Olive Oil Attenuates the Cholesterol-induced Development of Nonalcoholic Steatohepatitis Despite Increased Insulin Resistance in a Rodent Model

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Key words
- nonalcoholic steatohepatitis
- atherogenic diet
- insulin resistance

Abstract

It is indefinite whether nonalcoholic steatohepatitis (NASH) results as by-product from general metabolic perturbations and adipokine dysregulations or whether defined dietary factors also play a pathogenetic role. Here, we examine the effects of a modification of dietary lipids in a NASH inducing diet on metabolic changes as well as hepatic steatosis, inflammation, and fibrosis in rats. Male Wistar rats were fed with variations of the atherogenic diet (AD), which induces pathophysiological changes resembling human NASH. Dietary variants (AD without cholesterol, cholate, or choline; change of neutral fat to olive oil or coconut oil) were fed for 8 weeks. Insulin resistance, adipokine profile, liver histology, and lipid content as well as expression of proinflammatory and profibrogenic genes were examined. AD led to clear signs of hepatic steatosis and inflammation together with an increase in TNF and collagen type 1 expression. AD without cholesterol showed markedly less liver damage without changes of insulin action and adipokine profile. AD with olive oil and AD without cholate clearly attenuated hepatic inflammation, whereas fat deposition and features of the metabolic syndrome were increased in these animals. Insulin resistance and hepatic fat deposition per se do not cause significant hepatic inflammation in this rodent model. However, dietary cholesterol is an important causal agent for the development of NASH. Olive oil plays a protective role in this respect, which might be due to the high content of monounsaturated fatty acids.

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Introduction

In the last years, nonalcoholic steatohepatitis (NASH) has emerged not only as a new feature of the metabolic syndrome but also as one of the main underlying factors behind cryptogenic liver fibrosis and cirrhosis [1]. In a recent study, the prevalence of NASH in biopsies obtained after sonographic screening of middle-aged US-Americans was as high as 12% [2], which points to the tremendous impact of this disease in the general population. This is even more aggravated in obese subjects, where, depending on the histopathologic criteria used, NASH prevalence rates between 25 and 75% have been reported [3]. Although the association between diet-induced hepatic fat deposition, insulin resistance, and liver fibrogenesis has clearly been shown in multiple studies (recently reviewed in [4]) the exact causal relationship between these phenomena as a basis for future therapeutic concepts remains to be defined.

NASH development is thought to begin with hepatic steatosis due to diet-induced dysregulation of hepatic lipid metabolism with an overstraining of hepatic fatty acid metabolism, decreased fatty acid export, and increased lipogenesis; this is aggravated by an increased peripheral lipolysis in obese subjects [5–8]. Hepatic steatosis increases cellular susceptibility to various stressors, for example, fatty acid-derived oxidative stress, environmental toxins or proinflammatory cytokines arising from the portal venous system leading to endoplasmic reticulum stress and the activation of hepatic stellate cells, which is the key event of hepatic fibrosis [9]. The well-documented perturbations of insulin action and the altered adipokine profile associated with diet-induced obesity such as decreased adiponectin and elevated leptin levels add to the proinflammatory context [10].

One area of uncertainty in this hypothesis is the role of the fat type in hepatic fat deposition, development of hepatic inflammation and the

associated metabolic dysregulation. Clinical studies have demonstrated an increased dietary intake of saturated fatty acids and cholesterol as well as a decreased ratio of dietary polyunsaturated to saturated fatty acids in patients with NASH [11,12]. Saturated fatty intake also correlated with hepatic triglyceride accumulation and insulin resistance [11]. In high fat fed rodent models, differential effects with respect to insulin resistance and liver fat accumulation were detected depending on the type of dietary fat: saturated fat dominated diets led to both hepatic steatosis and insulin resistance, whereas monounsaturated fats only increased liver triglyceride content without significantly changing insulin action [13]. Experimental data at the cell culture level show increased apoptosis of hepatocyte [14] and disruption of endoplasmic reticulum homeostasis [15] induced by saturated fatty acids; this was not seen when employing polyunsaturated controls.

High fat feeding in itself does not reliably induce hepatic inflammation in mice or rats [13]. The lack of a purely diet-induced rodent NASH model has been a drawback for the further elucidation of NASH pathogenesis, as hepatitis models employing toxins, bile duct ligation or nutrient deficient diets do not reflect the clinical situation well. Recently, an atherogenic diet initially described by Paigen et al. [16,17] has been shown to induce hepatic fat deposition and inflammation, and appears to be suitable for the dissection of diet-phenotype interactions in NASH. In this study, we have therefore examined morphologic, metabolic, and molecular parameters of diet-induced nonalcoholic steatohepatitis in different variations of the atherogenic diet with respect to fat components and fatty acid subtypes.

Materials and Methods

Experimental animals

Male Wistar rats were purchased at the age of 6 weeks from Charles River (Sulzfeld, Germany). The rats were caged singly with free access to water and subjected to different dietary regimes as described below. The standardized diets were prepared in pellet form by ssniff GmbH (Soest, Germany) using purified components. Animals were held on a 12:12-h light-dark cycle. All animal procedures complied with the German Law on Animal Protection as well as the UFAW Handbook on the Care and Management of Laboratory Animals, 2010.

Experimental design

After 3 days of acclimatization, the rats (5 animals per group) were fed ad libitum with either a standard rodent chow (SC, fat content 11 energy percent), or the atherogenic diet (AD) first described by Paigen et al., containing approximately 15 weight percent of neutral fat, 1.25% cholesterol, and 0.5% cholate by mass [17], or with variants of AD. For this, different dietary components of AD were selectively omitted, resulting in the diet types (i) AD without cholesterol (AD-cholesterol), (ii) AD without choline (AD-choline), and (iii) AD without cholate (AD-cholate), respectively. In other AD variations, only the diet's predominant fatty acid was changed by using alternative neutral fat sources to achieve a pronunciation of either monounsaturated *cis*- or middle chain saturated fatty acids: (iv) AD with olive oil, (AD/O) and (v) AD with coconut oil (AD/C). All diets were stored at 4 °C, feeding quality was verified by measuring food intake on a daily basis. After 8 weeks, the animals were sacrificed after an overnight fast (16 h). Venous blood was drawn from the heart into EDTA-coated vials, and plasma was prepared and stored at −20 °C pending further analysis. The right ventral liver lobe was removed, partially fixed in 10% neutral buffered formaldehyde and partially frozen in liquid nitrogen. In H & E stained sections liver steatosis and inflammation were semiquantitatively assessed by a blinded liver pathology expert (E.G.). The degrees of steatosis and inflammation were scored separately (0 = no steatosis/inflammation, 1 = minimal steatosis/inflammation, 2 = moderate steatosis/inflammation, 3 = severe steatosis/inflammation). Ten fields of view were examined at a 400× magnification from each liver section, and the mean scores of steatosis and inflammation were calculated first for each individual animal and then for the different experimental groups. Unless otherwise stated, all reagents were purchased from Sigma (St. Louis, USA) or Merck Eurolab (Darmstadt, Germany) at the highest purity grade available.

Biochemical measurements

Plasma glucose was measured enzymatically with an appropriate commercial kit (Sigma); insulin, leptin, and adiponectin were measured using rat-specific ELISA kits (Mercodia, Uppsala, Sweden; Linco Research, St. Charles, USA). The HOMA-Index was calculated as follows: HOMA-Index = Glucose (mmol/l) × Insulin (pmol/l)/155 [18]. Plasma aminotransferases, cholesterol and triglycerides were measured by standard assays on the cobas 8000 analyzer (Roche Diagnostics, Mannheim, Germany) in the Clinical Chemistry laboratory of the Nuremberg Hospital. The liver tissue triglyceride content was determined after lipid extraction using a GPO-triglyceride kit (Sigma, St. Louis USA) as described previously [13].

mRNA analysis

Total RNA from liver was isolated using a Qiagen RNeasy^{R} kit (Qiagen, Hilden, Germany). Real-time RT-PCR was performed as described in detail elsewhere [6]. In brief, first strand complementary cDNA was synthesized from equal amounts of total RNA by priming with arbitrary hexamers. For subsequent PCR amplification (Standard RT-PCR and LightCycler^{TM} system, Roche Diagnostics, Germany), the following primer pairs (1 µmol/l, Metabion, Martinsried, Germany) were employed: i) rat collagen I, 5′-AAG AGG CGA GAC AGG TTT CC-3′ (sense)/5′-AGA ACC ATC AGC ACC TTT G-3′ (antisense); ii) rat tumor necrosis factor, 5′-GTC GTA GCA AAC CAC CAA GC-3′ (sense)/5′-TGT GGG TGA GGA GCA CAT AG-3′ (antisense), iii) rat 18 s rRNA, 5′-TCA AGA ACG AAA GTC GGA G-3′ (sense)/5′-GGA CAT CTA AGG GCA TCA CA-3′ (antisense). After verification of the RT-PCR products by gel electrophoresis, a LightCycler^{TM} analysis was performed with the same temperature protocol. The formation of primer-dimers was ruled out by melting curve analysis. The cDNA content for a specific gene in each sample was semiquantitatively assessed by comparing the experimentally determined crossing point with the crossing points and respective concentrations of a pooled standard cDNA.

Results

Weight gain and basal metabolic characterization

During the diet phase no significant differences in food intake were noted. The animals' mean body weight increased 2.9 ± 0.1-fold during the diet phase; significant differences between the diet groups with respect to relative weight gain or final body

Table 1 Weight, glucose metabolism, adipokines, and liver triglycerides after 8 weeks of the specified diet form.

	Weight	Plasma levels					Liver triglycerides
		Fasting glucose (mg/dl)	Insulin (µg/l)	Adiponectin (ng/ml)	Leptin (pg/ml)	HOMA-Index	(mg/g)
Atherogenic diet (AD)	464±25	80±6	0.7±0.3	4159±979	778±146	3.7±1.7	8.6±3.0
AD without cholesterol	411±45	82±5	0.7±0.4	4521±462	1031±255	3.6±2.0	5.6±2.2
AD without cholate	444±32	90±7	1.2±1.4	3696±667	1392±1101	7.2±8.5	17.0±3.8*
AD without choline	466±23	89±4	1.1±0.6	2791±685	707±143	6.1±3.1	6.5±2.5
AD with olive oil	456±51	95±7	1.5±0.5*	2549±502*	1013±164	9.0±3.0*	8.4±4.2
AD with coconut oil	478±31	83±3	0.6±0.1	3746±948	1032±210	3.1±0.5	8.6±2.3
Standard rodent chow	471±34	96±6	0.9±1.4	6469±698*	1096±223	5.4±9.1	2.9±1.1*

*$p<0.05$ when compared to AD

Table 2 Liver transaminases and serum lipids after 8 weeks of the specified diet form.

	Plasma levels				
	AST (U/l)	ALT (U/l)	AST/ALT	Cholesterol (mg/dl)	Triglycerides (mg/ml)
Atherogenic diet (AD)	226±82	53±13	4.5±2.0	211±77	0.5±0.1
AD without cholesterol	200±64	79±45	3.1±1.5*	102±12*	0.9±0.3
AD without cholate	124±59	34±12	3.9±1.5	111±37	1.0±0.7*
AD without choline	218±89	46±16	4.7±0.7	220±49	0.7±0.3
AD with olive oil	213±51	76±39	3.2±1.2*	201±32	1.0±0.4*
AD with coconut oil	149±61	45±22	3.6±0.9	150±31	1.0±0.4

*$p<0.05$ when compared to AD

weight (**Table 1**) were not noted. Fasting blood glucose and leptin did not differ between the different dietary regimes. The fasting insulin level was approximately doubled in AD/O rats when compared to AD ($p<0.05$), which resulted in an almost 3-fold increase in the HOMA-index in this specific group compared to AD ($p<0.05$), while the difference was not significant when comparing to SC. AD/O rats also showed the lowest adiponectin levels of all AD variations: compared to AD, adiponectin was lowered by approximately 35% ($p<0.05$), and compared to SD it decreased by almost 60% ($p<0.05$). While all AD variations showed higher liver triglyceride (TG) levels than SD, this difference was most pronounced in AD-cholate, where liver TG was elevated almost 6-fold compared to SD and almost doubled when compared to AD ($p<0.05$). Liver TG levels tended to be decreased in AD-cholesterol when compared to AD, but this difference did not reach statistical significance. Plasma cholesterol was approximately 2-fold higher in animals fed with original AD compared to AD-cholesterol and not significantly different between the other groups (**Table 2**). While the absolute plasma levels of the alanine (ALT) and aspartate (AST) aminotransferase where not clearly different between the groups, the deRitis quotient (AST/ALT) as a marker of hepatocellular damage was elevated in original AD and significantly lowered in AD-cholesterol as well as in AD/O.

Liver histology

The effect of the different atherogenic diet variations on hepatic steatosis and inflammation was first examined histologically. Representative H & E stained sections and the histological score values for these parameters are shown in **Fig. 1, 2**.

Animals fed with original AD diet revealed moderate to severe periportal inflammation with some single cell necrosis and severe hepatocellular steatosis, whereas standard chow fed controls showed neither signs of inflammation nor of steatosis. Omitting the choline component did not significantly affect hepatic inflammation or steatosis, but rats fed with AD lacking cholate (AD-Cholate) or lacking cholesterol (AD-Cholesterol) showed marked improvements of both parameters into the range of standard chow fed rats ($p<0.05$). When changing the dietary neutral fat to olive or coconut oil, we observed no change in the extent of hepatic fat deposition, while the inflammation score was significantly decreased from 2±0.75 to 0.5±0.5 in AD/O compared to AD ($p<0.05$).

Expression of proinflammatory and profibrogenic molecules

To verify the histologic changes described above, we examined the mRNA expression of collagen I as marker of fibrogenesis and of tumor necrosis factor as marker of inflammation in liver tissue from differentially fed rats. As can be seen in **Fig. 3**, collagen I expression was reduced to standard chow diet levels in rats fed with AD-cholesterol and AD-cholate, and it was reduced by 70±20% and 80±10%, respectively in rats fed with AD/O and AD/C ($p<0.05$). Tumor necrosis factor expression was lowered significantly only in AD-cholesterol by 60±30%; in AD-cholate, AD/O and AD/C there was a trendwise reduction of about 50%, which did not reach statistical significance. Omitting choline from the diet did not lead to any significant expression changes.

Discussion

In this study, we have demonstrated that dietary fat components, in particular cholesterol and certain acyl moieties, determine the degree of diet-induced hepatic steatosis and inflammation. Unexpectedly, these fat-induced effects on the liver appear to be independent of the metabolic profile in the rodent model of nonalcoholic steatohepatitis employed in this study.

Using a cholesterol enriched diet containing cholate and choline on the background of an only slightly elevated triacylglycerol component based on lard (17 energy%, so called atherogenic

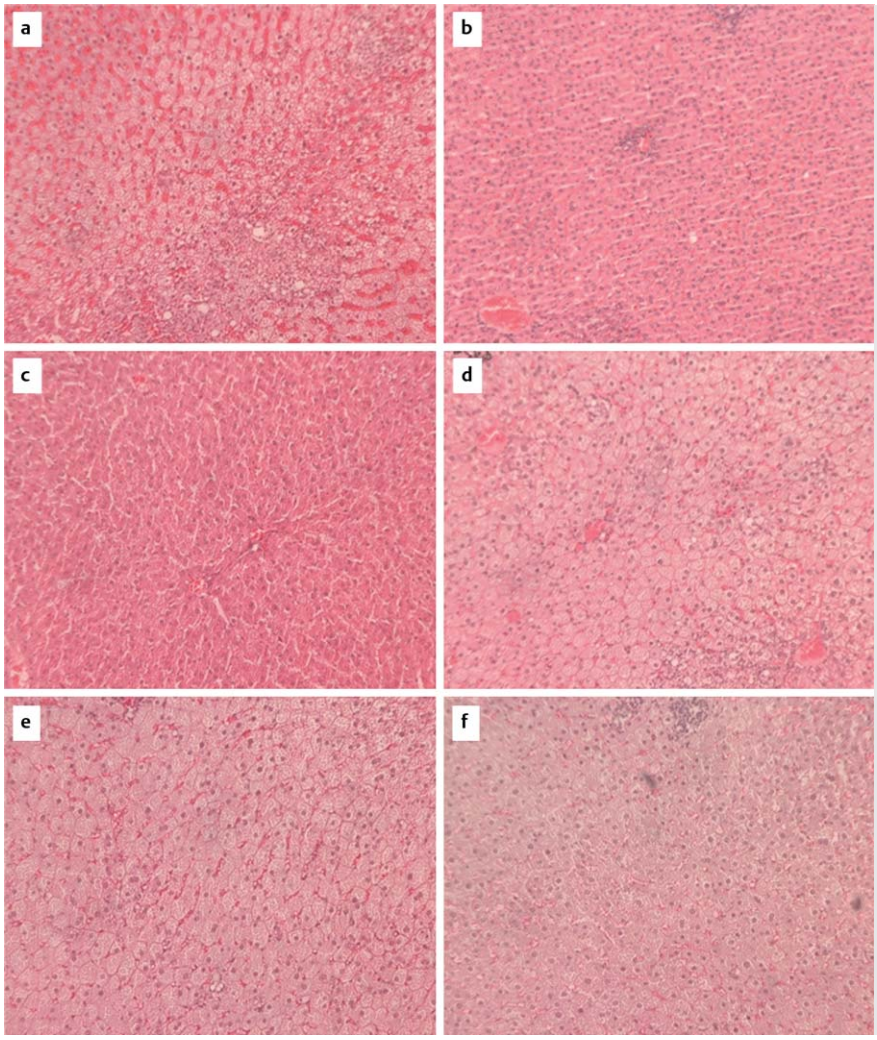

Fig. 1 Influence of specific diet components on liver histology in variations of the atherogenic diet. Rats were fed ad libitum with the proinflammatory atherogenic diet AD (panel **a**) or with AD without cholesterol (panel **b**), AD without cholate (panel **c**), AD without choline (panel **d**), AD with olive oil as main neutral fat component (panel **e**) and coconut oil as main neutral fat component (panel **f**). Representative H & E stains from liver tissue (magnification 400×) prepared according to standard procedures are shown.

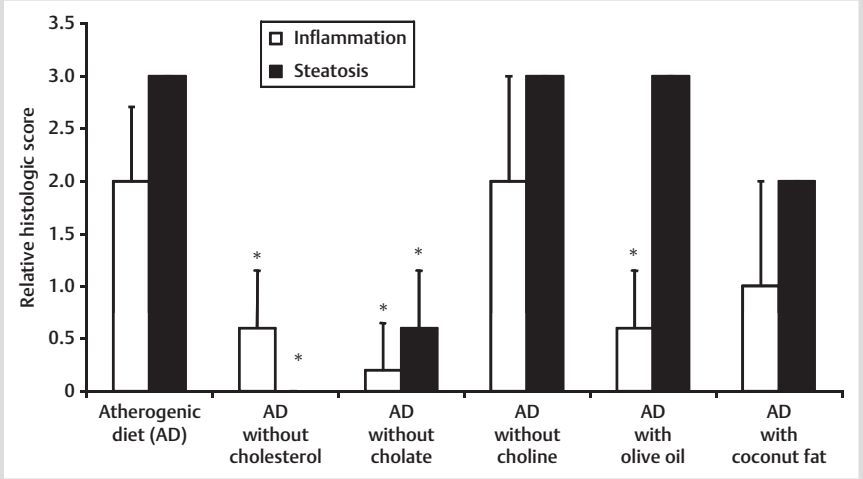

Fig. 2 Hepatic inflammation and steatosis scores in rats fed with variations of the atherogenic diet. Rats were fed ad libitum with the proinflammatory atherogenic diet AD, AD without cholesterol, AD without cholate, AD without choline, AD with olive oil as main neutral fat component, or AD with coconut oil as main neutral fat component. The mean semiquantitative histologic score from 6 animals ± SD is given for inflammation (white bars) and steatosis (black bars). *$p < 0.05$ compared to AD.

diet, AD) we observed marked hepatic fat deposition and periportal inflammation in comparison to conventionally fed rats. Furthermore, this diet leads to a significant upregulation of the proinflammatory cytokine TNF, which is known to play an important pathophysiological role in NASH progression. Moreover, rats fed with this diet revealed increased hepatic collagen type I expression, indicative of beginning fibrosis.

Variations of this diet have been routinely used up to now to generate and modulate atherosclerosis, respectively, but only few researchers have examined the effects of this dietary intervention on liver histology before. An AD-induced activation of the transcription factor NFKB in the liver tissue was observed as early as 1993 [19] but the link between steatohepatitis and the metabolic syndrome was not known at that time. Similar to our results, recent reports by Dorn et al. and Matuszawa et al. demonstrated hepatomegaly together with histologic features resembling human NASH such as steatosis, fibrosis, cellular ballooning and increased TNF and MCP-1 levels in C57/BL6 mice

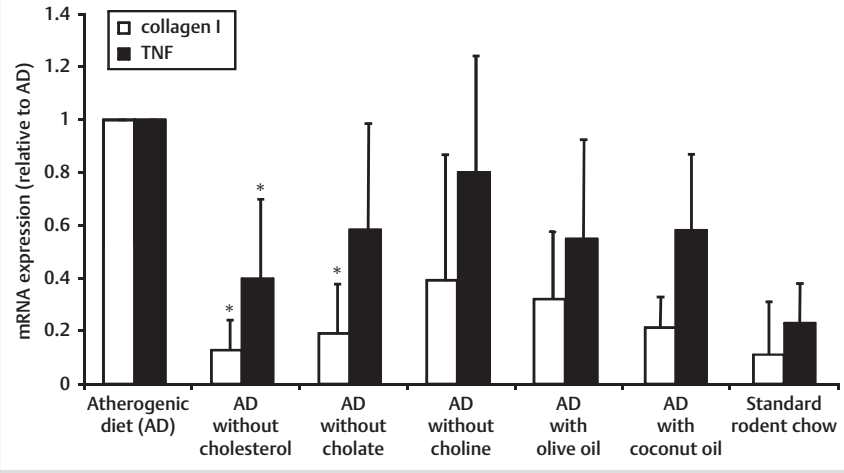

Fig. 3 Hepatic expression of markers of inflammation and fibrosis in rats fed with variations of the atherogenic diet. Rats were fed ad libitum with the proinflammatory atherogenic diet AD, AD without cholesterol, AD without cholate, AD without choline, AD with olive oil as main neutral fat component, AD with coconut oil as main neutral fat component, or standard rodent chow. The relative change of the hepatic mRNA expression of collagen type I (marker of fibrosis, white bars) and tumor necrosis factor TNF (marker of inflammation, black bars) as determined by RT-PCR from 6 animals ± SD is given. *$p < 0.05$ compared to AD.

after 12–24 weeks of an atherogenic diet composed nearly equal to the AD employed in this study [17,20].

In order to further characterize the individual pathophysiological impact of AD components on hepatic damage and metabolic perturbations we modified the different diet components such as the cholesterol concentration and the diet's fatty acid composition. The dietary triacylglycerol content itself was not increased, as this might lead to a severe nutritional imbalance in such an "atherogenic-high fat" diet, which could be argued to be of harm in itself. This approach led to several observations. An AD variation without cholesterol supplementation, but with same energy content derived from fat, did not cause relevant hepatic steatosis, inflammation or fibrosis. This clearly argues for an important pathophysiologic role of dietary cholesterol in this steatohepatitis model. Several recent animal studies and clinical observations underscore the assumption that dietary cholesterol is associated with hepatic inflammation. In patients suffering from NASH, mean cholesterol intake was elevated when compared to healthy controls and hepatic free cholesterol increased gradually from NAFLD to NASH [11,18,21]. In LDL receptor knockout mice, NMR-metabolomic studies indicate a causal association between dietary cholesterol and hepatic inflammation [22], which might be mediated by Kupffer cell scavenger receptors [23]. A further study showed only a slight inflammatory reaction in the liver as assessed by expression analysis of proinflammatory markers when using a high fat diet containing only 0.2% cholesterol [24], which is substantially less than in the AD used in this study. Interestingly, the inhibition of *endogenous cholesterol synthesis* does not seem to exert positive effects in human NASH, as a recent clinical study shows that cholesterol-synthesis blocking statins do not decrease the extent of hepatic inflammation in patients with NASH [25]. Taken together, these data together with our results hint to a possibly dose-dependent effect of dietary cholesterol on hepatic inflammation and argue against an unspecific toxic reaction. Therefore, dose-response effects as well as the effect of regulatory cholesterol intermediates (e.g., oxysterols) should be examined in further studies.

Notably, in our model, cholesterol did not appear to have an intrinsic steatogenic effect with respect to liver triglyceride content, as suggested by Basciano et al. when looking at a hamster model [26]. In contrast to our study, these authors employed a combination of a high fructose (40 weight%) and much more neutral fat enriched (30 weight%) dietary regimen, so it might be speculated that cholesterol facilitated hepatic triglyceride deposition from these external sources. Whether dietary reduction of cholesterol intake can influence human NASH progression should be determined in clinical studies.

There is ample evidence that the dietary fatty acid structure influences aspects of immune response and inflammation (reviewed in [27]). Therefore, we examined the effect of AD variations containing either mainly medium chain length saturated acyl moieties (derived from coconut oil) or long chain monounsaturated oleic acid (from olive oil) with the standard AD, which predominantly consists of the long chain saturated palmitic acid from lard. The cholesterol component was not changed between the diets. Here, we found clear histological and at least trendwise molecular evidence for a protective effect of dietary olive oil on hepatic inflammation, whereas the degree of steatosis itself was not decreased as previously described in rats fed with a methionine-choline deficient diet [28]. The coconut oil containing AD variation did not show significant changes to the original AD. Recent studies suggest several mechanisms, which could explain the differential dietary fatty acid effects observed here. Saturated fatty acids lead to endoplasmic reticulum stress and can induce *c-Jun NH(2)*-terminal kinase-mediated apoptosis, which is not the case for monounsaturated FFAs as oleate or palmitoleate [29–31]. In rats and rabbits challenged with an atherogenic diet, dietary olive oil has been reported to decrease microsomal lipid peroxidation and improve the hepatic antioxidant system [32,33]. Lipid peroxidation and tissue damage induced by chronic ethanol exposure in rats were reduced when the animals were fed with a virgin olive oil rich diet [34], showing that the protective effects of olive oil are – at least to some extent – independent of the underlying damaging cause. However, it cannot be ruled out that indirect actions of olive oil on cholesterol metabolism also contribute to these beneficial observations, as several reports have demonstrated that monounsaturated fatty acids also decrease hepatic cholesterol accumulation and increase biliary cholesterol clearance [35,36]. Additionally, it must be remembered that data coming from these experimental dietary regimes in rodents cannot be directly transferred to the human disease of steatohepatitis.

Whereas the omission of dietary choline did not lead to significant histologic or molecular changes, rats fed with AD lacking cholate showed a decrease of hepatic inflammation and collagen synthesis. As other authors have found decreased intracellular cholesterol levels when omitting cholate from cholesterol rich diets [37], it can be speculated that cholate contributes to intes-

tinal or hepatocellular cholesterol uptake. Further studies are needed to address the question as to how exactly oral bile acids interfere with cholesterol biosynthesis and the enterohepatic circulation. Interestingly, the changes in AD-cholate were noted although the absolute liver triglyceride content was clearly elevated, which argues against the notion of hepatic fat being a causal factor for the inflammatory process per se. When further examining the association between hepatic triglyceride content, metabolic changes, and inflammation we found that the diet group with the lowest histological hepatic inflammation index (AD-O) showed the lowest adiponectin and highest insulin levels together with an increase in hepatic triglyceride. This dissociation between early features of the metabolic syndrome and hepatic inflammation has been noted before [38]. These observations demonstrate that insulin resistance and hypoadiponectinemia do not uniformly lead to rapidly developing hepatic inflammation and point at a stronger causal role for singular dietary fat components than the general metabolic profile. In summary, this study for the first time demonstrates how individual diet components can influence differential aspects of fatty liver disease in rodents. In the experimental AD diet, the component mainly contributing to diet-induced *steatosis* is cholate, whereas variations of the neutral fat acyl moiety (at a fixed content of 15 weight percent) turned out not to change the extent of hepatic triglyceride deposition. The component mainly contributing to diet-induced *inflammation* was cholesterol, which could be counteracted most effectively by changing the neutral fat component from lard to olive oil. Inflammation was not dependent on the degree of steatosis. Based on these findings, variations of the atherogenic AD are a new unique model to study the different steps in the pathogenesis of steatohepatitis without using a nutritionally imbalanced high fat or high fructose diet. Whether these data apply similarly to human steatohepatitis should be examined in prospective studies linking the individual uptake of distinct lipids and liver histology.

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Conflict of Interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

References

1 *Poonawala A*, *Nair SP*, *Thuluvath PJ*. Prevalence of obesity and diabetes in patients with cryptogenic cirrhosis: a case-control study. Hepatology 2000; 32: 689–692
2 *Williams CH*, *Stengel J*, *Asike MI*, *Torres DM*, *Shaw J*, *Contreras M*, *Landt CL*, *Harrison SA*. Prevalence of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis Among a Largely Middle-Aged Population Utilizing Ultrasound and Liver Biopsy: A Prospective Study. Gastroenterology 2011; 140: 124–131
3 *de Oliveira CP*, *de Mello ES*, *Alves VA*, *Saviero SM*, *Strauss E*. Changes in histological criteria lead to different prevalences of nonalcoholic steatohepatitis in severe obesity. Ann Hepatol 2007; 6: 255–261
4 *Brunt EM*. Pathology of nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol 2010; 7: 195–203
5 *Wierzbicki AS*, *Oben J*. Nonalcoholic fatty liver disease and lipids. Curr Opin Lipidol 2012; 23: 345–352
6 *Fabbrini E*, *Mohammed BS*, *Magkos F*, *Korenblat KM*, *Patterson BW*, *Klein S*. Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. Gastroenterology 2008; 134: 424–431
7 *Neuschwander-Tetri BA*. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. Hepatology 2010; 52: 774–788
8 *Postic C*, *Girard J*. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. J Clin Invest 2008; 118: 829–838
9 *Jou J*, *Choi SS*, *Diehl AM*. Mechanisms of disease progression in nonalcoholic fatty liver disease. Semin Liver Dis 2008; 28: 370–379
10 *Marra F*, *Bertolani C*. Adipokines in liver diseases. Hepatology 2009; 50: 957–969
11 *Musso G*, *Gambino R*, *De Michieli F*, *Cassader M*, *Rizzetto M*, *Durazzo M*, *Faga E*, *Silli B*, *Pagano G*. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. Hepatology 2003; 37: 909–916
12 *Toshimitsu K*, *Matsuura B*, *Ohkubo I*, *Niiya T*, *Furukawa S*, *Hiasa Y*, *Kawamura M*, *Ebihara K*, *Onji M*. Dietary habits and nutrient intake in non-alcoholic steatohepatitis. Nutrition 2007; 23: 46–52
13 *Buettner R*, *Parhofer KG*, *Woenckhaus M*, *Wrede CE*, *Kunz-Schughart LA*, *Scholmerich J*, *Bollheimer LC*. Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. J Mol Endocrinol 2006; 36: 485–501
14 *Gentile CL*, *Pagliassotti MJ*. The role of fatty acids in the development and progression of nonalcoholic fatty liver disease. J Nutr Biochem 2008; 19: 567–576
15 *Wei Y*, *Wang D*, *Topczewski F*, *Pagliassotti MJ*. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. Am J Physiol Endocrinol Metab 2006; 291: E275–E281
16 *Paigen B*, *Morrow A*, *Brandon C*, *Mitchell D*, *Holmes P*. Variation in susceptibility to atherosclerosis among inbred strains of mice. Atherosclerosis 1985; 57: 65–73
17 *Matsuzawa N*, *Takamura T*, *Kurita S*, *Misu H*, *Ota T*, *Ando H*, *Yokoyama M*, *Honda M*, *Zen Y*, *Nakanuma Y*, *Miyamoto K*, *Kaneko S*. Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. Hepatology 2007; 46: 1392–1403
18 *Puri P*, *Baillie RA*, *Wiest MM*, *Mirshahi F*, *Choudhury J*, *Cheung O*, *Sargeant C*, *Contos MJ*, *Sanyal AJ*. A lipidomic analysis of nonalcoholic fatty liver disease. Hepatology 2007; 46: 1081–1090
19 *Liao F*, *Andalibi A*, *deBeer FC*, *Fogelman AM*, *Lusis AJ*. Genetic control of inflammatory gene induction and NF-kappa B-like transcription factor activation in response to an atherogenic diet in mice. J Clin Invest 1993; 91: 2572–2579
20 *Dorn C*, *Riener MO*, *Kirovski G*, *Saugspier M*, *Steib K*, *Weiss TS*, *Gabele E*, *Kristiansen G*, *Hartmann A*, *Hellerbrand C*. Expression of fatty acid synthase in nonalcoholic fatty liver disease. Int J Clin Exp Pathol 2010; 3: 505–514
21 *Caballero F*, *Fernandez A*, *De Lacy AM*, *Fernandez-Checa JC*, *Caballeria J*, *Garcia-Ruiz C*. Enhanced free cholesterol, SREBP-2 and StAR expression in human NASH. J Hepatol 2009; 50: 789–796
22 *Vinaixa M*, *Rodriguez MA*, *Rull A*, *Beltran R*, *Blade C*, *Brezmes J*, *Canellas N*, *Joven J*, *Correig X*. Metabolomic assessment of the effect of dietary cholesterol in the progressive development of fatty liver disease. J Proteome Res 2010; 9: 2527–2538
23 *Bieghs V*, *Wouters K*, *van Gorp PJ*, *Gijbels MJ*, *de Winther MP*, *Binder CJ*, *Lutjohann D*, *Febbraio M*, *Moore KJ*, *van Bilsen M*, *Hofker MH*, *Shiri-Sverdlov R*. Role of scavenger receptor A and CD36 in diet-induced nonalcoholic steatohepatitis in hyperlipidemic mice. Gastroenterology 2010; 138: 2477–2486 2486.e1–2486.e3
24 *Wouters K*, *van Gorp PJ*, *Bieghs V*, *Gijbels MJ*, *Duimel H*, *Lütjohann D*, *Kerksiek A*, *van Kruchten R*, *Maeda N*, *Staels B*, *van Bilsen M*, *Shiri-Sverdlov R*, *Hofker MH*. Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. Hepatology 2008; 48: 474–486
25 *Nelson A*, *Torres DM*, *Morgan AE*, *Fincke C*, *Harrison SA*. A pilot study using simvastatin in the treatment of nonalcoholic steatohepatitis: A randomized placebo-controlled trial. J Clin Gastroenterol 2009; 43: 990–994
26 *Basciano H*, *Miller AE*, *Naples M*, *Baker C*, *Kohen R*, *Xu E*, *Su Q*, *Allister EM*, *Wheeler MB*, *Adeli K*. Metabolic effects of dietary cholesterol in an animal model of insulin resistance and hepatic steatosis. Am J Physiol Endocrinol Metab 2009; 297: E462–E473
27 *Malhi H*, *Gores GJ*. Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. Semin Liver Dis 2008; 28: 360–369

28 *Hussein O*, *Grosovski M*, *Lasri E*, *Svalb S*, *Ravid U*, *Assy N*. Monounsaturated fat decreases hepatic lipid content in non-alcoholic fatty liver disease in rats. World J Gastroenterol 2007; 13: 361–368
29 *Cazanave SC*, *Mott JL*, *Elmi NA*, *Bronk SF*, *Werneburg NW*, *Akazawa Y*, *Kahraman A*, *Garrison SP*, *Zambetti GP*, *Charlton MR*, *Gores GJ*. JNK1-dependent PUMA expression contributes to hepatocyte lipoapoptosis. J Biol Chem 2009; 284: 26591–26602
30 *Malhi H*, *Bronk SF*, *Werneburg NW*, *Gores GJ*. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. J Biol Chem 2006; 281: 12093–12101
31 *Wei Y*, *Wang D*, *Pagliassotti MJ*. Saturated fatty acid-mediated endoplasmic reticulum stress and apoptosis are augmented by trans-10, cis-12-conjugated linoleic acid in liver cells. Mol Cell Biochem 2007; 303: 105–113
32 *Aguilera CM*, *Mesa MD*, *Ramirez-Tortosa MC*, *Quiles JL*, *Gil A*. Virgin olive and fish oils enhance the hepatic antioxidant defence system in atherosclerotic rabbits. Clin Nutr 2003; 22: 379–384
33 *Perona JS*, *Arcemis C*, *Ruiz-Gutierrez V*, *Catala A*. Effect of dietary high-oleic-acid oils that are rich in antioxidants on microsomal lipid peroxidation in rats. J Agric Food Chem 2005; 53: 730–735
34 *Kasdallah-Grissa A*, *Nakbi A*, *Koubaa N*, *El-Fazaa S*, *Gharbi N*, *Kamoun A*, *Hammami M*. Dietary virgin olive oil protects against lipid peroxidation and improves antioxidant status in the liver of rats chronically exposed to ethanol. Nutr Res 2008; 28: 472–479
35 *Lambert MS*, *Avella MA*, *Botham KM*, *Mayes PA*. The type of dietary fat alters the hepatic uptake and biliary excretion of cholesterol from chylomicron remnants. Br J Nutr 2000; 83: 431–438
36 *Li Y*, *Hou MJ*, *Ma J*, *Tang ZH*, *Zhu HL*, *Ling WH*. Dietary fatty acids regulate cholesterol induction of liver CYP7alpha1 expression and bile acid production. Lipids 2005; 40: 455–462
37 *Mawatari S*, *Ohnishi Y*, *Kaji Y*, *Maruyama T*, *Murakami K*, *Tsutsui K*, *Fujino T*. High-cholesterol diets induce changes in lipid composition of rat erythrocyte membrane including decrease in cholesterol, increase in alpha-tocopherol and changes in fatty acids of phospholipids. Biosci Biotechnol Biochem 2003; 67: 1457–1464
38 *Ferramosca A*, *Savy V*, *Zara V*. Olive oil increases the hepatic triacylglycerol content in mice by a distinct influence on the synthesis and oxidation of fatty acids. Biosci Biotechnol Biochem 2008; 72: 62–69