Original Article

Endogenously increased n-3 PUFA levels in fat-1 transgenic mice do not protect from non-alcoholic steatohepatitis

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Background: Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and fibrosis. Possible reasons for the NAFLD epidemic in industrialized countries are the high intake of pro-inflammatory n-6 polyunsaturated fatty acids (n-6 PUFAs) and low consumption of healthy n-3 PUFAs. Due to their anti-inflammatory properties, n-3 PUFAs may have the potential to alleviate chronic liver disease. Herein, we examined the therapeutic effect of increased n-3 PUFA tissue levels in fat-1 transgenic mice on progressive NASH.

Methods: Disease was induced in mice by streptozotocin and high fat diet (STZ/HFD) resulting in NASH. NAFLD in 6 and 8 weeks old wild type and fat-1 transgenic STZ/HFD treated mice was analyzed. Unlike all other mammals, fat-1 transgenic mice ubiquitously express an n-3 fatty acid desaturase, which converts n-6 to n-3 PUFAs, leading to increased n-3 and decreased n-6 PUFA tissue contents.

Results: Liver damage, NAFLD activity score (NAS), hepatic lipid accumulation and inflammation were significantly reduced in fat-1 transgenic STZ/HFD treated mice in the early (6 weeks) but not late (8 weeks) phase of NASH. Simultaneously, mRNA expression of genes involved in fatty acid uptake and storage (*Cd36* and *Plin3*, respectively) was significantly down-regulated in 6 week old but not 8 week old fat-1 transgenic STZ/HFD treated mice.

Conclusions: Endogenously elevated n-3 PUFA levels in fat-1 transgenic mice transiently delay the onset of STZ/HFD induced NASH but failed to efficiently protect from NASH development.

Keywords: Non-alcoholic fatty liver disease (NAFLD); non-alcoholic steatohepatitis (NASH); n-6/n-3; fat-1; steatosis

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Introduction

Affecting approximately 25% of the world's population, non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide (1) and is closely linked to obesity and metabolic syndrome. Due to its rapidly increasing prevalence, a 178% rise in NAFLD related liver deaths by 2030 is estimated (2). NAFLD ranges from "simple" steatosis to non-alcoholic steatohepatitis (NASH) which elevates the risk for progression to cirrhosis and hepatocellular carcinoma (3). Hence, NAFLD is becoming an increasingly threatening disease and understanding its progression is of great interest.

Over the past decades, high dietary uptake of pro-

inflammatory n-6 polyunsaturated fatty acids (n-6 PUFAs) and insufficient supply with salutary n-3 PUFAs in industrialized countries have led to a disadvantageous dietary n-3/n-6 PUFA ratio in favor of n-6 PUFAs (4). Numerous positive and protective effects, e.g., regarding tumorigenesis, diabetes, obesity and cardiovascular disease, were attributed to n-3 PUFAs (5). Furthermore, n-3 PUFAs have been shown to alleviate hepatic steatosis (6). Conversely, n-6 PUFAs induce platelet aggregation, vasoconstriction and inflammation (7). It is well known that the effects of PUFAs are mediated by bioactive lipid mediators such as resolvins and prostaglandins (7,8). As their synthesis from n-3 and n-6 PUFAs relies on the same converting enzymes (9,10), the n-3/n-6 PUFA ratio is crucial for the overall effect and has been hypothesized to be essential for the positive effects of n-3 PUFAs. Due to their anti-inflammatory and anti-steatotic properties, increasing endogenous n-3 PUFAs as well as the n-3/n-6 PUFA ratio might serve as effective therapeutic strategies alleviating NAFLD and NASH.

To date, apart from life style modification, no therapeutic interventions against NAFLD exist. Therefore, it is of utmost importance to understand NAFLD. Herein, we report that fat-1 transgenic mice with endogenously increased n-3 PUFA levels and n-3/n-6 PUFA ratios are not protected from NASH development despite a delayed onset of the disease.

Methods

Mice

Fat-1 transgenic breeder mice (11) with C57BL/6J background were provided by Dr. Weylandt (Charité University Medicine, Berlin, Germany) with kind permission of Dr. Kang (Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA). Fat-1 transgenic mice express the n-3 fatty acid desaturase (*fat-1*) gene from *C. elegans* which converts n-6 PUFAs to n-3 PUFAs (11). This leads to increased n-3 PUFA tissue levels and an increased n-3/n-6 PUFA ratio (12,13). The presence of the *fat-1* gene was confirmed by genotyping of each mouse (primer: forward 5'-CTTCACCATGCTTTCACCAACC-3' reverse 5'-ACACAGCAACAGATTCCAGAGATT-3'). Fat-1 transgenic (fat-1) and wildtype (wt) mice were housed on a 12 h/12 h light/dark cycle with *ad libitum* access to water and food. All animal experiments were approved by "Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern" (7221.3-1-022/15) and were executed in accordance with the German legislation and EU-directive 2010/63/EU.

Mouse model

NASH was induced by streptozotocin and high fat diet (STZ/HFD) (14) as described previously by our group (15,16). In short, male fat-1 transgenic and wt mice were injected with 200 µg streptozotocin (Sigma-Aldrich, St. Louis, Missouri, USA) in 10 µL of 0.05 M trisodium citrate (pH 4.5) (Merck, Darmstadt, Germany) intraperitoneally at day two postnatal. From 28 days of age the mice were fed a continuous HFD [fat content: 60 kJ%; D12492(II) modified experimental diet; Ssniff, Soest, Germany]. The fatty acid composition of the diet is displayed in *Table 1*. The mice develop NAFLD with progression from NASH to liver tumors within 20 weeks. To study the early phase of progressive NAFLD, blood and tissue was collected at the age of 6 and 8 weeks as described previously by our group (15). 14 wt mice and 11 fat-1 transgenic mice at an age of 6 weeks as well as 10 wt and 11 fat-1 transgenic 8 week old mice were sacrificed. In case of only a subset of samples being analyzed, the exact number of analyzed samples per group is indicated in the methods section in parentheses behind the corresponding parameter in the following order: wt 6 weeks, fat-1 6 weeks, wt 8 weeks, fat-1 8 weeks. The general state of health was monitored daily and blood glucose levels and body weight were measured weekly. Animals exhibiting normal blood glucose levels were excluded from the experiment.

Plasma analyses

Alanine aminotransferase (ALT) and glutamate dehydrogenase (GLDH) activity in EDTA plasma (sample size: 14, 11, 7, 11) and plasma triglyceride contents (sample size: 11, 11, 7, 11) were measured as described previously (15). Free fatty acids (sample size: 10, 10, 7, 10; ab65341, Abcam, Cambridge, UK) and total cholesterol (sample size: 11, 11, 7, 11; ab65390, Abcam, Cambridge, UK) assays were performed according to the manufacturer's instructions.

Histology and immunohistochemistry

Histological and immunohistochemical stainings were performed as described previously (15) and were analyzed in

Table 1 Fatty acid composition (in % of total fatty acids) of the livers of 6 and 8 week old STZ/HFD treated fat-1 transgenic and wt mice as well as of the HFD

Fatty acids	6 weeks		8 weeks		High fat diet
	wt	fat-1	wt	fat-1	
C18:2n-6	9.85	10.24	8.93	9.21	6.68
C18:3n-3	0.35	0.42*	0.31	0.52*	0.92
C20:4n-6	4.74	4.6	4.44	2.62**	n.d.
C20:5n-3	0.25	0.87***	0.24	0.77***	n.d.
C22:4n-6	0.23	0.09***	0.22	0.07***	0.04
C22:6n-3	4.33	8.28**	4.36	6.84***	n.d.
Sum SFA	36.06	34.48*	34.37	35.43	53.19
Sum MUFA	41.43	37.95*	44.58	41.62	38.81
Sum PUFA	22.51	27.57***	21.05	22.95	8.00
Sum n-3 PUFA	5.71	10.46***	5.58	9.23***	0.94
Sum n-6 PUFA	16.52	16.82	15.16	13.42	6.79
n-6/n-3 PUFA ratio	2.92	1.62***	2.78	1.48***	7.22

Group differences were tested by *t*-test within each time point. *P<0.05 *vs.* wt, **P<0.01 *vs.* wt, ***P<0.001 *vs.* wt. STZ/HFD, streptozotocin and high fat diet; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n.d., not detectable.

a blinded manner. Formalin fixed liver tissue was embedded in paraffin and cut into 5 µm thick sections. For assessment of NAFLD activity score (NAS), the sections were stained with hematoxylin and eosin (Merck, Darmstadt, Germany). NAS was determined as proposed by Kleiner *et al.* (17) and as described previously by our group (15). The score for each section was determined by three independent observers. Infiltration of granulocytes in liver tissue was assessed by staining for chloroacetate esterase (CAE) with Naphthol AS-D chloroacetate (Sigma-Aldrich, St. Louis, Missouri, USA) and counterstaining with hematoxylin. For quantification CAE positive cells were counted in 30 consecutive high power fields (HPF). Immunohistochemical staining against F4/80 was conducted as described previously by our group (15,18). For analysis of the staining at least 30 consecutive photomicrographs were taken using a 40x objective. The red stained F4/80-positive area was quantified using the color threshold tool in ImageJ 1.47v. Oil Red O staining of lipids in liver tissue was performed using 8 µm thick paraformaldehyde (ChemCruz, Dallas, Texas, USA) fixed frozen tissue sections that were subsequently stained with Oil Red O (sample size: 14, 11; 9, 11; Sigma-Aldrich, St. Louis, Missouri, USA) and counterstained with hematoxylin. For analysis at least 10 photomicrographs were taken per section using a 20× objective. The red stained area was quantified using ImageJ 1.47v.

qRT-PCR

Total RNA was isolated from snap frozen liver tissue lysates using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with the RNase-Free DNase Set (Qiagen, Hilden, Germany) for on column digestion of genomic DNA according to the manufacturer's instructions. For reverse transcription of 2 µg RNA to cDNA, the Oligo(dT)$_{18}$ mRNA Primer (New England Biolabs GmbH, Ipswich, Massachusetts, USA) and SuperScript™ II Reverse Transcriptase (Invitrogen, ThermoFisher Scientific, Waltham, Massachusetts, USA) were used. Quantitative Real-Time PCR gene expression analysis for the detection of amplified dsDNA strands was performed with the Bio-Rad iQ5 system (Bio-Rad Laboratories, Hercules, California, USA) using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, California, USA). The primers used for amplification are listed in *Table 2*. The results were calculated using the $2^{-\Delta\Delta Ct}$ method with normalization of the target gene expression levels to the housekeeping gene expression (*Rps18*) and to expression levels of a liver tissue pool obtained from healthy wt C57BL/6J mice. qRT-PCR was performed with randomly selected liver tissue samples

Table 2 Primers used for qRT-PCR analysis of mouse liver tissue

Gene	Forward (5'-3')	Reverse (5'-3')
Fatty acid transport protein 2 (*Fatp2*)	AACACATCGCGGAGTACCTG	CTCAGTCATGGGCACAAATG
Fatty acid transport protein 5 (*Fatp5*)	GACTTTTGATGGGCAGAAGC	GGGCCTTGTTGTCCAGTATG
cluster of differentiation 36 (*Cd36*)	GGTGATGTTTGTTGCTTTTATGATTTC	TGTAGATCGGCTTTACCAAAGATG
peroxisome proliferator activated receptor gamma (*Pparg*)	TCATGACCAGGGAGTTCCTC	CAGGTTGTCTTGGATGTCCTC
Fatty acid binding protein 1 (*Fabp1*)	AAGTGGTCCGCAATGAGTTC	GTAGACAATGTCGCCCAATG
Perilipin 2 (*Plin2*)	CCTCAGCTCTCCTGTTAGGC	CACTACTGCTGCTGCCATTT
Perilipin 3 (*Plin3*)	CCACAGGATGCTGAAAAGG	TGATGTCCCTGAACATGCTG
Perilipin 5 (*Plin5*)	GCCACAGTGAATGATCTTGCCTG	GTCCACCATGCCTGTGACACTT
Ribosomal protein S18 (*Rps18*)	AGGATGTGAAGGATGGGAAG	TTGGATACACCCACAGTTCG

of 6 wt and 5 fat-1 mice of each age.

Lipid extraction and fatty acid analysis

After homogenization of frozen liver samples and the addition of C19:0 as an internal standard, the total liver lipids were extracted in duplicate using chloroform/methanol (2:1, v/v) by the use of Ultra Turrax T25 (IKA, Staufen, Germany), 3×15 sec, 15,780 ×g, at room temperature. The detailed sample preparation procedure has been previously described (19). Briefly, the organic phase was separated and dried with Na_2SO_4 and K_2CO_3 (10:1, w/w), and the solvent was subsequently removed under gentle nitrogen at room temperature. Next, 2 mL of 0.5 M sodium methoxide in methanol was added to the samples, which were shaken in a 60 ℃ water bath for 10 min. Subsequently, 1 mL of 14% boron trifluoride in methanol was added to the mixture, which was then shaken for additional 10 minutes at 60 ℃. Saturated $NaHCO_3$ solution (2 mL) was added, and the fatty acid methyl esters (FAMEs) were extracted three times in 2 mL of n-hexane. The FAMEs were stored at –18 ℃ until used for gas chromatography (GC) analysis. The fatty acid analysis of the liver lipids was performed using capillary GC with a CP-Sil 88 CB column (100 m × 0.25 mm, Agilent, Santa Clara, CA, United States) that was installed in a PerkinElmer gas chromatograph CLARUS 680 with a flame ionization detector and split injection (PerkinElmer Instruments, Shelton, United States). The detailed GC conditions were recently described (20). Briefly, hydrogen was used as the carrier gas at a flow rate of 1 mL min^{-1}. The split ratio was 1:20, and the injector and detector were set at 260 and 280 ℃, respectively. The quantification of fatty acids was done by the use of C19:0 as internal standard. For the calibration procedure the reference standard mixture "Sigma FAME" (Sigma-Aldrich, Deisenhofen, Germany), the methyl ester of C18:1cis-11, C22:5n-3 and C18:2cis-9, trans-11 (Matreya, State College, PA, USA), C22:4n-6 (Sigma-Aldrich, Deisenhofen, Germany) and C18:4n-3 (Larodan, Limhamn, Sweden) were used. The five-point calibration of single fatty acids ranged between 16 and 415 mg/mL and was checked after GC analysis of five samples.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.05 (GraphPad Software, La Jolla, California, USA). The group difference within each time point was assessed by *t*-test and is indicated in the graphs as follows: *P<0.05, **P<0.01, ***P<0.001. Statistical significance was set at P<0.05. All data are presented as mean ± standard deviation (SD) in dot plots.

Results

General aspects

Liver fatty acid analysis revealed significantly increased hepatic contents of n-3 PUFAs and significantly decreased n-6/n-3 PUFA ratios in fat-1 transgenic STZ/HFD mice compared to wt at both time points. Compared to fat-1 transgenic STZ/HFD mice, livers of wt STZ/HFD mice

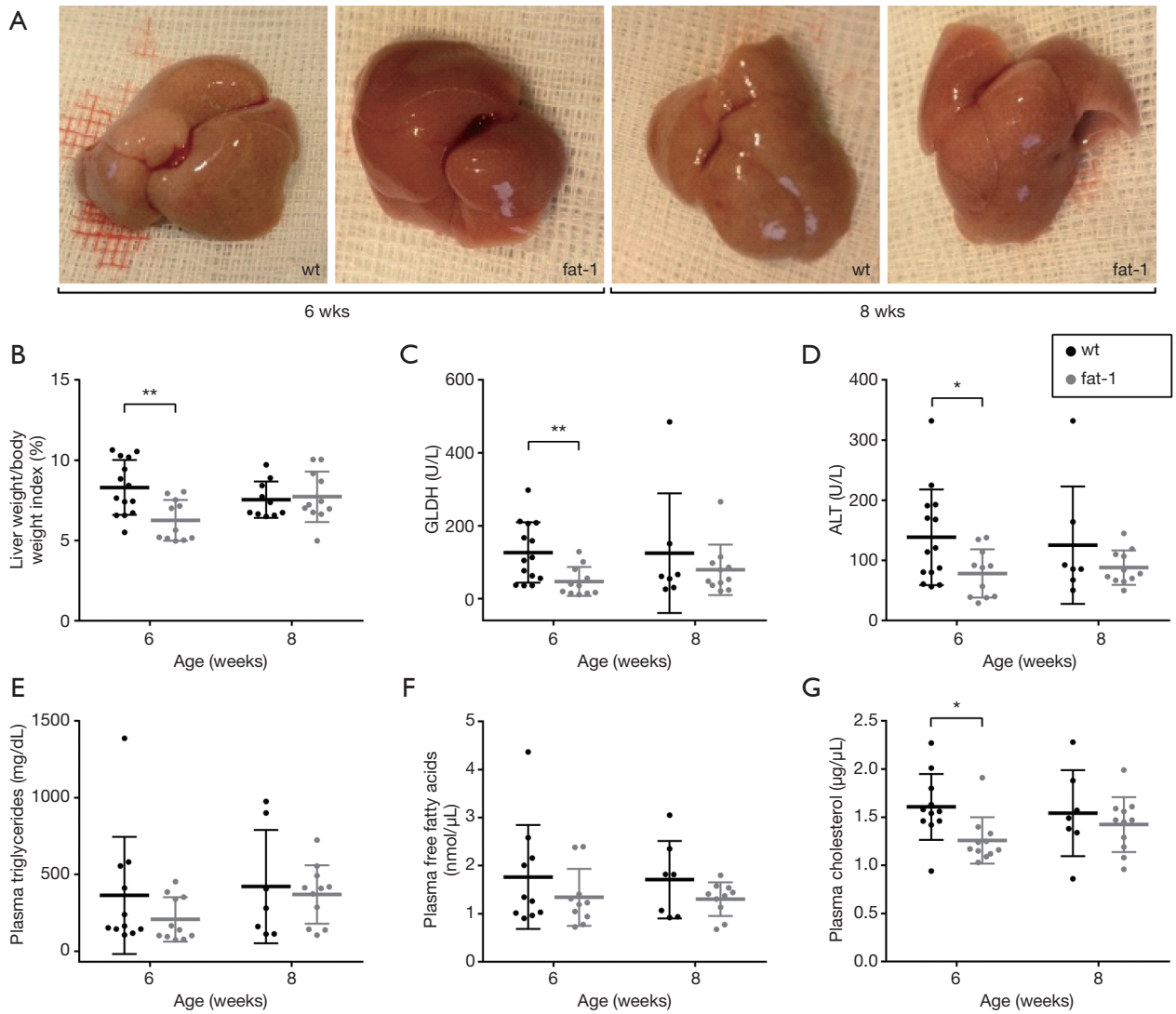

Figure 1 Representative images of livers of 6 and 8 weeks old STZ/HFD treated wt and fat-1 mice (A) and analysis of liver weight/body weight index (B), plasma activity of GLDH (C) and ALT (D) as well as plasma content of triglycerides (E), free fatty acids (F) and total cholesterol (G) of 6 and 8 week old STZ/HFD treated wt and fat-1 mice. Values are presented as mean ± SD. Group differences were tested by *t*-test within each time point. *P<0.05, **P<0.01, n=7–14.

appeared palish in color, particularly at 6 weeks (*Figure 1A*). Moreover, the liver weight/body weight index was significantly reduced by 2% in fat-1 transgenic STZ/HFD mice compared to wt STZ/HFD mice at 6 weeks but not at 8 weeks of age (*Figure 1B*). Liver damage, as assessed by ALT and GLDH activities in plasma, was transiently alleviated in fat-1 compared to wt STZ/HFD mice (*Figure 1C*,*D*), showing significantly reduced levels at 6 weeks of age. Plasma triglyceride (*Figure 1E*), free fatty acid (*Figure 1F*) and total cholesterol levels (*Figure 1G*) trended to be reduced in fat-1 compared to wt STZ/HFD mice. This reduction was more pronounced at 6 weeks of age and was significant for plasma cholesterol at this time point, but less prominent at 8 weeks of age. Despite these differences, blood glucose levels did not differ between the groups (data not shown).

Histopathology

The NAS was determined for histopathologic evaluation of

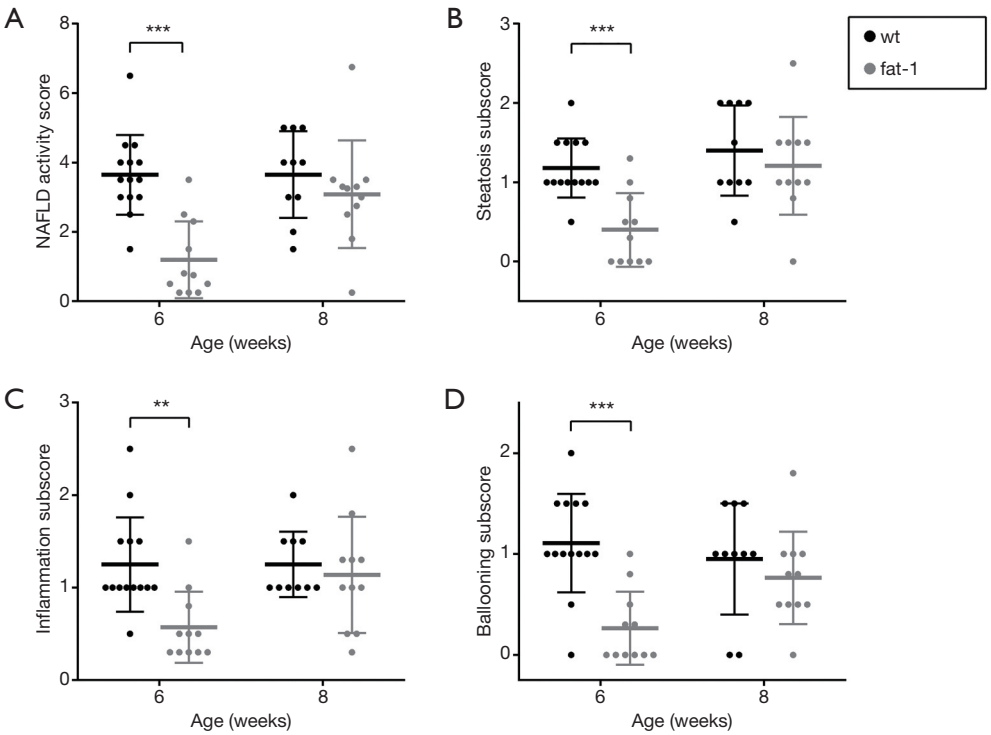

Figure 2 NAFLD activity score (A) and its subscores for steatosis (B), inflammation (C) and ballooning (D) of 6 and 8 week old STZ/HFD treated wt and fat-1 mice. Values are presented as mean ± SD. Group differences were tested by *t*-test within each time point. **P<0.01, ***P<0.001, n=10–14 per group.

NAFLD (*Figure 2A*). Interestingly, fat-1 STZ/HFD mice exhibited a highly significantly lower NAS than wt STZ/HFD mice at 6 weeks of age (wt: 3.6, fat-1: 1.2), whereas no difference was detected at an age of 8 weeks. The same trend was observed for all subscores (steatosis, inflammation and ballooning; *Figure 2B*,*C*,*D*, respectively), with the reduction in fat-1 STZ/HFD mice being highly significant at 6 weeks of age.

For further characterization of hepatic steatosis (*Figure 3*), Oil Red O stained liver sections were analyzed quantitatively (*Figure 3A*,*D* upper panel). While wt STZ/HFD mice revealed massive accumulation of hepatic fat, Oil Red O-positive area was markedly reduced in livers of fat-1 STZ/HFD mice. The difference was highly significant at 6 weeks of age, where in fat-1 STZ/HFD mice only ~13.5% of the liver was Oil Red O-positive compared to ~40% in wt STZ/HFD mice. At an age of 8 weeks the difference was less pronounced and did not reach statistical significance.

As inflammation is a key feature of NASH, immigrated granulocytes were assessed by CAE staining (*Figure 3B*,*D* lower panel). Interestingly, significantly fewer CAE positive cells per HPF were observed in fat-1 compared to wt STZ/HFD mice at the age of 6 weeks, whereas this difference disappeared at 8 weeks. Furthermore, we investigated the abundance of hepatic macrophages by F4/80 immunohistochemistry (*Figure 3C*). However, no difference between fat-1 and wt STZ/HFD mice was observed.

Fatty acid transport and storage

Hepatic mRNA expression of fatty acid transport protein (*Fatp*) 2 (*Figure 4A*) and 5 (*Figure 4B*) and fatty acid binding protein *Fabp1* (*Figure 4C*) did not differ between the genotypes. Nevertheless, the STZ/HFD-induced increase of hepatic *Cd36* mRNA expression of wt mice was significantly lower in fat-1 transgenic mice at 6 weeks of age (*Figure 4D*). However, no difference was detectable at 8 weeks of age. CD36 is a scavenger receptor involved in lipid signaling, uptake and transport. Its mRNA expression is known to be regulated by PPAR-γ. Analysis of hepatic *Pparg* mRNA expression levels revealed significantly decreased values for fat-1 STZ/HFD mice at both time points (*Figure 4E*).

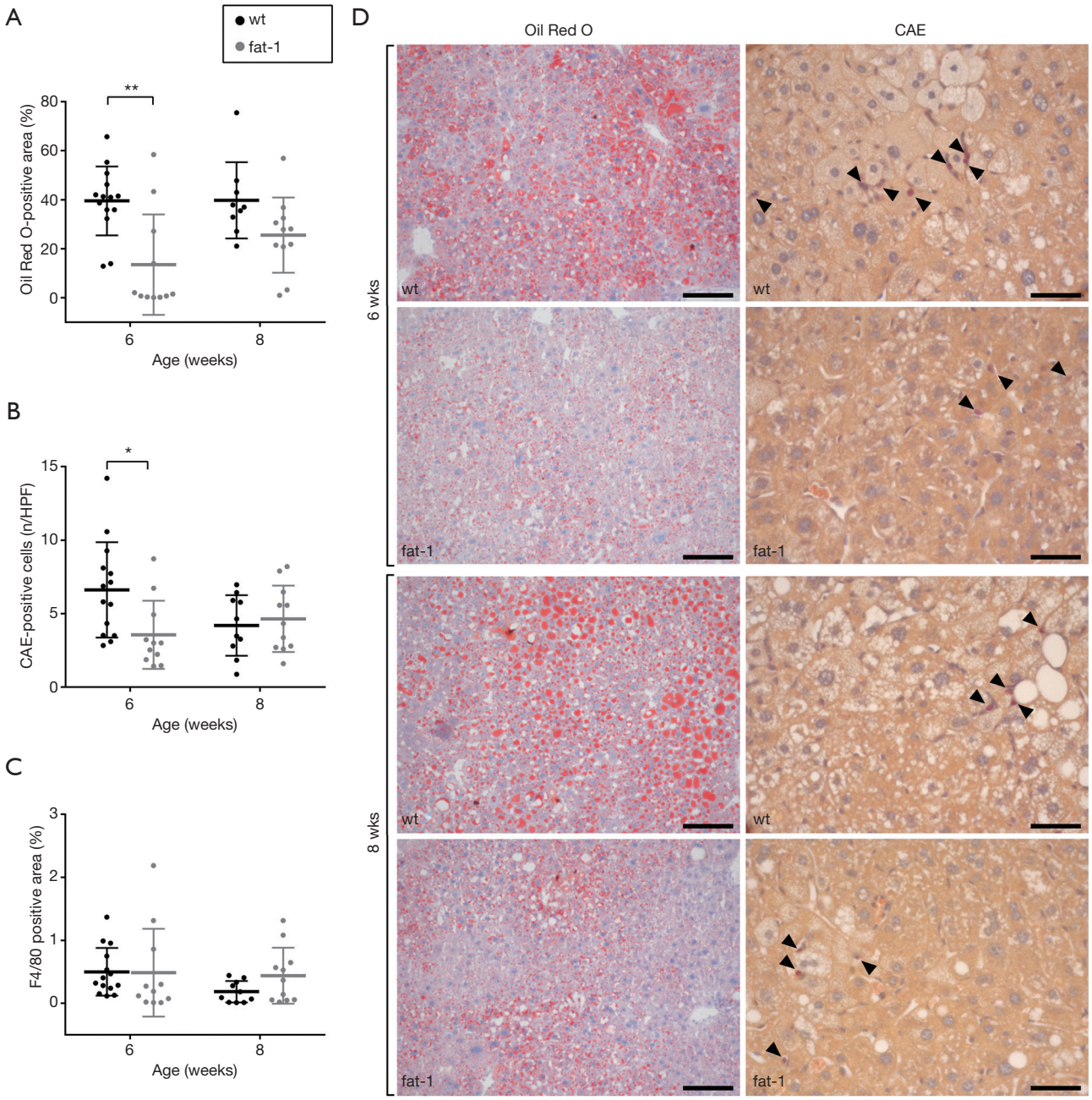

Figure 3 Quantitative analysis of Oil Red O (A), CAE (B) and F4/80 (C) stained liver sections of 6 and 8 week old STZ/HFD treated wt and fat-1 mice. Values are presented as mean ± SD. Group differences were tested by *t*-test within each time point. *P<0.05, **P<0.01, n=9–14 per group. (D) Representative photomicrographs of Oil Red O (left panel) and CAE (right panel) stained liver sections of 6 and 8 week old STZ/HFD treated wt and fat-1 mice. The scale bars mark 100 µm (Oil Red O) and 50 µm (CAE), respectively. CAE positive cells are marked by arrowheads.

NAFLD is known to be aggravated by the perilipin (Plin) protein family. As Plins play a role as structural proteins on lipid droplets, we analyzed mRNA expression of *Plin2* (*Figure 4F*), *Plin3* (*Figure 4G*) and *Plin5* (*Figure 4H*). Compared to wt STZ/HFD mice, 6 week old fat-1 transgenic STZ/HFD mice exhibited reduced *Plin2* and

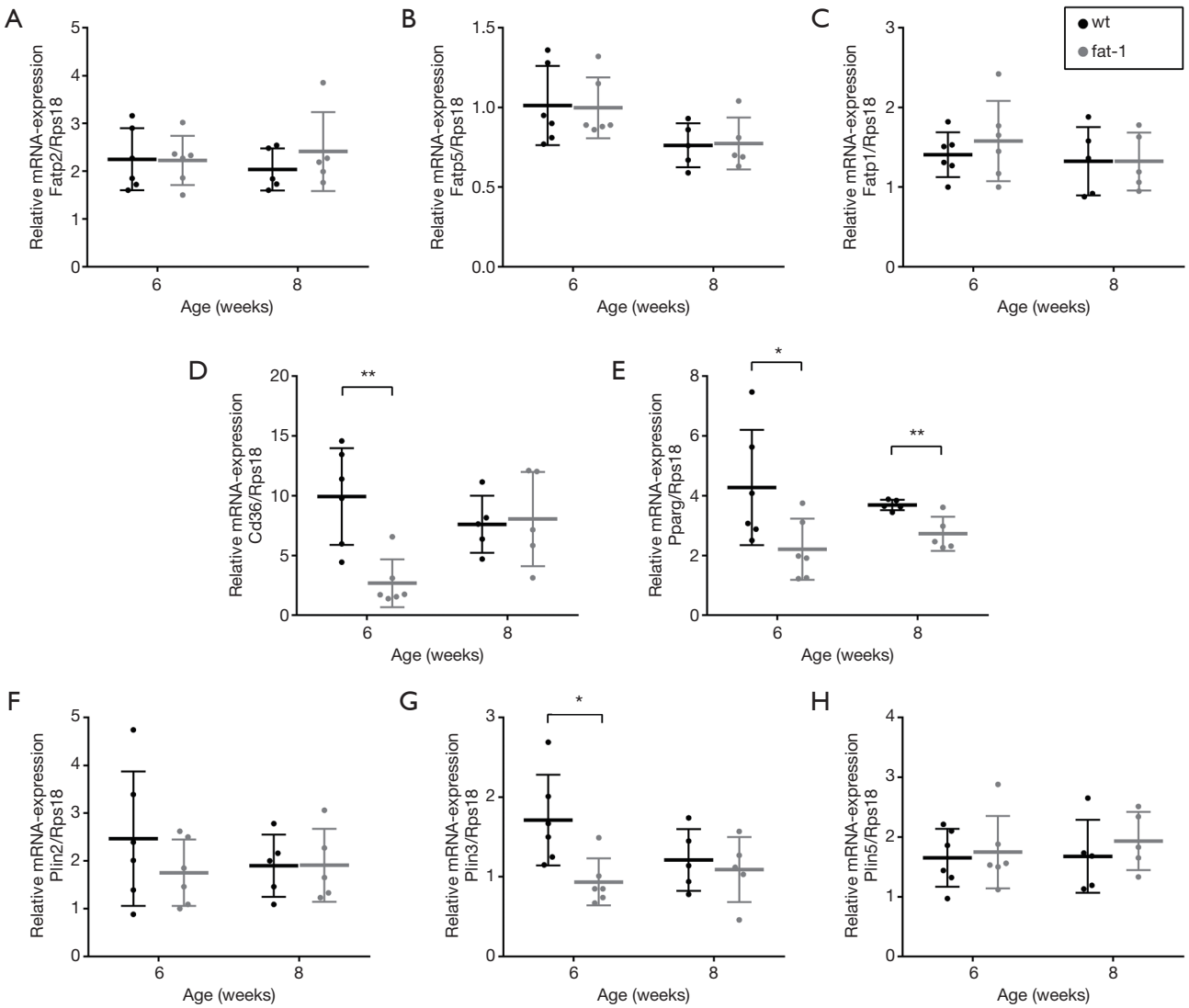

Figure 4 mRNA expression of *Fatp2* (A), *Fatp5* (B), *Fabp1* (C), *Cd36* (D), *Pparg* (E), *Plin2* (F), *Plin3* (G) and *Plin5* (H) relative to *Rps18* in liver tissue of 6 and 8 week old STZ/HFD treated wt and fat-1 mice. Values are presented as mean ± SD. Group differences were tested by *t*-test within each time point, *P<0.05, **P<0.01. n=5–6 per group.

Plin3 mRNA expression levels, which was significant for *Plin3*. However, at 8 weeks of age both genotypes showed similar mRNA expression levels. Interestingly, *Plin5* mRNA expression did not differ between the groups at both time points.

Discussion

Herein, we reported that endogenously elevated n-3 PUFA tissue contents in fat-1 transgenic mice may delay the onset of experimental NASH, but do not impede NASH development. While a positive effect of n-3 PUFAs on steatosis and NAFLD in general has been confirmed by numerous studies (21,22), the effect of n-3 PUFAs on NASH is discussed controversially. In accordance with our findings, a double-blind, randomized, placebo-controlled trial in NASH patients showed no impact of n-3 PUFA supplementation on liver injury, despite the liver fat content being significantly reduced (23). In another clinical trial, synthetic eicosapentaenoic acid (C20:5n-3) was unable to alleviate NASH pathology but reduced plasma triglyceride levels (24). Provenzano *et al.* even observed worsening of

NASH regarding inflammation and liver damage in n-3 PUFA supplemented methionine choline-deficient diet fed mice (25). These findings indicate that dietary n-3 PUFAs have a limited therapeutic potential to ameliorate NASH. However, the observed effects of n-3 PUFAs depend on multiple factors including dietary composition, n-3 PUFA tissue availability as well as liver disease model and thus, the inflammatory state. Most studies examining n-3 PUFA supplementation do not evaluate the overall n-3/n-6 PUFA ratio of the diet or tissue, which might significantly affect the outcome of the studies. Thus, studies using fat-1 transgenic mice, which diet-independently exhibit elevated tissue n-3 PUFA levels and n-3/n-6 PUFA ratio, are interesting as they allow linking the observed effects to the endogenous availability of n-3 PUFAs. Several studies that have examined HFD fed fat-1 transgenic mice, observed, i.e., reduced steatosis, ballooning, fibrosis, ALT, serum and liver triglycerides and total cholesterol (26,27). Additionally, in another disease model fat-1 transgenic mice seem to be protected from high fat plus high sugar diet induced NAFLD, exhibiting reduced steatosis compared to wt mice (28). Although we observed transiently alleviated NAFLD in fat-1 mice, n-3 PUFAs were unable to prevent progression to NASH. In contrast to the STZ/HFD mouse model, the other studies induced less progressive forms of NAFLD, which might be crucial for the overall outcome. These findings suggest that n-3 PUFAs hamper steatosis, but finally fail to prevent progression to more severe and complex forms of NAFLD. Hence, the effects described herein may help to understand the discrepancies between different studies.

In humans, NAFLD is often associated with the metabolic syndrome (including obesity and insulin resistance), which is incompletely reflected by the STZ/HFD mouse model. In fact, these mice are lean and the elevated blood glucose levels caused by STZ-induced beta-cell death rather reflect a 'type I-like' diabetes. To date, the lack of metabolic determinants of the metabolic syndrome in mouse models of NAFLD is a commonly known problem, limiting most mouse models of progressive NAFLD/NASH to reflect the human situation. Nevertheless, the STZ/HFD mouse model is a commonly used model of NAFLD as liver disease develops progressively in a short period of time.

CD36 is a scavenger receptor which is involved in fatty acid uptake and signal transduction (29). Herein, we reported upregulated *Cd36* mRNA expression in STZ/HFD treated wt mice and significantly lower values in fat-1 transgenic STZ/HFD mice at 6 weeks of age but not at 8 weeks. However, mRNA expression of other genes involved in lipid uptake and transport (*Fatp2*, *Ftp5*, *Fabp1*) was not affected by STZ/HFD treatment at any examined time point. It is known that hepatic CD36 protein and mRNA expression is elevated in NAFLD patients (30) and positively correlates with the liver fat content (31). Similarly, increased *Cd36* mRNA expression has been observed in mice with NASH (32). While mice protected from diet induced NAFLD display decreased CD36 (33) and PPAR-γ expression, other genes involved in lipid metabolism and transport like FATP5 were not altered (34). Furthermore, it has been shown that CD36 deficient mice are protected from NAFLD and insulin resistance (35). Therefore, the rise of *Cd36* mRNA expression in fat-1 STZ/HFD mice at 8 weeks might account for the lacking protection against NASH at that time point.

It is known that PPAR-γ regulates *Cd36* (34,36) and *Plin2* (37) mRNA expression. Herein, we showed that hepatic *Pparg* mRNA expression was significantly reduced in fat-1 transgenic STZ/HFD mice compared to wt STZ/HFD mice. However, PPAR-γ expression is found to be increased in NAFLD (38), and PPAR-γ polymorphisms are associated with NAFLD development and severity (39,40). Accordingly, knock out of PPAR-γ in hepatocytes or Kupffer cells has been shown to alleviate NAFLD in mice (41). Due to the role of PPAR-γ in fatty acids transport and storage, its down regulation might contribute to reduced liver fat accumulation in fat-1 transgenic STZ/HFD mice. By facilitating uptake of PPAR-γ agonists like fatty acids, CD36 controls its own expression in a feed-forward cycle (42). Therefore, increased fatty acid supply leads to increased fatty acid uptake. This mechanism might account for the delayed increase of *Cd36* mRNA expression in fat-1 STZ/HFD mice at 8 weeks. We suggest that, even though fatty acid uptake mechanisms were initially impaired in fat-1 STZ/HFD mice, continuous supply with fat and sustained insult of diabetes, still results in fatty acid delivery to the liver. Furthermore, beside protein facilitated fatty acid transport, fatty acid uptake by simple diffusion is possible (43). Therefore, upon continuous fatty acid oversupply passive diffusion might counteract regulatory mechanisms of protein assisted fatty acid transport.

Perilipins (Plin) are a protein family that is involved in lipid storage as coating proteins on lipid droplets. Herein, we observed increased levels of n-3 PUFAs reduce *Plin2* and *Plin3* but not *Plin5* mRNA expression in 6 week old but not 8 week old STZ/HFD treated mice. It has been shown that Plin2 expression correlates with the degree of

hepatic steatosis in human patients (44). Similarly, in an *in vitro* model of hepatic steatosis, cultured hepatocytes displayed increasing *Plin2* mRNA expression with rising degree of steatosis (45). Several knock down and knock out experiments have been conducted to reveal a causal link between Plin2 expression and NAFLD. Liver specific *Plin2* knock out results in alleviated steatosis and inflammation in methionine choline deficient diet fed mice (46). Similar effects were obtained with *Plin2* knock down in HFD fed mice, resulting in improved steatosis and insulin resistance (47). Whereas in humans Plin3 is expressed in non-steatotic as well as steatotic liver (44,48), its expression is upregulated in mice upon HFD feeding (49). Of interest, a *Plin3* knock down improves steatosis, triglyceride content, glucose tolerance and insulin sensitivity in mice (48,49). Thus, we suggest that down regulation of *Plin2* and *Plin3* expression at 6 weeks in fat-1 STZ/HFD mice might contribute to the transient protective effects of n-3 PUFAs observed at this time point.

In summary, an increased n-3 PUFA level and n-3/n-6 ratio in liver tissue of fat-1 transgenic STZ/HFD mice just transiently improved NASH pathology, but failed to prevent NASH development. These effects might be caused by a short-term reduction of genes involved in fatty acid uptake and lipid storage in the early phase of NASH. Thus, n-3 PUFAs might help to hamper NAFLD when the underlying cause is eliminated. However, under a continuous insult, like fatty acid over-supply and increased blood glucose levels, fat-1 transgenic STZ/HFD mice are not protected from NASH.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: All animal experiments were approved by "Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern" (7221.3-1-022/15) and were executed in accordance with the German legislation and EU-directive 2010/63/EU.

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