Redox Biology 64 (2023) 102803

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Prevention of colitis-induced liver oxidative stress and inflammation in a transgenic mouse model with increased omega-3 polyunsaturated fatty acids

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ARTICLE INFO

Keywords:
Colitis
Liver inflammation
Liver oxidative stress
n-3 PUFA
Fat-1 mice

ABSTRACT

Inflammatory bowel disease (IBD) is an immune-mediated gut dysfunction, which might also be associated with an inflammatory phenotype in the liver. It is known that the nutritional intake of omega-3 polyunsaturated fatty acids (n-3 PUFA) is inversely correlated to the severity and occurrence of IBD. In order to investigate whether n-3 PUFA can also reduce liver inflammation and oxidative liver damage due to colon inflammation, we explored the dextran sulfate sodium (DSS)-induced colitis model in wild-type and *fat-1* mice with endogenously increased n-3 PUFA tissue content. Besides confirming previous data of alleviated DSS-induced colitis in the *fat-1* mouse model, the increase of n-3 PUFA also resulted in a significant reduction of liver inflammation and oxidative damage in colitis-affected *fat-1* mice as compared to wild-type littermates. This was accompanied by a remarkable increase of established inflammation-dampening n-3 PUFA oxylipins, namely docosahexaenoic acid-derived 19,20-epoxydocosapentaenoic acid and eicosapentaenoic acid-derived 15-hydroxyeicosapentaenoic acid and 17,18-epoxyeicosatetraenoic acid. Taken together, these observations demonstrate a strong inverse correlation between the anti-inflammatory lipidome derived from n-3 PUFA and the colitis-triggered inflammatory changes in the liver by reducing oxidative liver stress.

1. Introduction

Chronic inflammation is a worldwide problem often related to the particular nutritional pattern prevalent in the Western world [1]. The inflammatory bowel diseases (IBDs), comprised of Crohn's disease (CD) and ulcerative colitis (UC) are characterized by an immune-mediated gut dysfunction, hypothesized to be connected to the nutritional pattern of the Western world [1]. The IBDs are chronic relapsing inflammatory diseases of colon and rectum in UC or the entire intestinal tract in CD, and can have also systemic manifestations e.g. in skin and joints. They can thus affect multiple organ systems, and treatment often involves systemic immune suppression with glucocorticosteroids or TNF antagonists, or other immune-modulating drugs. Interestingly, this general chronic inflammatory phenotype might also be linked to an inflammatory phenotype in the liver: A meta-analysis provided data that roughly a third of the IBD patients suffer also from nonalcoholic fatty liver disease and steatohepatitis (NAFLD/NASH) [2], with a 4-fold higher prevalence of NASH in IBD patients as compared to the general population [3]. In addition, a recent study has shown increased liver

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https://doi.org/10.1016/j.redox.2023.102803
Received 19 May 2023; Received in revised form 22 June 2023; Accepted 26 June 2023
Available online 26 June 2023

Abbreviations

AA	arachidonic acid
CD	Crohn's disease
CYP	cytochrome P450
DAI	disease activity index
DHA	docosahexaenoic acid
DSS	dextran sulfate sodium
EDP	epoxydocosapentaenoic acid
EEQ	epoxyeicosatetraenoic acid
EET	epoxyeicosatrienoic acid
EPA	eicosapentaenoic acid
GC	gas chromatography
HDHA	hydroxydocosahexaenoic acid
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
IBD	inflammatory bowel disease
IL-1β	interleukin 1 beta
IL-6	interleukin 6
LOX	lipoxygenase
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
n-3/6	omega-3/6
PUFA	polyunsaturated fatty acid
SFA	saturated fatty acid
TNF-α	tumor necrosis factor alpha
UC	ulcerative colitis
WT	wild-type

fibrosis in patients with CD [4]. A leaky gut syndrome as well as pro-inflammatory cytokines might be the molecular basis of IBD-associated liver damage [5–7]. Indeed, a recent study in mice using the dextran sulfate sodium (DSS)-induced colitis model and a high-fat diet has shown that even low-grade inflammation in the colon, probably in part also by changes in the gut microbiome, could contribute to a liver inflammatory phenotype and fibrosis [8]. In this context, age-related issues such as low-grade inflammation, changes in the microbiome and subsequent changes in gut mucosal integrity are probably converging [6]. Moreover, a recent study in mice was able to demonstrate that the combination of steatosis and even mild colitis increased risk of liver tumorigenesis substantially [9].

It is known, that the nutritional intake of long-chain omega-3 polyunsaturated fatty acids (n-3 PUFA) is inversely correlated to the severity and occurrence of IBD [10,11]. A wealth of animal studies has clearly shown inflammation-dampening effects of n-3 PUFA or their oxylipins in colitis models [12–19]. While there is also positive evidence from intervention studies in humans [20,21], several large studies have not found clinical benefit in either CD or UC, so that current Cochrane reviews do not support a role for n-3 PUFA in treatment or remission maintenance of either CD or UC [22–25]. It has been suggested that n-3 PUFA supplementation supports diversification of the gut microbiota composition and thus has an anti-aging effect at the level of the microbiome [26]. A previous study has also shown effects on protective short-chain fatty acid formation due to n-3 PUFA supplementation [27]. Furthermore, there are epidemiological data indicating that high n-3 PUFA intake protects from the development of hepatocellular carcinoma in humans [28–31].

Since oral application of PUFA is difficult in mice, due to low resistance of these fatty acids towards spontaneous oxidation by air oxygen, we used the established *fat-1* mouse model. The *fat-1* mouse is a transgenic animal model able to produce n-3 PUFA endogenously due to introduction of an ω-3 FA desaturase-1 (*fat-1*) [32]. It has previously been shown, by us and others, that expression of the *fat-1* gene and subsequent increase of endogenous n-3 PUFA protects mice from inflammation as well as tumorigenesis in the colon [33–38] and the liver [39–47].

On this background, we investigated whether DSS-colitis in mice with endogenously increased n-3 PUFA tissue content would lead to less severe liver inflammatory changes than in wild-type mice, and whether this could be due to changes in the lipidome and subsequently reduced oxidative liver damage.

2. Material and methods

2.1. Animals

C57BL/6J wild-type (WT) and heterozygous C57BL/6J wt/*fat-1* (*fat-1*) mice were maintained under standard conditions in a specific pathogen-free environment at 12 h day-night cycles according to the FELASA recommendation with food and water *ad libitum*. Mice were genotyped by PCR as described in Gu et al. [48]. Experiments were approved by the state animal care committee (Landesamt für Arbeitsschutz, Verbraucherschutz und Gesundheit, Teltow (Brandenburg), Germany).

2.2. DSS-colitis model

Female 8-week-old mice were divided randomly into homogeneous groups according to their weight and age and fed a corn oil-based diet (ssniff Spezialdiäten GmbH, Soest, Germany) for four weeks before and during experimental colitis. The corn oil based-diet contained 15% protein, 60% carbohydrates, and 25% fat (10% (w/w) corn oil) of energy. For induction of colitis, mice received 2.5% (w/v) DSS (molecular weight = 36.000–50.000; MP Biomedicals, Eschwege, Germany) in the drinking water *ad libidum* for 7 days followed by one day of normal drinking water. On the 8th day mice were sacrificed, the colon was prepared, colon length was determined and tissues were snap frozen. For histology, colons and livers were fixed in 10% neutral buffered formalin and paraffin-embedded. In order to assess colitis activity, a scoring system was applied [49].

2.3. Analysis of blood parameters

Blood concentrations of β-hydroxybutyrate as well as blood glucose levels were measured using a ketone and glucose meter. Plasma cholesterol was analyzed enzymatically as described [50] and plasma triglycerides were determined by the CHOD-PAP method, both by using the Cobas Mira auto-analyzer (Roche, Mannheim, Germany).

2.4. Fatty acid analysis

Fatty acid composition analysis was performed in tail biopsies by gas chromatography (GC) according to established protocols for transesterification, extraction of fatty acids, and GC [51–53].

2.5. Oxylipin analysis

Oxylipins were extracted from homogenized mouse tissue samples as described [54]. In brief, 15–50 mg tissue was homogenized in methanol in a ball mill, and the non-esterified oxylipins were extracted by solid-phase extraction as described [55,56]. The oxylipins were quantified by liquid chromatography-electrospray tandem mass spectrometry in selected reaction monitoring by external calibration using isotope-labeled oxylipins as internal standards [55–57].

Table 1
Primer sequences used for real-time transcription polymerase chain reaction.

Target	Primer sequences
Il1β	forward: 5′-CAA CCA ACA AGT GAT ATT CTC CAT G-3′ reverse: 5′-GAT CCA CAC TCT CCA GCT GCA-3′
Il6	forward: 5′-TGA GAA AAG AGT TGT GCA ATG GC-3′ reverse: 5′-GCA TCC ATC ATT TCT TTG TAT CTC TGG-3′
Tnfa	forward: 5′-CCA TTC CTG AGT TCT GCA AAG G-3′ reverse: 5′-AGG TAG GAA GGC CTG AGA TCT TAT C-3′

2.6. Histological and immunohistological evaluation of colitis severity and liver histology

Paraffin-embedded liver sections were stained with hematoxylin and eosin to evaluate status of liver damage using a NAFLD activity score including steatosis and inflammatory alterations [58]. For histopathological evaluation of colitis, intestinal paraffin-embedded sections were stained with hematoxylin and eosin and mucosal inflammation was graded as described [59]. All histological analyses were performed in a blinded manner. Immunohistochemical analysis for protein carbonyls and 3-nitrotyrosine was conducted on formalin-fixed paraffin-embedded tissues as described [60].

2.7. RNA extraction and quantitative PCR analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription into cDNA was performed with the iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, München, Germany). Quantitative real-time PCR analysis was conducted using the SsoFast EvaGreen Supermix (Bio-Rad Laboratories). Primer sequences are listed in Table 1.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (San Diego, California, USA). Statistical significance was determined by two-tailed Student's *t*-test for unpaired observations. Differences were considered statistically significant at $p < 0.05$. Data are expressed as individual values ± SEM.

3. Results

3.1. Baseline characterization

Healthy wild-type and *fat-1* mice were comparable with regard to their metabolic characteristics as demonstrated in Fig. 1A–C. Over a

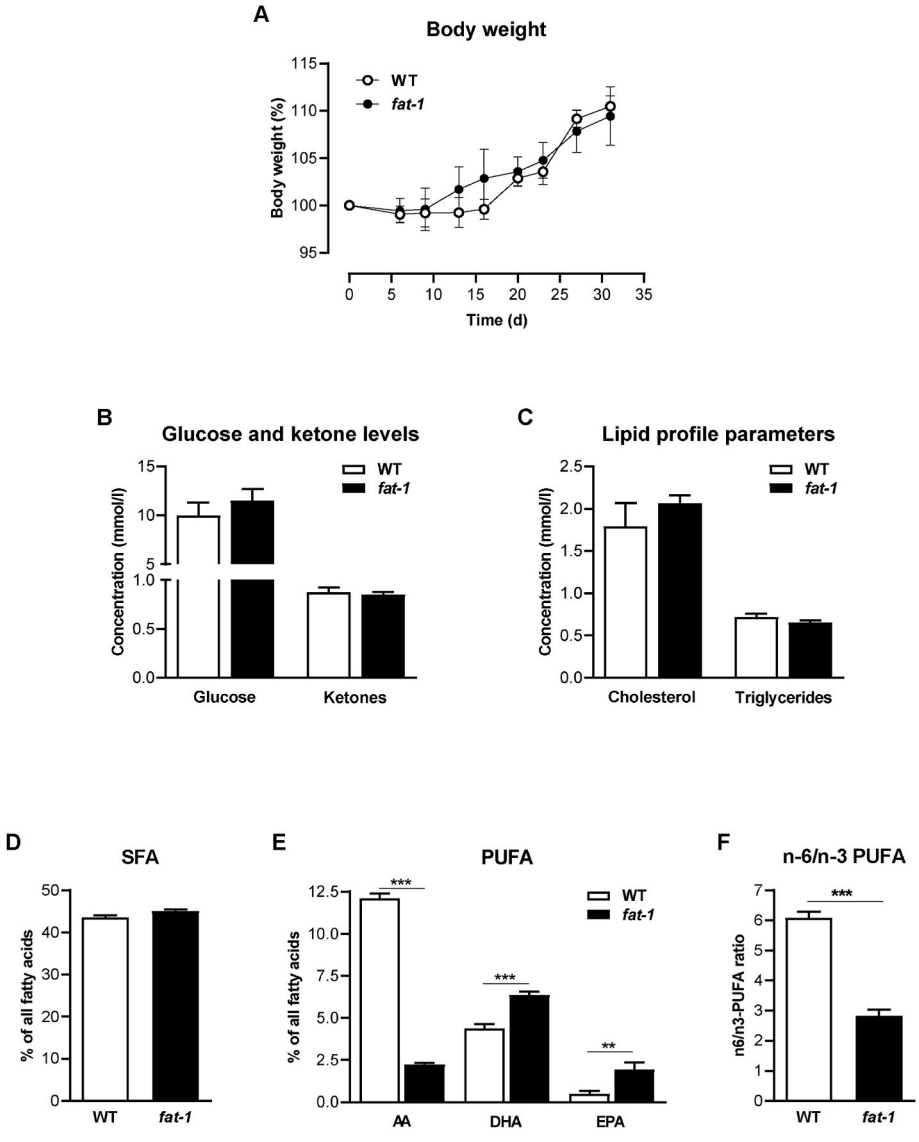

Fig. 1. Metabolic characterization and fatty acid profile of wild-type (WT) and *fat-1* mice. (A) Body weight change from 100% baseline over 28 days in WT and *fat-1* mice ($n = 4$/group) fed with a corn oil-based diet. **(B)** Blood glucose and β-hydroxybutyrate concentration, **(C)** plasma triglyceride and cholesterol levels in WT and *fat-1* mice ($n = 4$/group) fed with a corn oil-based diet for 4 weeks. Relative amounts of **(D)** saturated fatty acids (SFA) and **(E)** polyunsaturated fatty acids (PUFA) arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in tail biopsies of WT and *fat-1* mice ($n = 7$/group) after four weeks on a corn oil-based diet. **(F)** n-6/n-3 PUFA ratio in WT and *fat-1* mice ($n = 7$/group). The ratio was calculated as % (C18:2 n6, C18:3 n6, C20:3 n6, C20:4 n6, C22:4 n6)/% (C18:3 n3, C20:5 n3, C22:5 n3, C22:6 n3). Data are represented as mean ± SEM. Statistical significance was assessed by unpaired Student's *t*-test (**P < 0.01, ***$P < 0.001$).

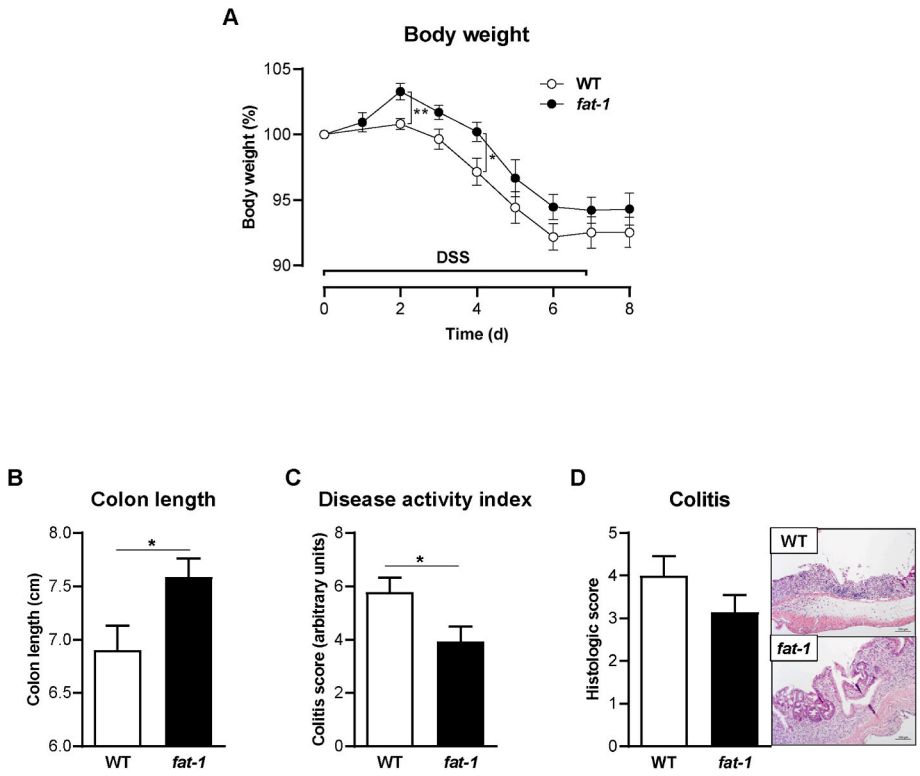

Fig. 2. Body weight change and hallmarks of colitis severity in wild-type (WT) and *fat-1* mice with DSS-induced colitis. (A) Body weight change of WT and *fat-1* mice ($n = 7$/group) treated with DSS for 7 days and fed with a corn oil-based diet 28 days prior and during DSS colitis. Weight change of individual mice was determined in relation to the initial body weight at day 0. **(B)** Colon length (average colon length in untreated mice without colitis 7.6 cm), **(C)** disease activity index (DAI, below 0.5 in untreated mice), **(D)** histologic scores (below 0.5 in untreated mice) and representative images (magnification x100) from hematoxylin/eosin-stained colon sections of WT and *fat-1* mice ($n = 7$/group) treated with DSS for 7 days and fed with a corn oil-based diet 28 days prior and during DSS colitis. Data are represented as mean ± SEM. Statistical significance was assessed by unpaired Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

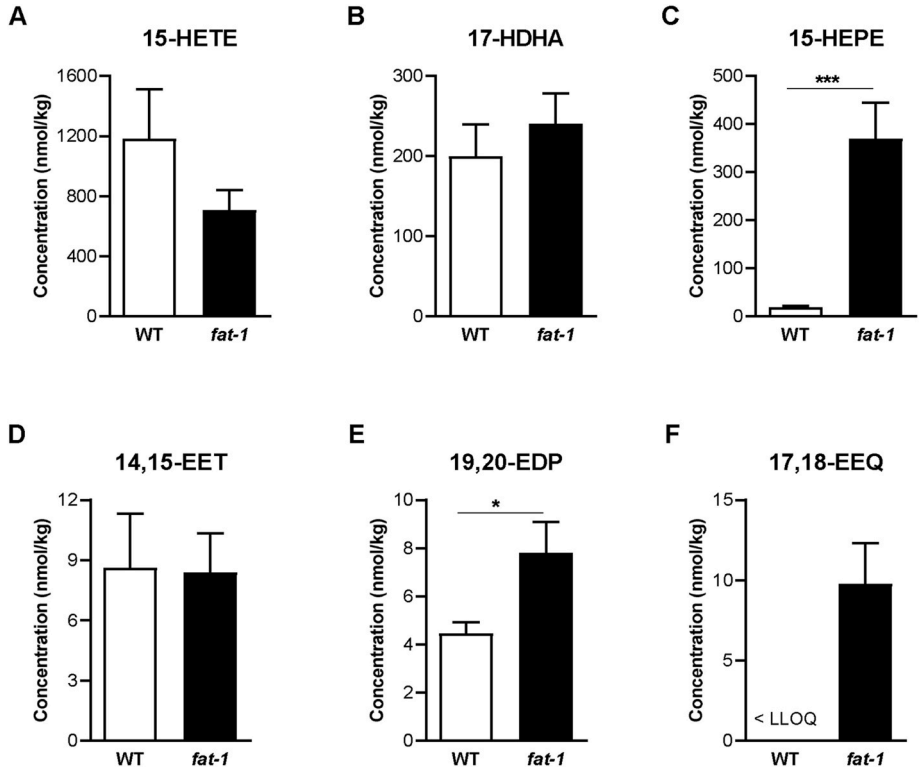

Fig. 3. Comparison of colonic 15-lipoxygenase (LOX)- and cytochrome P450 (CYP)-derived oxylipins in wild-type (WT) and *fat-1* mice with DSS-induced colitis. Shown are the concentrations of selected non-esterified 15-LOX monohydroxy fatty acids derived from AA **(A)**, DHA **(B)** and EPA **(C)** and CYP epoxy metabolites derived from AA **(D)**, DHA **(E)** and EPA **(F)** in colon tissues of WT and *fat-1* mice ($n = 7$/group) treated with DSS for 7 days and fed with a corn oil-based diet 28 days prior and during DSS colitis. The lower limit of quantification (LLOQ) for 17,18-EEQ was 1.25 nmol/kg tissue. Data are represented as mean ± SEM. Statistical significance was assessed by unpaired Student's t-test (*P < 0.05, ***P < 0.001).

HETE, hydroxyeicosatetraenoic acid; HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; EET, epoxyeicosatrienoic acid; EDP, epoxydocosapentaenoic acid; EEQ, epoxyeicosatetraenoic acid; LLOQ, lower limit of quantification.

period of 28 days, weight increased in both animal groups (Fig. 1A), but no significant differences were observed. Blood glucose, blood ketones, triglycerides, cholesterol (Fig. 1B + C), and total colon length showed no significant differences (data not shown, average 7.6 cm in both groups). While the relative amount of saturated fatty acids (Fig. 1D) was similar in tail tissues from both groups, n-6 PUFA arachidonic acid (AA) was significantly lower and n-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were significantly higher in *fat-1* mice (Fig. 1E). This resulted in a dramatic shift of the n-6/n-3 PUFA ratio in *fat-1* mice (Fig. 1F).

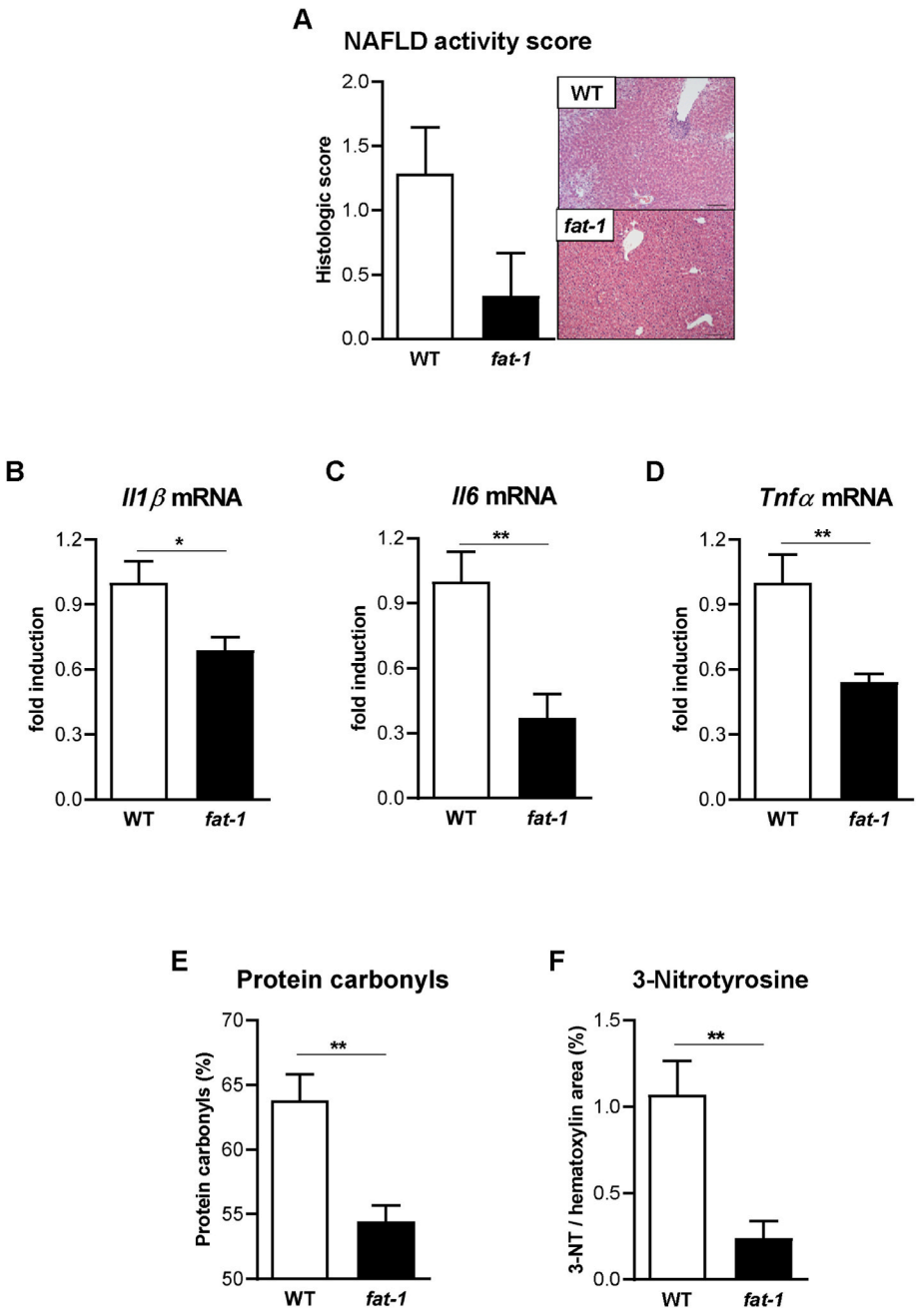

Fig. 4. Effect of DSS-induced colitis on inflammation and oxidative damage in livers of wild-type (WT) and *fat-1* mice. Mice were treated with DSS for 7 days and fed a corn oil-based diet 28 days prior and during DSS colitis. **(A)** Histologic NAFLD activity scores and representative images (magnification x100) from liver sections of WT and *fat-1* mice ($n = 7$/group). Relative mRNA expression of inflammatory marker genes **(B)** *Il1β*, **(C)** *Il6* and **(D)** *Tnfα* in liver tissues of WT and *fat-1* mice ($n = 7$ biological replicates with technical duplicates). **(E)** Relative amount of protein carbonyls in liver tissues of WT and *fat-1* mice ($n = 7$/group) analyzed by immunofluorescence. **(F)** Assessment of 3-Nitrotyrosine immunohistochemistry in liver tissues of WT and *fat-1* mice ($n = 7$/group). Data are represented as mean ± SEM. Statistical significance was assessed by unpaired Student's *t*-test ($^*P < 0.05$, $^{**}P < 0.01$).

3.2. Colitis induction and activity

We used the well-established DSS-model to induce colitis in both mouse groups. During the 7-day DSS treatment, both mouse strains lost body weight (Fig. 2A), indicating an active inflammatory process. Consistent with this, DSS treatment resulted in colon shortening (Fig. 2B) as well as an increased disease activity index (Fig. 2C) and histological colitis score (Fig. 2D). As described by us previously, all of these changes were significantly less pronounced in *fat-1* mice when compared to the wild-type animals [16,34].

3.3. Formation of anti-inflammatory oxylipins

PUFA-derived oxylipins in the colon tissue of colitis-affected mice were quantified. When analyzing 15-LOX products, we found a trend that AA-derived 15-hydroxyeicosatetraenoic acid (15-HETE) was lower in *fat-1* mice as compared to wild-type mice, while DHA-derived 17-hydroxydocosahexaenoic (17-HDHA) seemed to be elevated in *fat-1* mice, however, both changes were not significant (Fig. 3A+B). In contrast, EPA-derived 15-hydroxyeicosapentaenoic acid (15-HEPE) was significantly increased in *fat-1* mice compared to wild-type animals (Fig. 3C). With regard to CYP epoxy eicosanoids, we found no significant differences for AA-derived 14,15-epoxyeicosatrienoic acid (14,15-EET), while DHA-derived 19,20-epoxydocosapentaenoic acid (19,20-EDP) and EPA-derived 17,18-epoxyeicosatetraenoic acid (17,18-EEQ) was significantly higher in *fat-1* mice compared to wild-type animals (Fig. 3D–F). These findings demonstrate a relatively higher formation of EPA-derived oxylipins in *fat-1* mice in inflamed colons, that have established inflammation-dampening effects.

3.4. Liver damage in mice with DSS-induced colitis

Finally, we examined parameters of liver inflammation and damage caused by DSS-induced colitis. Interestingly, the NAFLD activity score was lower (Fig. 4A) and the expression of inflammatory cytokines was significantly reduced in *fat-1* mice compared to wild-type mice (Fig. 4B–D), indicating that the inflammatory response is less active in the livers of *fat-1* mice. To investigate whether this reduced liver inflammation in *fat-1* mice was also accompanied by a reduction of liver damage, we examined the livers of both mouse strains for oxidative damage. As shown by staining for protein carbonyls and 3-nitrotyrosine, we observed significantly less oxidative liver damage in *fat-1* mice compared to wild-type mice (Fig. 4E + F).

4. Discussion

The interplay between gut microbiome, low-grade inflammation in the gut, and metabolic diseases has been identified as important issue in the development of metabolic disease, of which nonalcoholic steatohepatitis and subsequent chronic liver damage is an important hallmark [6,61]. In this context, similar mechanisms of disease are considered for pathogenesis of IBD as well as chronic metabolic diseases and metabolic liver inflammation [62]. Dietary interventions with the aim to reduce the inflammatory environment might be a preventive option. One possibility is increased intake of n-3 PUFA to not only reduce local, intestinal inflammatory reactions but also to lower the whole-body inflammatory response. Several mechanisms for the action of n-3 PUFA are discussed, including the direct action of the fatty acids, the production of n-3 PUFA-derived inflammation-dampening oxylipins by action of PUFA-metabolizing enzymes, or the favorable modulation of the gut microbiota.

In this study, we assessed, for the first time, whether the established colitis-dampening effect of endogenously increased n-3 PUFA in the colons of *fat-1* mice can also affect liver inflammation and oxidative stress in colitis-affected animals. Indeed, besides confirming previous data of alleviated DSS-induced colitis in *fat-1* mice, we also found significantly lower liver inflammation and oxidative damage in *fat-1* animals with colitis as compared to wild-type littermates. The sum of %EPA + %DHA, a marker which is used to describe the endogenous n-3 PUFA status in humans (omega-3 index), was 8.3 ± 0.6% in the *fat-1* mice analyzed in the present study (data not shown). These values are comparable to the omega-3 index observed in healthy volunteers following EPA/DHA supplementation (0.46–1.6 g/day EPA and 0.38–1.1 g/day DHA for up to 12 weeks), which ranged from 8.4 to 11% [63–65]. This was accompanied by highly notable increases of established inflammation-dampening n-3 PUFA-derived oxylipins in *fat-1* colons, especially from EPA such as 15-HEPE and 17,18-EEQ, as previously shown by us in both healthy and colitis-affected mice [16,54]. Particularly, the epoxy compounds 19,20-EDP and 17,18-EEQ are established mediators to alleviate liver inflammation [41]. While we cannot show that these oxylipins are directly responsible for the lower inflammatory phenotype in the liver, we speculate that these oxylipin mediators have systemic effects to dampen concomitant liver inflammation and oxidative damage in the *fat-1* mice with colitis, leading to a healthier phenotype after DSS-induced colitis compared to control mice and fewer signs of oxidative damage. Given that chronic low-grade inflammation is a hallmark of pathogenesis in many western diseases ranging from colon cancer to atherosclerosis, these findings of systemic inflammatory reduction probably due to n-3 PUFA-derived inflammation-dampening oxylipins are important to develop future dietary treatment strategies. We propose that the anti-inflammatory lipidome derived from n-3 PUFA may contribute to the prevention of colitis-triggered inflammatory changes in the liver by reducing oxidative liver damage.

Declaration of competing interest

All authors declare that there are no conflicts of interest to be disclosed.

Data availability

Data will be made available on request.

Acknowledgements

This study was supported by research grants of the German Research Foundation (DFG) to TG (GR1240/20-1) and KHW (WE2908/13-1). The study was also supported by the Ministry of Science, Research and Culture of the State of Brandenburg. Publication costs were partially funded by the German Research Foundation – grant 491394008.

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