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

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ABSTRACT

**Background:** The cholesterol content of breast milk has been implied to affect the short- and long-term cholesterol homeostasis in the offspring. The mechanisms which regulate milk cholesterol concentrations are only partly understood. It is not known, for example, if maternal hypercholesterolemia affects milk cholesterol concentration. Using different mouse models, we set out to assess the impact of diet- or genetically-induced hypercholesterolemia on milk cholesterol levels.

**Methods:** At day 14 postpartum we determined milk, plasma and tissue lipids in wild-type, Ldlr-/-, and Abcg8-/- mice fed either chow or 0.5% high-cholesterol diet (HC). To detect possible compensatory changes, we quantified de novo cholesterol synthesis in mammary gland and liver via the deuterium incorporation method.

**Results:** In chow-fed mice, plasma cholesterol was similar between wild type and Abcg8-/- mice and higher in Ldlr-/- dams (4.2-fold, p<0.01). HC diet increased plasma cholesterol in all three mouse models compared to control diet (wild type, +48%, p<0.05; Abcg8-/-, +100%, p<0.05; Ldlr-/-, +380%, p<0.01; respectively). Despite the up to 4-fold increase in plasma cholesterol concentration, the various genetic and dietary conditions did not affect milk cholesterol levels (1.99 ± 0.46 mM). The de novo cholesterol synthesis in liver and mammary gland was strongly reduced in the various hypercholesterolemic conditions.

**Conclusions:** Milk cholesterol concentration in mice is not affected by conditions of maternal hypercholesterolemia and is maintained at stable levels via ABCG8- and LDLR-independent mechanism(s). The robustness of milk cholesterol levels is compatible with the concept of an important physiological function of cholesterol supply to the offspring.
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INTRODUCTION

Breast milk contains high levels of cholesterol in contrast to most infant formulas. The relatively high cholesterol concentration in breast milk has been implied to have a lasting impact on the cholesterol homeostasis of the offspring. Breastfed infants have high plasma cholesterol levels in early life, but lower plasma cholesterol in adulthood, compared to formula-fed individuals\(^{107}\). The lower serum cholesterol concentrations in adulthood may relate to long-term cardioprotective effects of breast milk, in accordance with the metabolic programming hypothesis\(^6\). The mechanisms involved in the regulation of milk cholesterol concentration are only partly understood. With the recent cardiometabolic disease pandemic, dyslipidemia and disturbances in cholesterol homeostasis are becoming increasingly common conditions in pregnant and lactating women. Maternal hypercholesterolemia during gestation has been associated with increased plasma cholesterol in the fetus\(^{79}\). However, it remains unclear to what extent maternal hypercholesterolemia, either caused by genetic or dietary factors, impacts cholesterol transport across the mammary gland and affects cholesterol concentration in milk with possible effects in the offspring.

Cholesterol in milk likely originates from different sources. The predominant fraction of cholesterol is considered to reach the milk via plasma\(^6\): either from preformed stores, from dietary origin or from de novo synthesis in the mammary gland epithelium cells\(^{349,350}\) or the liver\(^{351}\). The exact transport routes by which cholesterol is taken up by the mammary gland from the circulation have not been identified. There have been reports suggesting a predominant uptake of ApoB-containing lipoproteins\(^{352}\) and several receptors for their uptake are abundantly expressed in mammary epithelial cells, amongst which LDL-, VLDL- and LRP-receptors\(^{350}\). Other lipoproteins found in plasma, like the high-density lipoproteins, may serve as an alternative source for cholesterol uptake since respective receptors such as SRB1 are also expressed in the mammary epithelium\(^353\). In addition, mammary gland epithelial cells express the cholesterol efflux transporters, such as ABCG5/G8, ABCA1, and ABCG1, whose expression fluctuates depending on lactation stage\(^{354-356}\) and could possibly impact cholesterol levels in the milk. The ABC cassette G8 protein is a cholesterol transporter expressed on the apical membrane of hepatocytes and enterocytes, where it facilitates export of cholesterol\(^{168}\). Interestingly, Abcg8 is also expressed in mammary epithelial cells\(^{356}\). The LDL-receptor is the dominant transport protein involved in the uptake of apoB-containing lipoproteins from the plasma\(^{357}\). Humans with genetic loss of LDLR function have a severe hypercholesterolemia that is further increased upon dietary cholesterol exposure\(^{358}\).

Given the plausible importance of milk cholesterol for immediate and long-
term offspring health, we aimed to address the relationship between maternal hypercholesterolemia and milk cholesterol concentration in mouse models. We analyzed milk cholesterol concentrations in lactating mice with hypercholesterolemia of different severity, induced by dietary and/or genetic manipulations. The dietary means to manipulate plasma cholesterol concentrations consisted of feeding a high-cholesterol diet (0.5 % w/w), while genetic manipulation involved the ablation of either the \textit{Abcg8} or the \textit{Ldlr} gene. We assessed the relevance of cholesterol secretion into milk via the ABCG8 transporter and via mammary gland uptake of cholesterol via the LDL receptor. To gain insights into the origin of milk cholesterol in the different models of hypercholesterolemia, we measured the de novo cholesterol synthesis in the liver and mammary gland.

\textbf{MATERIALS AND METHODS}

\textbf{Animal studies}

Female C57BL6/J, \textit{Ldlr} knockout\textsuperscript{327}, and \textit{Abcg8} knockout\textsuperscript{359} mice were housed in temperature controlled-conditions with 12:12 hours light dark cycles and maintained on chow diet (RMH-B, ABDiets, Woerden, Netherlands). Breeding was initiated between 8-12 weeks of age. Due to accumulation of dietary plant sterols \textit{Abcg8-/-} mice are infertile, which is relieved upon ezetimibe treatment\textsuperscript{360}. Therefore, in order to facilitate fertilization, \textit{Abcg8-/-} females were pre-treated for 3 weeks with 0.005\% ezetimibe provided via the food, which was removed from the diet once pregnancy was confirmed in accordance with Solca \textit{et al}\textsuperscript{360}. The rest of the models were fed chow until E18 when half of the mice received 0.5\% cholesterol diet. Lactation day 1 (L1) was considered the day at which pups were born. On L14 the dams were injected i.p. with 2.3 mL 99\% 2H\textsubscript{2}O (deuterium oxide) per 100g BW, containing 0.9 \% NaCl. After 50 minutes, milk was collected for 10 minutes (details see below) directly followed by termination and harvesting of blood, liver, and mammary glands. All animal experiments were approved by the ethical committee for animal experimentation at the University of Groningen.

\textbf{Milk collection}

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

Thawed milk samples were homogenized by continuous vortexing. 25 to 50 µL of milk sample was subjected to lipid extraction according to Bligh & Dyer\(^{361}\). Unesterified cholesterol was subsequently derivatized to cholesteryl acetate and quantified by gas chromatography, using 5-alpha cholestane as internal standard\(^{225}\).

**Total plasma cholesterol and lipoprotein profiles**

Total plasma cholesterol was measured enzymatically using a commercially available kit (Roche Diagnostics GmbH, Mannheim, Germany). Lipoprotein fractions of pooled plasma samples (n=3-5) were separated via fast protein liquid chromatography gel filtration using a superose 6 column (GE Healthcare, Little Chalfont, UK) as published\(^{323}\). Samples were chromatographed at a flow rate of 0.5 ml/min, and lipoprotein fractions of 500 µl each were collected. Individual fractions were assayed for cholesterol concentrations using a commercial kit (Roche Diagnostics GmbH, Mannheim, Germany).

**Hepatic and mammary gland total cholesterol quantification**

Liver and mammary gland tissues were homogenized using RNase free-beads and the TissueLyser LT system (Qiagen GmbH, Hilden, Germany). Lipids were extracted according to Bligh&Dyer\(^{361}\). Cholesterol was de-esterified according to Ichihara et al\(^{362}\). Free cholesterol underwent acetylation followed by quantification using gas chromatography (GC, Agilent 6890, Amstelveen, the Netherlands)\(^{225}\).

**Organ-specific de novo cholesterol synthesis**

Liver- and mammary gland-specific de novo cholesterol synthesis were quantified using the deuterium incorporation method\(^{363}\). Briefly, at L14 the dams were injected i.p. with deuterated water (2.3 mL/100g BW, 99% 2H\(_2\)O, 0.9% NaCl) and terminated after 60 minutes by cardiac puncture. Plasma from a non-injected with 2H\(_2\)O control mouse was used for determining the number of hydrogen atoms incorporated in a single newly synthesized cholesterol molecule as measured by GC-MS. Following lipid extraction and de-esterification, the abundance of deuterium-substituted hydrogen atoms was determined by isotope ratio mass spectrometry (IRMС). Synthesis rates were determined as previously published\(^{247}\).

**Statistical analysis**

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

High-cholesterol diet increases plasma and hepatic cholesterol levels

To assess the isolated effect of ABCG8- or LDLR-deficiency we first measured cholesterol levels in plasma of dams on a chow diet. While ABCG8-deficiency did not affect basal plasma cholesterol, the LDLR-deficient dams displayed marked hypercholesterolemia (+4.2-fold, p<0.01, Fig. 1A), mostly due to increased cholesterol levels in LDL and VLDL (Figure 1B). Feeding the dams HC diet increased the levels of

![Graph A: Total plasma cholesterol levels](image)

![Graph B: Cholesterol in lipoprotein fractions](image)

**Figure 1**: Plasma lipids. A) Total plasma cholesterol levels were measured in whole plasma using commercially available enzymatic assay (WT, n=5; Abcg8 -/-, n=4-5; Ldlr -/-, n=4-8). Data are presented as median and interquartile range. Statistical significance was tested Kruskal-Wallis post-hoc Conover-Inman; non-different groups share a letter. The threshold of significance was p<.05. B) Cholesterol in lipoprotein fractions following separation by FPLC of pooled plasma samples (WT, n=5; Abcg8 -/-, n=4-5; Ldlr -/-, n=3-4).
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total plasma cholesterol in all models (Figure 1A). The size of the effect reached maximum in the Ldlr-/- mice (+380%, p<0.01) followed by Abcg8-/- (+100%, p<0.05) and wild-type (+48%, p<0.05). On the chow diet, hepatic cholesterol concentration corresponded with the differences in the plasma cholesterol levels: similar levels in wild-type and Abcg8 knockout mice and 0.6-fold higher in LDLR-deficient mice (p<0.01). The HC diet induced cholesterol accumulation in the hepatic tissues to similar levels in the three groups (Figure 2).

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

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

In order to further analyze the possible association between milk cholesterol levels

Figure 2: Hepatic cholesterol levels. A) Hepatic lipids were extracted according to Bligh & Dyer and measured by gas chromatography (WT, n=5; Abcg8 -/-, n=4-5; Ldlr -/-, n=5-8). Data are presented as median and interquartile range (Tukey). Statistical signi cance was tested with Kruskal-Wallis post- hoc Conover-Inman; non-different groups share a letter. The threshold of signi cance was p<.05.
and plasma and mammary gland cholesterol levels, we performed regression analysis. Cholesterol levels in mammary gland tissue were strongly and positively related to plasma cholesterol levels ($r^2 = 0.53$, $p<0.0001$), but not to milk cholesterol concentrations (n.s.). Reasonably, we found no significant correlation between milk and plasma cholesterol levels in the different conditions.

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

The increased plasma, hepatic and mammary gland cholesterol levels in the hypercholesterolemic models did not translate into increased milk cholesterol concentrations. We then tested the possibility that the stable concentrations were obtained by suppression of hepatic or mammary gland cholesterol synthesis. In all chow-

![Graph A](image1)

![Graph B](image2)

**Figure 3:** Mammary gland and milk cholesterol. A) The lipid content of mammary tissue was extracted according to Bligh & Dyer and measured by gas chromatography. B) Milk samples were obtained after i.p. injection with 1 IU oxytocin by using a modified electric human breast pump. Milk lipids were extracted according to Bligh & Dyer and cholesterol was quantified by gas chromatography (WT, $n=5$; Abcg8 $-/-$, $n=4-5$; Ldlr $-/-$, $n=4-6$). Data are presented as median and interquartile range. Statistical significance was assessed with Kruskal-Wallis post-hoc Conover-Inman test; non-different groups share a letter. The threshold of significance was $p<.05$. 

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... fed groups, the de novo hepatic and mammary gland cholesterol synthesis rates were negatively associated with plasma, liver and mammary gland cholesterol levels (Figure 4). Feeding the HC diet strongly reduced the cholesterol synthesis rate in liver (Figure 4A) and mammary gland (Figure 4B) in all three models.

**Figure 4**: De novo cholesterol synthesis. On L14 the dams received deuterium water i.p. one hour before harvesting the organs. The mammary gland was milked 10 minutes before harvesting. The fraction of deuterium-incorporated cholesterol in liver and mammary gland was assessed using isotope ratio mass spectrometry (IRMS). A) De novo cholesterol synthesis in the liver (%/h). B) De novo cholesterol synthesis in the mammary gland (%/h). (WT, n=5; Abcg8 -/-, n=4-5; Ldlr -/-, n=4-6) Data are presented as median and interquartile range (Tukey) Statistical significance was tested with Kruskal-Wallis post-hoc Conover-Inman; non-different groups share a letter. The threshold of significance was p<.05.
Chapter 3

To assess the possible relationship between the de novo synthesis in liver or in mammary gland and the milk cholesterol concentration, we used linear regression analysis. The milk cholesterol levels in chow-fed conditions correlated moderately with the fraction of de novo synthesized cholesterol in liver \( (r^2=0.38; p=0.03) \) and no significant correlation was found during HC diet feeding. On neither of the two diets, we observed a significant correlation between de novo cholesterol synthesis in mammary gland and milk cholesterol concentration in the different genetic models \( (r^2<0.3; \text{n.s.}) \).

**DISCUSSION**

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

The milk cholesterol concentration was not affected by genetic inactivation of two candidate genes with a possible role in cholesterol transport towards milk, nor by diet-induced hypercholesterolemia. This observation indicates that either the gene products are not involved, or that alternative transporting mechanisms ensure redundancy in the supply of cholesterol destined for secretion into the milk. The hypothesis that the LDL receptor is involved in milk cholesterol transport was based on findings describing an association between lactation and increased mammary gland expression of LDLR in human subjects\(^{350}\). In addition, lactation in rodents is characterized by an increase in circulating LDL\(^{364}\), compatible with a role for the low-density lipoproteins as a source for milk cholesterol. Our data indicate that uptake of cholesterol by the mammary gland can be conducted quantitatively by LDLR-independent mechanism(s). We cannot exclude that an alternative, LDLR-independent mechanism involves an alternative receptor for LDL uptake. In support of this notion, radioactivity studies in mice have shown the transfer of ApoB100 across the mammary epithelium towards the milk to take place at the same extent in both wild-type and LDLR-deficient mice\(^{352}\). Possibly VLDL and LRP receptors\(^{365}\), or even CD36\(^{366}\) can substitute for LDLR-deficiency. The hypothesis that ABCG8 is involved in milk cholesterol transport rests on the high expression levels of the heterodimer ABCG5/ABCG8 in lactating bovine mammary glands\(^{354,356}\). In hepatocytes
and intestinal epithelial cells, the ABCG5/ABCG8 dimer is expressed at the apical membrane\textsuperscript{168} where it is essential for the export of free cholesterol towards the bile and intestinal lumen, respectively\textsuperscript{174}. Our data, however, do not support a critically important role for ABCG8 in the process of cholesterol efflux across the mammary gland epithelium. Apparently, neither the LDL receptor nor ABCG8 is crucial for determining cholesterol transport towards milk in our experimental setup.

De novo cholesterol synthesis has been shown to contribute to milk cholesterol\textsuperscript{351}. For the dams, cholesterol demand is increased during lactation, corresponding with increased expression of cholesterol synthesis genes in both liver and mammary glands of bovines, rodents, and humans\textsuperscript{245,349,350}. We found 12-fold higher fractional cholesterol synthesis rates in liver compared to mammary gland, which is in agreement with previous studies demonstrating a larger contribution to milk cholesterol originating from hepatic than from mammary gland synthesis\textsuperscript{351}. The lower mammary gland cholesterol synthesis compared with hepatic synthesis also corresponds to the expression levels of the \textit{Hmgcr} gene in the two tissues, encoding the rate-limiting enzyme of cholesterol synthesis\textsuperscript{349}. In each of the three murine genotypes, dietary cholesterol supplementation strongly decreased de novo cholesterol synthesis in liver and in mammary gland, similarly to observations in rats\textsuperscript{246}. The decreased de novo synthesis rates in liver and mammary gland, however, did not decrease milk cholesterol levels. Thus, the cholesterol synthesis rate is apparently not a critical driver for the amount of cholesterol secreted into milk. Rather, it seems that milk cholesterol concentration is robust and “protected” against profound hypercholesterolemia despite strongly increased tissue cholesterol levels.

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

We would like to hypothesize on three physiological explanation(s) of the present findings. First, it is tempting to speculate that the apparent robustness of the cholesterol concentration in milk relates to physiological importance of its supply to the offspring. The importance of a stable milk cholesterol concentration could relate to the process of secretion of milk lipids, in particular triglycerides. Within the alveolar cells of the mammary gland the secretory lipids are shaped in a single phospholipid layer-wrapped lipid droplets. During exocytosis the lipid droplets acquire an additional cholesterol-rich phospholipid bilayer, resulting in the formation of the milk-fat globule (MFG)\textsuperscript{367}. Milk
cholesterol is mainly present as unesterified cholesterol in the MFG-membrane (85-90%) and the other part as cholesteryl esters in the MFG-core\textsuperscript{368,369}. The packaging of the lipid droplets with the MFG membrane, which is essential for their secretion, may, therefore translate into a rather stable cholesterol content in milk, based on its role as an emulsion-stabilizing component as part of the MFG-membrane. Second, the robust cholesterol concentration in milk could also underline the hypothesized physiological function of milk cholesterol for later health of the offspring. In contrast to breast milk, the fat globules of common infant milk formula are smaller in size and differ in composition, being coated with milk proteins instead of a phospholipid and cholesterol-rich membrane\textsuperscript{370,371}. Indeed, infant formulas hardly contain cholesterol. Cholesterol in early life is not considered an essential dietary component since infants are capable of de novo cholesterol synthesis, and thus do not critically depend on milk for their cholesterol supply. As expected, infants fed cholesterol-free formula have increased cholesterol synthesis rates compared to breastfed infants\textsuperscript{112}. Interestingly, however, adult individuals who had been breastfed as infant have lower total and pro-atherogenic LDL-cholesterol compared to previously formula-fed subjects\textsuperscript{107}. This has led to the hypothesis that early life cholesterol supply can program cholesterol homeostasis in later life. In support of this notion, we recently reported indications that dietary cholesterol availability in early life of mice determines the set-point for cholesterol absorption efficiency at adult age\textsuperscript{324}. The rather strictly regulated concentration of milk cholesterol found in this study could support the relevance of a stable cholesterol supply for its programming importance. Third, a stable supply of dietary cholesterol could theoretically be relevant for the development of intestinal microbiota in early life. The cholesterol synthesis rate, the biliary cholesterol secretion and the fecal cholesterol excretion have all been shown to correlate with abundance of certain bacterial taxa in hamsters\textsuperscript{372}. Additionally, conversion of cholesterol to the neutral sterol coprostanol by the intestinal microbiota is delayed in breastfed infants\textsuperscript{373}, indicating inhibited growth of certain bacterial groups. Yet, a recent study by ourselves does not support the possibility that stable cholesterol supply is relevant for the development of intestinal microbiota. We demonstrated in LDLR-deficient mice that dietary cholesterol supplementation (1.25%) did not affect either the composition or functionality of intestinal microbiota\textsuperscript{374}.

In conclusion, our results clearly demonstrate that the milk cholesterol concentration is very resistant to maternal diet- and genetically-induced hypercholesterolemia in mice. We speculate that the robust maintenance of stable milk cholesterol levels serves important physiological functions for the offspring, such as programming of long-term health benefits.
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