**Name of journal: World Journal of Gastroenterology**

**ESPS Manuscript NO: 10656**

**Columns:** **ORIGINAL ARTICLE**

**Elevated free cholesterol as a hallmark of non-alcoholic steatohepatitis in p62/insulin-like growth factor 2 mRNA binding protein 2-2 transgenic animals**

Simon Y *et al*. p62-induced free cholesterol aggravates murine NASH

Yvette Simon, Sonja M Kessler, Katja Gemperlein, Rainer M Bohle, Rolf Müller, Johannes Haybaeck, Alexandra K Kiemer

**Yvette Simon, Sonja M Kessler, Alexandra K Kiemer,** Saarland University, Department of Pharmacy, Pharmaceutical Biology, Saarbrücken 66123, Germany

**Katja Gemperlein, Rolf Müller,**Department of Pharmacy, Pharmaceutical Biotechnology, Saarland University and Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Centre for Infection Research (HZI), Saarbrücken 66123, Germany

**Rainer M Bohle,** Saarland University, Department of Pathology, Homburg, Saar 66421, Germany

**Sonja M Kessler, Johannes Haybaeck,** Medical University of Graz, Institute of Pathology, Graz 8036, Austria

**Author contributions:** SimonY, KesslerSMcontributed equally to this paper; Simon Y, Kessler SM, Haybaeck J and Kiemer AK designed experiments, analyzed data and wrote the manuscript; Kiemer AK initiated and directed the study; Kessler SM, Bohle RM and Haybaeck J scored the histological slides. Gemperlein K and Müller R performed GC-MS lipid analyses; all authors had full access to all of the data (including statistical reports and tables) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis.

**Supported by** An EASL Sheila Sherlock fellowship and a Bank Austria visiting scientist program fellowship to Kessler SM

**Correspondence to: Alexandra K Kiemer, PhD,** Department of Pharmacy, Saarland University, PO box 15 11 50, Saarbrücken 66041, Germany. pharm.bio.kiemer@mx.uni-saarland.de

**Telephone:** +49-681-30257301 **Fax:** +49-681-30257302

**Received:** April 11, 2014 **Revised:**  June 15, 2014

**Accepted:** July 11, 2014

**Published online:**

**Abstract**

**AIM:** To characterize mechanisms involved in the steatohepatitis-promoting actions of the insulin-like growth factor 2 (IGF2) mRNA binding protein p62/IMP2-2 in the absence of dietary cholesterol.

**METHODS:** Non-alcoholic steatohepatitis (NASH) was induced in wild-type mice and in mice overexpressing p62specifically in the liver by feeding a methionine and choline deficient diet for either two or four weeks. As a control, animals were fed the respective methionine and choline supplemented control diet. Serum triglycerides, cholesterol, glucose, aspartate aminotransferase (AST) and alanine transaminase (ALT) were determined by standard analytical techniques. Hepatic gene expression was determined by real-time reverse transcription-polymerase chain reaction (RT-PCR). Reactive oxygen species (ROS) generation in liver tissue was quantified as thiobarbituric acid reactive substances using a photometric assay and malondialdehyde as a standard. Tissue fatty acid profiles and cholesterol levels were analysed by gas chromatography-mass spectrometry (GC-MS) after hydrolysis. Hepatocellular iron accumulation was determined by Prussian blue staining in paraffin-embedded formalin-fixed tissue. Filipin staining of frozen liver tissue was used to quantify hepatic free cholesterol levels. Also nuclear staining of the nuclear factor neclear factor kappa B (NF-қB) subunit p62 was done on frozen tissue.

**RESULTS:** Liver-specific overexpression of the IGF2 mRNA binding protein p62/IMP2-2/ insulin-like growth factor 2 mRNA binding protein 2-2 (IGF2BP2-2) induces steatosis on regular chow and amplifies non-alcoholic steatohepatitis (NASH)-induced fibrosis in the MCD mouse model. Activation of nuclear factor NF-қB and expression of NF-қB target genes suggested an increased inflammatory response in *p62* transgenic animals. Deciphering hepatic lipid composition revealed an elevated presence of monounsaturated fatty acids as well as increased hepatic cholesterol. Also serum cholesterol was significantly elevated in *p62* transgenic mice. Dietary cholesterol represents a critical factor for the development from hepatic steatosis towards NASH. Filipin staining demonstrated increased free cholesterol in *p62* transgenic livers, which did not derive from the diet. As the mRNA levels of the rate-limiting enzyme for the cholesterol synthesizing enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase or HMGCR) were not significantly upregulated, increased cholesterol biosynthesis is suggested to be related to elevated sterol regulatory element binding transcription factor 2 (*SREBF2*) gene expression in transgenics, most likely due to pronounced hepatic iron accumulation. The latter might also be responsible for an elevated lipid peroxidation in transgenic livers.

**CONCLUSION:** Our data provide evidence that p62/IGF2BP2-2 drives the progression of NASH by an elevation of hepatic iron deposition and increasing the production of hepatic free cholesterol.

© 2014 Baishideng Publishing Group Inc. All rights reserved.

**Key words**: Insulin-like growth factor 2 mRNA binding protein 2-2; Methionine/choline deficient; Non-alcoholic fatty liver disease; Filipin; Iron

**Core tip:** Dietary cholesterol represents a critical factor for the development from hepatic steatosis towards non-alcoholic steatohepatitis (NASH). Liver-specific overexpression of the insulin-like growth factor 2 (IGF2) mRNA binding protein p62/IMP2-2/insulin-like growth factor 2 mRNA binding protein 2-2 (IGF2BP2-2) induces steatosis and amplifies NASH-induced fibrosis. We here show that p62 elevates monounsaturated fatty acids as well as hepatic cholesterol in the absence of exogenous cholesterol. Filipin staining demonstrates increased free cholesterol in *p62* transgenic livers. Srebf2-induced cholesterol biosynthesis in transgenics is most likely due to pronounced hepatic iron accumulation, which is also associated with lipid peroxidation in transgenic livers. In summary, our data show that p62/IGF2BP2-2 drives the progression of NASH by increasing hepatic free cholesterol.

Simon Y, Kessler SM, Gemperlein K, Bohle RM, Müller R, Haybaeck J, Kiemer AK. Elevated free cholesterol as a hallmark of non-alcoholic steatohepatitis in p62/insulin-like growth factor 2 mRNA binding protein 2-2 transgenic animals. *World J Gastroenterol* 2014; In press

**INTRODUCTION**

In industrialized countries non-alcoholic fatty liver disease (NAFLD) represent the most frequent hepatic manifestation of chronic liver diseases, whereby hepatic steatosis as first hit sensitizes the liver to the second hit that leads to hepatocyte injury, inflammation, and subsequent fibrotic changes[1]. Longstanding NAFLD can ultimately lead to hepatocellular carcinoma (HCC)[1], which is considered to be the sixth most common malignancy worldwide and the third leading cause of cancer-related deaths[2]. However, the pathophysiological mechanisms leading to the progression from NAFLD to end stage liver disease are as yet poorly understood.

The composition of fatty acids in the liver has emerged as a critical factor promoting the development of non-alcoholic steatohepatitis (NASH) and maybe also HCC[3-7] with a suggested pathophysiological role of monounsaturated fatty acids (MUFA)[6,8]. Recent observations also highlighted the accumulation of free cholesterol as important trigger for the progression from a simple steatosis to severe NASH[9-11]. In fact, dietary cholesterol was demonstrated to be a critical factor in the progression of NASH[10,12]. Cholesterol fed to LDLR-/- mice induced a prominent inflammatory response, whereas high fat feeding without cholesterol induced steatosis in the absence of inflammation[13].

The insulin-like growth factor (IGF) 2 mRNA binding protein p62/IMP2-2/insulin-like growth factor 2 mRNA binding protein 2-2 (IGF2BP2-2) displays a splice variant of IMP2/IGF2BP2 and was originally described as an autoantigen from an HCC patient[14]. p62 is upregulated in human HCC and its expression correlates with poor prognosis[15,16]. Physiological roles were as yet solely described for IMP2, which is required for proper nerve cell migration and morphological development during development by controlling cytoskeletal remodeling and dynamics (reviewed in[17]). We recently reported that mice with a liver-specific overexpression of p62 develop a fatty liver and show increased development towards NASH-induced fibrosis[4,18,19]. This amplification of an inflammatory response was observed in a feeding model omitting dietary cholesterol. We therefore aimed to investigate the mechanisms involved in the amplification of NASH by p62 in the absence of dietary cholesterol.

A methionine/choline deficient (MCD) diet without supplementation of cholesterol was fed to *p62* transgenic animals. The MCD diet is the most commonly used murine dietary model for acquired NASH, since in contrast to other models, it allows to study all stages of NASH, *i.e.* inflammation, oxidative stress, and fibrogenic changes[20].

Our data suggest p62 as a modulator of endogenous cholesterol synthesis leading to elevated levels of free cholesterol in the liver promoting inflammation *via* the activation of neclear factor kappa B (NF-қB). Moreover, the elevated levels of hepatocellular iron link lipid metabolism to the promotion of inflammatory reactions[21].

**MATERIALS AND METHODS**

***Material***

The methionine/choline deficient (MCD) diet (#960439) and the methionine/choline supplemented control (ctrl) diet (#960441), both containing 45% sucrose and 10% corn oil without cholesterol, were purchased from MP Biomedicals (Heidelberg, Germany). Polymerase chain reaction (PCR) primers were obtained from Eurofins MWG Operon (Ebersberg, Germany). The EvaGreen® qPCR Mix was obtained from Solis BioDyne (Tartu, Estonia). Antibodies for immunhistochemistry were purchased as indicated in Table 1.

***Animal treatment***

All animal procedures were performed under the guidelines of the local animal welfare committee (permission no.: 34/2010). Mice were maintained under 12-h dark-light cycles under controlled conditions (temperature 22 °C ± 2 °C and relative humidity of 55% ± 10%) with unrestricted access to food and water until the age of three weeks.

Mice were randomly divided into experimental groups at the age of 3 wk and fed an MCD diet or an MCD diet supplemented with choline bitartrate (2 g/kg) and DL-methionine (3 g/kg) for 2 or 4 wk; the latter was designated as a control diet (ctrl)[18]. Male and female wild-type or *p62* transgenic mice were used as described in[19].

***Serum parameters***

Animals were sacrificed and serum levels were determined at the “Zentrallabor des Universitätsklinikums des Saarlandes” (Homburg, Germany).

***Real-time reverse transcription-polymerase chain reaction***

Experiments and quantification were performed as previously described[22]. Primer sequences are given in Table 2.

***Quantification of thiobarbituric acid reactive substances***

Products of lipid peroxidation were measured by a fluorimetric assay. 10-20 mg liver tissues were homogenized in 1 × PBS (Na2HPO4 8.0 mmol/L, KH2PO4 1.5 mmol/L, NaCl 160 mmol/L in water) containing 1% phosphatase inhibitor cocktail II (Sigma-Aldrich, Taufkirchen, Germany), and centrifuged. For protein precipitation 100 µL lysate were mixed with 200 µL ice cold 10% trichloroacetic acid and after incubation on ice, centrifuged for 10 min at 14000 *g*. The clear supernatant was mixed with an equal volume of TBA [0.67% (w/v)], and heated for 15 min at 100 °C. After cooling down to room temperature the fluorescence intensity of the samples was measured in duplicate in a 96 well plate at λex/em = 530 nm/572 nm. Thiobarbituric acid reactive substances (TBARS) are expressed as malondialdehyde (MDA) equivalents as μmol per mg liver tissue. An MDA standard was used to create a standard curve, against which unknown samples were plotted.

***Fatty acid profile analysis***

Snap-frozen liver tissue samples were lyophilized to dryness (approximately 10 mg dry weight), and fatty acid extraction and alkaline hydrolysis was performed by the fatty acid methyl ester (FAME) method measured with GC-MS according to a published method[3,23].

***Histology and immunohistochemistry***

For histological examination, paraffin-embedded liver tissue specimens were cut and stained with Prussian blue for iron accumulation. Immunohistochemical staining was performed after demasking of the sections with the appropriate method listed in Table 3. The sections were immunostained with the appropriate antibody; concentration and incubation time and temperature are listed in Table 1. Subsequently, immunodetection was performed using distinct detection kits as listed in Table 1 according to the instructions of the manufacturer´s manual. Staining for unesterified “free” cholesterol was performed on frozen liver sections with filipin, which identifies free cholesterol according to a published protocol[11]. Quantification was done with Image J software on 5 randomly selcted pictures from each sample. NF-қB-p65 staining was performed according to a published protocol[24]. Counterstaining with either hematoxylin or (4’,6’-diamidine-2-phenylindol) DAPI for immunofluorescence (IF) was performed and sections were dehydrated and embedded with Entellan® (Merck, Germany, #107961). As negative controls sections were incubated without primary antibody.

Three investigators blinded to all experimental conditions (SMK, RMB, JH) examined the sections for hepatocellular iron and NF-қB-p65 nuclear translocation as shown in Table 3.

***Statistical analysis***

Data analysis and statistics were performed using Microsoft Office 2010 and OriginPro 8.6 G. Effect of genotype, MCD diet, and their interactions were displayed as mean values ± SEM with 10-12 animals per group. Statistical differences were estimated by Kruskal-Wallis-ANOVA for nonparametric samples followed by post-hoc-analysis with Mann-Whitney *U* test. Differences were considered statistically significant when *P* values were less than 0.05.

**RESULTS**

***General effects of the dietary manipulation***

Mice of both genotypes exhibited different characteristics typical for the MCD diet. These comprised a loss of body and relative liver weight through feeding the MCD diet (Table 4). Due to reduced VLDL secretion from the liver[25], serum triglycerides and cholesterol were reduced in MCD animals, as were serum glucose levels. *p62* transgenic animals exhibited even further reduced serum glucose levels as previously reported[18], most likely due to elevated IGF2 production. Elevated AST and ALT levels indicated liver damage induced by the MCD diet, as previously described[26](Table 4).

***p62 amplifies inflammation***

Since p62 promotes NASH-induced fibrosis paralleled by an increased expression of the chemokine monocyte chemoattractant protein 1 (MCP1)/chemokine ligand 2 (CCl2)[18], our previous data suggested that p62 elevates the inflammatory response during NASH. In fact, histological detection of an activation of the transcription factor NF-қB was assessed by nuclear translocation of its subunit p65, and suggested an elevated activation for *p62* transgenics (Figure 1A). This was confirmed by an NF-қB dependent gene expression profile with increased levels of tumor necrosis factor (TNF)(Figure 1B), inducible nitric oxide synthase 2 (Nos2)(Figure 1C), prostaglandin-endoperoxide synthase 2 (PTGS/COX2)(Figure 1D), and interleukin (IL) 1B in p62 transgenic mice (Figure 1E).

***p62 alters fatty acid pattern***

Animals of both genotypes developed steatosis on the MCD diet. However, previous histological analyses suggested an amplification of steatosis in *p62* transgenic animals compared to their wild-type littermates[18]. In fact, after 2 weeks, the relative liver weight was significantly increased in transgenics (*P* = 0.02)(Table 4) and therefore consistent with the histological changes. GC-MS analyses revealed significantly higher levels of hepatic fatty acids in *p62* transgenic mice (Figure 2A), whereas serum triglycerides were not changed (Table 4). The hepatic fatty acid pattern indicated strong alterations in *p62* transgenic animals compared to their wild-type littermates after 2 wk on MCD diet (Table 5). In particular, a more pronounced accumulation of monounsaturated (MUFA) compared to saturated (SFA) and polyunsaturated (PUFA) fatty acids was observed in transgenic animals (MUFA 68% increase *vs* SFA 40% and PUFA 45%)(Figure 2B). Both the distinct elevation of palmitoleic acid (C16:1) and of oleic acid (C18:1)(Figure 2C) indicated an increased desaturase activity. In fact, gene expression of the desaturase stearoyl-CoA desaturase (SCD) 1, being responsible for the formation of C16:1 and C18:1 fatty acids, tended to be increased in *p62* transgenic animals, despite a strong downregulation upon the MCD diet (Figure 2D). The gene expression of other lipogenic and fatty acid catabolism regulators, such as the fatty acid synthase (FASN), the lipolysis regulator peroxisome proliferator-activated receptor (PPAR) a, and the promotor of β-oxidation, carnitine palmytoyl transferase (CPT) 1a, were not altered upon p62 expression when fed the MCD diet (Figure 2D).

***Enhanced cholesterol in p62 transgenic animals***

Both liver cholesterol as well as serum cholesterol were distinctly elevated in *p62* transgenic mice (Figure 3A, B). Filipin staining for free cholesterol revealed a significant increase of free cholesterol in *p62* transgenic animals on the MCD diet (Figure 3C, D). While the mRNA levels of the rate-limiting enzyme for cholesterol synthesis Hmg-CoA reductase (HMGCR) were not significantly upregulated (Figure 3E), the expression of the cholesterol metabolism-related transcription factor sterol regulatory element binding transcription factor 2 (*SREBF2*) was significantly increased after 4 wk (Figure 3F).

***p62 induces hepatocellular iron deposition and lipid peroxidation***

Since cholesterol biosynthesis was previously reported to be induced by elevated hepatic iron[27], its deposition was determined. Both genotypes on the MCD diet had elevated hepatic iron deposition with a more distinct iron accumulation in *p62* transgenic mice (Figure 4A, B). Interestingly, iron deposition was also detected in transgenic, but not in wild-type mice on control diet (Figure 4B). Since hepatocellular iron is known as a promoter of oxidative stress, we assessed lipid peroxidation. Concordantly, p62 expression significantly increased lipid peroxidation as analyzed by TBARS assay on the control diet at 2 wk and on MCD a respective trend was observed at 4 wk (Figure 4C).

**DISCUSSION**

The fatty acid composition as well as the accumulation of free cholesterol are suggested to play a critical role in the development of NASH since an altered lipidome[3,11,12] as well as free cholesterol positively correlate with the severity of NASH[10,12,28,29]. A role of dietary cholesterol in the context of NAFLD has been suggested[30]. In the current study, we describe an increased production of free cholesterol in mice with a liver-specific overexpression of p62 in the absence of dietary cholesterol.

*p62* transgenic animals are more prone to the inflammatory response in this model of NASH as observed by an elevated activation of NF-қB in *p62* transgenic mice. Concordantly, gene expression of inflammatory mediators, such as tumor necrosis factor (TNF), were elevated in these animals as previously shown for MCP1/CCl2 [18]. TNF is strongly related to the fatty acid metabolism as it negatively regulates the expression of PPARa, leading to decreased catabolism[31]. In human NAFLD patients enhanced serum levels of TNF are a strong indicator for the progression from steatosis to NASH[32].

Surprisingly, we detected a lower apoptosis rate in *p62* transgenic mice by IHC of cleaved caspase-3, contrasting the apoptosis-inducing effect of TNF[33]. Additionally, *p62* transgenic animals did not show an induction of liver damage despite elevated fat and inflammation, which is in contrast to elevated AST and ALT levels in human NASH[34]. Both, less apoptosis and liver damage, confirm the cytoprotective properties of p62[16,19] and might even be linked to cytoprotective actions of MUFAs[6]. The increase of IL-1B mRNA as a result of NF-қB activation in *p62* transgenic animals might further link early lipidomic changes in steatosis with progression to a strong inflammatory response[35,36].

Interestingly, the rate of MUFA was increased to a higher extent than the SFA and PUFA in *p62* transgenic animals indicating alterations in the fatty acid metabolism as seen in both NASH and NASH-related HCC[3,6]. Increased MUFA are correlated to hypertriglyceridemia and obesity[37], even without exogenous ingestion, but due to hepatic synthesis. Notwithstanding, in animal studies exogenous MUFA were found to be protective against MCD-induced NASH[8].

Desaturases represent the rate-limiting enzymes for the production of palmitoleic (C16:1) and oleic acid (C18:1) with SCD1 being the predominant form in the liver[38,39]. In this study, an increase of SCD1 in *p62* transgenic animals, despite a strong downregulation through the MCD diet, was found. In human NASH-related HCC tissues an upregulation of SCD was found likewise[6]. The expression of Fasn was found to be downregulated upon the MCD diet without differences among the genotypes, similar to other murine models of steatohepatitis[31,40] and human NASH[28]. We also found no changes for the lipolysis regulators PPARa and CPT1 in *p62* transgenic mice.

*p62* transgenic mice fed the MCD diet showed hyperlipidemia with increased serum cholesterol levels. These findings are particularly interesting having in mind that the MCD diet is known for lowered serum triglyceride (TG) levels and in this particular respect differs from human NASH[41]. To our knowledge the observed increase in hepatic total and free cholesterol is the first time that a hepatic cholesterol accumulation is documented in a nutritional mouse model without exogenous cholesterol. Increased dietary cholesterol intake is associated with risk and severity of NAFLD and is paralleled by hepatic free cholesterol accumulation in human as well as in experimental settings[42] and even deciphers steatosis from NASH[43]. Besides dietary cholesterol intake cellular cholesterol accumulation might be a result of disturbed cholesterol homeostasis[42]. We here observed enhanced expression of the transcription factor SREBF2, but no distinct effect on the expression of the rate-limiting enzyme HMGCR. While a positive correlation between the severity of NASH with the expression of these genes was found[29], others reported no correlation of SREBF2 with hepatic cholesterol despite elevated HMGCR[27]. Interestingly, however, cholesterol biosynthesis was found to be positively correlated with iron accumulation: when additional iron was given, it led to deposition of free cholesterol and its upregulated biosynthesis[27]. Furthermore, hepatocellular iron deposition was reported to be elevated in human NASH patients[44] and NASH-related HCC patients[45]. Variations in hepatic iron levels can directly lead to a modulation of lipogenesis, lipid storage and secretion, as iron is an integral part of several lipid metabolism related enzymes[46]. In this context, SCD1 activity has been shown to be iron-dependent, as the protein contains iron as a cofactor[47]. Accordingly, the iron accumulation in *p62* transgenic mice might elevate SCD1 activity.

Enhanced iron accumulation is also related to enhanced lipid peroxidation[44] since iron is known to catalyze the production of reactive oxygen species, which can then initiate cellular damage and lipid peroxidation[48]. In fact, reactive oxygen species have been suggested as critical contributors to the second hit[49]. The elevated ROS production in *p62* transgenics on the control diet, which is in accordance with the iron accumulation in these animals, might predispose them towards the development of NASH.

Taken together, this study reveals that the liver-specific overexpression of p62 leads to an amplified progression of NAFLD towards NASH by increased production of hepatic free cholesterol driving the inflammatory response in liver disease.

**ACKNOWLEDGMENT**

We thank Eva Dilly for the technical assistance in all animal matters. We thank Christina Guth at the Leibniz Institute for New materials (INM) for support in microscopy.

**COMMENTS**

***Background***

In industrialized countries non-alcoholic fatty liver disease (NAFLD) represents the most frequent chronic liver disease and displays a potential risk factor for the development of hepatocellular carcinoma (HCC), the most common primary liver cancer. HCC is a highly aggressive cancer type with high mortality, which is difficult to detect and to cure. The insulin-like growth factor (IGF) 2 mRNA binding protein p62 was originally discovered in an HCC patient.p62 induces a fatty liver and promotes non-alcoholic steatohepatitis (NASH)-induced fibrosis.

***Research frontiers***

Dietary cholesterol represents a critical factor for the development from hepatic steatosis towards NASH. In this context, the accumulation of free cholesterol was recently highlighted as important trigger for the progression from a simple steatosis to severe NASH. In the area of NASH research, more recent research hotspots are the role of free cholesterol as well as iron accumulation in disease progression. Whether and how elevated free cholesterol can accumulate independently of dietary cholesterol is a timely topic of highest interest.

***Innovations and breakthroughs***

The authors show for the first time that free cholesterol can accumulate and promote NAFLD in the absence of dietary cholesterol. The IGF2 mRNA binding protein p62 facilitates increased levels of both free cholesterol and hepatic iron. Furthermore, hepatic iron accumulation was associated with lipid peroxidation. In summary, this study shows that p62 drives the progression of NASH by increasing hepatic free cholesterol.

***Applications***

The understanding of how p62 promotes NASH progression and further characterization of the role of specific lipid changes will increase knowledge about NASH pathogenesis and might therefore help to develop preventive strategies against NASH and NASH-associated HCC in the near future. This might also lead to new therapeutic options in NASH treatment.

***Peer review***

The data show that p62/IGF2BP2-2 drives the progression of NASH by increasing hepatic free cholesterol. This study was excellent.

**REFERENCES**

1 **Cohen JC**, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. *Science* 2011; **332**: 1519-1523 [PMID: 21700865 DOI: 10.1126/science.1204265]

2 **El-Serag HB**. Hepatocellular carcinoma. *N Engl J Med* 2011; **365**: 1118-1127 [PMID: 21992124 DOI: 10.1056/NEJMra1001683]

3 **Kessler SM**, Simon Y, Gemperlein K, Gianmoena K, Cadenas C, Zimmer V, Pokorny J, Barghash A, Helms V, van Rooijen N, Bohle RM, Lammert F, Hengstler JG, Mueller R, Haybaeck J, Kiemer AK. Fatty acid elongation in non-alcoholic steatohepatitis and hepatocellular carcinoma. *Int J Mol Sci* 2014; **15**: 5762-5773 [PMID: 24714086 DOI: 10.3390/ijms15045762]

4 **Laggai S**, Simon Y, Ranssweiler T, Kiemer AK, Kessler SM. Rapid chromatographic method to decipher distinct alterations in lipid classes in NAFLD/NASH. *World J Hepatol* 2013; **5**: 558-567 [PMID: 24179615 DOI: 10.4254/wjh.v5.i10.558]

5 **Laggai S**, Kessler SM, Boettcher S, Lebrun V, Gemperlein K, Lederer E, Leclercq IA, Mueller R, Hartmann RW, Haybaeck J, Kiemer AK. The IGF2 mRNA binding protein p62/IGF2BP2-2 induces fatty acid elongation as a critical feature of steatosis. *J Lipid Res* 2014; **55**: 1087-1097 [PMID: 24755648 DOI: 10.1194/jlr.M045500]

6 **Muir K**, Hazim A, He Y, Peyressatre M, Kim DY, Song X, Beretta L. Proteomic and lipidomic signatures of lipid metabolism in NASH-associated hepatocellular carcinoma. *Cancer Res* 2013; **73**: 4722-4731 [PMID: 23749645 DOI: 10.1158/0008-5472.CAN-12-3797]

7 **Kessler SM**, Laggai S, Barghash A, Helms V, Kiemer AK. Lipid metabolism signatures in NASH-associated HCC.--letter. *Cancer Res* 2014; **74**: 2903-2904 [PMID: 24778416 DOI: 10.1158/0008-5472.CAN-13-2852.]

8 **Larter CZ**, Yeh MM, Haigh WG, Williams J, Brown S, Bell-Anderson KS, Lee SP, Farrell GC. Hepatic free fatty acids accumulate in experimental steatohepatitis: role of adaptive pathways. *J Hepatol* 2008; **48**: 638-647 [PMID: 18280001 DOI: 10.1016/j.jhep.2007.12.011]

9 **Farrell GC**, van Rooyen D. Liver cholesterol: is it playing possum in NASH? *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G9-G11 [PMID: 22556144 DOI: 10.1152/ajpgi.00008.2012]

10 **Ioannou GN**, Haigh WG, Thorning D, Savard C. Hepatic cholesterol crystals and crown-like structures distinguish NASH from simple steatosis. *J Lipid Res* 2013; **54**: 1326-1334 [PMID: 23417738 DOI: 10.1194/jlr.M034876]

11 **Puri P**, Wiest MM, Cheung O, Mirshahi F, Sargeant C, Min HK, Contos MJ, Sterling RK, Fuchs M, Zhou H, Watkins SM, Sanyal AJ. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology* 2009; **50**: 1827-1838 [PMID: 19937697 DOI: 10.1158/0008-5472]

12 **Tomita K**, Teratani T, Suzuki T, Shimizu M, Sato H, Narimatsu K, Okada Y, Kurihara C, Irie R, Yokoyama H, Shimamura K, Usui S, Ebinuma H, Saito H, Watanabe C, Komoto S, Kawaguchi A, Nagao S, Sugiyama K, Hokari R, Kanai T, Miura S, Hibi T. Free cholesterol accumulation in hepatic stellate cells: mechanism of liver fibrosis aggravation in nonalcoholic steatohepatitis in mice. *Hepatology* 2014; **59**: 154-169 [PMID: 23832448 DOI: 10.1002/hep.26604]

13 **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 [PMID: 18666236 DOI: 10.1002/hep.22363]

14 **Zhang JY**, Chan EK, Peng XX, Tan EM. A novel cytoplasmic protein with RNA-binding motifs is an autoantigen in human hepatocellular carcinoma. *J Exp Med* 1999; **189**: 1101-1110 [PMID: 10190901 DOI: 10.1084/jem.189.7.1101]

15 **Lu M**, Nakamura RM, Dent ED, Zhang JY, Nielsen FC, Christiansen J, Chan EK, Tan EM. Aberrant expression of fetal RNA-binding protein p62 in liver cancer and liver cirrhosis. *Am J Pathol* 2001; **159**: 945-953 [PMID: 11549587 DOI: 10.1016/S0002-9440(10)61770-1]

16 **Kessler SM**, Pokorny J, Zimmer V, Laggai S, Lammert F, Bohle RM, Kiemer AK. IGF2 mRNA binding protein p62/IMP2-2 in hepatocellular carcinoma: antiapoptotic action is independent of IGF2/PI3K signaling. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G328-G336 [PMID: 23257922 DOI: 10.1152/ajpgi.00005.2012]

17 **Bell JL**, Wächter K, Mühleck B, Pazaitis N, Köhn M, Lederer M, Hüttelmaier S. Insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs): post-transcriptional drivers of cancer progression? *Cell Mol Life Sci* 2013; **70**: 2657-2675 [PMID: 23069990 DOI: 10.1007/s00018-012-1186-z]

18 **Simon Y**, Kessler SM, Bohle RM, Haybaeck J, Kiemer AK. The insulin-like growth factor 2 (IGF2) mRNA-binding protein p62/IGF2BP2-2 as a promoter of NAFLD and HCC? *Gut* 2014; **63**: 861-863 [PMID: 24173291 DOI: 10.1136/gutjnl-2013-305736]

19 **Tybl E**, Shi FD, Kessler SM, Tierling S, Walter J, Bohle RM, Wieland S, Zhang J, Tan EM, Kiemer AK. Overexpression of the IGF2-mRNA binding protein p62 in transgenic mice induces a steatotic phenotype. *J Hepatol* 2011; **54**: 994-1001 [PMID: 21145819 DOI: 10.1016/j.jhep.2010.08.034]

20 **Liu Y**, Meyer C, Xu C, Weng H, Hellerbrand C, ten Dijke P, Dooley S. Animal models of chronic liver diseases. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G449-G468 [PMID: 23275613 DOI: 10.1152/ajpgi.00199.2012]

21 **Corradini E**, Pietrangelo A. Iron and steatohepatitis. *J Gastroenterol Hepatol* 2012; **27 Suppl 2**: 42-46 [PMID: 22320915 DOI: 10.1111/j.1440-1746.2011.07014.x]

22 **Bouayed J**, Desor F, Rammal H, Kiemer AK, Tybl E, Schroeder H, Rychen G, Soulimani R. Effects of lactational exposure to benzo[alpha]pyrene (B[alpha]P) on postnatal neurodevelopment, neuronal receptor gene expression and behaviour in mice. *Toxicology* 2009; **259**: 97-106 [PMID: 19428949 DOI: 10.1016/j.tox.2009.02.010]

23 **Garcia R**, Pistorius D, Stadler M, Müller R. Fatty acid-related phylogeny of myxobacteria as an approach to discover polyunsaturated omega-3/6 Fatty acids. *J Bacteriol* 2011; **193**: 1930-1942 [PMID: 21317327 DOI: 10.1128/JB.01091-10]

24 **Koerber K**, Sass G, Kiemer AK, Vollmar AM, Tiegs G. In vivo regulation of inducible no synthase in immune-mediated liver injury in mice. *Hepatology* 2002; **36**: 1061-1069 [PMID: 12395315 DOI: 10.1053/jhep.2002.36155]

25 **Yao ZM**, Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J Biol Chem* 1988; **263**: 2998-3004 [PMID: 3343237]

26 **Yamazaki Y**, Kakizaki S, Takizawa D, Ichikawa T, Sato K, Takagi H, Mori M. Interstrain differences in susceptibility to non-alcoholic steatohepatitis. *J Gastroenterol Hepatol* 2008; **23**: 276-282 [PMID: 17868334 DOI: 10.1111/j.1440-1746.2007.05150.x]

27 **Graham RM**, Chua AC, Carter KW, Delima RD, Johnstone D, Herbison CE, Firth MJ, O'Leary R, Milward EA, Olynyk JK, Trinder D. Hepatic iron loading in mice increases cholesterol biosynthesis. *Hepatology* 2010; **52**: 462-471 [PMID: 20683946 DOI: 10.1002/hep.23712]

28 **Caballero F**, Fernández A, De Lacy AM, Fernández-Checa JC, Caballería J, García-Ruiz C. Enhanced free cholesterol, SREBP-2 and StAR expression in human NASH. *J Hepatol* 2009; **50**: 789-796 [PMID: 19231010 DOI: 10.1016/j.jhep.2008.12.016]

29 **Min HK**, Kapoor A, Fuchs M, Mirshahi F, Zhou H, Maher J, Kellum J, Warnick R, Contos MJ, Sanyal AJ. Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic fatty liver disease. *Cell Metab* 2012; **15**: 665-674 [PMID: 22560219 DOI: 10.1016/j.cmet.2012.04.004]

30 **Smits MM**, Ioannou GN, Boyko EJ, Utzschneider KM. Non-alcoholic fatty liver disease as an independent manifestation of the metabolic syndrome: results of a US national survey in three ethnic groups. *J Gastroenterol Hepatol* 2013; **28**: 664-670 [PMID: 23286209 DOI: 10.1111/jgh.12106]

31 **Glosli H**, Gudbrandsen OA, Mullen AJ, Halvorsen B, Røst TH, Wergedahl H, Prydz H, Aukrust P, Berge RK. Down-regulated expression of PPARalpha target genes, reduced fatty acid oxidation and altered fatty acid composition in the liver of mice transgenic for hTNFalpha. *Biochim Biophys Acta* 2005; **1734**: 235-246 [PMID: 15893958 DOI: 10.1016/j.bbalip.2005.02.011]

32 **Abiru S**, Migita K, Maeda Y, Daikoku M, Ito M, Ohata K, Nagaoka S, Matsumoto T, Takii Y, Kusumoto K, Nakamura M, Komori A, Yano K, Yatsuhashi H, Eguchi K, Ishibashi H. Serum cytokine and soluble cytokine receptor levels in patients with non-alcoholic steatohepatitis. *Liver Int* 2006; **26**: 39-45 [PMID: 16420507 DOI: 10.1111/j.1478-3231.2005.01191.x]

33 **Feldstein AE**, Canbay A, Angulo P, Taniai M, Burgart LJ, Lindor KD, Gores GJ. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology* 2003; **125**: 437-443 [PMID: 12891546 DOI: 10.1016/S0016-5085(03)00907-7]

34 **Albano E**, Mottaran E, Vidali M, Reale E, Saksena S, Occhino G, Burt AD, Day CP. Immune response towards lipid peroxidation products as a predictor of progression of non-alcoholic fatty liver disease to advanced fibrosis. *Gut* 2005; **54**: 987-993 [PMID: 15951547 DOI: 10.1136/gut.2004.057968]

35 **Yu J**, Ip E, Dela Peña A, Hou JY, Sesha J, Pera N, Hall P, Kirsch R, Leclercq I, Farrell GC. COX-2 induction in mice with experimental nutritional steatohepatitis: Role as pro-inflammatory mediator. *Hepatology* 2006; **43**: 826-836 [PMID: 16557554 DOI: 10.1002/hep.21108]

36 **Stienstra R**, Saudale F, Duval C, Keshtkar S, Groener JE, van Rooijen N, Staels B, Kersten S, Müller M. Kupffer cells promote hepatic steatosis via interleukin-1beta-dependent suppression of peroxisome proliferator-activated receptor alpha activity. *Hepatology* 2010; **51**: 511-522 [PMID: 20054868 DOI: 10.1002/hep.23337]

37 **Paillard F**, Catheline D, Duff FL, Bouriel M, Deugnier Y, Pouchard M, Daubert JC, Legrand P. Plasma palmitoleic acid, a product of stearoyl-coA desaturase activity, is an independent marker of triglyceridemia and abdominal adiposity. *Nutr Metab Cardiovasc Dis* 2008; **18**: 436-440 [PMID: 18068341 DOI: 10.1016/j.numecd.2007.02.017]

38 **Miyazaki M**, Bruggink SM, Ntambi JM. Identification of mouse palmitoyl-coenzyme A Delta9-desaturase. *J Lipid Res* 2006; **47**: 700-704 [PMID: 16443825 DOI: 10.1194/jlr.C500025-JLR200]

39 **Ntambi JM**, Miyazaki M. Regulation of stearoyl-CoA desaturases and role in metabolism. *Prog Lipid Res* 2004; **43**: 91-104 [PMID: 14654089 DOI: 10.1016/S0163-7827(03)00039-0]

40 **Matsuzaka T**, Atsumi A, Matsumori R, Nie T, Shinozaki H, Suzuki-Kemuriyama N, Kuba M, Nakagawa Y, Ishii K, Shimada M, Kobayashi K, Yatoh S, Takahashi A, Takekoshi K, Sone H, Yahagi N, Suzuki H, Murata S, Nakamuta M, Yamada N, Shimano H. Elovl6 promotes nonalcoholic steatohepatitis. *Hepatology* 2012; **56**: 2199-2208 [PMID: 22753171 DOI: 10.1002/hep.25932]

41 **Anstee QM**, Goldin RD. Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. *Int J Exp Pathol* 2006; **87**: 1-16 [PMID: 16436109 DOI: 10.1111/j.0959-9673.2006.00465.x]

42 **Musso G**, Gambino R, Cassader M. Cholesterol metabolism and the pathogenesis of non-alcoholic steatohepatitis. *Prog Lipid Res* 2013; **52**: 175-191 [PMID: 23206728 DOI: 10.1016/j.plipres.2012.11.002]

43 **Farrell GC**, van Rooyen D, Gan L, Chitturi S. NASH is an Inflammatory Disorder: Pathogenic, Prognostic and Therapeutic Implications. *Gut Liver* 2012; **6**: 149-171 [PMID: 22570745 DOI: 10.5009/gnl.2012.6.2.149]

44 **Fujita N**, Miyachi H, Tanaka H, Takeo M, Nakagawa N, Kobayashi Y, Iwasa M, Watanabe S, Takei Y. Iron overload is associated with hepatic oxidative damage to DNA in nonalcoholic steatohepatitis. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 424-432 [PMID: 19190144 DOI: 10.1158/1055-9965.EPI-08-0725]

45 **Sorrentino P**, D'Angelo S, Ferbo U, Micheli P, Bracigliano A, Vecchione R. Liver iron excess in patients with hepatocellular carcinoma developed on non-alcoholic steato-hepatitis. *J Hepatol* 2009; **50**: 351-357 [PMID: 19070395 DOI: 10.1016/j.jhep.2008.09.011]

46 **Ahmed U**, Latham PS, Oates PS. Interactions between hepatic iron and lipid metabolism with possible relevance to steatohepatitis. *World J Gastroenterol* 2012; **18**: 4651-4658 [PMID: 23002334 DOI: 10.3748/wjg.v18.i34.4651]

47 **Pigeon C**, Legrand P, Leroyer P, Bouriel M, Turlin B, Brissot P, Loréal O. Stearoyl coenzyme A desaturase 1 expression and activity are increased in the liver during iron overload. *Biochim Biophys Acta* 2001; **1535**: 275-284 [PMID: 11278167 DOI: 10.1016/S0925-4439(01)00024-2]

48 **Philippe MA**, Ruddell RG, Ramm GA. Role of iron in hepatic fibrosis: one piece in the puzzle. *World J Gastroenterol* 2007; **13**: 4746-4754 [PMID: 17729396]

49 **Seki S**, Kitada T, Yamada T, Sakaguchi H, Nakatani K, Wakasa K. In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases. *J Hepatol* 2002; **37**: 56-62 [PMID: 12076862 DOI: 10.1016/S0168-8278(02)00073-9]

**P-Reviewers:** Vespasiani-Gentilucci U **S-Editor:** Ding Y **L-Editor: E-Editor:**

**Figure 1 p62 expression amplifies inflammation.** A: Immunofluorescent staining with anti-nuclear factor kappa B (NF-қB)-p65 (red, left panel), 4',6-diamidino-2-phenylindole (DAPI) for nuclei (blue, middle panel), and merge (right panel) shows activation of NF-қB after 4 wk on the methionine-choline deficient (MCD) diet through translocation to the nucleus (white arrows)(original magnification 400×); B-E: Gene expression analysis of NF-қB target genes with tumor necrosis factor (TNF)(B), inducible nitric oxide synthase 2 (NOS2)(C), prostaglandin-endoperoxide synthase 2 (PTGS/COX2)(D), and interleukin 1B (IL-1B)(E) from whole liver by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) are expressed as ratio against the housekeeping gene 18S. Data are represented as mean ± SEM (*n* = 10-12). Ctrl: Control.

**Figure 2 p62 alters fatty acid pattern.** A: Sum of all fatty acids of mice fed the methionine-choline deficient (MCD) or ctrl diet for 2 and 4 wk. Liver tissues were lyophilized, lipids were hydrolyzed, and fatty acid (FA) were analyzed by gas-chromatography mass-spectrometry (GC-MS); B: Sum of saturated fatty acids (SFA), sum of monounsaturated fatty acids (MUFA), sum of polyunsaturated fatty acids (PUFA), and sum of all fatty acids (sum FA) from animals fed the MCD diet for 2 wk are represented as mean ± SEM (*n* = 9-12). Data is displayed as percentage of MCD-fed wt mice, which were set to 100%, each; C: Pamlitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), as well as oleic acid (C18:1) are represented as mean ± SEM (*n* = 9-12); D: Relative hepatic mRNA expression of stearoyl-CoA desaturase (Scd) 1, fatty acid synthase (FASN), peroxisome proliferator-activated receptor (PPAR) a, and carnitine palmitoyl transferase (CPT) 1a from mice fed the MCD diet for 2 and 4 wk were normalized against the housekeeping gene 18S and are shown as percentage of MCD-fed wt mice, which were set to 100%, each. Data are represented as mean ± SEM (*n* = 10-12). tg: Transgenic; wt: Wild-type; ctrl: Control.

**Figure 3 p62 expression elevates serum and liver cholesterol.** Hepatic (A) and serum (B) cholesterol concentrations in mice fed the respective diet for 2 or 4 wk. Representative cryo sections stained with Filipin for hepatic free cholesterol in mice fed the methionine-choline deficient (MCD) diet for 4 wk (original magnification 400×)(C) with corresponding quantification (D)(mean out of 5 randomly picked sections on the slide). Relative hepatic mRNA expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase)(HMGCR)(E) and sterol regulatory element binding transcription factor 2 (SREBF2)(F) are shown as ratio against the housekeeping gene 18S. Data are represented as mean ± SEM (*n* = 10-12). tg: Transgenic; wt: Wild-type; ctrl: Control.

**Figure 4 p62 expression leads to increased iron accumulation and reactive oxygen species production.** Representative paraffin-embedded liver sections stained with Prussian blue for iron accumulation from animals fed the respective diet for 4 wk (original magnification 200× and 500× for inserts)(A) with the corresponding hepatocellular iron score (B) for all time points (for scoring see supplement S1); C: Hepatic thiobarbituric acid reactive substances (TBARS) were measured to indicate lipid peroxidation and are represented as mean ± SEM (*n* = 10-12). tg: Transgenic; wt: Wild-type; ctrl: Control.

**Table 1 Antibody dilutions, demasking, incubation time, temperature, and immunodetection used for immunofluorescence**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Antibody**  **(source)** | **Demasking of antigens** | **Dilution** | **incubation** | **Detection system** |
| NF-қB p65  (Neomarkers, USA) | Citrate buffer pH 6.0, 95 °C, 10 min, water bath | 1:1000 | 18 h at 4°C | IF with Alexa Fluor 546 (Invitrogen, Germany) as secondary antibody |

NF-қB: Nuclear factor kappa B; IF: Immunofluorescence.

**Table 2 Primer sequences as used for real-time reverse transcription-polymerase chain reaction**

|  |  |  |  |
| --- | --- | --- | --- |
| **mRNA** | **Accession No.** | **Primer sense, 5'🡪 3'** | **Primer antisense, 5'🡪 3'** |
| 18S | NR\_003278.1 | GTAACCCGTTGAACCCCATT | CCATCCAATCGGTAGTAGCG |
| PPARa | NM\_001113418.1 | CCTTCCCTGTGAACTGACG | CCACAGAGCGCTAAGCTGT |
| IL-1B | NM\_008361.3 | GAGAGCCTGTGTTTTCCTCC | GAGTGCTGCCTAATGTCCC |
| TNF | NM\_013693.2 | CCATTCCTGAGTTCTGCAAAGG | AGGTAGGAAGGCCTGAGATCTTATC |
| HMGCR | NM\_008255.2 | atccaggagcgaaccaagagag | cagaagccccaagcacaaac |
| SCD1 | NM\_009127.4 | agatctccagttcttacacgaccac | ctttcatttcaggacggatgtct |
| CPT1a | NM\_013495.2 | CTCAGTGGGAGCGACTCTTCA | GGCCTCTGTGGTACACGACAA |
| NOS2 | NM\_010927.3 | ctcactgggacagcacagaa | gatgtggccttgtggtgaa |
| PTGS/COX2 | XM\_192868 | tgacccccaaggctcaaatat | tgaacccaggtcctcgctta |
| FASN | NM\_007988.3 | GGCTGCTACAAACAGACCAT | CACGGTAGAAAAGGCTCAGT |
| SREBF2 | NM\_033218.1 | ACCTAGACCTCGCCAAAGGT | CGGATCACATTCCAGGAGA |

PPARa: Peroxisome proliferator-activated receptor a; IL-1B: Interleukin 1B; TNF: Tumor necrosis factor; HMGCR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; SCD1: Stearoyl-CoA desaturase 1; CPT1a: Carnitine palmitoyl transferase 1a; NOS2: Nitric oxide synthase 2; PTGS/COX2: Prostaglandin-endoperoxide synthase 2; FASN: Fatty acid synthase; SREBF2: Sterol regulatory element binding transcription factor 2.

**Table 3 Scoring system for hepatocellular iron and nuclear factor kappa B-p65 nuclear translocation**

|  |  |  |  |
| --- | --- | --- | --- |
| **Scoring system** | | | **Assessed by** |
| Hepatocellular iron | score 0 | no granules | Prussian blue |
| score 1 | zone 1, granules seen at 40× |  |
| score 2 | granules seen at 20× |  |
| score 3 | granules seen at 10× |  |
|  | score 4 | granules seen at 10× in zone 1 and 2 |  |
| Nuclear translocation of NF-қB-p65 | score 0 | none | NF-қB-p65 IF |
| score 1 | 1-5 positive nuclei/20× |  |
| score 2 | 6-12 positive nuclei/20× |  |
| score 3 | > 13 positive nuclei/20× |  |

NF-қB: Nuclear factor kappa B; IF: Immunofluorescence.

**Table 4 Liver weight and serum parameters of *p62* transgenic and wild-type mice fed the methionine-choline deficient or control diet for 2 and 4 wk**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **2 wk** | | | | **4 wk** | | | |
|  | **ctrl** | | **MCD** | | **ctrl** | | **MCD** | |
|  | **wt** | **tg** | **wt** | **tg** | **wt** | **tg** | **wt** | **tg** |
| Number of animals | 10 | 10 | 12 | 12 | 10 | 10 | 12 | 12 |
| Relative liver weight  (% of body weight) | 4.8 ± 0.1 | 4.6 ± 0.1 | 3.4 ± 0.1c | 4.0 ± 0.2a,c,e | 4.2 ± 01 | 4.1 ± 0.1 | 3.4 ± 0.1c | 3.5 ± 0.2c,e |
| Serum ALT  (U/L) | 289 ± 83 | 233 ± 20 | 418 ± 36c | 469 ± 73c,e | 189 ± 38 | 222 ± 25 | 235 ± 40 | 209 ± 46 |
| serum AST (U/L) | 1568 ± 224 | 1535 ± 162 | 2404 ± 125c | 2544 ± 207c,e | 1845 ± 204 | 1712 ± 253 | 2813 ± 286c | 3057 ± 346c |
| Serum triglycerides (mg/dL) | 244 ± 19 | 219 ± 17 | 107 ± 5c | 128 ± 11c,e | 211 ± 18 | 203 ± 21 | 95 ± 7c | 106 ± 5c,e |
| Serum HDL  (mg/dL) | 94 ± 6 | 98 ± 4 | 24 ± 2c | 21 ± 4c,e | 112 ± 8 | 119 ± 8 | 15 ± 2c | 18 ± 2c,e |
| Serum glucose (mg/dL) | 234 ± 27 | 179 ± 15 | 67 ± 10c | 59 ± 7c,e | 193 ± 13 | 253 ± 36 | 59 ± 7c | 40 ± 5a,c,e |

a*P* < 0.05 *vs* wild-type; c*P* < 0.05 *vs* control diet; e*P* < 0.05 *vs* wild-type on control diet; Values are expressed as mean ± SEM; tg: Transgenic; wt: Wild-type; ctrl: Control; MCD: Methionine-choline deficient.

**Table 5 Gas-chromatography mass-spectrometry fatty acid analyses of mice fed for 2 wk the methionine-choline deficient or control diet**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Fatty acid** | **ctrl wt** | **ctrl tg** | ***P* value1** | **MCD wt** | **MCD tg** | ***P* value2** | ***P* value3** |
| 14:0 | 0.25 ± 0.04 | 0.25 ± 0.05 | 0.970 | 0.24 ± 0.04 | 0.43 ± 0.04 | 0.003 | 0.005 |
| 15:0 | 0.01 ± 0.003 | 0.01 ± 0.003 | 0.113 | 0.02 ± 0.01 | 0.05 ± 0.01 | 0.035 | 0.002 |
| 16:0 | 17.23 ± 0.79 | 16.08 ± 1.43 | 0.623 | 17.16 ± 1.63 | 23.85 ± 1.65 | 0.006 | 0.0033 |
| 16:1 | 1.69 ± 0.29 | 1.51 ± 0.30 | 0.570 | 0.57 ± 0.10 | 1.13 ± 0.12 | 0.005 | 0.138 |
| 17:0 | 0.11 ± 0.01 | 0.06 ± 0.01 | 0.021 | 0.23 ± 0.03 | 0.34 ± 0.03 | 0.01 | 0.0001 |
| 17:1 | 0.02 ± 0.01 | 0.01 ± 0.01 | 0.046 | 0.003 ± 0.003 | 0.01 ± 0.01 | 0.514 | 0.117 |
| 18:0 | 12.64 ± 0.57 | 9.41 ± 0.81 | 0.006 | 13.08 ± 0.97 | 18.15 ± 1.00 | 0.0014 | 0.0009 |
| 18:1 | 20.13 ± 2.51 | 17.98 ± 2.07 | 0.678 | 24.11 ± 1.28 | 39.66 ± 3.27 | 0.001 | 0.0009 |
| 18:2 | 17.88 ± 0.87 | 16.42 ± 1.60 | 0.241 | 23.90 ± 2.15 | 36.28 ± 6.34 | 0.040 | 0.004 |
| 18:3 | 0.23 ± 0.05 | 0.27 ± 0.10 | 0.571 | 1.50 ± 0.28 | 2.99 ± 0.46 | 0.003 | 0.00009 |
| 20:0 | 0.22 ± 0.05 | 0.10 ± 0.03 | 0.045 | 0.14 ± 0.03 | 0.33 ± 0.05 | 0.0007 | 0.138 |
| 20:1 | 0.40 ± 0.03 | 0.30 ± 0.04 | 0.045 | 0.81 ± 0.14 | 1.99 ± 0.27 | 0.002 | 0.00009 |
| 20:2 | 0.45 ± 0.04 | 0.53 ± 0.06 | 0.385 | 1.67 ± 0.17 | 2.94 ± 0.25 | 0.0006 | 0.00009 |
| 20:3 | 1.09 ± 0.09 | 0.89 ± 0.14 | 0.186 | 2.87 ± 0.37 | 5.15 ± 0.44 | 0.0006 | 0.00009 |
| 20:4 | 13.69 ± 0.58 | 10.37 ± 0.80 | 0.011 | 16.49 ± 1.15 | 23.20 ± 1.81 | 0.006 | 0.00015 |
| 22:0 | 0.59 ± 0.11 | 0.29 ± 0.07 | 0.031 | 0.31 ± 0.03 | 0.50 ± 0.03 | 0.0007 | 0.921 |
| 22:1 | 0.01 ± 0.003 | 0.003 ± 0.003 | 0.387 | 0.01 ± 0.01 | 0.04 ± 0.01 | 0.091 | 0.129 |
| 22:4 | 0.57 ± 0.05 | 0.51 ± 0.08 | 0.273 | 4.24 ± 0.48 | 6.25 ± 0.56 | 0.0036 | 0.00009 |
| 22:6 | 4.72 ± 0.28 | 3.82 ± 0.29 | 0.054 | 12.08 ± 0.90 | 14.06 ± 1.05 | 0.214 | 0.00009 |
| 23:0 | 0.08 ± 0.01 | 0.04 ± 0.004 | 0.0003 | 0.08 ± 0.01 | 0.10 ± 0.01 | 0.194 | 0.223 |
| 24:0 | 0.42 ± 0.02 | 0.31 ± 0.02 | 0.003 | 0.45 ± 0.02 | 0.63 ± 0.05 | 0.0013 | 0.0009 |

1*P* value of comparison of ctrl wt and ctrl tg; 2*P* value of comparison of MCD wt and MCD tg; 3*P* value of comparison of wt ctrl and tg MCD; Values [µg/mg dry liver tissue] are expressed as mean ± SEM. Liver tissues were lyophilized and analyzed by gas-chromatography mass-spectrometry. tg: Transgenic; wt: Wild-type; ctrl: Control; MCD: Methionine-choline deficient.



