ondersteunt de hypothese dat de prebiotica die van nature in moedermelk voorkomen bijdragen aan een grotere galzouten pool niet.

CONCLUSIE

Het doel van het onderzoek beschreven in dit proefschrift, was om de effecten van verschillende voedings- en farmacologische interventies tijdens en na de zwangerschap op de vetstofwisseling en op het galzout metabolisme te beschrijven. Het nabootsen van foetale ondervoeding met behulp van eiwitbeperking tijdens de zwangerschap in muizen was niet van invloed op de vetstofwisseling in de foetale periode. De toediening van een synthetische LXR agonist aan zwangere muizen had wel invloed op het lipiden metabolisme bij de foetus. Deze effecten waren op de volwassen leeftijd echter nog maar in geringe mate aanwezig. Intra-uteriene groeivertraging bij kinderen was niet wezenlijk van invloed op de vetstofwisseling zoals werd aangetoond bij premature pasgeborenen.

Deze studies tonen aan dat foetale ondervoeding niet leidt tot significante foetale aanpassingen in het lipiden metabolisme. De huidige studies geven ook aan dat de toegepaste technieken met stabiele isotopen technieken adequaat zijn voor het onderzoeken van gevolgen van vroege omgevingsfactoren op het lipiden metabolisme. Gezien de toenemende incidentie van obesitas, zou het interessant zijn om het onderzoeksfocus ook te richten op de metabole gevolgen van maternale of vroeg postnatale overvoeding.

FREQUENTLY USED ABBREVIATIONS

Abca1	ATP binding cassette, sub family A, member 1
Abcg1	ATP binding cassette, sub family G, member 1
Abcg5	ATP binding cassette, sub family G, member 5
Abcg8	ATP binding cassette, sub family G, member 8
Acc1	acetyl-Coenzyme A carboxylase 1
AGA	appropriate for gestational age
ASBT	apical sodium bile salt transporter
CA	cholate
CDCA	chenodeoxycholate
Cyp7a1	cholesterol 7α-hydroxylase
Cyp27a1	sterol-27-hydroxylase
Fasn	fatty acid synthase
HDL	high-density lipoprotein
Hmgcr	3-hydroxy-3-methylglutaryl-coenzyme A reductase
IUGR	intrauterine growth restriction
IV	intravenous
LDL	low-density lipoprotein
Lxrα	liver x receptor alpha
Lxrα	liver x receptor beta
MIDA	mass isotopomere distribution analysis
Rxr	retinoid x receptor
Scd1	steaoryl-coenzym A desaturase 1
SGA	small-for-gestational age
Srebp1c	sterol regulatory element binding protein-1c
VLDL	very-low-density lipoprotein

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CURRICULUM VITAE

Hester van Meer werd op 3 februari 1972 geboren in Baarn. Haar lagere en middelbare schooltijd bracht zij voor een groot deel door in het Friese Oosterwolde. Na het behalen van haar atheneum B diploma aan het Amsterdams Lyceum begon zij in 1991 met de studie Technische Bedrijfswetenschappen aan de Rijksuniversiteit te Groningen. In 1993 maakte zij de overstap naar de studie Geneeskunde aan dezelfde universiteit. Haar coschappen werden verricht in het toenmalige Academisch Ziekhuis Groningen. Hester deed haar afstudeeronderzoek in het Sint Elisabeth Hospitaal op Curaçao onder begeleiding van mr.dr. AAE Verhagen. In 2000 behaalde zij haar artsexamen cum laude.
Hierna werkte zij anderhalf jaar als arts-assistent kindergeneeskunde in de Isala Klinieken te Zwolle en het Martini Ziekenhuis te Groningen. In 2002 startte zij met haar opleiding tot kinderarts in het Beatrix Kinderziekenhuis (opleider: prof. PJJ Sauer en vervolgens mr.dr. AAE Verhagen). Haar perifere stage werd verricht in het Medisch Centrum Leeuwarden. Tijdens haar opleiding begon ze met het promotieonderzoek dat heeft geleid tot dit proefschrift (promotor: prof. HJ Verkade). Zij werd geregistreerd als kinderarts in april 2010. Oktober 2009 startte zij als fellow kinder maag-, darm- en leverziekten (opleider: prof. E.H.H.M. Rings) in het Beatrix Kinderziekenhuis. In juli 2012 zal zij haar fellowship kinder maag-, darm- en lever ziekten afronden.

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