Choline Supplementation Protects against Liver Damage by Normalizing Cholesterol Metabolism in Pemt/Ldlr Knockout Mice Fed a High-Fat Diet^{1,2}

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Abstract
Dietary choline is required for proper structure and dynamics of cell membranes, lipoprotein synthesis, and methyl-group metabolism. In mammals, choline is synthesized via phosphatidylethanolamine N-methyltransferase (Pemt), which converts phosphatidylethanolamine to phosphatidylcholine. Pemt^{−/−} mice have impaired VLDL secretion and developed fatty liver when fed a high-fat (HF) diet. Because of the reduction in plasma lipids, Pemt^{−/−}/low-density lipoprotein receptor knockout (Ldlr^{−/−}) mice are protected from atherosclerosis. The goal of this study was to investigate the importance of dietary choline in the metabolic phenotype of Pemt^{−/−}/Ldlr^{−/−} male mice. At 10–12 wk of age, Pemt^{−/−}/Ldlr^{−/−} (HF^{+}/+) and half of the Pemt^{−/−}/Ldlr^{−/−} (HF^{−}/−) mice were fed an HF diet with normal (1.3 g/kg) choline. The remaining Pemt^{−/−}/Ldlr^{−/−} mice were fed an HF diet supplemented (5 g/kg) with choline (HFCS^{−}/−) mice. The HF diet contained 60% of calories from fat and 1% cholesterol, and the mice were fed for 16 d. HF^{−}/− mice lost weight and developed hepatomegaly, steatohepatitis, and liver damage. Hepatic concentrations of free cholesterol, cholesterol-esters, and triglyceride (TG) were elevated by 30%, 1.1-fold and 3.1-fold, respectively, in HF^{−}/− compared with HF^{+}/+ mice. Choline supplementation normalized hepatic cholesterol, but not TG, and dramatically improved liver function. The expression of genes involved in cholesterol transport and esterification increased by 50% to 5.6-fold in HF^{−}/− mice when compared with HF^{+}/+ mice. Markers of macrophages, oxidative stress, and fibrosis were elevated in the HF^{−}/− mice. Choline supplementation normalized the expression of these genes. In conclusion, HF^{−}/− mice develop liver failure associated with altered cholesterol metabolism when fed an HF/choline diet. Choline supplementation normalized cholesterol metabolism, which was sufficient to prevent nonalcoholic steatohepatitis development and improve liver function. Our data suggest that choline can promote liver health by maintaining cholesterol homeostasis. J. Nutr. 144: 252–257, 2014.

Introduction
Nonalcoholic fatty liver disease (NAFLD)^6 is a term that describes a wide spectrum of hepatic disorders that feature fat accumulation in the liver unrelated to alcohol consumption. The spectrum of NAFLD ranges from simple steatosis (TG accumulation in hepatocytes) to nonalcoholic steatohepatitis [NASH (steatosis with inflammation and fibrosis)], cirrhosis (replacement of hepatocytes by scar tissue), and eventually liver failure (1–5). NAFLD is an emerging hepatic illness related to obesity, dyslipidemia, and insulin resistance (5). The estimated worldwide prevalence of NAFLD and NASH range from 6.3% to 33% and 3% to 5%, respectively (6).

Choline is an essential nutrient that is required for neurotransmitter synthesis, methyl-group metabolism, cell-membrane structure and signaling, and lipid transport (7–9). Phosphatidylethanolamine (PE) is synthesized in all nucleated mammalian cells via the CDP–choline pathway; this pathway uses choline as the initial substrate, and thus it mainly depends on dietary choline intake (7–10). The liver is unique in that it possesses a second pathway for PC synthesis; phosphatidylethanolamine N-methyltransferase (PEMT) converts phosphatidylethanolamine (PE) to PC via 3 sequential methylations using 5-adenosylmethionone
as the methyl donor (11–13). Importantly, PEMT is the only de novo pathway for choline biosynthesis in mammals.

When fed an unpurified diet, Pemt−/− mice have a normal liver morphology with minimally affected concentrations of hepatic PC and PE (9,14,15). However, when fed a high-fat (HF) diet for 3 wk, Pemt−/− mice develop hepatic steatosis attributable to impaired TG secretion (16). In a separate study, Pemt−/− mice fed an HF diet for 10 wk presented with hepatomegaly, steatohepatitis, and a decreased PC:PE ratio (17). Furthermore, Pemt−/− mice developed end-stage liver failure within 3 d of consuming a choline-deficient diet attributable to a significant decrease in the PC:PE ratio (18–20).

Jacobs et al. (17) reported recently that Pemt−/− mice were protected from HF-diet–induced obesity and insulin resistance that could be reversed by supplementing the diet with choline. When the Pemt−/− mice were bred with the low-density lipoprotein receptor knockout (Ldlr−/−) mice, the resulting Pemt−/−/Ldlr−/− mice had decreased hepatic VLDL secretion and were protected from atherosclerosis when fed an HF–cholesterol diet (21). The initial purpose of this study was to determine whether the reduction in atherosclerosis in the Pemt−/−/Ldlr−/− mice could be modulated by dietary choline supplementation. Contrary to what we predicted, HF-diet–fed Pemt−/−/Ldlr−/− mice presented with severe weight loss and liver damage within 3 wk. To explain this phenomenon, we evaluated the choline requirement in the Pemt−/−/Ldlr−/− mice. Choline supplementation prevented weight loss in the mice lacking Pemt and Ldlr. Furthermore, we show that increased choline consumption prevented steatohepatitis and liver failure, but not TG accumulation, in Pemt−/−/Ldlr−/− mice fed an HF diet. Our data provide a novel association between choline availability and hepatic cholesterol metabolism in a context of NAFLD.

Materials and Methods

All procedures were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval by the University of Alberta Health Sciences Animal Care and Use Committee. Male mice (n = 8–10 per group) were maintained on an unpurified diet (PICO Laboratory Rodent Diet 20; LabDiet). At 10–12 wk of age, Pemt−/−/Ldlr−/− (HF++) and half of the Pemt−/−/Ldlr−/− (HF−−) mice were fed an HF diet with normal (1.3 g/kg) choline. The remaining Pemt−/−/Ldlr−/− mice were fed an HF diet supplemented (5 g/kg) with choline (HFCs−/− mice). The HF diet (F6535; Bio-Serv) contained 60% of calories from fat and 1% cholesterol (wt:wt), and mice consumed food ad libitum for 16 d. Choline bitartrate (C1629; Sigma-Aldrich) was used as the source of choline. A pilot study was performed to investigate the long-term consequences of HF-diet feeding in the Pemt−/−/Ldlr−/− mice. The HF−− mice lost >20% body weight, and the feeding trial was terminated after only 21 d (data not shown). On examination, the HF−− mice presented with severe hepatomegaly and NASH. All mice were food deprived for 12 h before being killed by exsanguination under isoflurane anesthesia. Plasma was collected and frozen at −80°C. The liver was snap frozen in liquid nitrogen and then stored at −80°C, or preserved in 10% phosphate-buffered formalin, pH 7.0. The extent of steatosis, hepatocyte ballooning, and both lobular and portal inflammation were graded by a pathologist using the Brunt criteria (22). Plasma alanine aminotransferase [ALT (Biotron Diagnostics)] and bile acids (Trinity Biotech) were determined using a commercially available kit. Total phospholipids (PLs), TG, free cholesterol (FC), and cholesteryl ester (CE) were measured by GLC (20,23). The extraction and quantification of PLs and choline-related compounds were performed according to the method by Xiong et al. (24). RNA extraction, cDNA preparation, and qPCR analysis were performed as described previously (25). A standard curve was used to calculate mRNA level relative to the control gene, 18S.

Data are reported as means ± SDs. Data were subjected to Bartlett’s test for homogeneity of variances; unequal variances were stabilized by log transformation. Data were compared using 1-factor ANOVA, followed by a Tukey’s post hoc test. Histologic grading was compared using a Kruskal-Wallis ANOVA of rank, followed by Dunn’s multiple-comparison test. The level of significance was set at P < 0.05 in all analyses.

Results

In an attempt to explain the sensitivity of the Pemt−/−/Ldlr−/− mice to the HF diet, we performed a 16-d feeding trial. By day 16 of feeding, the HF−− mice weighed significantly less than the HF++ and HFCs−/− mice (Fig. 1A). In addition, HFCs−/− mice had lowered liver weight (6.7 ± 0.3% body weight) compared with HF−− (10.2 ± 0.7% body weight), but remained higher when compared with the HF++ (4.0 ± 0.2% body weight) mice.

Histologic examination of the liver revealed the development of severe steatosis, as indicated by enlarged lipid droplets in the liver that coincided with hepatomegaly, hepatocyte ballooning, and inflammation in HF−− mice compared with HF++ mice (Fig. 2A, B). The size of the lipid droplets and hepatocyte inflammation were reduced in the HFCs−/− mice (Fig. 2A, B) compared with HF−− mice. Plasma ALT activity, a measure of liver damage, was 6.4-fold higher in HF−− mice compared with HF++ mice; choline supplementation significantly reduced the increase in plasma ALT caused by Pemt deletion (Fig. 2C).

mRNA markers of macrophages [EGF-like module containing, mucin-like, hormone receptor-like sequence 1 (F4/80) and cluster of differentiation (CD)-68], oxidative stress [mitochondrial uncoupling protein 2 (Ucp2) and NADPH oxidase 2 (Nox2)], and fibrosis [collagen type I α 1 (Col1a1)] were elevated in HF−− compared with HF++ and HFCs−/− mice (Fig. 2D). Hepatic TG (Fig. 3A), CE (Fig. 3B), and FC (Fig. 3C) concentrations were significantly higher in HF−− compared with HF++ mice (Fig. 3A-C). Hepatic CE and FC concentrations were normal in the HFCs−/− mice, but hepatic TG concentrations remained elevated compared with HF++ mice (Fig. 3A-C). Interestingly, HF−/− and HFCs−/− mice had significantly lowered plasma TG (Fig. 3D), CE (Fig. 3E), and FC (Fig. 3F) compared with HF++ mice. Plasma CE and TG were lower in HF−/− than HFCs−/− mice. HF−/− mice had a

![FIGURE 1](https://academic.oup.com/jn/article-abstract/144/3/252/4571666/Downloaded on 12 April 2018)

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significant reduction in hepatic lysophosphatidylcholine, glycerophosphocholine, PC, and PE compared with HF<sup>++</sup> mice (Table 1). HFCS<sup>2</sup> mice had higher lysophosphatidylcholine, PC, and PE compared with HF<sup>-/-</sup> mice, but these metabolites remained lower compared with the HF<sup>++</sup> mice. The concentration of hepatic lysophosphatidylethanolamine was reduced in HF<sup>-/-</sup> mice compared with both HF<sup>++</sup> and HFCS<sup>2</sup> mice. The concentration of sphingomyelin and choline and the PC:PE ratio was lower in both HF<sup>-/-</sup> and HFCS<sup>2</sup> mice compared with HF<sup>++</sup> mice (Table 1).

The expression of ATP-binding cassette subfamily A, member 1 (Abca1), Sr-b1 (scavenger receptor class B type 1), multidrug resistant protein 2, and sterol O-acyltransferase 1 (Soat1) was greater in HF<sup>-/-</sup> mice compared with HF<sup>++</sup> mice (Table 2). Choline supplementation normalized the expression of Abca1, Sr-b1, and Soat1 but not multidrug resistant protein 2 expression caused by Pemt deletion. The concentration of 3-hydroxy-3-methylglutaryl-CoA reductase mRNA was not altered by experimental treatments. Plasma bile acids were elevated in HF<sup>-/-</sup> mice (22.5 ± 4.1 μmol/L) compared with HF<sup>++</sup> mice (12.8 ± 2.9 μmol/L) but did not significantly differ from HF<sup>++</sup> mice. The hepatic mRNA concentrations of cholesterol 7 α-hydroxylase (Cyp7a1), sterol 12 α-hydroxylase (Cyp8b1), organic anion transporting polypeptide 1 (Oatp1), sodium taurocholate cotransporting polypeptide (Ntcp), bile salt export pump (Bsep), and small heterodimer partner (Shp) genes were lower in HF<sup>-/-</sup> compared with HF<sup>++</sup> mice. Choline supplementation normalized the abundance of Cyp7a1, Ntcp, Bsep, and Shp genes, increased the expression of Oatp1 compared with HF<sup>-/-</sup>, and increased the expression of Cyp8b1 compared with the other 2 groups (Table 2).

**Discussion**

The current research has provided strong evidence that Pemt deletion increases choline requirements in HF<sup>-/-</sup> mice. HF<sup>-/-</sup> mice lost significant body weight and liver function, although...
they consumed the recommended dietary amount of choline (1.3 g choline/kg diet). HF−/− mice had significantly lower hepatic PC and PE concentrations; in addition, they developed hepatomegaly and hepatic inflammation, accumulated more hepatic TG, CE, and FC, and presented a 6.4-fold increase in plasma PC and PE concentrations; in addition, they developed hepatic inflammation and liver damage (26). Jacobs et al. (17) reported that Pemt−/− mice were protected from HF (60% of calories as fat, 0% cholesterol, 1.3 g choline/kg diet) diet–induced obesity and insulin resistance. This protection was reversed when the HF diet was supplemented with choline. In a separate study, HF [40% of calories as fat, 1.25% cholesterol (wt:wt), 3.43 g choline/kg diet] diet–fed Pemt−/−/Ldr−/− mice were dramatically protected from atherosclerosis (21). In both studies (17, 21), mice did not suffer excessive weight loss throughout the feeding period. The data from the current study indicate that the Pemt−/−/Ldr−/− mice are susceptible to the hepatic consequences of HF diet feeding and, as such, have a higher requirement for dietary choline.

PC accounts for >50% of PLs in most mammalian membranes and is crucial for cellular integrity, differentiation, and proliferation (27, 28). It is well established that a decreased hepatic PC:PE ratio is related to NAFLD progression in mice (17, 29). In humans, a reduced hepatic PC:PE ratio was observed in patients with NASH (20). The appropriate distribution of PC and PE is essential for cellular integrity and for transport of molecules across intracellular membranes (30, 31). When this ratio is decreased, leakage of cellular contents into the extracellular space activates resident Kupffer cells, leading to increased inflammation, impaired mitochondrial respiration, and hepatocyte injury (20, 29). In the current study, the HF−/− mice presented with lower hepatic PC and PC:PE ratio compared with the HF+/+ mice (Table 1); the consequent reduction in VLDL secretion (21) increased hepatic TG and lowered plasma TG in these mice (Fig. 3, A, D). Surprisingly, hepatic and plasma TG remained elevated in the HFCs−/− mice (Fig. 3, A, D), which could be attributed to the failure of choline supplementation to completely reverse the reduction in PC concentrations or increase the PC:PE ratio (Table 1). However, the progression of NAFLD from steatosis to NASH was prevented in HFCs−/− mice (Fig. 2B).

The HF−/− mice had elevated hepatic CE and FC compared with HF+/+ mice (Fig. 3B, C). Similarly, HF−/− mice presented an induced expression of 2 cholesterol transport genes, Abca1 and Sb-r1, as well as a 5.6-fold increase in the expression of Soat1, the gene coding for acyl-CoA:cholesterol acyltransferase that converts cholesterol to CE (Table 2). The expression of cholesterol metabolism-related genes (Table 2) and CE and FC concentrations (Fig. 3B, C) was normalized in HFCs−/− mice. From these novel findings, we proposed that elevated concentrations of hepatic free cholesterol play a significant role in the progression of NAFLD. Cholesterol is a substrate for bile acid synthesis; as such, an excess of free cholesterol in the HF−/− mice could increase bile acid formation. In support of this hypothesis, plasma bile was elevated 4.4-fold in the HF−/− mice; this increase was prevented by choline supplementation. Increased intracellular concentrations of hydrophobic bile acids are believed to play an important role in liver injury by inducing apoptosis or necrosis of hepatocytes (32). Hydrophobic bile acids induce injury to cultured human hepatocytes (33), isolated rat hepatocytes (34), and whole rat liver (35). Through their detergent actions, bile acids may compromise the structural integrity of cell membranes, leading to ROS. ROS induce oxidative damage, causing mitochondrial dysfunction, endoplasmic reticulum stress, and cell death (37). Bile acids can also activate Kupffer cells to produce ROS, exacerbating liver injury (38).  

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### TABLE 1 Liver choline and phospholipid concentrations in HF+/+ , HF−/− , and HFCs−/− mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>HF+/+</th>
<th>HF−/−</th>
<th>HFCs−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline, μg/mg liver</td>
<td>0.017 ± 0.001a</td>
<td>0.009 ± 0.002b</td>
<td>0.008 ± 0.001c</td>
</tr>
<tr>
<td>LPC, μg/mg liver</td>
<td>0.32 ± 0.03a</td>
<td>0.11 ± 0.01c</td>
<td>0.18 ± 0.03b</td>
</tr>
<tr>
<td>LPE, μg/mg liver</td>
<td>0.19 ± 0.02a</td>
<td>0.14 ± 0.01b</td>
<td>0.20 ± 0.01a</td>
</tr>
<tr>
<td>SM, μg/mg liver</td>
<td>1.45 ± 0.29a</td>
<td>0.78 ± 0.04b</td>
<td>0.72 ± 0.01b</td>
</tr>
<tr>
<td>GPC, μg/mg liver</td>
<td>0.11 ± 0.02b</td>
<td>0.06 ± 0.01c</td>
<td>0.08 ± 0.01a</td>
</tr>
<tr>
<td>PC, μg/mg liver</td>
<td>15.0 ± 0.57b</td>
<td>5.78 ± 0.63a</td>
<td>9.70 ± 1.42b</td>
</tr>
<tr>
<td>PE, μg/mg liver</td>
<td>9.98 ± 0.63a</td>
<td>5.71 ± 0.77a</td>
<td>8.54 ± 0.93b</td>
</tr>
<tr>
<td>PC:PE ratio</td>
<td>1.51 ± 0.07a</td>
<td>1.02 ± 0.09a</td>
<td>1.16 ± 0.10a</td>
</tr>
</tbody>
</table>

1 Values are means ± SDs; n = 4–5 per group. For a variable, means in a row with superscripts without a common letter differ, P < 0.05. GPC, glycerophosphocholine; HF+/+, Pemt+/+/Ldr+/+ mice fed a high-fat diet; HF−/−, Pemt−/−/Ldr−/− mice fed a high-fat diet; HFCs−/−, Pemt−/−/Ldr−/− mice fed a high-fat diet supplemented with choline; Ldr, LDL receptor; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Pemt, phosphatidylethanolamine N-methyltransferase; SM, sphingomyelin.

### TABLE 2 Hepatic expression of genes involved in cholesterol and bile acid metabolism in HF+/+ , HF−/− , and HFCs−/− mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>HF+/+</th>
<th>HF−/−</th>
<th>HFCs−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>αAbca1</td>
<td>1.00 ± 0.10b</td>
<td>1.54 ± 0.20a</td>
<td>0.97 ± 0.37b</td>
</tr>
<tr>
<td>Sr-b1</td>
<td>1.00 ± 0.12b</td>
<td>1.63 ± 0.29a</td>
<td>1.16 ± 0.28a</td>
</tr>
<tr>
<td>Soat1</td>
<td>1.00 ± 0.21b</td>
<td>6.58 ± 1.30a</td>
<td>1.54 ± 1.37a</td>
</tr>
<tr>
<td>HMG-CoA-Red</td>
<td>1.00 ± 0.14b</td>
<td>1.25 ± 0.28a</td>
<td>1.21 ± 0.31a</td>
</tr>
<tr>
<td>Cyp7a1</td>
<td>1.00 ± 0.25b</td>
<td>0.04 ± 0.02b</td>
<td>0.80 ± 0.39a</td>
</tr>
<tr>
<td>Cyp8b1</td>
<td>1.00 ± 0.42b</td>
<td>0.03 ± 0.01c</td>
<td>2.01 ± 0.40a</td>
</tr>
<tr>
<td>Oatp1</td>
<td>1.00 ± 0.23b</td>
<td>0.003 ± 0.002c</td>
<td>0.42 ± 0.01c</td>
</tr>
<tr>
<td>Ntcp</td>
<td>1.00 ± 0.10b</td>
<td>0.17 ± 0.05b</td>
<td>1.07 ± 0.16c</td>
</tr>
<tr>
<td>Bsep</td>
<td>1.00 ± 0.18b</td>
<td>0.39 ± 0.07b</td>
<td>1.10 ± 0.15b</td>
</tr>
<tr>
<td>Mr2</td>
<td>1.00 ± 0.11b</td>
<td>1.55 ± 0.19b</td>
<td>1.83 ± 0.55b</td>
</tr>
<tr>
<td>Shp</td>
<td>1.00 ± 0.34b</td>
<td>0.38 ± 0.23b</td>
<td>1.25 ± 0.11b</td>
</tr>
</tbody>
</table>

1 Data are means ± SDs; n = 4–5 per group. Relative mRNA expression was normalized to 18S expression. For a variable, means in a row with superscripts without a common letter differ, P < 0.05. Abca1, ATP-binding cassette subfamily A, member 1; Bsep, bile salt export pump; Cyp7a1, cholesterol 7α-hydroxylase; Cyp8b1, sterol 12α-hydroxylase; HMG−/−, Pemt−/−/Ldr−/− mice fed a high-fat diet; HF−/−, Pemt−/−/Ldr−/− mice fed a high-fat diet supplemented with choline; HMG-CoA-Red, 3-hydroxy-3-methylglutaryl-CoA reductase; Ldr, LDL receptor; Mr2, multigland resistant protein 2; Ntcp, sodium taurocholate cotransporting polypeptide; Oatp1, organic anion transporting polypeptide 1; Pemt, phosphatidylethanolamine N-methyltransferase; Shp, small heterodimer partner; Soat1, sterol Oacyltransferase 1; Sb-r1, scavenger receptor class B type 1.
To investigate the significance of hepatic free cholesterol in the progression of NAFLD, we measured the expression of genes involved in bile acid synthesis (Table 2). Cyp7a1 is the rate-limiting enzyme for the conversion of cholesterol to bile acids via the classical pathway (39,40). Cyp8b1 controls the ratio of cholic acid-to-chenodeoxycholic acid and thus plays a crucial role in bile acid synthesis via the alternative pathway (41). Both Cyp7a1 and Cyp8b1 are subject to a feedback inhibition when bile acids are elevated (42–44). Likewise, Ntcp (45,46) and Oatp1 (47), 2 proteins responsible for the reuptake of bile acids by the liver (47), were dramatically reduced in HF/−/− mice. It is possible that the reduction in these bile acid metabolism genes is attributable to increased farnesoid X receptor signaling (42,43,47); however, we observed a reduction in Shp and Bsep mRNA in the livers of HF/−/− mice. SHP feeds back and inhibits its own expression (42,43), which could explain the reduction in Shp mRNA levels in HF/−/− mice. Diminished Bsep expression was reported previously under obstructive cholestasis, characterized by bile acid accumulation in serum and liver, and several other pathophysiologic conditions (48). A reduction in bile acid metabolism genes could also be attributable to the activation of liver X receptor (49). Interestingly, we observed an upregulation of 2 liver X receptor target genes, Abca1 and Soat1, in HF/−/− mice (50). Together, the changes in cholesterol and bile acid metabolism in response to Pemt deletion and choline supplementation (Table 2) support our hypothesis that elevated hepatic free cholesterol concentrations play a significant role in the progression of NAFLD, potentially via providing the substrate for and increasing hepatic bile acids in HF/−/− mice.

In conclusion, Pemt/Ldlr/−/− mice require extra dietary choline to maintain a healthy liver. Choline supplementation did not prevent steatosis in the Pemt/Ldlr/−/− mice but was able to normalize cholesterol metabolism, which was sufficient in improving liver function and preventing the progression to NASH and liver failure. An abundant single nucleotide polymorphism in the human PEMT gene (V175M) is associated with NAFLD; the V175M PEMT is ~40% less active compared with wild-type PEMT. This single nucleotide polymorphism can decrease FC formation and VLDL secretion, leading to hepatic steatosis (51). Our study suggests that people with the V175M mutation may require dietary choline supplementation, especially if they eat a diet high in fat. Our data also suggest that the provision of dietary choline or 1 of its metabolites can maintain liver health by regulating cholesterol metabolism.

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**Literature Cited**


