Dietary cholesterol does not break your heart but kills your liver
Gerhard P. Püschel, MD*, Janin Henkel, PhD

Abstract
It is increasingly accepted that dietary cholesterol has a much lower impact on the progression of cardiovascular disease than previously assumed. However, both animal experiments and human studies seem to support the view that dietary cholesterol may contribute to the transition from benign steatosis to the potentially fatal non-alcoholic steatohepatitis. Cholesterol esters and cholesterol accumulate in the hepatocyte and impair its function. This leads to oxidative stress and endoplasmic reticulum stress triggering the release of pro-inflammatory cytokines and rendering the hepatocyte more susceptible to apoptotic or necrotic cell death. Kupffer cells group around dying hepatocytes and phagocytose the hepatocyte debris and lipids. In addition, they are exposed to lipid peroxidation products released from hepatocytes. Kupffer cells, thus activated, release pro-inflammatory, chemotactic and profibrotic cytokines that promote inflammation and fibrosis. Therefore, dietary cholesterol may be harmful to the liver, in particular when administered in combination with polyunsaturated fatty acids that favor lipid peroxidation.

Keywords: non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, poly-unsaturated fatty acids, Western-type diet

Atherosclerosis and dietary cholesterol: a historical overview
At the beginning of the last century the impact of dietary lipids on the development of cardiovascular diseases was recognized.1 In the 1950s, the comparison of the diet composition at the beginning of the century with post World-War II diets revealed that, among others, increased consumption of saturated fat and cholesterol coincided with the increasing prevalence of cardiovascular disease.2 While it was emphasized early on that the ingestion of saturated fatty acids in particular might drive the elevation of plasma cholesterol levels, a reduction of cholesterol consumption was regarded as an effective intervention to reduce plasma cholesterol levels and hence the risk for cardiovascular disease.3 This view was supported by a large number of animal experimental models (references in4–6), in which high cholesterol diets were used to induce atherosclerotic alterations. In some studies, atherosclerotic lesions could be partially reverted by subsequently feeding a cholesterol-free diet, for example.7 In humans, large epidemiological studies revealed high plasma cholesterol, in particular LDL cholesterol, as a major risk factor for the development of atherosclerosis and it was shown that an increase in cholesterol consumption resulted in a proportional increase in plasma cholesterol.8 However, the dependency of plasma cholesterol was particularly prominent at very low dietary cholesterol intake, far below the quantities normally found in a typical diet in industrialized countries. In addition, although dietary cholesterol intake resulted in an increase in plasma cholesterol levels, the relative changes were in the range of merely 10%. These considerations shed some doubt on the validity of the recommendation to reduce plasma cholesterol levels by dietary interventions.9

Current view on dietary cholesterol and cardiovascular disease
Critical reevaluation of older data together with new studies that were corrected for potential confounders, which were not considered in the early epidemiological studies, refuted the hypothesis that dietary cholesterol has a major impact on the development of cardiovascular disease,10 although this view is not un-contradicted.11 Rather than dietary cholesterol itself, other nutritional factors that coincide with the uptake of dietary cholesterol in a diet rich in animal protein appear to be of relevance.12 Therefore, current dietary recommendations include a reduction of the intake of animal products and an increase in the intake of whole grains. Notably, the replacement of saturated fatty acids by mono- and polyunsaturated fatty acids in the diet is part of the current recommendations (eg, see healthy eating at http://www.heart.org).13–15

Physiological role of liver in cholesterol metabolism
The liver plays a central role in cholesterol metabolism. Dietary cholesterol is delivered to the circulation via the chylomicron pathway. The majority of the triglycerides of the chylomicrons are hydrolyzed by lipoprotein lipase that releases fatty acids for

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Department of Nutritional Biochemistry, University of Potsdam, Institute of Nutritional Science, Nuthetal, Germany.
*Corresponding author. Department Nutritional Biochemistry, University of Potsdam, Institute of Nutritional Science, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany. E-mail address: gpusche@uni-potsdam.de (Gerhard P. Püschel).
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their use primarily in adipose tissue and skeletal muscle. The remaining remnant particles, the chylomicron remnants, are rich in cholesterol. Most of these remnant particles are taken up by hepatocytes by receptor-mediated endocytosis among other routes via ApoE and the LDL receptor related protein (Fig. 1). After lysosomal degradation, cholesterol is funneled into different pathways in the hepatocyte. Besides degradation and elimination (see below), cholesterol and cholesterol esters are incorporated into VLDL particles, which are secreted by the hepatocyte. In the periphery, lipoprotein lipase hydrolyzes most of the triglycerides in VLDL as described for chylomicrons and another remnant particle, the IDL, is generated. IDL travels to the liver and is subject to 2 completely different fates: (1) it can be taken up by receptor mediated endocytosis via the LDL receptor or the LDL receptor-related protein as described for the chylomicron remnant or (2) hepatic lipase hydrolyzes a large part of the triglycerides remaining in the IDL particle. While the fatty acids thus liberated are either re-incorporated in triglycerides of VLDL or oxidized by the hepatocyte, the extracellular remnants of the IDL are converted into cholesterol-rich LDL particles, which, after leaving the liver, may serve as a source for cholesterol in any cell of the body. If the supply of cholesterol in cells exceeds their demand, they may rid themselves of excess cholesterol by transferring it on HDL. HDL in turn is delivered to the hepatocyte, which can either take up the entire HDL particle by receptor-mediated endocytosis for example via the LDL receptor or extract cholesterol from the cholesterol esters contained in the HDL particle.

Next to the intestinal epithelial cells, the hepatocyte is probably the only site at which significant quantities of cholesterol may be removed from the body either by excretion in form of free cholesterol or by secretion after conversion into bile acids. If the supply with cholesterol exceeds the hepatocyte’s capacity for bile acid synthesis and cholesterol secretion, the only safe mode of disposing cholesterol is the formation of cholesterol esters that are transiently stored in the hepatocyte.

**Evidence for the impact of dietary cholesterol on NASH development**

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome. Its prevalence is increasing as a result of the increasing proportion of overweight and obese patients in the population. While simple steatosis,
of clinical significance, appears to be fully reversible, more severe forms of the disease, the non-alcoholic steatohepatitis (NASH), is a chronically progressive disease leading to fibrosis, cirrhosis, and eventually hepatocellular carcinoma. Currently, NASH is the most common reason for terminal hepatic failure in western societies.25 Despite intense research, it is not clear (1) whether NAFLD and NASH are different temporal stages of the same disease and if so (2) what are the molecular mechanisms that trigger the progression. Recent evidence suggests that dietary cholesterol might play a critical role in this process.

The impact of dietary cholesterol on liver pathology was actually described prior to its role in the development of atherosclerosis.1 In his seminal work on atherosclerosis Antischkow describes previous work in which the feeding of egg yolk to rabbits resulted in “an extraordinarily rich infiltration of the liver parenchyma with fat-like substances” that was always accompanied by “strongly pronounced areas of parenchymal degeneration”. However, this aspect of dietary cholesterol largely fell into oblivion. Only with the recent surge of NASH and the search for an appropriate rodent model of NASH, renewed interest in the impact of dietary cholesterol on hepatic steatosis and inflammation awoke. While many animal models that are based on diets which induce conditions resembling the metabolic syndrome also result in hepatic steatosis in rodents, most of these diets fail to cause hepatic inflammation and fibrosis in animals. On the other hand, dietary interventions that reproducibly induce hepatic inflammation and fibrosis, such as a choline-methionine-deficient diet, fail to reproduce the symptoms of the metabolic syndrome, indicating that the mechanisms that trigger fibrosis development differ from those in human NASH.18

Recently, fructose and cholesterol have been shown to be crucial components in so called Western-type diets for the induction of NASH-like hepatic pathologies in rodents.19–22 Feeding a “fast food” diet to mice, which is rich in saturated fat and cholesterol and fructose, resulted in a steady accumulation of cholesterol in the liver over a period of 36 weeks that was accompanied by inflammation and fibrosis.23 Notably, insulin resistance preceded hepatic inflammation in these animals. The combination of butter fat with cholesterol in the diet resulted in a NASH-like phenotype in mice with an atherosclerosis-prone genetic background.24 While feeding a high fat diet consisting mostly of saturated and mono-unsaturated fatty acids resulted in steatosis, only the combination of the same high fat diet with cholesterol caused inflammation and fibrosis, as such a choline-methionine-deficient diet, fail to reproduce the symptoms of the metabolic syndrome, indicating that the mechanisms that trigger fibrosis development differ from those in human NASH.18

Dietary cholesterol accumulates preferentially in the liver.27 Although the hepatic accumulation appears to be independent of food composition and can be observed in animals fed cholesterol on a chow-based diet, the accumulation is particularly pronounced in animals receiving a soybean-based diet rich in polyunsaturated fatty acids61 (Table 1). Initially, the primary site of cholesterol accumulation is the hepatocyte. In a healthy hepatocyte the endogenous production of cholesterol is reduced when the exogenous supply increases by retaining the inactive form of SREBP2, the key transcription factor controlling the expression of enzymes involved in cholesterol synthesis, in the endoplasmic reticulum (ER). This feedback regulation may be impaired in patients with NASH.42 The hepatocyte can handle cholesterol in 3 ways (Fig. 1): (1) cholesterol can be excreted into the bile by active transport via the ABCG5/ABCG8 heterodimer in the apical membrane.43 (2) Alternatively, cholesterol can be oxidized by CYP7A1 and/or CYP27A1 to initiate bile acid synthesis.44 Synthesis of bile acids and biliary secretion of bile acids and cholesterol is the major route by which the body can dispose of cholesterol. Of note, a large proportion of bile acids, and also cholesterolescreted into the bile, reenter the circulation after resorption in the gut. The main impact of inhibitors of the

Potential molecular mechanisms underlying NASH-induction by cholesterol

Cholesterol accumulation in the hepatocyte

Dietary cholesterol accumulates preferentially in the liver.27
intestinal cholesterol uptake, like ezetimibe, is the interruption of cholesterol re-uptake.36 (3) Thirdly, cholesterol can be converted into cholesterol esters which either are incorporated into VLDL together with free cholesterol or may be stored transiently in lipid droplets of the hepatocyte. The latter fate is the only safe way the hepatocyte can dispose of excess cholesterol when the supply exceeds the capacity of turnover in the other routes. Accordingly, the hepatocyte seems to redirect fatty acids from triglyceride synthesis to the synthesis of cholesterol esters, which contributes to the drop of plasma triglycerides after cholesterol feeding observed in many studies.18,26,27,46 (Table 1) as well as the increase in cholesterol in the VLDL and remnant fractions.46 Accumulation of cholesterol ester is further favored because the hepatocyte may react with an increase in de novo fatty acid synthesis in order to ensure a sufficient supply of fatty acids for cholesterol esterification. To this end, cholesterol, after enzymatic or non-enzymatic conversion into oxysterols, may induce SREBP1c and thus enzymes of fatty acid synthesis by activation of the liver X receptor (LXR) (Fig. 1).67 However, the accumulation of cholesterol esters per se is unlikely to account for the transition from steatosis to NASH and fibrosis. Rather, oxidative stress resulting from the excessive lipid accumulation or the accumulation of free cholesterol might be relevant in this respect.

**Oxidative stress**

Oxidative stress has been proposed as a possible contributor to the transition from benign steatosis to NASH with inflammation and fibrosis.48 Fatty acid oxidation products are elevated in the circulation of patients with NASH in comparison to patients with blunt steatosis69 and lipid peroxidation products like malondialdehyde or 4-hydroxynonenal are capable of triggering inflammation and fibrosis.50,51 by directly activating non-parenchymal cells (see below). Oxidative stress may result from an imbalance between the antioxidative defense systems and the increasing production of reactive oxygen species and lipid peroxidation products in mitochondrial, peroxisomal and microsomal fatty acid oxidation that result from lipid accumulation in hepatic steatosis (Fig. 1).52 Cholesterol contributes to the increase in oxidative stress. In particular when administered in combination with ω-6 PUFA, dietary cholesterol appears not only to be a strong trigger of hepatic steatosis, but also for oxidative stress and subsequent inflammation and fibrosis in rodent liver.26 Malondialdehyde, which is produced during peroxidation of PUFA under relatively mild oxidative conditions, was increased in livers of mice fed a diet rich in ω-6 PUFA, irrespective of the presence of cholesterol. By contrast, the additional presence of cholesterol caused a strong increase in oxidized peroxiredoxins and protein carbonyls, which are indicative of severe oxidative stress. Notably, it was the combination of PUFA and cholesterol that apparently was responsible for the strong oxidative stress because the same quantity of cholesterol in combination with saturated fat caused steatosis, but only mild signs of inflammation, and no signs of fibrosis (Table 1).

Cholesterol itself is also subject to oxidative modifications. Oxysterols are elevated in NASH patients53 and appear to be causative in NASH development.49 While oxysterols via the LXR induce pathways that eliminate cholesterol from the cell and thereby reduce the cell’s cholesterol burden (Fig. 1), depending on the species oxysterols also may have adverse effects. Thus, 25-hydroxy-cholesterol has been shown to enhance the inflammatory response in hepatocytes by NFκB activation54 whereas its conjugation product, 25-hydroxycholesterol-3-sulfate attenuated inflammation. Several oxysterols can induce apoptosis by triggering the mitochondrial apoptotic pathway55 in hepatoma cells or primary rat hepatocytes if cells were exposed to a combination of oxysterols and fatty acids. In addition, oxysterols appear to contribute to cell death by antagonizing Akt-dependent survival pathways (Fig. 1).56 Although in a different study, oxysterols apparently did not reduce cell viability of hepatocytes,57 they still might contribute to NASH development by acting on non-parenchymal liver cells (see below).

**Free cholesterol as trigger of hepatocyte apoptosis and necrosis**

Cholesterol may be safely stored in cholesterol esters. However, this storage is impaired in NASH patients. In addition to the impaired feedback inhibition of cholesterol synthesis (see above), an increase in the activity of cholesterol ester hydrolase may contribute to the increase in free cholesterol.58 The concentration of free cholesterol increases as liver damage advances.59 Changes in free cholesterol may result in ER stress,42,60 ER stress induced activation of the IRE1α-XBP-1 pathway can further promote steatosis by inducing key enzymes of triglyceride

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**Table 1**

| Parameter                          | STD      | WD       | WD + Cho | HFD     | HFD + Cho |
|-----------------------------------|----------|----------|----------|---------|-----------|
| Serum lipids                      |          |          |          |         |           |
| Serum triglycerides, mmol/L       | 1.01 ± 0.04 | 0.92 ± 0.13 | 0.55 ± 0.03 | 2.60b | 6.04 ± 0.03 | 10.6c |
| Serum cholesterol, mmol/L         | 1.97 ± 0.06 | 2.56 ± 0.32 | 3.81 ± 0.13 | 2.60b | 3.89 ± 0.13 | 24b |
| Liver triglycerides, mg/g tissue  | 23.86 ± 2.43 | 26.05 ± 2.99 | 145.53 ± 8.09 | 31ab | 129.41 ± 19.97 | 16ab |
| Liver cholesterol, mg/g tissue    | 47.80 ± 1.41 | 47.55 ± 3.57 | 433.03 ± 14.58 | 31ab | 65.56 ± 7.97 | 16ab |
| Hepatic inflammation markers      |          |          |          |         |           |
| Cd11b mRNA, arbitrary units       | 1.13 ± 0.12 | 0.28 ± 0.14 | 7.40 ± 0.28 | 231ab | 7.02 ± 0.12 | 32b |
| C2c2 (Mcp1) mRNA, arbitrary units | 1.22 ± 0.20 | 2.30 ± 0.43 | 8.79 ± 0.50 | 231ab | 1.67 ± 0.23 | 20b |
| Hepatic fibrosis marker           |          |          |          |         |           |
| Col1a1 mRNA, arbitrary units      | 1.06 ± 0.08 | 1.86 ± 0.26 | 10.34 ± 0.95 | 31ab | 3.07 ± 0.31 | 20b |

Mice were fed standard chow (STD), a soybean oil based Western-type diet rich in polyunsaturated fatty acids (WD), a lard based high fat diet containing predominantly saturated fatty acids (HFD) or WD + Cho containing in addition 0.75% cholesterol (WD + Cho, HFD + Cho). The following parameters were determined after 20 weeks of diet intervention: Serum lipids, hepatic lipids, hepatic inflammation markers Cd11b and Mcp1 (monocyte chemotactic protein 1, C2c2), marker of hepatic fibrosis Col1a1 (collagen type 1a1). Statistics: one-way-ANOVA with Dunnett’s or Tukey’s post hoc test for multiple comparison: *P < 0.05 vs STD; **P < 0.05 vs WD; ***P < 0.05 vs HFD; ****P < 0.05 vs WD + Cho. J. Henkel, preliminary data.
biosynthesis. In addition, ER stress may result in the activation of the inflammasome (Fig. 1) and a subsequent increase in IL-1β production in hepatocytes, directly linking cholesterol accumulation to the induction of an inflammatory response. Furthermore, cholesterol-elicited ER stress may trigger hepatocyte apoptosis or sensitize hepatocytes to other proapoptotic signals. In a different study, no ER stress-mediated activation of apoptotic pathways was observed. Rather, accumulation of free cholesterol in mitochondria caused a depletion of mitochondrial reduced glutathione and sensitized hepatocytes against TNFs or FAS-induced apoptosis and necrosis thereby fostering NASH development.

Recent evidence suggests that an increase in intracellular free cholesterol may affect the regulation of lipid turnover by interfering with the function of proteins in the lipid droplet coat. Formation of cholesterol crystals within the phospholipid monolayer surrounding the lipid droplet was observed and correlated with the progression of steatosis to NASH. While the initial formation of cholesterol crystals within the hepatocyte appeared to promote hepatocyte death, the remnant lipid droplets of dead hepatocytes were surrounded by Kupffer cells in crown-like structures. While cholesterol crystals were found only in the outer layer of lipid droplets within the hepatocytes, presumably due to further hydrolysis of cholesterol esters by Kupffer cell lysosomal enzymes lipid droplet remnants within the crown-like structures contained cholesterol crystals not only in the lipid droplet coat but also in their core. The Kupffer cells phagocytosing the cholesterol crystals evolve into foam cells and react with an inflammatory response (Fig. 2).

Kupffer cell and stellate cell activation

Cholesterol crystals may trigger the inflammatory response in THP macrophages or primary Kupffer cells phagocytosing lipid droplets of apoptotic or necrotic hepatocytes. Lipolytic enzymes released in the zone of inflammation may release cholesterol from cholesterol esters and thereby enhance cholesterol crystal formation (Fig. 2). Transwell experiments showed that direct contact and phagocytosis of the crystals was mandatory. Cholesterol crystals can activate the NLRP3 inflammasome and thereby promote the activation of IL-1β and IL-18 from their precursors. Consequently, inhibition of the NLRP3 inflammasome reduced the severity of liver inflammation and fibrosis in genetic or diet-induced mouse models of NASH. Cholesterol has been shown to favor the transdifferentiation of hepatic stellate cells into myofibroblasts (Fig. 2) and thereby might contribute to the development of hepatic fibrosis.

As noted above, oxidative stress is a crucial factor in the development of NASH. Apart from direct damage to the hepatocyte, lipid oxidation products may activate the inflammatory response in Kupffer cells. Thus, 27-hydroxycholesterol in combination with 4-hydroxynonenal, both of which are products of lipid oxidation, may activate TLR4 signaling and cause NFkB activation in animal models of atherosclerosis. A similar mechanism has been proposed as potential mechanism contributing to the inflammation in NASH (Fig. 2). In addition, oxysterols increased TGFβ and MCP1 expression in Kupffer cells as well as IL-8 and TIMP secretion from hepatic stellate cells and thereby may contribute to inflammation and fibrosis.

Concluding remark

While dietary cholesterol apparently has a much lower impact on the progression of cardiovascular disease than previously assumed, both animal experiments and human studies seem to support the view that dietary cholesterol may contribute to the transition from benign steatosis to the potentially fatal NASH. Dietary cholesterol may be harmful to the liver, in particular...
when administered in combination with polyunsaturated fatty acids, which favor lipid peroxidation. This finding is of particular relevance, considering recent recommendations to replace saturated fat with polyunsaturated fat for the prevention of cardiovascular disease without explicitly suggesting a concurrent reduction of cholesterol intake.

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The literature list in this short review is far from comprehensive. Many authors’ relevant work was not cited. Please accept our sincere apologies.

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