BBA - Molecular Basis of Disease 1865 (2019) 298–307

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

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

Effect of alcohol on the interleukin 6-mediated inflammatory response in a new mouse model of acute-on-chronic liver injury

Ersin Karatayli[a,*], Rabea A. Hall[a], Susanne N. Weber[a], Steven Dooley[b], Frank Lammert[a]

[a] Department of Medicine II, Saarland University Medical Center, Saarland University, Homburg, Germany
[b] Department of Medicine II, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

ARTICLE INFO

Keywords:
Acute-on-chronic liver failure (ACLF)
Abcb4 knock-out mouse
Inflammation
liver fibrosis
alcoholic liver disease (ALD)

ABSTRACT

Background and Aims: ACLF is usually associated with a precipitant in the setting of a chronically damaged liver. We aim to combine a mouse model with a pre-injured liver (Abcb4/Mdr2$^{-/-}$) with a recently standardized ethanol feeding model to dissect alcohol-related inflammatory responses in this model.

Method: Ten (n = 64) and 15 (n = 64) week old wild-type (WT) C57BL/6 J and Abcb4$^{-/-}$ knock-out (KO) mice were either fed control (WT/Cont and KO/Cont groups) or liquid ethanol diet (5% v/v) followed by an ethanol binge (4 mg/kg) (WT/EtOH and KO/EtOH groups). Hepatic mRNA levels of IL6, IFN-G, IL-1B, TGFB1, TNF-A, CCL2, HGF, CRP, RANTES, PNPLA3 and COL3A1 were evaluated using the $2^{-\Delta\Delta Ct}$ method. IL6 and HGF plasma levels were quantified by ELISA.

Results: Older mice in KO/EtOH group displayed higher IL6 expressions compared to KO/Cont, WT/EtOH and WT/Cont groups of the same age, whereas HGF did not differ. Significant over-expression of CCL2 also corresponded to the same group. Males in KO/EtOH group exhibited higher IL6 expression than females. Lipid droplets were observed in about 80% of mice challenged with ethanol. There was a profound downregulation in PNPLA3 and RANTES levels after ethanol exposure. Mean size of the LDs was inversely correlated with hepatic PNPLA3 levels.

Conclusion: We propose a novel promising approach to model alcohol-related *ACLI*. Acute inflammatory IL6-driven response might help transition from a stable chronic state to a progressive liver damage in Abcb4$^{-/-}$ mice. Repression of PNPLA3 resulted in a notable expansion in size of lipid droplets, indicating lipid remodeling in this model.

1. Introduction

Alcoholic liver disease (ALD), as the name implies, is generally regarded as the liver injury resulting from alcohol abuse. Alcohol consumption is globally common and thus, alcohol is one of the major causes of liver diseases worldwide with a wide spectrum of liver related disorders ranging from fatty liver to severe types of liver injury such as cirrhosis, liver failure, and hepatocellular carcinoma [1,2]. These diseases are chronic and characterized by persistent hepatic inflammation with complex pathophysiological mechanisms. An acute hepatic insult in this setting, on the other hand, may result in the aggravation of pre-existing liver injury. The clinical outcome of such an acute insult may either be the deterioration of liver function, which further worsens the pathobiochemical and histopathological abnormalities as in the case of acute-on-chronic liver injury (*ACLI*) or the failure of one or more organs with a high short and medium term mortality rate of 50–90%, which is the case in acute-on-chronic liver failure (ACLF) [3,4]. Liver injury in these acute-on-chronic complications is usually associated with underlying precipitating events, with bacterial infections and/or acute alcohol excess being the most frequent precipitants [5]. In a recent study, severe systemic inflammatory response (SIRS) has been suggested to be associated with the rapid progression of liver disease from compensated cirrhosis to ACLF, in which different cytokine profiles have been identified depending on the precipitant [6]. SIRS was found to be more frequently present in patients with active alcoholism/acute alcoholic hepatitis in this study, implying the significance of alcohol as the trigger event of excessive inflammatory responses. Other studies in patients with acute deterioration of alcoholic cirrhosis also confirmed the presence of SIRS in these patients [7,8]. Despite the evidence of a dysregulated immune response playing a key role in the development of the disease, alcohol related *ACLI* and/or ACLF have not been described and characterized *in vivo* so far. Even though the underlying mechanism is

* Corresponding author.
E-mail address: Ersin.Karatayli@uks.eu (E. Karatayli).

https://doi.org/10.1016/j.bbadis.2018.11.008
Received 10 April 2018; Received in revised form 9 November 2018; Accepted 13 November 2018
Available online 15 November 2018

still unknown, not only alcohol consumption itself but also the drinking pattern of an individual has been documented to profoundly affect alcoholic liver injury [9,10].

The recently developed chronic-plus-binge alcohol feeding model (NIAAA mouse model) by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) mimics the drinking pattern of alcoholic liver disease patients who have a background of chronic drinking for years and a recent history of excessive alcohol intake (binge) [11,12]. This chronic-plus-binge model was shown to induce hepatic steatosis, liver injury and inflammation in the settings of elevated blood alcohol concentrations in C57BL/6 inbred mice, which is an alcohol-preferring strain and therefore the preferred strain for *ad libitum* ethanol challenge. *Abcb4* (or *Mdr2*) knock-out mice (*Abcb4*$^{-/-}$) on the other hand, which lack the hepatobiliary phosphatidylcholine translocator, have been shown to develop chronic liver inflammation and progressive hepatic fibrosis, eventually followed by hepatocellular carcinoma (HCC) at later age [13–15]. ABCB4 is the ATP-dependent phosphatidylcholine (PC) translocating protein that flops PC across the canalicular membrane of hepatocytes. PC is an essential component of forming vesicles to reduce the toxic activity of bile acids, the absence of which contributes to the pathogenesis of liver injury in *Abcb4*$^{-/-}$ mice. During the past years, the knockout has been transferred from the original FVB/NJ to the C57BL6/J background and has been extensively characterized in our group to allow comparison with the alcohol-preferring C57BL/6 inbred line mice as wild-type controls.

The current study, therefore, aims to adopt/combine the NIAAA chronic-plus binge ethanol feeding model with the *Abcb4*$^{-/-}$ model in order to evaluate the deleterious effects of alcohol-induced mechanisms in the setting of pre-injured liver and dissect alcohol-related inflammatory responses in this novel pre-clinical model.

2. Materials and methods

2.1. Mouse model

A total of 128 mice were included in the study. Ten and 15 week old C57BL/6 J wild-type mice (n = 32 for each age) and *Abcb4*$^{-/-}$ mice (n = 32 for each age) were housed under environmentally controlled conditions (temperature, humidity 12 h/12 h dark/light cycle) and were divided in two groups. Equal numbers of mice from each gender are included in the study (8 males, 8 females) in each group. Mice were weighed daily (Supplementary Figs. 1 and 2). Diets and sample processing was adapted from the previously established ethanol feeding model (NIAAA model) [11]. Briefly, all mice were first fed with an *ad libitum* liquid diet for acclimatization to tube feeding with control liquid diet (Rodent liquid diet, Lieber-Decarli´82, Bio-Serv, Frenchtown, NJ) for a period of five days. At the end of 5th day, mice were either fed control liquid diet (WT/Cont and KO/Cont groups) for a period of 10 days followed by isocaloric maltose dextrin gavage (9 g/kg body weight) or Lieber-De Carli ethanol liquid diet (Rodent liquid diet, Lieber-Decarli´82-Ethanol, Bio-Serv, Frenchtown, NJ, 4% v/v) followed by an acute ethanol binge (4 mg/kg body weight, WT/EtOH and KO/EtOH groups). Mice were sacrificed 7–9 h after gavage. Plasma samples were collected from blood taken from the inferior vena cava and liver tissue samples were frozen in liquid nitrogen and stored at −80 °C for further use. All experimental protocols were carried out in line with the relevant welfare regulations and the Animal Care and Use Committee for Saarland University and were approved by Saarland University Animal Ethics Committee (TV 44/2015).

2.2. Hepatic gene expression and relative quantification analyses

Total RNA was extracted from snap-frozen liver tissue samples by RNeasy Mini Kit (Qiagen, Hilden, Germany). The High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) was used for cDNA transcription of 1 µg of extracted RNA. Interleukin 6 (*Il-6*; Mm00446191_m1), C-reactive protein (*Crp*; Mm00432680_g1), Collagen 3α1 (*Col3a1*;Mm01254476_m1), Hepatocyte Growth Factor (*Hgf*, Mm01135184_m1), Regulated upon activation, normal T cell expressed and secreted (*Rantes*, Mm013002427_m1), Patatin-like phospholipase domain-containing protein 3 (*Pnpla3*, Mm00504420_m1), Monocyte chemo-attractant protein 1 (*Mcp1/Ccl2*, Mm00441242_m1), Interferon-γ (*Ifng*, Mm01168134_m1), Interleukin-1β (*Il1b*, Mm00434228-m1), Transforming growth factor-β1 (*Tgfb1*, Mm01178820_m1), Tumor necrosis factor-α (*Tnfa*, Mm00443258_m1) and Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, Mm99999915_g1) pre-designed Taqman gene expression assays (Applied Biosystems/Thermo Scientific, Foster city, CA, USA) were carried out on the Taqman 7500 Fast Real-Time PCR System. ΔΔCt algorithm was used to analyze the relative changes in expressions, where *Gapdh* served as endogenous control.

2.3. Determination of plasma biochemical markers

The plasma samples were diluted with 0.9% (v/v) NaCl and levels of alanine aminotransferase ALT, aspartate aminotransferase AST, and alkaline phosphatase AP (Roche Cobas 8000 c702 modular analyzer) as well as ethanol (EtOH) (Roche Cobas 8000 c502 modular analyzer) were determined in the central laboratory of Saarland University Medical Center.

2.4. Characterization of liver fibrosis and inflammation

Collagen accumulation in liver was evaluated by relative quantification of *Col3A1* (see above), Sirius Red histochemistry and the collagen specific amino acid hydroxyproline (Hyp) content. For histopathological analysis, 5 µm thick formalin-fixed (4%, v/v), paraffin-embedded liver sections were stained by Sirius Red [16]. Liver fibrosis was then quantified using a histomorphometric semi-automatic system for image analysis (Leica microscope, equipped with Leica application suite software; Wetzlar, Germany). The percentage of collagenous area was calculated from five randomly chosen microscopic fields in each liver section. Hyp was measured photometrically from liver hydrolysates with a protocol adapted from Jamall et al. [17].

For assessment of inflammation, hepatic *Crp* levels were relatively quantified as described above and 5 µm formalin-fixed (4%, v/v), paraffin-embedded liver sections were stained with hematoxylin-eosin (H&E) [16]. H&E stained sections were also used to measure the diameters of lipid droplets by light microscopy. Briefly, the average diameter of lipid droplets for each sample was calculated from 20 to 30 lipid droplets randomly chosen in H&E stained microscopic sections with the help of Leica application suite software.

2.5. Determination of plasma IL-6 and HGF levels

A subset of mice (n = 4–5 for each group) was used to assay the IL-6 and HGF levels in plasma by ELISA. Plasma HGF levels were determined with the Mouse/Rat HGF Quantikine ELISA kit (R&D Systems, Cat # MHG00), and plasma IL-6 levels were measured with the mouse IL-6 Quantikine ELISA Kit (R&D Systems, Cat # M6000B).

2.6. Immunohistochemistry (IHC)

Inflammatory infiltrates were identified by IHC staining of paraffin-embedded liver tissue sections for CD45 (Biolegend), CD4 (Abcam), CD11b (Abcam) and F4/80 (Abcam). In brief, 3 µm thick paraffin slides were deparaffinised in a descending alcohol series, followed by antigen retrevial in 10 mM citrate buffer (pH 6.0) in a microwave oven (600 W, 15 min). Endogenous peroxidase was blocked by incubation for 20 min in 3% (v/v) H_2O_2 in PBS. Blocking with an avidin and biotin system (Dako) was followed by washing steps with PBS. Samples were then incubated with the primary antibody (1:100, 1:4000, 1:1000 and 1:400

dilutions in 2% (w/v) milk powder in PBS for CD45, CD4, CD11b and F4/80, respectively) over night at 4 °C (for CD45 and CD4) or 1 h at 37 °C (for CD11b and F4/80). Secondary antibody (biotinylated-goat-anti-rabbit, 1:100 diluted in 2% (w/v) milk powder in PBS) and ABC complex (Vectastain) were applied afterwards. Finally, nuclei were counter-stained with Mayer's hematoxylin.

2.7. Statistical analysis

Statistical analysis was performed by SPSS v18.0 (IBM Corp., Armonk, NY, USA). Group differences were estimated by analysis of variance (ANOVA) [18], including *post hoc* (Bonferroni) correction for multiple testing. All values are presented as mean ± standard error [19]. *p*-Values < 0.05 were regarded as significant.

3. Results

3.1. Assessment of liver fibrosis

Regardless of age, fibrosis stages ranging from none (wild-type mice) to bridging fibrosis (knock-out mice challenged with ethanol) were observed in our mice as shown by Sirius red staining of liver sections (Fig. 1A). Relative hepatic mRNA levels of *Col3a1* (Fig. 1B) and hydroxyproline contents (Fig. 1D) also demonstrated higher expressions of collagen in the knock-out compared to wild-type mice. Panel C of Fig. 1 illustrates that collagen areas (%), calculated by histological evaluation of paraffin-embedded liver sections with a semi-quantitative scoring system (fibrosis scores), were also in line with hydroxyproline contents and relative quantification of *Col3a1* expression, showing significant collagen accumulation in *Abcb4* knock-out mice. Although hydroxyproline assay and collagen area calculation revealed higher collagen levels in mice challenged with ethanol (Fig. 1C and D), this increase was not significant when compared to control diet fed counterparts.

3.2. Plasma biochemical markers and ethanol levels

Plasma ethanol levels were found to be highly increased in ethanol challenged knock-out and wild-type mice of both groups as expected ($p < 0.001$) Although not significant, higher plasma activities of ALT and AST were observed in ethanol challenged mice (Supplementary Fig. 3).

3.3. Inflammation

Hepatic CRP levels, which is an indicator of inflammation, were found to be upregulated in 15 week old ethanol challenged knock-out mice as compared to wild-type mice on control diet ($p = 0.001$) (Fig. 2B). This finding was also supported by the infiltrating inflammatory cells shown by H&E stained liver sections (Fig. 2A).

3.4. Relative expression levels of inflammatory mediators and PNPLA3

No significant difference in hepatic expression of *IL-6* was observed in 10 week-old mice, whereas the older knock-out mice fed the ethanol diet showed a significant induction in comparison to wild-type mice on control diet ($p < 0.001$) or ethanol diet ($p = 0.002$), and knock-out mice on control diet ($p = 0.003$) (Fig. 3A). In line with hepatic expression levels, IL-6 was also found to be upregulated in plasma of the same group relative to the others, as measured by ELISA (Fig. 4). On the other hand, hepatic and plasma HGF levels did not differ either in 10 or 15 week-old mice (Figs. 3B and 4B). Interestingly, higher levels *IL-6* were observed in 15 week-old male ethanol-fed mice as compared to their female counterparts ($p = 0.011$) (Fig. 5). A similar increase was also observed for *Hgf*, but without reaching significance (Fig. 5). *Rantes* levels in liver samples of the knock-out mice were upregulated in both age groups ($p = 0.033$ and $p = 0.037$, respectively), however this increase was replaced with repression of *Rantes* upon addition of ethanol to the diet in 15 week-old wild-type ($p = 0.038$) and knock-out ($p = 0.006$) mice (Fig. 3C). Similarly, a decrease in *Pnpla3* levels was observed in ethanol-challenged older mice regardless of the genotype ($p = 0.002$ for wild-type and $p = 0.044$ for knock-out mice) (Fig. 3D). Besides, a strong down-regulation of *Pnpla3* was observed in 15 week-old ethanol-challenged mice when compared with control diet-fed wild-type mice of the same age ($p < 0.001$) (Fig. 3D). Whereas ABCB4 deficiency was found to have no effect on hepatic *Tnf-α* expression, an upregulation was observed with alcohol administration both in wild-type and knock-out mice at 15 weeks of age ($p = 0.014$ and $p = 0.046$, respectively) (Fig. 3E). *Ccl2* expression was upregulated by alcohol exposure both in wild-type and knock-out 15 week-old mice. The knock-out mice also showed higher levels of *Ccl2* in comparison to wild-type mice. The induction was most significant in the 15 week-old *Abcb4*$^{-/-}$ mice challenged with ethanol ($p < 0.001$) (Fig. 3F). Hepatic *IL-1β*, *Tgf-β* and *Ifn-γ* expression did not differ (Fig. 3G–I).

3.5. Lipid droplets

Lipid droplets (LD) were observed in 80–90% of 15 week old mice challenged with ethanol regardless of whether they were pre-injured (knock-out) or not (13/16 in WT-EtOH and 14/16 in KO-EtOH group; Fig. 6A), whereas no LDs were seen in mice on control diet. In these mice, hepatic *Pnpla3* mRNA expression was significantly ($p < 0.001$) downregulated (Fig. 3D), which correlated with a notable expansion in LD size. Of note, no significant difference was found in terms of LD size between ethanol treated wild-type and knock-out mice. A non-linear regression analysis revealed an estimated correlation formula of "mean LD size(μm) = 2.70 − 3.01 ln [relative PNPLA3 mRNA level]" ($r^2 = 0.46$ and $p < 0.01$) (Fig. 6B).

4. Discussion

The details of acutely worsening mechanisms of a chronically pre-injured liver after an inflammatory insult in *ACLI* and/or ACLF patients still remain to be clarified and current knowledge on the pathophysiological progress of the disease provided by animal models has been very limited so far. Although aiming to mimic ACLF in a preclinical model with ethanol challenge as the precipitating event for better understanding of alcohol-induced acute inflammatory responses in chronically injured liver, the current study models alcohol-induced acute liver injury rather than liver failure on the background of pre-existing chronic liver disease in *Abcb4*$^{-/-}$ mice, and thus represents an "*ACLI* model". Several studies, each of which had a different methodology to develop fibrosis and to administer a precipitating acute insult, have recently been carried out to develop an *in vivo* ACLF model, such as bile duct ligation (BDL) [3], intravenous concanavalin A (ConA) injection [20], carbontetrachloride (CCl_4) [21] and heat stable antigen (HSA) [22] administration with subsequent D-gal/LPS injection and porcine serum (PS) administration [23], followed by LPS injection. Adopting the NIAAA ethanol feeding protocol [11] to *Abcb4*$^{-/-}$ mice offers a novelty, since no *ACLI*/ACLF model has so far been developed in which alcohol is used as an acute insult. An additional superiority over current models requiring laborious and time consuming protocols to create fibrosis in healthy animals is due to spontaneous development of severe fibrosis in *Abcb4*$^{-/-}$ mice at young age and short (15 day) protocol of NIAAA feeding. In this regard, the knockout genetic background of the original FVB/NJ strain has been transferred to the alcohol preferring C57BL6/J inbred strain in the past years [24]. Moreover, less stressful applications and low mortality rates in this disease model due to no need of further interventions to create liver injury make it a more appropriate model in terms of animal welfare and ethics. ABCB4-deficient mice have been studied extensively over the years, because they spontaneously develop chronic liver injury, which resembles the human

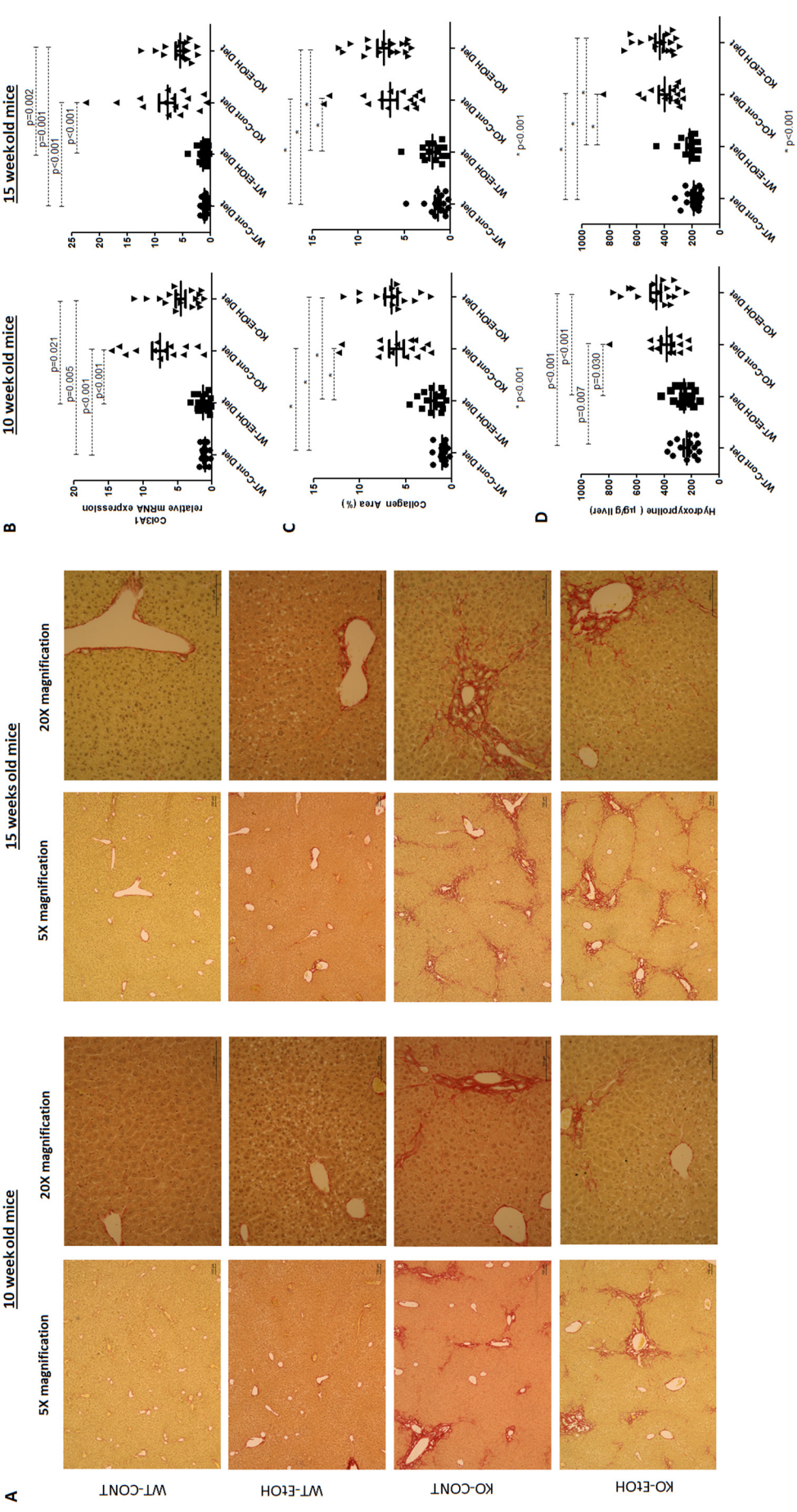

Fig. 1. Assessment of fibrosis. Representative Sirius Red stained liver sections with 5× and 20× magnification (A), hepatic relative *Col3A1* expression determined by qPCR (B), semiquantitative analysis of hepatic collagen area (C) and liver hydroxyproline content (D) in 10 and 15 week-old mice showed the accumulation of collagen in liver. Each scale bar is 100 µm. $p < 0.05$ was considered significant. CONT, Control diet; EtOH, ethanol diet; KO, *Abcb4* knock-out mice; WT, C57BL/6 J wild-type mice.

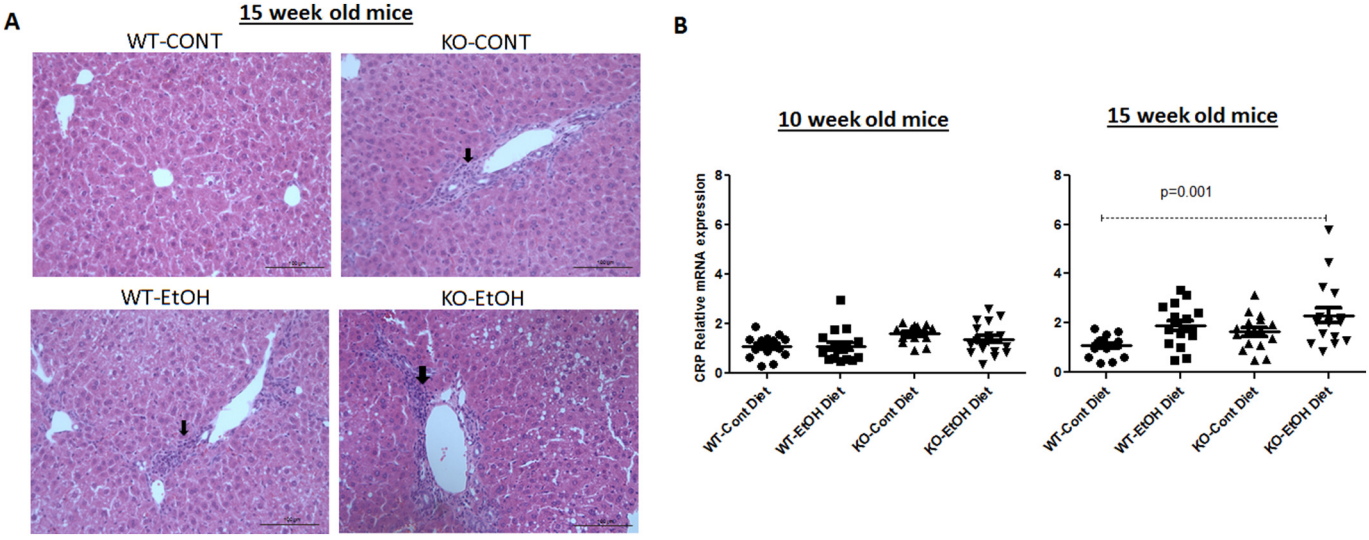

Fig. 2. Evaluation of inflammation. Inflammation reached significant levels only in 15 weeks old knock-out mice challenged with ethanol diet as evidenced by representative H&E stained liver sections (A) and relative quantification of hepatic *Crp* expression determined by qPCR (B). Black arrows show infiltrating inflammatory cells. Each scale bar is 100 µm. $p < 0.05$ was considered significant. CONT, Control diet; *Crp*, C reactive protein; EtOH, Ethanol diet; KO, *Abcb4* knock-out mice; WT, C57BL/6 J wild-type mice. Magnification 20×.

disease progressive familial intrahepatic cholestasis (type 3) with sclerosing cholangitis, liver fibrosis and lesions progressing slowly to liver cancer [13]. Moreover, this model is particularly interesting, because in addition to several studies showing the association between mutations of the orthologous human *ABCB4* gene and a wide clinical spectrum of liver disease [25–28], a recent large-scale whole-genome analysis of the Icelandic population revealed that common and rare *ABCB4* variants aggravate the progression of chronic liver diseases, which correlates well with the ABCB4-knockout model [29].

Both ABCB4 deficiency and alcohol diet resulted in increased inflammation in 15-week old mice, however, significant inflammation has been achieved only when older mice were challenged with ethanol, as presented by the increasing numbers of inflammatory cells and elevated CRP levels (Fig. 2A and B). These inflammatory infiltrates in the liver were previously shown to be predominated by portal inflammatory infiltrates composed of $Cd45^+$ neutrophils and CD11b and CD4-positive lymphocytes [15,30]. Immunohistochemistry of liver samples in our model revealed a similar profile of inflammatory infiltrates with additional $F4/80^+$ macrophages (Supplementary Fig. 4).

Progression of liver injury in *Abcb4*$^{-/-}$ mice is a dynamic process that depends on strain and time course [13,24,31,32]. The first abnormalities in *Abcb4* $^{-/-}$ mice were detected in liver at the age of 3 weeks, and the whole liver appears to be fibrotic at the age of 12 weeks [32]. Moreover, an age-dependent deregulation of pathways related to oxidative stress and proliferation, lipid metabolism, modifications of DNA methylation and a rational link between inflammation and tumorigenesis in this model have already been documented by molecular and metabolic profiling of diseased livers [19,33–35]. In this regard, the older mice (15 weeks) in our experimental settings served as a better model, since we obtained more significant results in terms of inflammation and IL-6 expression in liver, which might be due to the differences in disease progression depending on time course (Fig. 2B and 3A).

Interleukins are a class of immunomodulatory cytokines critically involved in the regulation of immune responses and thus play important roles in the pathogenesis of acute and chronic disorders, including alcoholic liver diseases [36,37]. Depending on the inflammatory stimulus, some interleukins may not only have pro- but also anti-inflammatory functions. IL-6 is among such cytokines, which exhibits two contrasting functions, *i.e.* it acts as a pro-inflammatory cytokine in models of chronic inflammatory diseases [38,39], and contrarily shows anti-inflammatory effects in acute inflammation [40]. Acute inflammation is characterized by initial infiltration of neutrophils at the site of inflammation, which are then replaced by monocytes and T cells to prevent tissue damage due to accumulation of neutrophil-secreted proteases [41,42]. Several lines of evidence indicate that IL-6 plays a crucial role in suppressing primarily neutrophil-attracting (CXCL1, CXCL8, CX3CL1) and enhancing mainly monocyte-attracting chemokines (CCL2, CCL8, CXCL5, CXCL6) [43–45]. In fact, this transition from neutrophil infiltration to monocyte recruitment has been suggested to be the major switch from acute to chronic inflammation [45]. This feature of IL-6 may be particularly relevant in the *Abcb4*$^{-/-}$ model, which provides a suitable experimental framework for acute on chronic liver injury. Therefore, IL-6 is a primary regulator of both acute and chronic inflammation. In our experimental set-up, we observed IL-6 upregulation in liver samples of 15 weeks old *Abcb4*$^{-/-}$ mice, which were challenged by ethanol (Fig. 3A). This elevation was even more prominent in plasma, reaching about 10-fold increase of IL-6 levels as compared with the 15 week old wild-type mice on control diet. In this regard, several therapeutic options for blockade of IL-6 signalling pathways are available [46]. However, neutralization of IL-6 has been shown to have protective and detrimental effects in different disease models. These results might be due to the ambivalent effects of IL-6, which may act as both pro- and anti-inflammatory cytokine depending on the stimulus [47–50]. Hence, it would be interesting to study the effects of blocking IL-6 in this preclinical model. Besides, a molecular mechanism explaining the lower HCC susceptibility in females by Naugler et al. [51], in which estrogen steroid hormones had a protective effect against HCC through inhibition of IL-6 production, was reported. Consistently, a gender bias was also observed in our model. Similar differences were also observed for inflammation as reflected by relative hepatic CRP expression ($p = 0.01$), whereas no significance was observed in terms of liver injury when collagen contents were compared between male and female knock-out mice on the ethanol diet (data not shown).

CCL2 (MCP-1) is an important chemokine proposed to be a master regulator of macrophage recruitment and activation in chronic inflammatory diseases [52]. Several lines of evidence suggest a close interaction of IL-6 and CCL2 in various disease conditions [53–57]. Relevant to our model, CCL2 was shown to be induced by IL-6 in acute inflammation [57], and IL-6 and CCL2 were shown to constitute a positive feedback loop to maintain and amplify STAT3 signalling in cancer

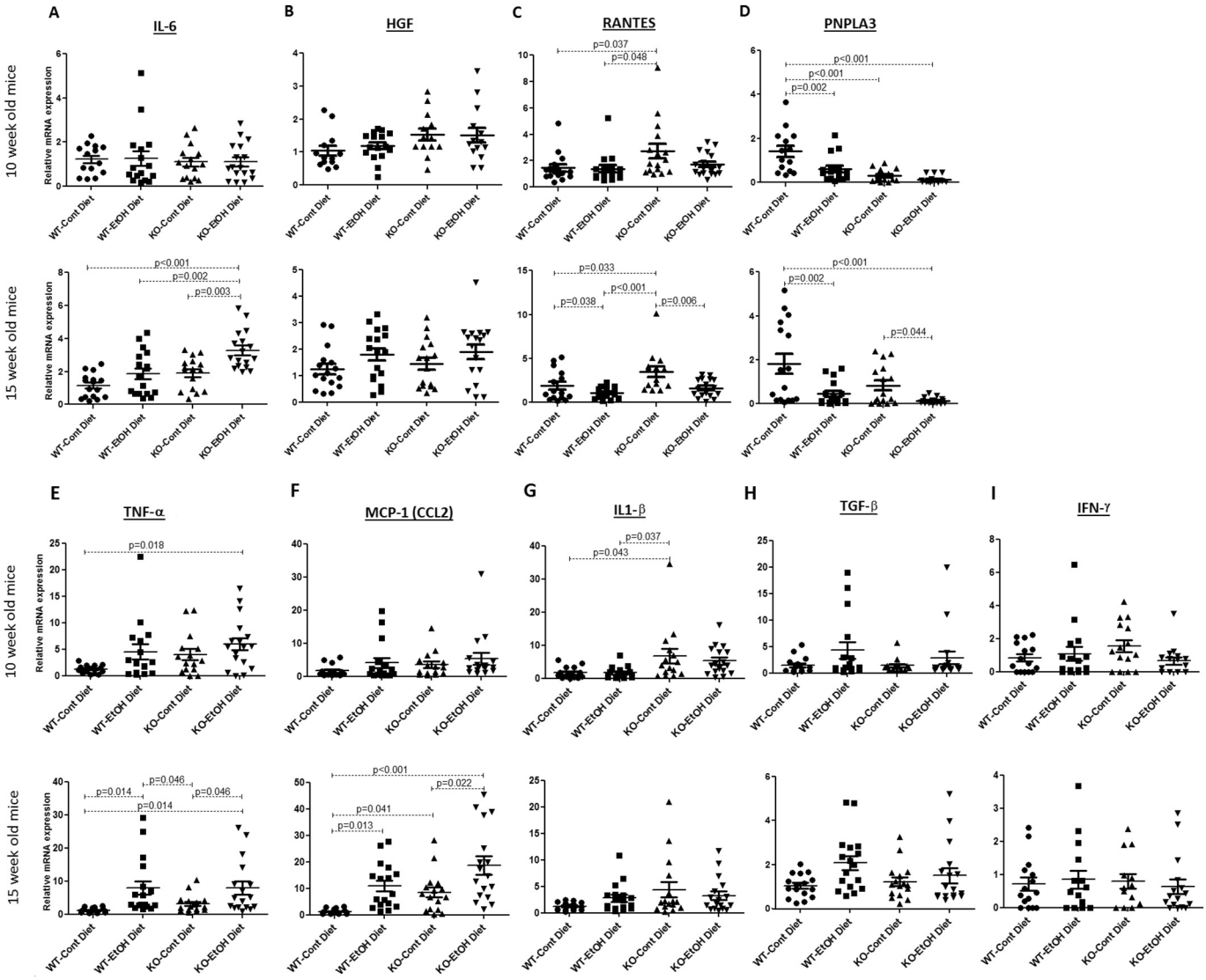

Fig. 3. Relative quantification of mRNA levels in liver. Hepatic transcriptional levels of *IL-6* (A), *Hgf* (B), *Rantes* (C), *Pnpla3* (D), *Tnf-α* (E), *Ccl2* (F), *IL-1β* (G), *Tgf-β* (H) and *Ifn*-γ (I) were determined by qPCR in 10 (upper panel) and 15 week-old mice (lower panel). $p < 0.05$ was considered significant. CONT, control diet; EtOH, ethanol diet; HGF, hepatocyte growth factor; IFN-γ, interferon-γ; IL1-β, interleukin-1β; IL-6, interleukin-6; KO, *Abcb4* knock-out mice; MCP-1 (CCL2), monocyte chemoattractant protein-1 (C-C motif chemokine ligand 2); PNPLA3, patatin-like phospholipase domain-containing 3; RANTES, regulated on activation, normal T cell expressed and secreted; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor α; WT, C57BL/6 J wild-type mice.

[53]. Furthermore, CCL2-deficient mice are protected, at least in part, from alcoholic liver injury [58]. Although not documented for liver, aging has been shown to enhance the CCL2 expression in various tissue types including vascular smooth muscle and alveolar epithelium [59–61]. A similar effect of aging in our model might also explain the more pronounced effects in the older animals in this study.

Acting as a growth factor for a broad spectrum of tissues and cell types, HGF is a potent mitogen for mature parenchymal hepatocyte cells. HGF has been proposed to be used as one of the three novel serum markers to predict the liver stiffness and fibrosis stages in chronic liver disease patients [62]. Moreover, a close interaction of HGF and IL-6 in liver related, inflammatory mediated diseases and cancer have been reported in many studies so far [63–68]. A feedback loop wherein the anti-inflammatory properties of IL-6 mediated *via* HGF, which is produced in response to IL-6 stimulation, has been documented in inflammatory diseases [63]. Therefore, in search for such a crosstalk, HGF was studied in parallel to IL-6, however no significant difference for HGF was observed in our model, either in plasma or in liver (Fig. 2B and 3B).

RANTES, which is a key player in inflammatory processes during progression of liver fibrosis [69], has been shown to activate T cell signalling pathways through activation of several immune cells including T cells, monocytes, basophils, eosinophils, natural killer cells, and dendritic cells [26] [70]. Moreover, It has been reported to have an increased expression in murine models of chronic liver inflammation leading to HCC including *Abcb4*$^{-/-}$ mice [1,71], which is also in line with our results. However, the hepatic expression levels of *RANTES* were down-regulated both in knock-out mice and wild-type mice upon ethanol challenge, when compared to their counterparts fed with control diet. Similarly, in a cohort of 55 patients with decompensated cirrhosis, 26 of which were presented with ACLF, it has recently been demonstrated that levels of *RANTES* were lower in patients with ACLF compared to patients without ACLF [72].

A profound decrease in the Pnpla3 expression levels was observed in the ethanol treated mice in our study. A previous *in vitro* study provided evidence that PNPLA3-mediated retinol release may protect against liver fibrosis by inducing a specific signature of proteins involved in extracellular matrix remodeling [73]. Therefore, the downregulation of

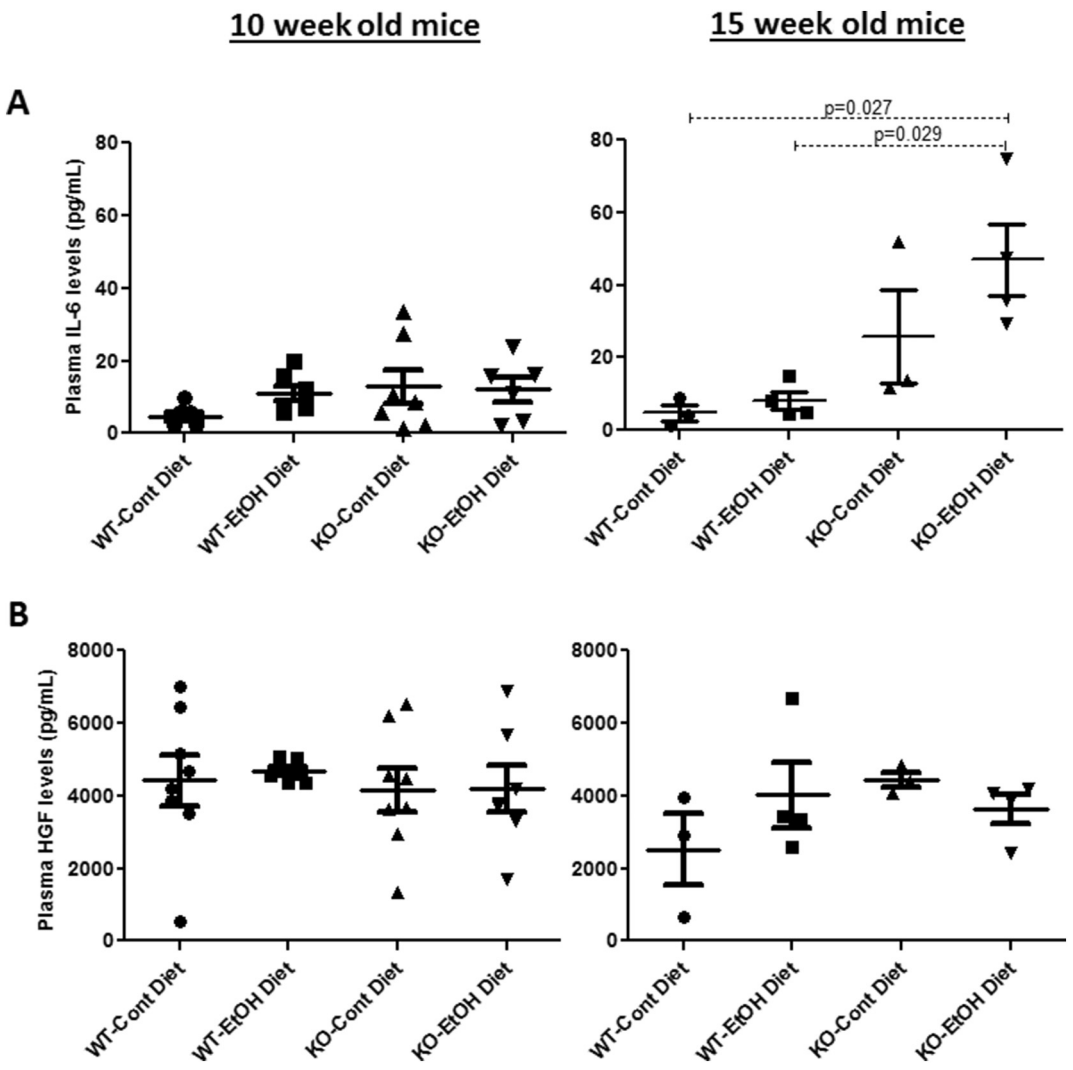

Fig. 4. Plasma levels of IL-6 and HGF. Plasma levels quantified of IL-6 (A) and HGF (B) by ELISA in 10 (left) and 15 week-old mice demonstrated approximately 10-fold increase in ethanol-challenged older mice ($p = 0.027$), whereas HGF did not differ. CONT, control diet; EtOH, ethanol diet; HGF, hepatocyte growth factor; IL-6, interleukin 6; KO, *Abcb4* knock-out mice; WT, C57BL/6 J wild-type mice. $p < 0.05$ was considered significant.

hepatic *Pnpla3* expression observed in our model after ethanol administration may interfere with the protective pathways linking PNPLA3 to liver fibrosis, further increasing the present liver injury in *Abcb4*$^{-/-}$ mice. On the other hand, Hoekstra et al. reported that a 23-fold increase in Pnpla3 expression in liver upon feeding with the western type diet (WTD) was strikingly abolished after a subsequent 16 h fasting period in C57BL/6 mice [74]. Therefore, the PNPLA3 response in our model might be due to fasting of animals rather than exposure to ethanol,

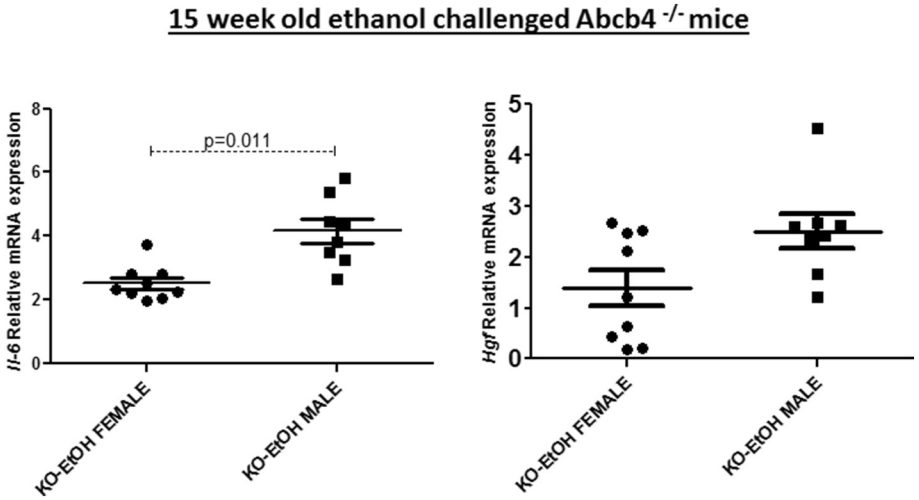

Fig. 5. Gender disparity. Comparison of males and females in the ethanol-challenged 15 week-old knock-out mice revealed that hepatic expression of *IL-6* (left) is significantly ($p = 0.011$) higher in males than females. Although a similar increase was observed in *Hgf* expression levels (right) in liver, it was not significant. EtOH, ethanol diet; HGF, hepatocyte growth factor; IL-6, interleukin-6; KO, *Abcb4* knock-out mice.

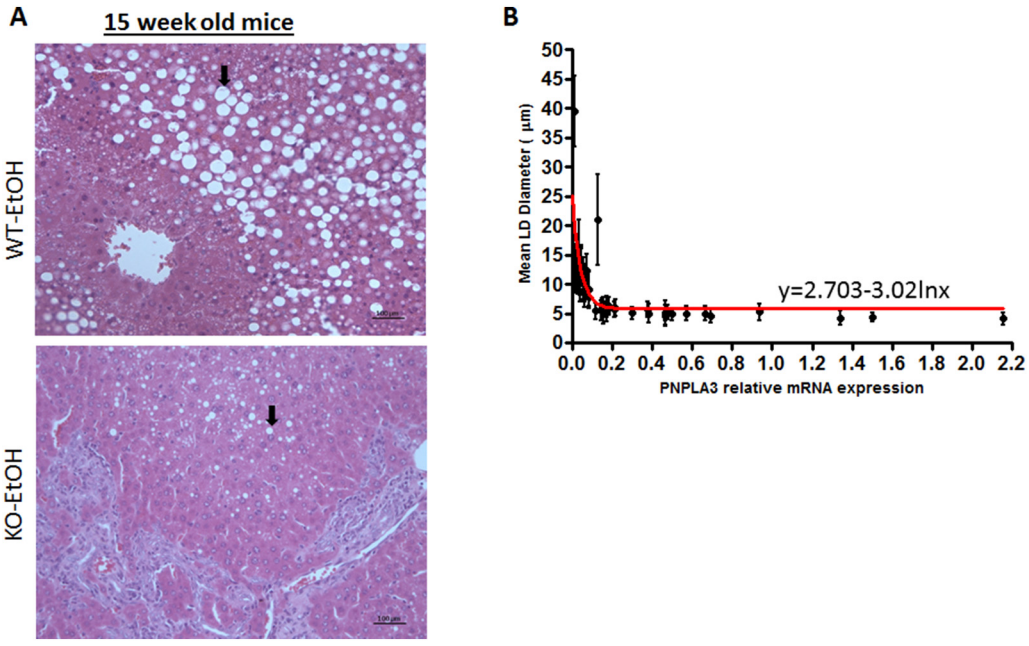

Fig. 6. Formation of lipid droplets. Hepatic steatosis was observed in ethanol challenged mice regardless of the genotype as shown by accumulation of lipid droplets observed in representative H&E stained histological slides (A). Repression of *Pnpla3* expression resulted in a notable expansion of lipid droplet size as demonstrated by a non-linear regression analysis estimating a correlation formula of "mean LD size (µm) = 2.703 − 3.02 ln [relative PNPLA3 mRNA level]" ($r^2 = 0.46$ and $p < 0.01$). EtOH, ethanol diet; KO, *Abcb4* knock-out mice, LD, lipid droplet; *Pnpla3*, patatin-like phospholipase domain-containing 3; WT, C57BL/6 J wild-type mice. Each dot represents a single mouse. Scale bar is 100 µm. Magnification 20×.

since the alcohol treated mice were hardly conscious after ethanol gavage and not able to eat for 7–9 h till they were sacrificed. Using the liver tissue samples available from one of our previous studies [75], this possibility was further supported by comparing the relative quantification of PNPLA3 expression in glucose administered and fasted BALB-Abcb4$^{-/-}$ mice, where the former were found to have about 5-fold more hepatic Pnpla3 levels compared to the latter (data not shown). Irrespective of the cause, the repression of hepatic Pnpla3 expression levels resulted in a notable expansion in size of lipid droplets observed in mice challenged with ethanol suggesting a possible role for remodeling of lipid droplets in this model. Of note, the correlation between *Pnpla3* expression and lipid droplet size was observed in both wild-type and ABCB4-deficient mice in our study. Whether the well-known PNPLA3 p.I148M variant results in a loss or gain of function of the PNPLA3 protein is still contradictory in the literature. Kumari et al. showed an increased hepatic triglyceride synthesis in the presence of this variant due to a gain of function in the lysophosphatidic acid-acyltransferase activity [76], whereas a study by He et al. on purified human PNPLA3 protein [77] and a cell culture study with a stable transfection of PNPLA3 variant by Pirazzi et al. [78] suggested a loss of function effect for this variant. Interestingly, several studies in mouse models have shown that carriers of the PNPLA3 p.I148M allele were also characterized by larger lipid droplets [77,79,80]. Therefore, down-regulation of PNPLA3 expression, and thereby the possible decrease in enzymatic activity in our model resulted in a similar phenotype with models bearing PNPLA3 p.I148M variant favoring the loss of function side of this controversial issue.

This study proposes a novel promising approach to model alcohol related acute-on-chronic liver injury by combining a mouse model with pre-existing liver injury with the well-established chronic-binge ethanol exposure [11]. In the current model, the up-regulation of CRP and IL-6 appears only in the 15 weeks old knock-out mice on ethanol diet, pointing out the importance of an acute insult (alcohol) on a pre-injured liver (knock-out). The significant over-expression of CCL2 also corresponds to the same group, suggesting that IL-6/CCL2 interaction might orchestrate acute inflammatory responses in pre-injured liver that could promote the transition from a stable chronic state to progressive liver injury after ethanol exposure. Additionally, combined metabolic dysregulation and inflammation was also evident by significant alterations in expression of chemokines and the critical triacylglycerol hydrolase PNPLA3 in this model. Furthermore, repression of PNPLA3 resulted in a notable expansion in size of lipid droplets, indicating lipid remodeling in this *ACLI* model. Even though no animal model is ideal for a particular disease due to its own limitations and advantages, such models are necessary and useful tools to help clarify the underlying mechanisms of the diseases, especially when successful therapeutic options are not available due to lack of knowledge.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2018.11.008.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgement

This study was supported by the Homburg Research Promotion Program HOMFOR 2017 grant (Saarland University Medical Center, Homburg, Germany) (to EK) and BMBF LiSyM projects (031 L0051 and 031 L0043) (to FL and SD, respectively). The authors would like to thank Mrs. Christina Meng at the Central Laboratory, Saarland University Medical Center, for analyzing biochemical parameters and ethanol concentrations in plasma. Abcb4-deficient mice on the C57BL/6 J genetic background were kindly provided by R. Barikbin and D. Goldenberg.

References

[1] N. Barashi, I.D. Weiss, O. Wald, H. Wald, K. Beider, M. Abraham, S. Klein, D. Goldenberg, J. Axelrod, E. Pikarsky, R. Abramovitch, E. Zeira, E. Galun, A. Peled, Inflammation-induced hepatocellular carcinoma is dependent on CCR5 in mice, Hepatology (Baltimore, Md.) 58 (2013) 1021–1030.

[2] R.S. O'Shea, S. Dasarathy, A.J. McCullough, D. Practice Guideline Committee of the American Association for the Study of Liver, G. Practice Parameters Committee of the American College of, Alcoholic liver disease, Hepatology (Baltimore, Md.) 51 (2010) 307–328.

[3] V. Balasubramaniyan, D.K. Dhar, A.E. Warner, W.Y. Vivien Li, A.F. Amiri, B. Bright, R.P. Mookerjee, N.A. Davies, D.L. Becker, R. Jalan, Importance of Connexin-43 based gap junction in cirrhosis and acute-on-chronic liver failure, J. Hepatol. 58 (2013) 1194–1200.

[4] R. Jalan, R. Williams, Acute-on-chronic liver failure: pathophysiological basis of therapeutic options, Blood Purif. 20 (2002) 252–261.

[5] R. Moreau, R. Jalan, P. Gines, M. Pavesi, P. Angeli, J. Cordoba, F. Durand, T. Gustot, F. Saliba, M. Domenicali, A. Gerbes, J. Wendon, C. Alessandria, W. Laleman, S. Zeuzem, J. Trebicka, M. Bernardi, V. Arroyo, Acute-on-chronic liver failure is a

distinct syndrome that develops in patients with acute decompensation of cirrhosis, Gastroenterology 144 (2013) 1426–1437 (1437.e1421-1429).
[6] J. Claria, R.E. Stauber, M.J. Coenraad, R. Moreau, R. Jalan, M. Pavesi, A. Amoros, E. Titos, J. Alcaraz-Quiles, K. Oettl, M. Morales-Ruiz, P. Angeli, M. Domenicali, C. Alessandria, A. Gerbes, J. Wendon, F. Nevens, J. Trebicka, W. Laleman, F. Saliba, T.M. Welzel, A. Albillos, T. Gustot, D. Benten, F. Durand, P. Gines, M. Bernardi, V. Arroyo, Systemic inflammation in decompensated cirrhosis: characterization and role in acute-on-chronic liver failure, Hepatology (Baltimore, Md.) 64 (2016) 1249–1264.
[7] R. Jalan, R.P. Mookerjee, Acute-on-chronic liver failure: an early biopsy is essential? Gut 59 (2010) 1455–1456.
[8] A. Katoonizadeh, W. Laleman, C. Verslype, A. Wilmer, G. Maleux, T. Roskams, F. Nevens, Early features of acute-on-chronic alcoholic liver failure: a prospective cohort study, Gut 59 (2010) 1561–1569.
[9] T.K. Li, Quantifying the risk for alcohol-use and alcohol-attributable health disorders: present findings and future research needs, J. Gastroenterol. Hepatol. 23 (Suppl. 1) (2008) S2–S8.
[10] S. Stranges, J.L. Freudenheim, P. Muti, E. Farinaro, M. Russell, T.H. Nochajski, M. Trevisan, Differential effects of alcohol drinking pattern on liver enzymes in men and women, Alcohol. Clin. Exp. Res. 28 (2004) 949–956.
[11] A. Bertola, S. Mathews, S.H. Ki, H. Wang, B. Gao, Mouse model of chronic and binge ethanol feeding (the NIAAA model), Nat. Protoc. 8 (2013) 627–637.
[12] S.H. Ki, O. Park, M. Zheng, O. Morales-Ibanez, J.K. Kolls, R. Bataller, B. Gao, Interleukin-22 treatment ameliorates alcoholic liver injury in a murine model of chronic-binge ethanol feeding: role of signal transducer and activator of transcription 3, Hepatology (Baltimore, Md.) 52 (2010) 1291–1300.
[13] T.H. Mauad, C.M. van Nieuwkerk, K.P. Dingemans, J.J. Smit, A.H. Schinkel, R.G. Notenboom, M.A. van den Bergh Weerman, R.P. Verkruisen, A.K. Groen, R.P. Oude Elferink, et al., Mice with homozygous disruption of the mdr2 P-glycoprotein gene. A novel animal model for studies of nonsuppurative inflammatory cholangitis and hepatocarcinogenesis, Am. J. Pathol. 145 (1994) 1237–1245.
[14] J.J. Smit, A.H. Schinkel, R.P. Oude Elferink, A.K. Groen, E. Wagenaar, L. van Deemter, C.A. Mol, R. Ottenhoff, N.M. van der Lugt, M.A. van Roon, et al., Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease, Cell 75 (1993) 451–462.
[15] P. Fickert, A. Fuchsbichler, M. Wagner, G. Zollner, A. Kaser, H. Tilg, R. Krause, F. Lammert, C. Langner, K. Zatloukal, H.U. Marschall, H. Denk, M. Trauner, Regurgitation of bile acids from leaky bile ducts causes sclerosing cholangitis in Mdr2 (Abcb4) knockout mice, Gastroenterology 127 (2004) 261–274.
[16] K. Hochrath, S. Ehnert, C.L. Ackert-Bicknell, Y. Lau, A. Schmid, M. Krawczyk, J.G. Hengstler, J. Dunn, K. Hiththetiya, B. Rathkolb, K. Micklich, W. Hans, H. Fuchs, V. Gailus-Durner, E. Wolf, M.H. de Angelis, S. Dooley, B. Paigen, B. Wildemann, F. Lammert, A.K. Nussler, Modeling hepatic osteodystrophy in Abcb4 deficient mice, Bone 55 (2013) 501–511.
[17] I.S. Jamall, V.N. Finelli, S.S. Que Hee, A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues, Anal. Biochem. 112 (1981) 70–75.
[18] A. Baranova, P. Lal, A. Birerdinc, Z.M. Younossi, Non-invasive markers for hepatic fibrosis, BMC Gastroenterol. 11 (2011) 91.
[19] E. Pikarsky, R.M. Porat, I. Stein, R. Abramovitch, S. Amit, S. Kasem, E. Gutkovich-Pyest, S. Urieli-Shoval, E. Galun, Y. Ben-Neriah, NF-kappaB functions as a tumour promoter in inflammation-associated cancer, Nature 431 (2004) 461–466.
[20] R.R. Zhou, H.B. Liu, J.P. Peng, Y. Huang, N. Li, M.F. Xiao, H. Wang, X.G. Fan, High mobility group box chromosomal protein 1 in acute-on-chronic liver failure patients and mice with ConA-induced acute liver injury, Exp. Mol. Pathol. 93 (2012) 213–219.
[21] W. Hou, Z.F. Piao, H.Y. Zhang, Z. Liu, Q.H. Meng, The approaches for making acute-on-chronic liver failure in rat, Zhonghua shi yan he lin chuang bing du xue za zhi = Zhonghua shiyan he linchuang bingduxue zazhi = Chinese Journal of Experimental and Clinical Virology 23 (2009) 394–396.
[22] X. Li, L.K. Wang, L.W. Wang, X.Q. Han, F. Yang, Z.J. Gong, Blockade of high-mobility group box-1 ameliorates acute on chronic liver failure in rats, Inflammation Research: Official Journal of the European Histamine Research Society … [et al.] 62 (2013) 703–709.
[23] F. Li, L. Miao, H. Sun, Y. Zhang, X. Bao, D. Zhang, Establishment of a new acute-on-chronic liver failure model, Acta Pharm. Sin. B 7 (2017) 326–333.
[24] T. Potikha, E. Stoyanov, O. Pappo, A. Frolov, L. Mizrahi, D. Olam, T. Shnitzer-Perlman, I. Weiss, N. Barashi, A. Peled, G. Sass, G. Tiegs, F. Poirier, G.A. Rabinovich, E. Galun, D. Goldenberg, Interstrain differences in chronic hepatitis and tumor development in a murine model of inflammation-mediated hepatocarcinogenesis, Hepatology (Baltimore, Md.) 58 (2013) 192–204.
[25] C. Gendrot, Y. Bacq, M.C. Brechot, J. Lansac, C. Andres, A second heterozygous MDR3 nonsense mutation associated with intrahepatic cholestasis of pregnancy, J. Med. Genet. 40 (2003) e32.
[26] E. Jacquemin, J.M. De Vree, D. Cresteil, E.M. Sokal, E. Sturm, M. Dumont, G.L. Scheffer, M. Paul, M. Burdelski, P.J. Bosma, O. Bernard, M. Hadchouel, R.P. Elferink, The wide spectrum of multidrug resistance 3 deficiency: from neonatal cholestasis to cirrhosis of adulthood, Gastroenterology 120 (2001) 1448–1458.
[27] J.F. Lucena, J.I. Herrero, J. Quiroga, B. Sangro, J. Garcia-Foncillas, N. Zabalegui, J. Sola, M. Herraiz, J.F. Medina, J. Prieto, A multidrug resistance 3 gene mutation causing cholelithiasis, cholestasis of pregnancy, and adulthood biliary cirrhosis, Gastroenterology 124 (2003) 1037–1042.
[28] O. Rosmorduc, B. Hermelin, R. Poupon, MDR3 gene defect in adults with symptomatic intrahepatic and gallbladder cholesterol cholelithiasis, Gastroenterology 120 (2001) 1459–1467.
[29] D.F. Gudbjartsson, H. Helgason, S.A. Gudjonsson, F. Zink, A. Oddson, A. Gylfason, S. Besenbacher, G. Magnusson, B.V. Halldorsson, E. Hjartarson, G.T. Sigurdsson, S.N. Stacey, M.L. Frigge, H. Holm, J. Saemundsdottir, H.T. Helgadottir, H. Johannsdottir, G. Sigfusson, G. Thorgeirsson, J.T. Sverrisson, S. Gretarsdottir, G.B. Walters, T. Rafnar, B. Thjodleifsson, E.S. Bjornsson, S. Olafsson, H. Thorarinsdottir, T. Steingrimsdottir, T.S. Gudmundsdottir, A. Theodors, J.G. Jonasson, A. Sigurdsson, G. Bjornsdottir, J.J. Jonsson, O. Thorarensen, P. Ludvigsson, H. Gudbjartsson, G.I. Eyjolfsson, O. Sigurdardottir, I. Olafsson, D.O. Arnar, O.T. Magnusson, A. Kong, G. Masson, U. Thorsteinsdottir, A. Helgason, P. Sulem, K. Stefansson, Large-scale whole-genome sequencing of the Icelandic population, Nat. Genet. 47 (2015) 435–444.
[30] A. Ghallab, U. Hofmann, S. Sezgin, N. Vartak, R. Hassan, A. Zaza, P. Godoy, K. Schneider, G. Guenther, Y. Ahmed, A. Abbas, V. Keitel, L. Kuepfer, S. Dooley, F. Lammert, C. Trautwein, M. Spiteller, D. Drasdol, A.F. Hoffmann, P.L.M. Jansen, J.G. Hengstler, R. Reif, Bile micro-infarcts in cholestasis are initiated by rupture of the apical hepatocyte membrane and cause shunting of bile to sinusoidal blood, Hepatology (2018) (Epub ahead of print).
[31] M. Katzenellenbogen, L. Mizrahi, O. Pappo, N. Klopstock, D. Olam, J. Jacob-Hirsch, N. Amariglio, G. Rechavi, E. Domany, E. Galun, D. Goldenberg, Molecular mechanisms of liver carcinogenesis in the mdr2-knockout mice, Mol. Cancer Res.: MCR 5 (2007) 1159–1170.
[32] M. Roderfeld, T. Rath, F. Lammert, C. Dierkes, J. Graf, E. Roeb, Innovative immunohistochemistry identifies MMP-9 expressing macrophages at the invasive front of murine HCC, World J. Hepatol. 2 (2010) 175–179.
[33] M. Katzenellenbogen, O. Pappo, H. Barash, N. Klopstock, L. Mizrahi, D. Olam, J. Jacob-Hirsch, N. Amariglio, G. Rechavi, L.A. Mitchell, R. Kohen, E. Domany, E. Galun, D. Goldenberg, Multiple adaptive mechanisms to chronic liver disease revealed at early stages of liver carcinogenesis in the Mdr2-knockout mice, Cancer Res. 66 (2006) 4001–4010.
[34] T. Moustafa, P. Fickert, C. Magnes, C. Guelly, A. Thueringer, S. Frank, D. Kratky, W. Sattler, H. Reicher, F. Sinner, J. Gumhold, D. Silbert, G. Fauler, G. Hofler, A. Lass, R. Zechner, M. Trauner, Alterations in lipid metabolism mediate inflammation, fibrosis, and proliferation in a mouse model of chronic cholestatic liver injury, Gastroenterology 142 (2012) (140–151.e112).
[35] E. Stoyanov, G. Ludwig, L. Mizrahi, D. Olam, T. Schnitzer-Perlman, E. Tasika, G. Sass, G. Tiegs, Y. Jiang, T. Nie, J. Kohler, R.F. Schinazi, P.M. Vertino, H. Cedar, E. Galun, D. Goldenberg, Chronic liver inflammation modifies DNA methylation at the precancerous stage of murine hepatocarcinogenesis, Oncotarget 6 (2015) 11047–11060.
[36] M.G. Neuman, Cytokines and inflamed liver, Clin. Biochem. 33 (1999) 601–605.
[37] M.G. Neuman, Cytokines–central factors in alcoholic liver disease, Alcohol Res. Health 27 (2003) 307–316.
[38] T. Alonzi, E. Fattori, D. Lazzaro, P. Costa, L. Probert, G. Kollias, F. De Benedetti, V. Poli, G. Ciliberto, Interleukin 6 is required for the development of collagen-induced arthritis, J. Exp. Med. 187 (1998) 461–468.
[39] M. Yamamoto, K. Yoshizaki, T. Kishimoto, H. Ito, IL-6 is required for the development of Th1 cell-mediated murine colitis, J. Immunol. 164 (2000) 4878–4882.
[40] Z. Xing, J. Gauldie, G. Cox, H. Baumann, M. Jordana, X.F. Lei, M.K. Achong, IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses, J. Clin. Invest. 101 (1998) 311–320.
[41] D.E. Doherty, G.P. Downey, G.S. Worthen, C. Haslett, P.M. Henson, Monocyte retention and migration in pulmonary inflammation. Requirement for neutrophils, laboratory investigation, A Journal of Technical Methods and Pathology 59 (1988) 200–213.
[42] M.J. Melnicoff, P.K. Horan, P.S. Morahan, Kinetics of changes in peritoneal cell populations following acute inflammation, Cell. Immunol. 118 (1989) 178–191.
[43] S.M. Hurst, T.S. Wilkinson, R.M. McLoughlin, S. Jones, S. Horiuchi, N. Yamamoto, S. Rose-John, G.M. Fuller, N. Topley, S.A. Jones, Il-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation, Immunity 14 (2001) 705–714.
[44] S.A. Jones, Directing transition from innate to acquired immunity: defining a role for IL-6, J. Immunol. 175 (2005) 3463–3468.
[45] G. Kaplanski, V. Marin, F. Montero-Julian, A. Mantovani, C. Farnarier, IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation, Trends Immunol. 24 (2003) 25–29.
[46] S.A. Jones, J. Scheller, S. Rose-John, Therapeutic strategies for the clinical blockade of IL-6/gp130 signaling, J. Clin. Invest. 121 (2011) 3375–3383.
[47] J. Gewiese-Rabsch, C. Drucker, S. Malchow, J. Scheller, S. Rose-John, Role of IL-6 trans-signaling in CCl(4)induced liver damage, Biochim. Biophys. Acta 1802 (2010) 1054–1061.
[48] N.C. Riedemann, T.A. Neff, R.F. Guo, K.D. Bernacki, I.J. Laudes, J.V. Sarma, J.D. Lambris, P.A. Ward, Protective effects of IL-6 blockade in sepsis are linked to reduced C5a receptor expression, Journal of Immunology (Baltimore, Md. : 1950) 170 (2003) 503–507.
[49] K. Yamaguchi, Y. Itoh, C. Yokomizo, T. Nishimura, T. Niimi, H. Fujii, T. Okanoue, T. Yoshikawa, Blockade of interleukin-6 signaling enhances hepatic steatosis but improves liver injury in methionine choline-deficient diet-fed mice, Laboratory Investigation; A Journal of Technical Methods and Pathology 90 (2010) 1169–1178.
[50] K. Yamaguchi, Y. Itoh, C. Yokomizo, T. Nishimura, T. Niimi, A. Umemura, H. Fujii, T. Okanoue, T. Yoshikawa, Blockade of IL-6 signaling exacerbates liver injury and suppresses antiapoptotic gene expression in methionine choline-deficient diet-fed db/db mice, Laboratory Investigation; A Journal of Technical Methods and Pathology 91 (2011) 609–618.
[51] W.E. Naugler, T. Sakurai, S. Kim, S. Maeda, K. Kim, A.M. Elsharkawy, M. Karin, Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6

production, Science 317 (2007) 121–124.
[52] S.L. Deshmane, S. Kremlev, S. Amini, B.E. Sawaya, Monocyte chemoattractant protein-1 (MCP-1): an overview, Journal of Interferon & Cytokine Research: The Official Journal of the International Society for Interferon and Cytokine Research 29 (2009) 313–326.
[53] W. Chen, Q. Gao, S. Han, F. Pan, W. Fan, The CCL2/CCR2 axis enhances IL-6-induced epithelial-mesenchymal transition by cooperatively activating STAT3-Twist signaling, Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine 36 (2015) 973–981.
[54] L. de Leseleuc, M. Orlova, A. Cobat, M. Girard, N.T. Huong, N.N. Ba, N.V. Thuc, R. Truman, J.S. Spencer, L. Adams, V.H. Thai, A. Alcais, E. Schurr, PARK2 mediates interleukin 6 and monocyte chemoattractant protein 1 production by human macrophages, PLoS Negl. Trop. Dis. 7 (2013) e2015.
[55] U.J. Jung, M.S. Choi, Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease, Int. J. Mol. Sci. 15 (2014) 6184–6223.
[56] Z.Y. Lin, Y.H. Chuang, W.L. Chuang, Cancer-associated fibroblasts up-regulate CCL2, CCL26, IL6 and LOXL2 genes related to promotion of cancer progression in hepatocellular carcinoma cells, Biomed. Pharmacother. = Biomedecine & Pharmacotherapie 66 (2012) 525–529.
[57] X.M. Wang, M. Hamza, T.X. Wu, R.A. Dionne, Upregulation of IL-6, IL-8 and CCL2 gene expression after acute inflammation: correlation to clinical pain, Pain 142 (2009) 275–283.
[58] P. Mandrekar, A. Ambade, A. Lim, G. Szabo, D. Catalano, An essential role for monocyte chemoattractant protein-1 in alcoholic liver injury: regulation of proinflammatory cytokines and hepatic steatosis in mice, Hepatology (Baltimore, Md.) 54 (2011) 2185–2197.
[59] C.L. Krone, K. Trzcinski, T. Zborowski, E.A. Sanders, D. Bogaert, Impaired innate mucosal immunity in aged mice permits prolonged Streptococcus pneumoniae colonization, Infect. Immun. 81 (2013) 4615–4625.
[60] Y. Song, H. Shen, D. Schenten, P. Shan, P.J. Lee, D.R. Goldstein, Aging enhances the basal production of IL-6 and CCL2 in vascular smooth muscle cells, Arterioscler. Thromb. Vasc. Biol. 32 (2012) 103–109.
[61] M.S. Valentine, P.A. Link, J.A. Herbert, F.J. Kamga Gninzeko, M.B. Schneck, K. Shankar, J. Nkwocha, A.M. Reynolds, R.L. Heise, Inflammation and monocyte recruitment due to aging and mechanical stretch in alveolar epithelium are inhibited by the molecular chaperone 4-phenylbutyrate, Cell. Mol. Bioeng. 11 (2018) 495–508.
[62] M. Krawczyk, S. Zimmermann, G. Hess, R. Holz, M. Dauer, J. Raedle, F. Lammert, F. Grunhage, Panel of three novel serum markers predicts liver stiffness and fibrosis stages in patients with chronic liver disease, PLoS One 12 (2017) e0173506.
[63] G.M. Coudriet, J. He, M. Trucco, W.M. Mars, J.D. Piganelli, Hepatocyte growth factor modulates interleukin-6 production in bone marrow derived macrophages: implications for inflammatory mediated diseases, PLoS One 5 (2010) e15384.
[64] R. Kariv, A. Enden, I. Zvibel, G. Rosner, S. Brill, D.A. Shafritz, Z. Halpern, R. Oren, Triiodothyronine and interleukin-6 (IL-6) induce expression of HGF in an immortalized rat hepatic stellate cell line, Liver International: Official Journal of the International Association for the Study of the Liver 23 (2003) 187–193.
[65] Z. Liu, T. Sakamoto, T. Ezure, S. Yokomuro, N. Murase, G. Michalopoulos, A.J. Demetris, Interleukin-6, hepatocyte growth factor, and their receptors in biliary epithelial cells during a type I ductular reaction in mice: interactions between the periductal inflammatory and stromal cells and the biliary epithelium, Hepatology (Baltimore, Md.) 28 (1998) 1260–1268.
[66] A. Prystupa, P. Kicinski, J. Sak, A. Boguszewska-Czubara, A. Torun-Jurkowska, W. Zaluska, Proinflammatory cytokines (IL-1alpha, IL-6) and hepatocyte growth factor in patients with alcoholic liver cirrhosis, Gastroenterol. Res. Pract. 2015 (2015) 532615.
[67] Y. To, M. Dohi, K. Matsumoto, R. Tanaka, A. Sato, K. Nakagome, T. Nakamura, K. Yamamoto, A two-way interaction between hepatocyte growth factor and interleukin-6 in tissue invasion of lung cancer cell line, Am. J. Respir. Cell Mol. Biol. 27 (2002) 220–226.
[68] L. Zhu, J. Shi, H. Jin, A.B. Liu, H. Wang, H. Pyo, T.C. Wang, C.S. Yang, Y. Wu, J. Ye, Y.B. Zhu, S. Tu, The IL-6/STAT3/HGF and TGF-β1 signaling loop mediates crosstalk between cancer cells and bone marrow-derived myofibroblasts that regulate cancer stem cells and gastric tumorigenesis, Gastroenterology 144 (Supplement 1) (2013) (Page S-70).
[69] M.L. Berres, R.R. Koenen, A. Rueland, M.M. Zaldivar, D. Heinrichs, H. Sahin, P. Schmitz, K.L. Streetz, T. Berg, N. Gassler, R. Weiskirchen, A. Proudfoot, C. Weber, C. Trautwein, H.E. Wasmuth, Antagonism of the chemokine Ccl5 ameliorates experimental liver fibrosis in mice, J. Clin. Invest. 120 (2010) 4129–4140.
[70] K.B. Bacon, B.A. Premack, P. Gardner, T.J. Schall, Activation of dual T cell signaling pathways by the chemokine RANTES, Science (New York, N.Y.) 269 (1995) 1727–1730.
[71] A. Mohs, N. Kuttkat, J. Reissing, H.W. Zimmermann, R. Sonntag, A. Proudfoot, S.A. Youssef, A. de Bruin, F.J. Cubero, C. Trautwein, Functional role of CCL5/RANTES for HCC progression during chronic liver disease, J. Hepatol. 66 (2017) 743–753.
[72] C. Sole, E. Sola, M. Morales-Ruiz, G. Fernandez, P. Huelin, I. Graupera, R. Moreira, G. de Prada, X. Ariza, E. Pose, N. Fabrellas, S.G. Kalko, W. Jimenez, P. Gines, Characterization of inflammatory response in acute-on-chronic liver failure and relationship with prognosis, Sci. Rep. 6 (2016) 32341.
[73] P. Pingitore, P. Dongiovanni, B.M. Motta, M. Meroni, S.M. Lepore, R.M. Mancina, S. Pelusi, C. Russo, A. Caddeo, G. Rossi, T. Montalcini, A. Pujia, O. Wiklund, L. Valenti, S. Romeo, PNPLA3 overexpression results in reduction of proteins predisposing to fibrosis, Hum. Mol. Genet. 25 (2016) 5212–5222.
[74] M. Hoekstra, Z. Li, J.K. Kruijt, M. Van Eck, T.J. Van Berkel, J. Kuiper, The expression level of non-alcoholic fatty liver disease-related gene PNPLA3 in hepatocytes is highly influenced by hepatic lipid status, J. Hepatol. 52 (2010) 244–251.
[75] K. Hochrath, M. Krawczyk, R. Goebel, M. Langhirt, B. Rathkolb, K. Micklich, J. Rozman, M. Horsch, J. Beckers, M. Klingenspor, H. Fuchs, V. Gailus-Durner, E. Wolf, M. Acalovschi, D.A. Volmer, M. Hrabe de Angelis, F. Lammert, The hepatic phosphatidylcholine transporter ABCB4 as modulator of glucose homeostasis, FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology 26 (2012) 5081–5091.
[76] M. Kumari, G. Schoiswohl, C. Chitraju, M. Paar, I. Cornaciu, A.Y. Rangrez, N. Wongsiriroj, H.M. Nagy, P.T. Ivanova, S.A. Scott, O. Knittelfelder, G.N. Rechberger, R. Birner-Gruenberger, S. Eder, H.A. Brown, G. Haemmerle, M. Oberer, A. Lass, E.E. Kershaw, R. Zimmermann, R. Zechner, Adiponutrin functions as a nutritionally regulated lysophosphatidic acid acyltransferase, Cell Metab. 15 (2012) 691–702.
[77] S. He, C. McPhaul, J.Z. Li, R. Garuti, L. Kinch, N.V. Grishin, J.C. Cohen, H.H. Hobbs, A sequence variation (I148M) in PNPLA3 associated with nonalcoholic fatty liver disease disrupts triglyceride hydrolysis, J. Biol. Chem. 285 (2010) 6706–6715.
[78] C. Pirazzi, M. Adiels, M.A. Burza, R.M. Mancina, M. Levin, M. Stahlman, M.R. Taskinen, M. Orho-Melander, J. Perman, A. Pujia, L. Andersson, C. Maglio, T. Montalcini, O. Wiklund, J. Boren, S. Romeo, Patatin-like phospholipase domain-containing 3 (PNPLA3) I148M (rs738409) affects hepatic VLDL secretion in humans and in vitro, J. Hepatol. 57 (2012) 1276–1282.
[79] J. Boursier, A.M. Diehl, Patatin-like phospholipase domain-containing protein 3 and liver disease: opportunities to unravel mechanisms underlying statistical associations, Hepatology (Baltimore, Md.) 61 (2015) 18–20.
[80] E. Smagris, S. Basuray, J. Li, Y. Huang, K.M. Lai, J. Gromada, J.C. Cohen, H.H. Hobbs, Pnpla3I148M knockin mice accumulate PNPLA3 on lipid droplets and develop hepatic steatosis, Hepatology (Baltimore, Md.) 61 (2015) 108–118.