Lipocalin 2 drives neutrophilic inflammation in alcoholic liver disease

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Abstract

Background: Alcoholic liver disease (ALD) is a healthcare issue worldwide. Especially, alcoholic steatohepatitis (ASH), a disease entity that emerges from various degrees of ALD and that is driven by neutrophilic inflammation, is associated with high mortality partly due to limited treatment options. Recently, the siderophore binding peptide Lipocalin 2 (LCN2) has been shown to exert protective functions in liver homeostasis. This study aimed to investigate the role of LCN2 in ALD.

Methods: Mouse and human blood and liver samples with confirmed ALD were analysed for LCN2 expression. Further, wild-type (WT) and *Lcn2*-deficient (*Lcn2*^{-/-}) mice were fed the alcohol supplemented Lieber-DeCarli diet for 2 weeks to induce ALD. Experimental liver injury was characterised clinically, histologically and by expression analysis. Mechanistically, *in vivo* neutrophil transfers were established to track neutrophil migration into the inflamed liver. Genetic experiments were corroborated by pharmacologic LCN2 blockade before an acute alcohol challenge.

Results: LCN2 was highly elevated in blood and livers of patients with ALD similar to ethanol-fed WT mice. Notably, *Lcn2*^{-/-} mice were protected from ALD which was associated with diminished hepatic neutrophil infiltration. Mechanistically, adoptive transfer experiments revealed that LCN2 was required for neutrophil infiltration into the liver in ALD. Further, pharmacological blockade of LCN2 protected WT mice from hepatic injury and neutrophilic inflammation after acute alcohol challenge.

Conclusion: In contrast to previous studies about LCN2 in hepatic diseases, here we show that LCN2 drives neutrophilic inflammation induced by ethanol toxicity. Our data suggests that blockade of LCN2 represents an appealing therapeutic target in ALD.

Zusammenfassung

Hintergrund: Die alkoholische Lebererkrankung (ALD) stellt ein weltweites Gesundheitsproblem dar. Besonders die akute alkoholische Steatohepatitis (ASH), für die es kaum Therapiemöglichkeiten gibt und welche in jedem Stadium der ALD auftreten kann, ist mit einer hohen Mortalität assoziiert. Bisherige Arbeiten zeigten, dass Lipocalin 2 (LCN2) eine schützende Funktion in der Leber ausübt. In dieser Arbeit untersuchten wir die Rolle von LCN2 in der Entstehung der ALD.

Methoden: In murinen und humanen Blut- und Leberproben mit nachgewiesener ALD wurde die Expression von LCN2 untersucht. Weiters wurden Wildtyp (WT) und *Lcn2*^{-/-} Mäuse mit einer alkoholischen Lieber-DeCarli Diät für 2 Wochen gefüttert. Der Leberschaden wurde klinisch, histologisch und mittels RNA-Expressionsanalyse charakterisiert. Neutrophilen-Transfers wurden etabliert, um die Migration Neutrophiler Granulozyten vom Blut in die Leber zu verfolgen. Zudem testeten wir die Effektivität eines anti-LCN2 Antikörpers in einem akuten Alkoholmodell.

Resultate: LCN2 Expression war im Blut und in der Leber von Patienten und Mäusen mit ALD erhöht. *Lcn2*^{-/-} Mäuse waren vor ALD geschützt, was mit einer verminderten Neutrophilen-Infiltration in die Leber assoziiert war. Transferexperimente zeigten, dass *Lcn2*^{-/-} Neutrophile eine reduzierte Lebensdauer und Infiltrationsneigung in die Leber haben. Pharmakologische LCN2-Blockade schützte die Mäuse vor Ethanol-induziertem Leberschaden.

Konklusion: Im Gegensatz zu bisherigen Studien über LCN2 in Lebererkrankungen, finden wir, dass LCN2 die Alkohol-induzierte Leberentzündung vorantreibt. Unsere Daten suggerieren, dass die pharmakologische Blockade von LCN2 eine vielversprechende Behandlungsoption für Patienten mit ALD ist.

Declaration

Herewith I, Verena Wieser, ensure that

- 1) the presented data is the result of my own work.
- 2) this paper has not been handed in for any other academic degree.
- 3) the present paper form is identical with the digital form.

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Introduction

Alcoholic liver disease: A worldwide problem

Alcohol is a psychoactive substance with dependence-producing properties that is commonly consumed in most ethnicities, age groups and in both genders. The abuse of alcohol causes disease that leads to social and economic problems as outlined below [4].

Individual injury from alcohol depends on the volume of consumed alcohol (dose-dependency), the pattern of drinking, and also the quality of consumed alcohol. The toxic abuse of alcohol causes multiple diseases ranging from alcohol dependence, liver disease, cancers and injuries. In 2010 the worldwide *per person* consumption was 6.2 litres of pure alcohol (aged 15 years or older), which corresponds to 13.5 grams of pure alcohol per day. It is now appreciated that more than 20 grams per day are harmful for women and 40 grams for men. The greater the economic wealth of a country, the more alcohol is consumed and the smaller the number of abstainers. As such, high-income countries demonstrate the highest alcohol per capita consumption (APC) [4].

As reported by the WHO, in 2012 alcohol consumption was responsible for about 3.3 million deaths, or 5.9 % of all global deaths. There are significant sex differences, for example 7.6 % of deaths among males were attributable to alcohol compared with only 4.0% of deaths among females. Moreover, 139 million disability-adjusted life years (DALYs), or 5.1 % of the global burden of disease and injury, were due to alcohol consumption [4].

Clinical presentation and disease progression: Steatosis – hepatitis – fibrosis and cirrhosis

Chronic consumption of alcohol leads to different forms of liver disease, ranging from simple steatosis up to steatohepatitis and cirrhosis. Alcohol induced **simple liver steatosis** often presents asymptomatic and is rapidly reversible after a certain period of abstinence. About 20-25% of heavy drinkers develop alcoholic **steatohepatitis (ASH)**, Fig. 1). Why only a smaller part of these heavy drinkers is affected is still unknown. It is assumed that various patient specific risk factors such as genetic risk, obesity, exposition to other toxins and co-infection with hepatitis C play a role. Severe ASH is a distinct disease which is characterised through massive liver inflammation and cell death[5]. ASH develops from **liver cirrhosis** but can similarly occur in every stage of disease such as steatosis, **fibrosis and hepatocellular carcinoma (HCC)**. ASH patients can present only mild symptoms with only a slight elevation of liver transaminases but also severe and vital inflammation. Severe ASH is associated with 30% mortality and treatment options in this stage are rare [5].

Figure 1. Progression of alcoholic liver disease (adopted from [5])

In 80-90% heavy drinkers (women who consume more than 20 grams per day, men who consume more than 40 per day) develop alcoholic steatohepatitis which in 20-40% ends up in fibrosis. 8-20% of these patients who develop fibrosis progress to cirrhosis and again 3-10% of these patients end up in HCC. Notably, in all stages of disease 20-25% of affected people can develop acute steatohepatitis [5].

Patients with ASH clinically present with fever, hepatomegaly, jaundice, anorexia, encephalopathy, leucocytosis (representing neutrophilic infiltration into the liver), elevated liver transaminases and co-infections. The diagnosis is made clinically and can be ensured only by liver biopsy. Severe ASH often ends up in sepsis and multi-organ failure within few days whereas renal function determines the prognosis [5].

To define the prognosis of severe ASH various scores have been established. The most established score is the Maddrey Score (or Maddrey Discriminant Function Formula (DF)), which is composed of the serum bilirubin and the prothrombin time and indicates if steroid treatment is sensible [5].

Treatment options are scarce for severe ASH. Immediate alcohol abstinence is the most important step [5][5]. Most of the patients are severely immune-compromised, such that the alertness for infections (pneumonia, urinary tract infections or peritonitis) is pivotal [5][5]. Further basic measures include parenteral substitution with energy and

vitamins, especially vitamin B and the avoidance of nephrotoxic substances such as contrast agents and painkillers [5].

In various studies, it has been shown that patients with a Maddrey score over 32 (especially those with encephalopathy and leucocytosis) profit from the treatment with corticosteroids. Nevertheless, only short-time survival is positively influenced by steroids and complications, such as severe bacterial and fungal infections, limit steroid treatment. Therefore, alternative treatment options are desperately awaited [5, 6].

It is now appreciated that tumour necrosis factor alpha (TNF- α) plays a crucial role in the pathogenesis of ALD and thus, clinical anti-TNF trials (i.e. Infliximab, Etanercept) were initiated with divergent outcomes. On one hand two studies which used a single infusion of Infliximab could show an improvement in Maddrey score and liver histology after one month; on the other hand, a French study which used a higher dose of Infliximab in combination with corticosteroids observed more infectious complications and as a consequence also a higher mortality in the treatment group [7-9]. Another study from the US also showed more infectious complications and missing benefit in the anti-TNF group [10]. Taken together, anti-TNF strategies have not yielded a major breakthrough so that alternative treatment options have to be considered for the future. Pentoxifyllin is a weak TNF- α inhibitor and shows moderate immune-modulatory functions. In various studies, it has been shown that the substance has a positive impact onto renal function, although treatment resulted in a higher mortality compared to the corticosteroid group. Therefore, Pentoxifyllin exhibits a renoprotective effect, without benefit on mortality caused by ASH [11, 12].

Also N-acetylcystein seems to have no therapeutical effect: In one study, high doses of intravenous N-acetylcysteine therapy for 14 days conferred neither survival benefits

nor early biological improvement in patients with severe ASH undergoing adequate nutritional support [13].

For a selected patient population with a first episode of severe alcoholic hepatitis not responding to medical therapy, early liver transplantation (within 6 months) can improve survival. Unfortunately, liver transplantation is also a rare treatment option because of two major limitations: Donor organs are limited and a 6-month alcohol abstinence is usually required before patients with severe ASH are considered for liver transplantation [14].

Genetic risk factors

There are three hints that suggest a genetic impact on susceptibility for ALD [15]: First, women are more prone to suffer from ALD (when drinking the same amounts compared to men); second, Hispanics subjects are also more susceptible compared to white and black people; and third, monozygotic twins show a higher prevalence of alcoholic cirrhosis than monozygotic twins.

Genome wide association studies have shown that certain polymorphisms in the gamma-aminobutyric acid (*GABA*) and in alcohol-degrading enzymes such as alcohol dehydrogenase (*ADH*) and acetaldehyde dehydrogenase (*ALDH*) are associated with a higher risk for alcoholism, which means a higher risk for alcohol dependence (*ADH1B*1*, *ADH1C*2* and *ALDH2*1* with odds ratios of 1.89, 1.32 and 4.35). On the other side, the *ALDH2*2* is found in 30 to 50% of Asians who are known to be susceptible for alcohol effects. Asians and also homozygote carriers of *ALDH2*2* are protected from ALD, because drinking of alcohol leads to a “flush syndrome”

(tachycardia, nausea and vomiting) which usually keeps carriers away from alcohol consumption [15].

Several studies have also investigated whether there are genetic alterations which prone individuals to disease progression. Therefore, genes, which encode proteins that are involved in oxidative stress, inflammation and fibrosis have been examined. Surprisingly, robust data could only be found for polymorphisms in the genes for *TNF- α* (*TNF- α -238A*) and for Patatin-like-Phospholipase-3 (*PNPLA3 rs738409 G*) [15].

Pathogenesis

Ethanol metabolism

Nearly 100% of the consumed alcohol is absorbed in the upper gastrointestinal tract (20% in the stomach and 80% in the small intestine) and only a small part undergoes first pass metabolism. The liver is the main organ which metabolizes ingested alcohol. There are two pathways involved in ethanol metabolism: Cytosolic alcohol dehydrogenase (ADH), which is the major enzyme degrading alcohol, and the microsomal monooxygenase cytochrome P450 2E1 (CYP2E1), which was termed inducible microsomal ethanol-oxidizing system (MEOS) previously and acts in the oxidative pathway (Fig. 2). The peroxisomal catalase (Cat) is the main non-oxidative enzyme degrading ethanol [16].

ADH catalyses the reaction from ethanol to acetaldehyde thereby reducing nicotinamide adenine dinucleotide (NAD⁺) to NADH. For further ethanol degradation it is necessary to re-oxidize NADH to NAD⁺ in the mitochondria. During this step reactive

oxygen species (ROS) are generated. When the re-oxidation system is overloaded, accumulating NADH suppresses energy production and β -oxidation of fatty acids thereby promoting liver steatosis (Fig. 2) [16].

Also during CYP2E1 activity ROS are generated (Fig. 2). In one study it was shown that *CYP2E1* is induced by ethanol and that the absence of CYP2E1 in mice reverses steatosis and oxidative stress but not liver inflammation, whereas another study demonstrated that CYP2E1 is not involved in alcohol-induced liver injury [17-19]. *In vitro* it has been shown that the specific inhibition of CYP2E1 or ADH and the combined inhibition of ADH and CYP2E1 greatly decreases the formation of protein aldehyde adducts (see below) [20].

To a lesser extent, ethanol is metabolized by the peroxisomal catalase, a non-oxidative pathway, which generates fatty acid ethyl esters (FAEE). These FAEE are a marker for chronic alcohol consumption and may play a role in the pathogenesis of alcoholic liver steatosis [21].

Acetaldehyde (AA) seems to be the most toxic by-product during degradation of alcohol: Since over 30 years AA has been classified as a carcinogen because of damaging DNA ranging from point mutations up to DNA adducts, which are the formations of chemicals bound to DNA [22]. DNA adducts have been shown to promote carcinogenesis, for example the development of colorectal cancer [23]. Furthermore, it has also been shown that AA interacts with the enzymes involved in DNA repair [24]. It has been demonstrated that AA induces mitochondrial damage and thereby promoting oxidative stress [25].

In a next step AA is oxidized by the mitochondrial ALDH to acetate (Fig. 2). Recently, it was shown that the lack of ALDH2 ameliorates alcoholic fatty liver but worsens liver inflammation and fibrosis in mice indicating a toxic role for AA and a lipogenic role for

acetate or rather accumulating NADH [26]. Acetate is further metabolised in the citric cycle [27].

Alcohol Degradation and Steatosis: Lipogenesis vs. beta-oxidation

Figure 2. Degradation of ethanol, lipogenesis and β -oxidation (adopted from [16, 21, 27-31])

Ethanol is mainly degraded by ALD or CYP2E1 to AA, which in turn is metabolized by ALDH to acetate. Acetate is established in the citric cycle, in which ACC favours the metabolisation to Malonyl-CoA. Malonyl-CoA gets converted to Acetyl CoA via β -oxidation (blue coloured) or used as brick for triglycerides (which is takes place when the β -oxidation system is overloaded, red coloured). Master regulators favouring β -oxidation are AMPK, SIRT-1 and PPAR α which upregulate mitochondrial enzymes such as CPT1 and enzymes recovering Malonyl-CoA to Acetyl CoA in the citric cycle. SREBP-1c and XBP1 are enzymes counteracting β -oxidation and promoting lipogenesis via the up-regulation of lipogenic enzymes such as ACC, FAS and SCD-1.

Steatosis in ALD: Lipogenesis vs. β -oxidation

Lipogenesis is the biochemical process of rigging triglycerides and especially takes place in the liver and adipose tissue. One consequence of a shift towards lipogenesis

is liver steatosis. Up to 90% of the patients with heavy alcohol consumption develop liver steatosis which is reversible upon alcohol withdrawal. Until now it is not completely understood how ethanol can cause liver steatosis. Hepatocytes isolated from rats fed an ethanol-containing liquid diet showed higher rates of fatty acid and triacylglycerol synthesis, but a decreased rate of fatty acid oxidation, concomitant to a lower activity of carnitine palmitoyltransferase I (CPT1) [32]. Various studies have shown that ethanol induces sterol regulatory element-binding protein 1c (*SREBP1c*) and downregulates adenosine monophosphate-activated protein kinase (*AMPK*), Sirtuin 1 (*SIRT1*) and peroxisome proliferator-activated receptor alpha (*PPAR α*), all three important transcription factors in lipid synthesis [29].

SREBP1-c is an isoform of SREBP and known to induce fatty liver via the upregulation of Acetyl-CoA carboxylase (**ACC**), fatty acid synthase (**FAS**) and stearoyl-coenzyme A desaturase 1 (**SCD1**), all three enzymes synthesizing fatty acids. ACC is the rate-limiting enzyme in the synthesis of triglycerides and an inhibitor of CPT1, the initial enzyme in the β -oxidation of fatty acids (Fig. 2). Ethanol was shown to induce the fatty acid synthesis pathway by activation of SREBP *in vitro*. Probably, this activation is mediated by acetaldehyde, because the effect was only seen in cell lines expressing alcohol dehydrogenase. Feeding mice an ethanol-supplemented Lieber-DeCarli diet results in a significant increase in levels of the active form of SREBP -1c. This is associated with increased expression of hepatic lipogenic genes as well as the accumulation of triglycerides in the livers [33]. In addition to this, ob/ob mice (a mouse strain which is prone to obesity due the lack of leptin and therefore eating excessively) lacking *Srebp-1c* show less steatosis compared to ob/ob mice expressing *Srebp-1c* [34].

XBP-1 is known to play an essential role in the endoplasmic reticulum stress response but was recently discovered as a novel transcription factor promoting hepatic lipogenesis. XBP-1 activity is increased after a high carbohydrate diet and induced the transcription of lipogenic genes such as *Scd1* and *Acc* (Fig. 2). *Xbp-1*-deficient mice are protected from steatosis, hypertriglyceridemia and hypercholesterolemia [30].

AMPK is known to reduce fatty acid synthesis via inactivation of ACC (see above) and to promote fatty acid oxidation via the activation of malonyl Co-A decarboxylase (MCD), an enzyme counteracting the ACC (Fig. 2). *In vitro*, AMPK activity is inhibited by ethanol in rat hepatoma cell lines expressing *Adh* and *Aldh2*. Conversely, the activation of AMPK by AMPK-activators such as metformin largely blocks the ability of ethanol to increase levels of mature SREBP-1 protein. Along with this, *in vivo* ethanol feeding also reduces AMPK activity [35].

PPAR α is activated by increased intracellular free fatty acids and in turn dimerises with its co-receptor retinoid X receptor (RXR) and activates enzymes involved in β -oxidation such as MCD and CPT1 therefore counteracting steatosis [36]. Fischer and colleagues [37] have shown that in mice fed an ethanol diet the ratio PPAR α /retinoid X receptor alpha binding is decreased and also PPAR α regulated genes are reduced. *In vitro* experiments by Galli *et al.* [38] demonstrate that acetaldehyde is the reason for reducing the transcriptional and DNA binding activity of PPAR α . Furthermore, it has been shown that *Ppar α* knockout mice fed an ethanol diet exhibit marked hepatomegaly, hepatic inflammation, cell toxicity, fibrosis, apoptosis, and mitochondrial swelling indicating a protective role of PPAR α in steatosis and alcoholic steatohepatitis [39].

Adiponectin (ADIPOQ) is exclusively produced and secreted by the adipose tissue and exerts its functions via two distinct receptors (ADIPOR1/2) which are seven-transmembrane domain proteins with an internal N-terminus and an extracellular C-terminus. Both receptors, but especially ADIPOR2, are highly expressed in the liver and this organ also seems to be the most important target for adiponectin [40]. Xu and colleagues [41] have shown that circulating concentrations of adiponectin decrease after chronic ethanol feeding in mice whereas the administration of recombinant adiponectin significantly improves liver steatosis and inflammation. The authors suggest that adiponectin increases CPT1 activity and thereby fatty acid oxidation. On the other side, adiponectin also decreases ACC and FAS activity as well as TNF- α production therefore counteracting not only lipogenesis but also hepatic inflammation [41].

It has been shown that sirtuins especially **SIRT1** play a crucial role in lipid metabolism. Sirtuins are a family of NAD⁺ dependent deacetylases and have been shown to play a role in longevity, DNA repair and metabolism. SIRT1 seems to be a master regulator in hepatic lipogenesis and fatty acid β -oxidation: it decreases fatty acid synthesis via the degradation of SREBP-1c and increases β -oxidation via the activation of Ppar gamma coactivator 1alpha (PGC-1 α). Lieber and colleagues [42] have shown that alcohol feeding reduces hepatic *Sirt1* mRNA by 50% and *Pgc-1 α* mRNA by 46% and also inhibits the protein expression of SIRT1 and PGC-1 α indicating that ethanol consumption downregulates important master regulators of metabolism which counteract lipogenesis therefore inducing steatosis. Recently, it has been shown that microRNA-217 promotes ethanol-induced fat accumulation in hepatocytes by down-regulation of *Sirt1* [43]. Down-regulation of *Sirt1* is associated with up-regulation of lipogenic enzymes such as *Scd1* and *Fas* and down-regulation of genes taking part in

β -oxidation such as *Pgc1 α* , *Ppara* and acyl-CoA oxidase (*Aox*) demonstrating its antilipogenic effects [44].

β -oxidation

β -oxidation is the biochemical process of fatty acid degradation and most of the processes necessary for β -oxidation take place in the mitochondria. Mitochondria also play a critical role in cellular energy supply (ATP production via the citric acid cycle and the electron transport chain) and in the formation of ROS. Especially because the liver is a predominant organ in the metabolism, hepatocytes are enriched with mitochondria. In the first step of β -oxidation, fatty acids ranging from the diet are oxidized in mitochondria by CPT1 into fatty acyl-carnitine which in turn is oxidised during several steps to acetyl-CoA. As described above, acetyl-CoA is also an accumulating metabolite from ethanol degradation. Acetyl-CoA is further metabolised in the citric acid cycle thereby reducing NADH and generating ATP (Fig. 2). Reduced NADH passes electrons to oxygen when getting oxidised again to NAD⁺. Excessive transport of electrons onto oxygen (as a consequence of accumulating acetate from ethanol degradation) and following reduction of oxygen to superoxide results in the production of ROS. Moreover, chronic ethanol administration alters mitochondrial oxidative phosphorylation by inhibiting the synthesis of proteins of the respiratory complexes. As a consequence, the oxidation system is overloaded and in combination with the reducing environment induced by ethanol, it results in an increased production of ROS. When the β -oxidation system is overloaded, fatty acids accumulate and get synthesized to triglycerides which also amass and form liver steatosis [31].

Oxidative stress: A disbalance between ROS and antioxidants

During ethanol degradation (especially CYP2E1 activity) and because of the inhibition of mitochondrial proteins ROS occur and cause massive cell damage (see above). Another source of ROS is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which is expressed in hepatocytes, hepatic stellate cells, and especially in Kupffer cells and neutrophils [45]. Occasionally, free radicals are responsible for apoptosis and DNA damage; thereby worst hit the mitochondrial DNA (mtDNA), which encodes the subunits of the electron transport. As mentioned above, in mice *Cyp2e1* deficiency and assumed diminished ROS production protects from hepatic steatosis. Two studies have also shown that *p47(phox)* knockout mice (which lack a critical subunit of NADPH oxidase) are protected from ROS production and liver steatosis. Bone marrow transplantation experiments have indicated that NADPH oxidase in parenchymal cells plays a major role compared to NADPH oxidase originating from bone marrow derived cells [46, 47].

The antioxidant enzyme Cu/Zn-superoxide dismutase (SOD1) is able to convert the toxic superoxide to H₂O₂. It has been shown that the delivery of *Sod1* with adenovirus reduces early alcohol-induced liver injury in rats and that mice lacking *Sod1* are prone to ALD [48, 49].

Another antioxidant enzyme is heme oxygenase-1 (HO-1) which is induced by oxidative stress and seems to play a protective role in ALD. An *in vitro* study by Nussler *et al.* [50] has demonstrated that primary human hepatocytes stimulated with ethanol produce high amounts of ROS. Pre- and co-incubation of cells with HO-1 inducers such

as nifedipine, verapamil and quercetin significantly reduce oxidative stress and cellular damage suggesting a protective role of HO-1 induction in ALD [50]. *In vivo*, the induction of *Ho-1* using Cobalt protoporphyrin (CoPP) ameliorates liver inflammation when mice feeding an alcohol diet [51].

Apoptosis and fibrosis: An association through the Hedgehog pathway

Apoptosis is the programmed cell death, which is characterised by blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. It can either be induced by mitochondria-independent, also called extrinsic (via activation of death receptors) or by mitochondria-dependent, also called intrinsic (DNA damage and ROS formation) pathways [52]. In ALD, the combination of both pathways induces apoptosis: On the one hand *TNF- α* is massively upregulated (see below) and activates the TNF-receptor 1 caspase cascade (via caspase 8), which in turn induces apoptosis. On the other hand, intrinsic signals such as DNA damage and ROS (as described above) induce the release of cytochrome c from permeabilized mitochondria into the cytoplasm. Cytochrome c initiates the ‘apoptosome’ and also the activation of the caspase cascade (via caspase 9) [53].

In ALD, fibrosis, which is the consequence of chronic liver injury, can also occur in 20-40 %. Hepatic stellate cells (HSC; also called Ito cells) are resident liver cells placed in the space of Disse (between hepatocytes and sinusoid) and are in a quiescent state in the healthy liver. When getting activated, they are involved in the process of liver fibrosis. In ALD, they are activated by either acetaldehyde directly or in a paracrine manner by apoptotic hepatocytes, activated KCs, leukocytes or by their released

fibrogenic mediators, such as transforming growth factor beta (TGF- β) and ROS [54]. A recent study by Jung *et al.* [55] demonstrates that the Hedgehog signalling pathway could play an important role in the pathogenesis of alcoholic fibrosis. The Hedgehog signalling pathway, which has its name from its ligand Hedgehog (Hh) is a master regulator in animal development thereby regulating apoptosis and the expansion and proliferation of various progenitor cells. Apoptotic hepatocytes produce Hh ligands, which is associated with apoptosis. Hh ligands in turn not only activate HSCs but also promote the transformation of hepatocytes to myofibroblastoids. This process is called epithelial to mesenchymal transition (EMT). It has been shown that Hh-responsive cells are increased in ALD: As described above TGF- β is upregulated in ALD and TGF- β treatment selectively promotes the viability of Hh-responsive immature liver cells and stimulates surviving hepatocytes to produce Hh ligands. These findings implicate that fibrosis is going along with apoptosis and also that parenchymal cells play an active role in hepatic fibrogenesis [55].

Gut permeability

In healthy individuals, the mucosal barrier is intact and only low levels of endotoxin (products from gram-negative bacteria especially lipopolysaccharide (LPS)) can be detected in the peripheral blood. After alcohol exposure, intestinal bacterial composition and its metabolism is altered: non-protective microorganisms such as *Proteobacteria* are overwhelming protective microorganisms such as *Bacteroidetes*. Additionally, breakdown of the gut barrier by direct and indirect alcoholic effects, as will

be explained below, results in the translocation of microbial products such as LPS into the circulation that promote liver disease by activating pro-inflammatory pathways [56]. Already years ago, various studies have shown that acute and chronic alcohol ingestion increases levels of endotoxin (especially LPS) from the intestinal microbiota in the blood of humans and mice [57]. Another study also has demonstrated that the endotoxin-binding capacity of whole blood in patients with alcoholic liver disease is decreased [58]. Furthermore, in ALD a positive correlation between LPS levels and the grade of liver injury is observed [59]. Concerning the chicken-and-egg problem, a study by Keshavarzian *et al.* [60] has been shown suggestive evidence that intestinal oxidative stress, intestinal hyperpermeability and endotoxemia occur before the development of ASH rather than to be the consequence of liver disease. They demonstrate that preventing gut leakiness (assessed by urinary excretion of lactulose and mannitol) by oats also prevents from endotoxemia and improves liver injury [60]. Whether endotoxemia emerges because of barrier defects, dysbiosis of the gut microbiota or reduced endotoxin clearance and binding capacity remains unclear: Various studies have highlighted that chronic alcohol ingestion increases intestinal permeability via a direct damage of the intestinal junctions (such as occludin) by ethanol and its degradation product AA (degradation of ethanol via bacterial alcohol dehydrogenase), intestinal oxidative stress or zinc deficiency [61-63]. Various studies have shown that the microbial composition of the intestine influences the progression of multiple extraintestinal diseases such as obesity and diabetes and also psychiatric disease such as schizophrenia and infectious diseases such as HIV infection [64-66]. Microbiota diversity and changes have been focus of current studies. Mutlu and colleagues [67] have been shown that a subpopulation of alcoholics has abnormal alterations in their colonic microbiome termed “dysbiosis”. These patients

have lower median abundances of *Bacteroidetes* and higher abundances of *Proteobacteria* and these alterations seem also to correlate positively with endotoxemia [67]. To my knowledge, there are no studies which have investigated the effect of administrating these bacteria on ALD progression, but treating intestinal dysbiosis by oral administration of antibiotics (e.g. polymyxin B and neomycin) reverses liver injury which is accompanied with lower LPS levels in mice [68]. Also germ free mice (which are mice without any microorganisms in their gut or on their skin) fed an alcohol-supplemented drinking water are protected from liver injury compared to conventional mice fed an alcohol diet. Interestingly, also the conventionalization (which means the installing of foreign microbiota into an organisms) of germ-free mice with intestinal contents from alcohol-fed conventional mice induces liver and gut inflammation further indicating that alcohol induces disease progressing dysbiosis [69]. Two studies have also shown that the oral treatment with either the supernatants of the probiotic *Lactobacillus rhamnosus* Gorbach-Goldin or the live bacteria ameliorates acute alcohol-induced intestinal permeability and liver injury [70, 71]. This indicates a pivotal role for the misbalance of the intestinal microbiome and gut leakiness in ASH.

Hepatitis/Inflammation

As described in the prior chapter, endotoxemia is a pathophenomen in both, human and murine ALD. The most studied endotoxin LPS binds to lipopolysaccharide binding protein (LBP) or soluble cluster of differentiation 14 (CD14). LBP is necessary to initiate the binding of LPS to its cell membrane receptor toll like receptor 4 (TLR4) and its two

co-receptors CD14 and myeloid differentiation factor 2 (MD2). TLR4, CD14 and LBP play a critical role in the pathogenesis of ASH as the deficiency in a (co-) receptor ameliorates ASH: *Tlr-4* deficient mice, *Cd14*-deficient and *Lbp*-deficient mice are protected from alcohol-induced liver injury [72-74].

LPS activates **hepatic macrophages**, so called KCs which represent the main source of pro-inflammatory cytokines. KC depletion by gadolinium chloride (GdCl₃), a selective KC toxicant, protects from alcohol-induced ALT elevation, inflammation, steatosis and necrosis indicating a pivotal involvement of KCs in the pathogenesis of ALD [75]. Once activated, the LPS/TLR4 signalling ends up in the activation of various transcription factors such as the nuclear factor κ B (NF- κ B) and activator protein 1 (AP1). These transcription factors initiate the production of many pro-inflammatory cytokines such as TNF- α and Interleukin-1 β (IL-1 β) and in turn IL-6, IL-8/ chemokine (C-X-C motif) ligand 1 (CXCL-1) and other chemokines leading to the migration and activation of other immune cells like neutrophils, monocytes and lymphocytes [76].

Macrophages can not only be activated but can also be polarized towards a specific phenotype: either the pro-inflammatory M1 (also called “classically activated”) or the anti-inflammatory M2 (also called “alternatively activated”) phenotype [77]. A study by Louvet *et al.* [78] has shown that the activation of the cannabinoid receptor 2 (CB₂), which shows potent anti-inflammatory properties, inhibited M1 polarization and favours the transition to an M2 phenotype which in turn displays beneficial effects on alcohol-induced inflammation and steatosis [78]. Further, a recent study has demonstrated that patients with lower hepatic injury exhibit higher hepatic expression of M2 genes compared to patients with more severe liver lesions. Interestingly, these patients show a significant induction of macrophage apoptosis indicating that limiting macrophage/KC activation and their survival protects from alcohol induced liver injury. *In vivo* and *in*

vitro studies suggested that IL-10 released from M2 polarized cells induce apoptosis of M1 polarized cells [79].

Neutrophils are phagocytes normally found in the circulation and recruited to tissue in case of infection or inflammation. During inflammation neutrophils are mobilized massively from the bone marrow and get activated by pro-inflammatory cytokines and chemokines, such as IL-17 and especially IL-8/CXCL-1 (so called “priming”). After priming, neutrophils accumulate in the liver sinusoids and transmigrate into the liver parenchyma along a chemokine gradient. Transmigration requires the interaction between adhesion molecules on neutrophils with endothelial cells via so called integrins such as vascular cell adhesion protein 1 (VCAM-1). Once migrated in the liver tissue, neutrophils kill pre-sensitized hepatocytes mostly by oxidant stress and proteases. ASH is characterised by neutrophilic infiltration, a process which correlates with disease severity and the upregulation of chemokines especially IL-8/CXCL-1 is associated with enhanced neutrophilic infiltration in the liver and disease [80, 81].

A recent study by Bertola and colleagues [82] has shown that E-selectin is induced in a murine model of ASH and also that elevated E-selectin plays an important role in hepatic neutrophil infiltration: *E-selectin*-deficient mice are protected from neutrophil infiltration and inflammation, but not from liver steatosis. The detrimental role of neutrophils in ALD is supported by the finding that the depletion of neutrophils with an anti-Ly6G antibody protects against disease [82].

TNF- α appears to be a key cytokine in the pathogenesis of ALD. Already more than twenty years ago it has been shown that TNF- α is increased in plasma as well as in liver tissue of patients with severe ASH. Moreover, the elevation of TNF- α levels in the blood is associated with a higher mortality [83, 84]. Also plasma levels of the soluble TNF- α receptors p55 (TNF-R1) and p75 (TNF-R2) have been shown to increase with

the severity of liver disease, reaching a maximum in patients with severe acute alcoholic hepatitis within liver cirrhosis [85]. Yin *et al.* [86] have shown that ethanol feeding causes liver injury in wild-type (WT) and *Tnf-R2* knockout mice but not in *Tnf-R1* knockout mice, providing solid evidence that TNF- α plays an important role in the development of early alcohol-induced liver injury via the TNF-R1 pathway [86]. So it has been obvious to test if TNF- α blockade could be a therapeutical option and could improve human disease. Against expectation blocking TNF- α in severe ASH has revealed divergent results in clinical trials (as discussed above).

IL-1 has been one of the first cytokines discovered. The IL-1 superfamily has substantial gained interest over the years, actually consisting of 11 cytokines which act in a pro- or anti-inflammatory manner. IL-1 β is the most prominent representative part of this family and plays a crucial role in many human diseases such as rheumatoid arthritis, gout and type 2 diabetes. Similarly, patients with alcoholic hepatitis have increased serum IL-1 activity [87]. A recent study by Petrasek and colleagues [88] has highlighted the relevance of IL-1 and the inflammasome activity (which regulates IL-1 β activation) in ALD. Mice lacking either subunits of the inflammasome (Caspase 1 or PYD and CARD domain containing (*Pycard*)) which activate IL-1 β or mice lacking the IL-1 receptor (*Il-1R*) are protected from ALD. Similarly, the IL-1 receptor antagonist Anakinra potently protects from ASH [88]. These data demonstrate the importance of the IL-1 signalling pathway in the pathogenesis of ALD and hold out hope of new therapeutic options. A clinical phase 2/phase 3 study is ongoing.

IL-6 seems to be a hepato-protective cytokine in the pathogenesis of ASH. Various studies have shown that *Il-6*-deficiency in mice aggravates ethanol-induced liver injury. It has been shown that IL-6 protects from oxidative stress and mitochondrial dysfunction by mediating mitochondrial DNA repair, induces anti-apoptotic proteins as

well as PPAR α and decreases liver and serum levels of TNF- α . Also an oral treatment with the IL-6 inducer ME3738 improves ALD in rats [89-92].

IL-8/CXCL-1 (also known as neutrophil chemotactic factor) induces chemotaxis especially in neutrophils but also in other granulocytes and stimulates their migration to the site of infection or inflammation [93]. In early studies, it has already been shown that IL-8 is upregulated in ASH and levels correlate with disease severity, mortality and neutrophil infiltration. In contrast, there has been detected only a moderate elevation of IL-8 levels in alcoholic cirrhosis or while alcohol withdrawal and levels in non-alcoholic liver disease are comparable low [94-96].

Our group has already studied IL-8 signalling in a murine model of alcoholic liver disease. We have been able to show that blocking of the neutrophilic receptor for IL-8 (chemokine (C-X-C motif) receptor (CXCR) 1/2) and in turn blocking neutrophil migration with so called pepducins improves hepatic inflammation in murine ALD [97]. Also **IL-17** is known to attract neutrophils: IL-17 plasma levels and IL-17 producing T lymphocytes as well as hepatic IL-17-secreting cell infiltrates (including T lymphocytes and neutrophils) are dramatically increased in patients with ALD compared to patients with other liver diseases indicating a pivotal role of IL17 in liver neutrophil recruitment and disease progression of ASH [98].

Monocyte chemoattractant protein-1 (MCP-1)/CXCL2 is elevated in patients with ALD. Deficiency of *Mcp-1* protects mice from alcoholic liver injury, independent of its receptor chemokine (C-C motif) receptor (CCR) 2, by inhibition of pro-inflammatory cytokines and induction of genes related to fatty acid oxidation. Therefore, MCP-1 has been suggested as a link from chemokines to hepatic lipid metabolism [99].

In summary, many pathogenic pathways in parenchymal as well as myeloid cells converge on the expression of cytokines that regulate the susceptibility to ALD with its broad spectrum of disease pathology. The next chapter deals with a molecule which has not been anticipated with ALD yet.

Lipocalin 2

Lipocalin 2 (LCN2) also known as neutrophil gelatinase associated lipocalin (NGAL) oncogene 24p3, uterocalin or siderocalin, is small 24kDa glycoprotein and belongs to the lipocalin superfamily. Lipocalins are characterised by their similar structure rather than by their sequence identity (often less than 20%). All the same is the typical cup-shaped cavity which enables lipocalins to bind their specific ligands [100].

At first, LCN2 has been purified from murine kidney cells which were infected with the simian virus. Later it has been shown, that in humans, LCN2 is co-located with myeloperoxidase (MPO) in the granules of neutrophils and is also expressed in other organs such as the breast, bone marrow, kidney, liver, trachea, lungs, intestine and adipose tissue. Previous studies have demonstrated that LCN2 is dramatically elevated in inflammatory and metabolic diseases. As such, it is considered as an acute phase protein [100]. In the following chapters, I want to discuss the various biological functions of LCN2.

LCN2 in infection and inflammation

Initially, LCN2 has been discovered as a molecule binding to so called siderophores. Siderophores are enzymes produced by gram-negative bacteria which bind to free iron and transport it into the microorganisms where iron promotes bacterial proliferation. LCN2 is upregulated and secreted from immune and epithelial cells after TLR4 activation. It binds to siderophores and these complexes are transported into the host cells thereby removing essential iron from bacteria in a bacteriostatic manner as it has been demonstrated for *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumonia* and *mycobacteria* [101-105]. Srinivasan *et al.* [106] have shown, that LCN2 is highly upregulated after LPS injection and second, that *Lcn2*-deficient mice show delayed hypoferrremia, elevated leukocyte apoptosis and increased mortality during endotoxin-induced sepsis indicating that TLR4 ligation induces LCN2 to prevent from invading pathogens by iron deprivation [106]. Further, it has been demonstrated that cells (i.e. macrophages) lacking the functional TLR4 receptor expressed low levels of *Lcn2* upon the stimulation with LPS highlighting that functional TLR4 receptor signalling is necessary for *Lcn2* induction [107].

Recently, it has been shown that LCN2 also plays a siderophore-independent role in bacterial infections: Endogenous LCN2 deactivates macrophages during pneumococcal infection thereby exerting anti-inflammatory functions. Deactivated macrophages are a subtype of macrophages down-regulating pro-inflammatory mediators and inducing anti-inflammatory cytokines especially IL-10 which mediates the anti-inflammatory effects of LCN2. Furthermore, in an infection with gram-positive pneumococci, high LCN2 levels are a sign of macrophage deactivation and indicative of a poor outcome because of an impaired immune response [108].

Recent studies have also demonstrated that LCN2 was upregulated by proinflammatory cytokines such as IL-1 β . It has been known that neutrophils

accumulate at site of inflammation and promote inflammation by respiratory burst and emptying of their granules. As described above, LCN2 is contained in neutrophilic granula and released at site of infection. A murine heart transplantation study by Aigner *et al.* [109] has demonstrated that *Lcn2* deficient (*Lcn2*^{-/-}) grafts exhibit reduced neutrophil infiltration after transplantation compared to WT grafts suggesting that LCN2 is required for the recruitment of neutrophils. Schroll *et al.* [110] have further investigated in the role of LCN2 in neutrophil function and demonstrated that recombinant LCN2 stimulates the chemotaxis of murine and human neutrophils *in vitro*. Furthermore, they have been able to show that *Lcn2*^{-/-} neutrophils exert a diminished chemotactic activity *per se* probably due reduced expression of adhesion molecules.

LCN2 in oxidative stress

Neutrophils produce ROS and oxidative stress by NADPH oxidase activity, but LCN2 itself seems to act in an antioxidant manner: *In vitro* studies have demonstrated that the upregulation of *LCN2* results in the upregulation of various antioxidant enzymes such as *SOD1* and *HO-1*, which is associated with decreased NF-κB expression. Concordantly, these *LCN2*-overexpressing cells are more resistant to toxic substances [111]. Furthermore, a study by Roudkenar *et al.* [112] has demonstrated that *in vitro* silencing of *LCN2* made cells more susceptible to ROS toxicity indicating that LCN2 is necessary to handle with oxidative stress.

LCN2 in apoptosis

The pro- or anti-neoplastic role of LCN2 in carcinogenesis seems to depend on the cancer entity. On the one hand, *LCN2* is induced in various cancers (e.g. breast, stomach, oesophagus, brain) and promotes survival and growth via the sequestration of iron and the stimulation of metastasis via the promotion of the EMT (described above) *in vitro*. On the other hand, LCN2 inhibits the synthesis of vascular endothelial growth factor (VEGF) and also the pro-neoplastic transcription factor hypoxia inducible factor 1 α (HIF 1 α) thereby acting anti-neoplastic shown for other malignities such as colon, ovary and pancreas cancer *in vitro* [113, 114].

In a human hepatoma cell line, *LCN2* overexpression induces cell apoptosis via cleavage of caspases. Caspase inhibitors again abolish LCN2-induced apoptosis and an LCN2 neutralizing antibody also significantly attenuates LCN2-induced apoptosis indicating that *LCN2* overexpression can promote apoptosis in liver cells. This study also demonstrates that *LCN2* and *LCN2R* are downregulated in tumour tissues of 16 HCC patients showing that the cellular context is important for the determination of LCN2-mediated effects. As described above, apoptosis is a pathomechanism activated in ASH and LCN2-deficiency could protect from alcohol induced apoptosis in ALD [115].

LCN2 in liver function and steatosis

Recent studies have demonstrated that various stimuli such as fructose, high fat diet, liver damage due to toxic substances (such as tetrachloromethane (CCl₄), LPS and Concanavalin A) or partial hepatectomy induce LCN2 expression. This appears to impact on disease severity in several of these models as *Lcn2*^{-/-} mice suffer from more severe disease [116, 117]. Recently, it has been shown that LCN2 seems to play a

protective role in liver steatosis: Feeding a methionine choline-deficient (MCD) diet induces more lipid accumulation in *Lcn2*^{-/-} mice compared to WT controls. The authors suggest that the loss of *Lcn2* blocks normal intracellular lipid droplet formation via reduction of the lipid droplet coat protein Perilipin 5 (PLIN5) both *in vitro* and *in vivo* [118]. A study by Guo *et al.* [119] in 2010 also has showed that *Lcn2*^{-/-} mice fed a high fat diet over 16 weeks are more susceptible to insulin resistance and obesity compared to WT mice. In later studies the same authors have demonstrated that LCN2 is a serious selective modulator of PPAR γ activation and plays a role in adipose tissue remodelling [119, 120].

Thus, we conclude that LCN2 exerts many functions in various biological processes starting from infections and inflammation to malignancies and steatosis. These data present evidence that LCN2 plays an anti-inflammatory, anti-infectious and anti-oxidative role in various cellular settings and is essential for the function and migration of neutrophils and macrophages.

Aims of the study

Alcoholic liver disease leads to a dramatic comorbidity with a high mortality when reaching the stage of alcoholic steatohepatitis. Treatment options of this disease entity are rare. A previous study has already shown that LCN2 is upregulated in ALD and plays a crucial role in liver function, but it has never been studied whether LCN2 plays a role in the pathogenesis of ALD [116, 121]. LCN2 is an enzyme which exerts multifactorial functions and could play an essential role in the pathogenesis of ALD for the following reasons: LCN2 is necessary to cope with endotoxemia [106], which is a key feature of ASH. Further, LCN2 could act as antioxidant [112], protects from steatosis [118] and deactivates macrophages [108]. On the other side lack of LCN2 could prevent from alcohol induced apoptosis [115] and hepatic neutrophil recruitment [110].

The aim of this study is to show that multifactorial acting LCN2 is regulated in human ASH as well as in our mouse model of ALD. Second, we want to further investigate if and by which mechanisms LCN2 plays a role in the pathogenesis of ALD thereby maybe opening a new treatment option. To address this issue we feed WT and *Lcn2*^{-/-} mice an established alcohol-containing or an isocaloric control diet for 14 days and evaluate liver inflammation, steatosis, fibrosis and apoptosis. *Lcn2*^{-/-} mice are missing the gene encoding for Lipocalin 2 protein. To evaluate neutrophil migration *in vivo* we establish a neutrophil specific tracking setting.

Material and Methods

Animal experiments and mouse strains

We use C57Bl/6 wild-type (WT; Charles River Laboratories, Wilmington, Massachusetts) and Lipocalin 2 knock-out (*Lcn2*^{-/-}) mice. *Lcn2*^{-/-} were kindly provided from Prof. Günther Weiss (Medical University Innsbruck). All animal experiments are approved by the ethic committee as well as the ministry of science and research. Mice are bred in the „Zentrale Tierversuchsanlage“ of the Medical University of Innsbruck: Animals are housed in sterilised Type II long cages with air filters adhering to day and night rhythm of a 12 hour cycle.

Characteristics of the *Lcn2*^{-/-} mouse strain [105]:

***Lcn2*^{tm1Aade}**

Name: lipocalin 2; targeted mutation 1, Alan Aderem

MGI ID: MGI:3525578 Synonyms: *Lcn2*-

Phenotype: involves: 129P2/OlaHsd * C57BL/6 *Lcn2*^{tm1Aade}/*Lcn2*^{tm1Aade}

Mortality/aging: increased sensitivity to induced morbidity/mortality (within 42 hours after *E.coli* H9049 infection, more than 80% of homozygotes died compared to 0% of wild-type)

Chronic alcohol model

Six to eight weeks old female C57BL/6 WT and *Lcn2*^{-/-} mice are used for the acute or chronic model of ALD. For the chronic alcohol model, two mice per cage are housed and fed the liquid Lieber-DeCarli diet (Bioserv, Flemington, NJ; Cat. No. F1258; see Table 1) ad libitum with first 0 % (vol/vol) ethanol for two days, afterwards 1 % (vol/vol) ethanol for further 2 days, followed by 3 % (vol/vol) ethanol for further 2 days, and from day 6 on with 5 % (vol/vol) ethanol (36 % ethanol-derived calories) for overall two weeks; in the control diet, alcohol is replaced with an isocaloric amount of maltose [88, 122]. Special liquid diet feeding tubes are used (Bioserv, Flemington, NJ; Cat. No. 9019). Mice are monitored and weighed daily. At the beginning and on day 10 of the experiment about 30 µl of blood is taken via puncture of the facial vein to determine the baseline liver transaminases. Mice are euthanatized in the morning of day 15.

Figure 3. Feeding tubes and feeding protocol for inducing ALD in mice.

(A) Special feeding tubes are used to feed the liquid Lieber De-Carli diet (left photo). (B) WT and *Lcn2*^{-/-} mice are fed the Lieber De-Carli diet for 14 days, starting with a maltose-supplemented formula. After two days, half of the mice is used to ethanol, starting from 1% (vol/vol) on day 3 and 4, 3% (vol/vol) on day 5 and 6 and 5% (vol/vol) from day 6 on. Blood specimens (illustrated as red drops) are collected by facial vein puncture before starting the experiment and on day 10. Mice are sacrificed on day 15.

Table 1. Nutritional Profile of the Lieber De-Carli diet (adopted from [123])

Delivering Solutions™

◆ Nutritional ◆ Enrichment ◆ Medicated ◆ Special Needs

Nutritional Profile

Product# F1258 - Rodent Liquid Diet, Lieber-DeCarli '82, Ethanol, 4 Liters/Bag

Proximate Profile

Protein	%	28.8
Fat	%	30.5
Fiber	%	7.3
Ash	%	5.5
Moisture	%	<10
Carbohydrate	%	25.8

Caloric Profile

Protein	kcal/Liter	151
Fat	kcal/Liter	359
Carbohydrate	kcal/Liter	135
Ethanol	kcal/Liter	355
Total	kcal/Liter	1000

Amino Acids

Alanine	gm/Liter	1.0
Arginine	gm/Liter	1.3
Aspartic Acid	gm/Liter	2.3
Cystine	gm/Liter	0.6
Glutamic Acid	gm/Liter	7.4
Glycine	gm/Liter	0.9
Histidine	gm/Liter	1.0
Isoleucine	gm/Liter	2.0
Leucine	gm/Liter	3.0
Lysine	gm/Liter	2.7
Methionine	gm/Liter	1.2
Phenylalanine	gm/Liter	1.6
Proline	gm/Liter	3.7
Serine	gm/Liter	2.1
Threonine	gm/Liter	1.6
Tryptophan	gm/Liter	0.4
Tyrosine	gm/Liter	2.1
Valine	gm/Liter	2.4

Carbohydrates

Monosaccharides	gm/Liter	2.1
Disaccharides	gm/Liter	5.3
Polysaccharides	gm/Liter	23.8

Fatty Acids

C18:2 Linoleic	gm/Liter	9.3
C18:3 Linolenic	gm/Liter	0.3
Total Saturated	gm/Liter	5.2
Total Monounsaturated	gm/Liter	23.5
Total Polyunsaturated	gm/Liter	9.7

Minerals

Calcium	gm/Liter	1.3
Chloride	gm/Liter	0.40
Copper	mg/Liter	1.5
Chromium	mg/Liter	0.50
Fluoride	mg/Liter	0.25
Iodine	mg/Liter	0.05
Iron	mg/Liter	9.3
Magnesium	gm/Liter	0.13
Manganese	mg/Liter	14.7
Phosphorus	gm/Liter	1.2
Potassium	gm/Liter	0.90
Selenium	mg/Liter	0.05
Sodium	mg/Liter	267
Sulfur	mg/Liter	84.8
Zinc	mg/Liter	8.9

Vitamins

Ascorbic Acid	mg/Liter	0.0
Biotin	mg/Liter	0.05
Choline	mg/Liter	206
Folic Acid	mg/Liter	0.5
Niacin	mg/Liter	7.5
Pantothenic Acid	mg/Liter	3.7
Pyridoxine	mg/Liter	1.4
Riboflavin	mg/Liter	1.5
Thiamin	mg/Liter	1.5
Vitamin A	IU/Liter	6029
Vitamin B ₁₂	mcg/Liter	25
Vitamin D ₃	IU/Liter	400
Vitamin E	IU/Liter	39.3
Vitamin K ₃ (Menadione)	mg/Liter	0.13

Ingredients

Casein, Olive Oil, Maltodextrin, Corn Syrup Solids, Cellulose, Mineral Mix, Corn Oil, Suspending Aid, Safflower Oil, Vitamin Mix, L-Cystine, Choline Bitartrate, DL-Methionine

These are typical amounts of nutrients calculated from available information. Actual assay results may vary. For more information contact Jaime Lecker, Ph.D. Phone: 800-996-9908 ext. 112 (U.S. and Canada) 908-996-2155 (International) Email: jlecker@bio-serv.com.

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The Lieber De-Carli diet (BioServ, Cat. No. F1258) is supplemented with ethanol or an isocaloric amount of maltose (Nutricia, Vienna, Austria) and used to induce ALD in mice.

Acute alcohol model model

It has already been shown that a single ethanol administration causes an elevation of liver transaminases, steatosis and inflammation [88]. For this so called model of “binge drinking” we administer a bolus of ethanol (5 g/kg body weight; 33 % diluted in drinking water) into the stomach of the mice using a 24-gauge stainless steel feeding tube (“gavage”) [122]; control mice receive an isocaloric amount of maltose resolved in drinking water. Mice are euthanatized four hours later [122].

Harvesting

Mice are anesthetized with Xylazin (LLOYD Inc., Shenandoah, Iowa; 5 mg/kg body weight) and Ketamin (Vétoquinol, Ravensburg, Germany; 100 mg/kg body weight). Total body weight is measured before taking blood samples. Blood is collected with heparinised capillaries (BRAND, Wertheim, Germany; Cat. No. 749311) via the retro orbital sinus and centrifuged (10 min, 2000 g, 20° C) to extract plasma. Plasma is used to determine liver transaminases, blood ethanol concentration, blood LPS concentration and plasma cytokine levels.

In a next step livers are excised: After taken the weight to determine the liver-to-body ratio livers are split for snap freezing, RNA stabilization Reagent (QIAGEN, Hilden, Germany; Cat. No. 1018087) samples, frozen section and histological examination. Further, spleens, lymph nodes and the intestine are collected for histological examination and are snap frozen in liquid nitrogen.

For histological examination, tissue pieces are embedded in 10% formalin solution in cassettes (SIMPORT, Beloeil, Canada; Cat. No. M493-6) to fix tissue for histological sections. An additional liver piece is embedded in Tissue Tek® O.C.T (Sakura Finetek Europe B.V., Alphen aan Den Rijn, The Netherlands; Cat. No.1015400010) in tissue embedding molds (Polysciences Europe, Eppelheim, Germany; Cat. No. 18986) and frozen in Methylbutan (Merck, Darmstadt, Germany; Cat. No. 1.06056.1000) to fix tissue for frozen sections for TUNEL and Oil Red O staining.

Determination of the alanine aminotransferase (ALT)

Blood samples are defrosted on ice before performing the assay. ALT (or glutamate pyruvate transaminase (GPT)) is measured with the ALT reagent (BQ Kits, San Diego, California; Cat. No. BQ004C-CR), which catalyses a chemical reaction in the presence of ALT and coupled NADH oxidation gets measured. Reagent is reconstituted in 12 ml of Aqua destillata (A. d.) and incubated at 37° C. 10 µl of samples are pipetted in a 96well plate and incubated until 37° C. Then, 100 µl of ALT reagent is added per well and measured in the pre-heated photometer after one minute. Zero absorbance is measured and four additional absorbance readings are measured at one minute intervals for all specimens. Afterwards, mean absorbance change per minute is calculated (DA/min) and multiplied by 1768 to determine IU/L of ALT activity.

Determination of LPS

Blood samples are defrosted on ice before performing the assay. Blood endotoxin levels are measured using Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, Massachusetts, Cat. No. 88282). Specimens are diluted 1:50 in Endotoxin-free water (A. d.) and heat-inactivated before performing the assay. E. coli standard contains 34 EU/vial after dilution in 1 ml of Endotoxin-free water. Limulus Amebocyte Lysate (LAL; an extract from amoebocytes (Limulus polyphemus) which reacts with endotoxins) is dissolved in 1,4 ml of A. d., chromogenic substrate (which changes its colour in presence of a specific enzyme) is dissolved in 6,5 ml A. d. (end concentration ~2 mM). All components are incubated at 37° C prior performing the assay. 50 µl of standards and pre-diluted samples are pipetted into a 96well plate and also incubated at 37° C. 50 µl of the LAL is added and the plate is shaken for 10 sec on a plate shaker and gets incubated for another 10 min at 37° C. Substrate solution is added after incubation time and incubated for 7 min at 37° C. Afterwards, 50 µl of Stop Reagent are added to stop the reaction with substrate solution. After shaking for another 10 sec, absorbance is measured at 405-410 nm. Standard curve is calculated with Excel (Microsoft, Redmond, US) and according to that also concentrations of the samples are determined.

Determination of ethanol

Blood samples are defrosted on ice before performing the assay. Blood ethanol levels are measured using the EnzyChrom Ethanol Assay Kit (BioAssay Systems, Hayward, CA; Cat. No. ECET-100). Enzyme A is reconstituted by adding 120 µl Enzyme Buffer. Ethanol standard is prepared by 25 µl of 1 % Standard with 225 µl A. d.

(0,1% Ethanol). Also 0,06 % and 0,03 % Ethanol standards are prepared. For each sample, working reagent is prepared by mixing as following: 80 µl Assay Buffer, 1 µl Enzyme A, 1 µl Enzyme B, 2,5 µl NAD and 14 µl MTT. 10 µl of samples are pipetted into a 96 well plate and 90 µl of Working solution is added quickly. Plate gets incubated 30 min at room temperature. After incubation time, 100 µl of stop solution are added and plate is measured at 565 nm (520-600 nm). Standard curve is calculated with Excel (Microsoft, Redmond, US) and according to that also concentrations of the samples are determined.

Determination of plasma cytokines

Blood samples are defrosted on ice before performing the assay. Standards (Bio-Rad, Hercules, California; Cat. No. 171-I50001) are dissolved in 500 µl diluent buffer (Bio-Rad, Hercules, California; Cat. No. 171-304070M) and incubated on ice for 30 min and further prepared as 1:4 dilutions as recommended in the protocol. Beads (Bio-Rad, Hercules, California) are diluted 1:10 in assay buffer. Wells of a 96well plate are filled with 100 µl of assay buffer and aspirated again using a vacuum pump. Beads are vortexed, 50 µl of them are pipetted into a well and aspirated again. Plate is washed with 100 µl wash buffer per well twice and 50 µl of sample are added and incubated for 1 hour on a shaker in the dark. During incubation time antibodies (Bio-Rad, Hercules, California; listed below) are prepared to 1:10 dilutions. Samples are aspirated and the plate is washed for a total of three washes. Afterwards, 25 µl of the antibody mix are added per well and incubated for another 30 min on a shaker and protected from light. Antibody mix is aspirated and the plate is washed for a total of

three washes. 50 µl of Streptavidin are pipetted per well and incubated for 10 min on a shaker. Streptavidin, which is necessary for signal amplification, is aspirated and the plate is washed for a total of three washes again. 125 µl of assay buffer are added per well and measurement is started immediately after adding the buffer using the Bio-Plex MAGPIX multiplex reader (Bio-Rad, Hercules, California).

Antibodies used for Bio-Plex cytokine analysis (Bio-Rad, Hercules, California):

Reagent kit (Bio-Rad, Hercules, California; Cat. No. 171-304070M)

Bio-Plex Pro™ Mouse Cytokine TNF-α Set #171-G5023M

Bio-Plex Pro™ Mouse Cytokine IL-1β Set #171-G5002M

Bio-Plex Pro™ Mouse Cytokine IL-6 Set #171-G5007M

Bio-Plex Pro™ Mouse Cytokine IL-10 Set #171-G5009M

Bio-Plex Pro™ Mouse Cytokine KC Set #171-G5018M

Bio-Plex Pro™ Mouse Cytokine MCP-1 (MCAF) Set #171-G5019M

Plasma *Lcn2* was measured by ELISA (see below)

Determination of liver triglycerides

Liver lipid extraction: Before measuring liver triglyceride levels it is necessary to extract total liver lipids. Fatty-free bovine serum albumin (BSA; 2,5 %) pre-coated tubes are used for all steps of extraction. Frozen liver pieces are weighted and immediately put into an equal amount of ice-cold phosphate buffered saline (PBS; 5 ml per 1 g liver tissue) and placed on ice. Liver is homogenised with a tissue disruptor for 10 sec and afterwards put on 95° C thermoblock for 30 min. In a next step, samples are

centrifuged at 12000 g for 15 min at 20° C whereby lipid phases are centrifuged above cell debris. Supernatants containing dissolved lipids are collected and stored at -80° C until measurements.

Triglyceride measurement: Samples are defrosted on ice and mixed for 15 sec before performing the assay. TG reagent (Roche Diagnostics, Risch, Schweiz; Cat. No. 10016648 122) is used for the determination of triglyceride (TG) contents, Präzinorm U [122 mg/dl] (Roche Diagnostics, Risch, Schweiz; Cat. No. 10171735 122) is used as standard. TG reagent is incubated at 37° C before use. 10 µl of sample are pipetted into a 96 well plate, 100 µl of TG reagent are added and incubated at 37° C for 15 min. After incubation time, extinction values are measured at 450 nm. Standard curve is calculated with Excel (Microsoft, Redmond, US) and according to that also triglyceride concentrations per g liver tissue are determined.

Measurement of liver proteins (ELISA)

Protein extraction: Liver proteins are extracted according to the recommended protocol of the MPO-Elisa (Hycult Biotech, PB Uden; The Netherlands). In short: Samples are lysed using the lysis buffer specified below (Table 2; pH 7.4), which is necessary to optimize protein stabilisation. 200 µl of lysis buffer are added to 10 mg of liver tissue before homogenisation with the tissue disruptor. After homogenization for 15 sec, samples are centrifuged twice with 1500 g at 4° C for 15 minutes) to avoid contamination of cell debris. All steps are performed on ice and tissue homogenates are stored at -80° C.

Table 2. Lysis Buffer for protein extraction

NaCl (Carl Roth, Karlsruhe, Germany, Cat. No. 3957.1)	200 mM
TRIS (biomol, Hamburg; Germany; Cat. No. 08003.1)	10 mM
EDTA (Dinatriumsalz Dihydrat, Carl Roth, Karlsruhe, Germany; Cat. No. 8043.1)	5 mM
Glycerin (MERCK, Darmstadt, Germany; Cat. No. 4095, 250ml)	10 %
PMSF (Phenylmethylsulfonylfluoride, (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. P-7626)	1 mM
Leupeptin (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. L2884)	1 µg/ml
Aprotinin (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. A1153)	28 µg/ml

Protein measurements using ELISA: On the day before doing the assay, NUNC ELISA plates (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. 439454) are coated with the capture antibody diluted in PBS as recommended in the data sheet, covered with an adhesive strip and incubated overnight (O/N) at room temperature or on 4° C depending on the ELISA kit.

NOTE: For all non-pre-coated ELISA kits only half of the recommended amount is used. For example, plates are coated only with 50 µl of capture antibody instead of recommended 100 µl.

On the next day, each well is aspirated and washed with wash buffer (0.05% Tween20 in PBS, pH 7.2-7.4) for a total of three washes. After the last wash, remaining Wash Buffer is removed by inverting the plate and blotting it against clean paper towels. Plates are blocked by adding 300 µL of Reagent Diluent (1% BSA in PBS or 10% fetal calf serum (FCS) in PBS regarding to the manufacture's recommendation; pH 7.2-7.4) to each well. Plates are covered with an adhesive strip and incubated for at least 1 hour. After this blocking step, plates are washed again for three times. In case of pre-coated ELISA kits, coating and blocking can be skipped and assay procedures are started at the following step (using the amounts as indicated in the protocol).

Supernatants are defrosted on ice and mixed for 15 sec prior performing the assay. Standards are prepared as recommended (diluted in Reagent Diluent). 50 µl of sample or standard are added per well, plate is covered again and incubated for two hours at room temperature. Afterwards plate is washed three to five times. After washing, 50 µl of the Detection Antibody, diluted in Reagent Diluent, are added to each well and the plate is covered with a new adhesive strip and incubated two hours at room temperature. Three to 50 µL of the working dilution of Streptavidin-HRP are added to each well. Plates are covered and incubated for 20 min at room temperature in the dark. Three last wash steps are performed before adding 50 µL of the TMB Substrate Solution (Genemed Biotechnologies, South San Francisco, CA; Cat. No. 10-0009) to each well. The plate is incubated for another 20 min at room temperature in the dark. After adding 25 µl of Stop Solution (60 % sulfuric acid) to each well, the plate is gently tapped to ensure thorough mixing. Optical density of each well is immediately measured at 450 nm. Standard curve is calculated with Excel (Microsoft, Redmond, US) and according to that also protein concentrations per g liver tissue are determined.

Following ELISA kits are used:

Mouse MPO ELISA kit (Hycult Biotech, PB Uden; The Netherlands; Cat. No. HK210-02)

Mouse CXCL1/KC DuoSet (R&D Systems, Minneapolis; Cat. No. DY453)

Mouse Lipocalin-2/NGAL DuoSet (R&D Systems, Minneapolis; Cat. No. DY1857)

Mouse TNF- α ELISA Set (Becton Dickinson, Franklin Lakes, New Jersey; Cat. No. 555268)

Mouse IL-6 ELISA Set (Becton Dickinson, Franklin Lakes, New Jersey; Cat. No. 555240)

Mouse IL-1 β ELISA Set (Becton Dickinson, Franklin Lakes, New Jersey; Cat. No. 559603)

Determination of liver RNA levels (qPCR)

RNA isolation: RNA is isolated with TRIzol Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, Cat. No. 15596-018), protocol is used as recommended in the data sheet. Tissue samples are stored in RNA later at 4° C as described above and are homogenised in 1500 μ l of TRIzol Reagent for about 15 sec with a tissue disrupter. Cells are directly lysated in TRIzol by pipetting about 30 times in the culture plate. RNase-free pipette tips and tubes are used in all steps to avoid RNase contaminations.

Homogenised tissue or cells are incubated for 5min at room temperature to permit complete dissociation of the nucleoprotein complex. Afterwards, 0,3 ml of chloroform are added and capped tubes are vortexed for 15 sec. Samples are incubated for 2-3 min at room temperature and centrifuged at 12000 g for 15 min at 4° C. During centrifugation, the mixture separates into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase, which is taken up and put into a new vial. An isovolemic amount of 100 % isopropanol is added to the RNA and mixed for another 15 sec. After incubation at room temperature for 10min, samples are centrifuged at 12000 g for 10 min at 4° C. After centrifugation, the RNA forms a small pellet sitting on the side or bottom of the tube. Supernatant is removed from the tube, leaving only the RNA pellet. Then, the pellet is washed with 1 ml of ethanol and the tube is centrifuged at 7500 g for 5 min at

4° C. Supernatant is removed again and RNA pellet gets air-dried in the tube for about 5-10 min. After drying, the pellet gets dissolved in an appropriate amount of distilled water to a concentration of about 1 µg/µl. Samples are incubated in a water bath or heat block set at 55-60° C for 10-15 min.

Determining yield of RNA: To determine the concentration of isolated RNA, 2 µl of RNA are dissolved in 98 µl of A. d. and measured in the photometer at 260 nm and 280 nm. A concentration about 1 µg/µl is favoured and if necessary RNA is diluted once more in RNase-free water.

Reverse transcription for cDNA synthesis using M-MLV (Thermo Fisher Scientific, Waltham, Massachusetts, Cat. No. 28025-21): A 20 µl reaction volume is used for 1 µg of total RNA (dissolved in 8 µl of A. d.) as described in Table 3.

Table 3. Reaction mix for Reverse transcription

M-MLV Kit (Thermo Fisher Scientific, Waltham, Massachusetts, Cat. No. 28025-21)	
Reverse Transkriptase	1 µl
5 X Puffer	4 µl
0,1 M DTT	2 µl
RNase OUT , Ribonuclease inhibitor (Thermo Fisher Scientific, Waltham, Massachusetts; Cat. No. 10777-019)	0,5 µl
p(dT) Primer (Roche Diagnostics, Risch, Schweiz; Cat. No. 10 814 270 001)	1 µl
dNTP (GE Healthcare Life Sciences, Buckinghamshire, UK; Cat. No. 28-4065-52)	2 µl
A. d.	1,5 µl
RNA (diluted in A. d.)	8 µl

Rainbow strips (Grainer Bio-One, Holzkirchen, Germany; Cat. No. 673271) are used for the PCR and a “no template” control (consisting of 8 µl A. d.) is also transcribed to exclude contamination. For PCR a thermocycler with the following conditions is used:

Annealing and elongation (arising of cDNA): incubation for 60 min at 37° C

Denaturation of the Reverse Transcriptase: incubation for 10 min at 95° C

After PCR cDNA is stored at 4° C or -20° C until Sybr-Green PCR.

Quantitative Sybr-Green PCR: To determine up-regulation of specific genes, cDNA sequences are amplified by polymerase chain reaction. For these reactions, specific primers for genes of interest are designed and produced by Microsynth (Balgach, Switzerland) and mixed with the mastermix and cDNA as recommended in Table 4 in special strips (Stratagene, California, US; Cat. No. 410022).

Table 4. Sybr-Green PCR Mix (volume for 1 duplicate)

MESA GREEN qPCR Mastermix Low Rox Assay (Eurogentec, Seraing, Belgium; Cat. No. RT-Sy2X-06+W0U2R)	28,75 µl
Forward Primer (Microsynth, Balgach, Switzerland)	0,86 µl
Reverse Primer (Microsynth, Balgach, Switzerland)	0,86 µl
A. d.	24,73 µl

qPCR was performed with the MX3000 P cycler (Stratagene, California, US) and cycles are set as follows:

1. Denaturation for 10 min at 95° C
2. Primer annealing for 30 sec at 60° C
3. Elongation for 30 sec at 72° C

Steps 2 and 3 are repeated for a total of 40 cycles, each time at the end of primer annealing fluorescence is detected. Primer sequences are detailed in Table 5.

Table 5. Primer sequences

Target gene	Forward Primer sequence	Reverse Primer sequence
<i>c-βActin</i>	5'-GATGCTCCCCGGGCTGTATT-3'	5'-GGGGTACTTCAGGGTCAGGA-3'

Cat1	5'-AGCGACCA ATGAAGCAGTG-3'	5'-TCCGCTCTCTGTCAAAGTGTG-3'
Cd206	5'-GCTTCCGTCACCCTGTATGC-3'	5'-TCATCCGTGGTTCATAGACC-3'
Cd3	5'-CGTCCGCCATCTTGGTAG-3'	5'-CCTGGCAAGTGCCAACAG-3'
Cd45	5'-CAGATATGACCATGGGTTTGTG-3'	5'-TTCCACTCCAAACAAATTGAAG-3'
Cd68	5'-CATGGCGCAGAATTCATCT-3'	5'-AGAGAAACATGGCCCGAAGT-3'
Cldn1	5'-GGGGACAACATCGTGACCG-3'	5'-AGGAGTCGAAGACTTTGCACT-3'
Cldn3	5'-ACCAACTGCGTACAAGACGAG-3'	5'-CACCAACGGGTTATAGAAATCCC-3'
Cldn4	5'-GTCCTGGGAATCTCCTTGGC -3'	5'-TCTGTGCCGTGACGATGTTG-3'
Cldn7	5'-GGCCTGATAGCGAGCACTG-3'	5'-GTGACGCACTCCATCCAGA-3'
Ck19	5'-GACCTAGCCAAGATCCTGAGT-3'	5'-TCAGCTCCTCAATCCGAGCA-3'
Cxcl-1	5'-CTGGGATTCACCTCAAGAACATC-3'	5'-CAGGGTCAAGGCAAGCCTC-3'
Cyp2e1	5'-GAGGTGCTACTGAACCACAAG-3'	5'-CGGAGGATACTTAGGGAAAACC-3'
F4/80	5'-GACAATTGGGATCTGCCCTA-3'	5'-GGCCCTCCTCCACTAGATTC-3'
Fas	5'- AGGTGGTGATAGCCGGTATGT-3'	5'-TGGGTAATCCATAGAGCCCAG-3'
Gpx	5'-GGGCAAGGTGCTGCTCATTG-3'	5'-AGAGCGGGTGAGCCTTCTCA-3'
Gr-1	5'-GCTGTCCTCTTAACAGTGCTG-3'	5'-AGGTACTTGTTTAGTGGGAGGG-3'
Ho-1	5'-AAGCCGAGAATGCTGAGTTCA-3'	5'-GCCGTGTAGATATGGTACAAGGA-3'
Icam1	5'-TCCGCTACCATCACCGTGTAT-3'	5'-TAGCCAGCACCGTGAATGTG-3'
IL-1β	5'-GCAACTGTTCTGAACTCAACT-3'	5'-ATCTTTTGGGGTCCGTCAACT-3'
IL-6	5'-TGTTCTCTGGGAAATCGTGGA-3'	5'-AAGTGCATCATCGTTGTTCATACA-3'
IL-10	5'-ACCAGCTGGACAACATACTGCTAA -3'	5'-TAAAACTGGATCATTTCGGATAAGG -3'
Inos	5'-TAGTCAACTGCAAGAGAACGGAGA-3'	5'-CTTTCAGGTCACTTTGGTAGGATT-3'
Itga4	5'-TGGGCACAGGTGGAAAAATA-3'	5'-ATTCCAGCTTGACATGATGC-3'
Itgb1	5'-ATGCCAAATCTTGCGGAGAAT-3'	5'-TTTGCTGCGATTGGTGACATT-3'
Itgb7	5'-CTGCTGCCTCTCCATCTGTA-3'	5'-AGGTCAGGATCCTCCCATTC-3'
Lcn2	5'-TGGCCCTGAGTGTCATGTG-3'	5'-CTCTTGAGCTCATAGATGGTGC-3'
Lcad	5'-TCTTTTCCTCGGAGCATGACA-3'	5'-GACCTCTCTACTCACTTCTCCAG-3'
Madcam1	5'-TGTTCCGAATGACACAACGCT-3'	5'-AAGCTGCCAATCCATAGGACG-3'
Mcp-1	5'-GCTGTAGTTTTTGTACCAAGC-3'	5'-GCTGAAGACCTTAGGGCAGAT-3'
Mip-2	5'-CCAACCACCAGGCTACAGG-3'	5'-GCGTCACACTCAAGCTCTG -3'
Ocln	5'-TTGAAAGTCCACCTCCTTACAGA -3'	5'-CCGGATAAAAAGAGTACGCTGG-3'
Pecam1	5'-GGTGATGGCGTATCCAAG-3'	5'-TGGAGGTCTTATCTATCCTTCGC-3'
Pgc-1a	5'-TATGGAGTGACATAGAGTGTGCT-3'	5'-CCACTTCAATCCACCCAGAAAG-3'

<i>Pparaα</i>	5'-CTGTCATCACAGACACCCTCTC-3'	5'-TATTCGACACTCGATGTTTCAGG-3'
<i>Ppary</i>	5'-GTCTCACAATGCCATCAGGTT-3'	5'-GTTTCAGCTGGTTCGATATCACTG-3'
<i>Srebp1c</i>	5'-CCGGGGAACTTTTCCTTAAC-3'	5'-TGGTTGTTGATGAGCTGGAG-3'
<i>Sirt-1</i>	5'-GCTGACGACTTCGACGACG-3'	5'-TCGGTCAACAGGAGGTTGTCT-3'
<i>Socs3</i>	5'-GATTTTCGCTTCGGGACTAGCT-3'	5'-GGTACTCGCTTTTGGAGCTG-3'
<i>Sod1</i>	5'-AACCAGTTGTGTTGTCAGGAC-3'	5'-CCACCATGTTTCTTAGAGTGAGG-3'
<i>Tgf-β</i>	5'-CTCCCGTGGCTTCTAGTGC-3'	5'-GCCTTAGTTTGGACAGGATCTG -3'
<i>Tjp-1</i>	5'-GCTTTAGCGAACAGAAGGAGC-3'	5'-TTCATTTTTCCGAGACTTCACCA-3'
<i>Tnf-α</i>	5'-GGGAGTAGACAAGGTACAACCC-3'	5'-ATCTTCTCAAAATTCGAGTGACAA-3'
<i>Vcam-1</i>	5'-AGTTGGGGATTTCGGTTGTTCT -3'	5'-CCCCTCATTCTTACCACCC-3'
<i>Srebp1c</i>	5'-CCGGGGAACTTTTCCTTAAC-3'	5'-TGGTTGTTGATGAGCTGGAG-3'
<i>Ym1</i>	5'-CAGGTCTGGCAATTCTTCTGAA-3'	5'-GTCTTGCTCATGTGTGTAAGTGA-3'
<i>Xbp1</i>	5'-ACACGCTTGGAATGGACAC-3'	5'-CCATGGGAAGATGTTCTGGG-3'

Note: Primers are tested using a melting curve and have been plotted on an agarose gel electrophoreses to test for unspecific bands.

Oil Red O-Staining: Detection of lipid droplets on liver tissue slides

For Oil Red O-Staining, 5-10 μ m frozen liver sections are cut and dried for 30-60 min at room temperature on Menzel Gläser Super Frost ® Plus (VWR, Darmstadt, Germany; Cat. No. J1800AMNZ). Slides are fixed in ice-cold 10 % formalin solution for 5-10 min and air-dried for 30-60 min. Afterwards, slides are rinsed with distilled water, air-dried for a few min and incubated in 100 % propylene glycol for 10 min. Next, slides are incubated in 60° C pre-heated Oil Red Solution (0,5 % in propylene glycol; Amresco, Solon, OH; Cat. No. 0684) for 8-10 min and incubated in 85 % propylene glycol for 2-5 min. Then, slides are rinsed in distilled water twice and counter-stained

in Mayer's Hematoxylin Solution (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. MHS32) for 30 sec. Slides are blued beyond running tap water for 1 min and rinsed in water one before tiling in liquid Kaisers Glyceringelatine (MERCK, Darmstadt, Germany; Cat. No. 1.09242.0100) and topping with cover slips (Langenbrinck, Emmendingen).

Staining of neutrophils

The 91C Naphthol AS-D Chlorazetate Kit (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. 91C-1KT) was used to stain neutrophils on liver tissue slides. Previously, paraffin slides have been cut by the department of Pathology of the Medical University of Innsbruck and got de-coated and dehydrated before starting the procedure as detailed below:

- 2x5 min in Xylol
- 2x4 min in 100 Vol% Ethanol
- 1x4 min in 90 Vol% Ethanol
- 1x4 min in 80 Vol% Ethanol
- 1x4 min in 70 Vol% Ethanol
- A. d.

After de-coating from paraffin, slides are fixed in CAF-fixing solution (Table 6) for 30 sec at room temperature and rinsed beyond running tap water afterwards. Then, slides get incubated in the staining solution (Table 6) for 15 min at 37° C and rinsed with water for 2 min. Slides get counter-stained with Mayer's Hematoxylin Solution (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. MHS32) for 2 min and rinsed beyond

running tap water afterwards for another 3min. After rinsing in A. d., the slides are tiled with Kaisers Glyceringelatine (MERCK, Darmstadt, Germany; Cat. No. 1.09242.010). For sealing, slides are topped with cover slips (Langenbrinck, Emmendingen).

Table 6. Solutions for the Naphthol AS-D Chlorazetate Kit (SIGMA-ALDRICH, St. Louis, Missouri)*

CAF-fixer solution	Citrate solution	12,5 ml
	Aceton (Scharlab, Sentmenat, Barcelona, Spain; Cat. No. AC0310)	32,5 ml
	Formaldehyde 37% (Carl Roth, Karlsruhe, Germany; Cat. No. 4979.1)	4 ml
Staining solution** (components are contained in the kit)	A. d.	40 ml
	Sodium nitrite solution	1 ml
	Fast Red Violet LB standard solution	1 ml
	TRIZMAL	5 ml
	Naphthol AS-D Chloracetate solution	1 ml

*Note: For a volume of 50 ml.

**Note: A. d. was pre-incubated at 37° C. Natriumnitrit-Lösung und Fast Red Violet were mixed gently and incubated for 2 min, afterwards A. d., TRIZMAL und Naphthol AS-D Chlorazetate were added.

Staining of lymphocytes, macrophages, LCN2, VCAM-1 and Claudin1:

Immunohistochemistry (IHC) of CD3, MAC2, LCN2, VCAM-1 and Claudin1

Paraffin sections are used paraffin gets de-coated as follows:

- 2x5 min in Xylol
- 2x4 min in 100 Vol% Ethanol
- 1x4 min in 90 Vol% Ethanol
- 1x4 min in 80 Vol% Ethanol

- 1x4 min in 70 Vol% Ethanol
- A. d.

Antigen retrieval: After de-coating, slides are incubated in Tris-EGTA-buffer (TEG; 10 mM Tris, 0,5 mM EGTA, pH 9.0) for 30 min in a “Braun MultiGourmet Dampfgarer” (pre-heated for 20min) to break the protein-protein interactions. After cooling for 20 min slides are rinsed in A. d. and tissue sections are surrounded by fatty rings.

Blocking endogenous peroxidase: To reduce unspecific background, tissue sections are incubated for 10 min in Peroxidase-Blocking Solution (Dako, Santa Clara, California, Cat. No. S2023) and rinsed in buffer for 5 min. Unspecific binding sites are blocked by incubation in serum-free protein block (Dako, Santa Clara, California, Cat. No. X0909) for 10 min at room temperature.

Immunohistochemical staining: Tissue sections are incubated with primary antibody in antibody diluent (Dako, Santa Clara, California, Cat. No. S0809) for 1 h or overnight at recommended temperatures (room temperature or 4° C, respectively). On the next day, tissue sections are washed for 5min with wash buffer (Standard Tris-buffered saline (NaCl) with Tween20) and incubated with secondary antibody in antibody diluent as recommended. Again, tissue sections are rinsed for 5 min with the buffer and developed using ImmPACT AMEC Red Peroxidase (HRP) Substrate (Vector Laboratories, Burlingame, Ca; Cat. No. SK-4285) for recommended times depending on the antibody (listed in Table 7). Reaction is stopped within tap water and slides get counterstained with Mayer’s Hematoxylin Solution (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. MHS32) for 30 sec and rinsed in tap water afterwards. Slides are

coverslipped with VectaMount AQ aqueous mounting medium (Vector Laboratories, Burlingame, Ca; Cat. No. H-5501).

Table 7. Antibodies for IHC

Target	First Antibody and preferred incubation	Secondary Antibody and preferred incubation
LCN2	Anti-rat Lipocalin-2/NGAL antibody (R&D Systems, Minneapolis; Cat. No. AF3508) 1:50, O/N, 4° C	Polyclonal rabbit anti goat-Immunglobulins/HRP (Dako, Santa Clara, California; Cat. No. P0449) 1:100, 1 h
CD3	Anti-CD3 antibody (Dako, Santa Clara, California; Cat. No. #A0452) 1:200, 1 h, room temperature	ImmPRESS Reagent anti-rabbit Ig (Vector Laboratories, Burlingame, Ca; Cat. No. MP-7401) 30 min
MAC2	Anti-mouse/human MAC2 antibody (cedarlane, Burlington, Ontario, Canada; Cat. No. CL8942AP) 1:1000-1:2000, 1 h, room temperature	ImmPRESS Reagent anti-rat Ig (Vector Laboratories, Burlingame, Ca; Cat. No. MP-7444) 30 min
Ly-6B.2	Anti-mouse Ly-6B.2 antibody (AbDSerotec, Kidlington, UK; Cat. No. MCA771G) 1:50, 1 h, room temperature	ImmPRESS Reagent anti-rat Ig (Vector Laboratories, Burlingame, Ca; Cat. No. MP-7444) 30 min

FACS analysis of infiltrating and resident liver immune cells

Isolation of myelocytes from liver tissue for fluorescence-activated cell sorting (FACS) analysis: The digestion mix (Table 8) is prepared before collecting liver samples. After anesthetising the mouse and taking blood samples, whole liver is taken out and stored in ice-cold RPMI 1640 medium (life technologies, Cat. No. 61870-044) on ice (until up to eight livers have been harvested). Afterwards, livers are put on the Petri dishes and cut into pieces (2-5 mm) with a scissor or scalpel blade. Half of the liver is transferred into a 2 ml tubes and incubated in 1500 µl of digestion mix on a thermoblock at 37° C and 1000 rpm shaking. Digested livers are put onto a wire mesh and rubbed with the

backside of a 20 ml syringe. Cell suspension is collected with a pipette in 10 ml of RPMI and centrifuged with 300 g at 4° C for 5 min. Supernatant is thrown away and the pellet is resuspended in 5 ml of ACK lysis buffer (Table 9), shortly vortexed and kept at room temperature for 3 min to lyse red blood cells. Afterwards, the suspension is centrifuged at 300 g at 4° C for 5 min again and supernatant is discarded again. The cell pellet is resuspended in 5 ml of FACS buffer (PBS 0,5 % FCS, 2 µM EDTA) and strained through a 70 µm cell strainer (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. CLS431751) into a 50 ml falcon tube.

Isolation of blood cells for FACS analysis: Blood samples are collected as described above and red blood cells are lysed as described above (by adding 5 ml of ACK lysis buffer and 3 min incubation at room temperature). Afterwards, blood samples are centrifuged with 300 g at 4° C for 5 min. Pellets are resuspended in FACS buffer and stored on ice.

Table 8. Digestion mix for leukocyte isolation from liver tissue

Digestion mix (for 4 livers)	RPMI 1640 medium (life technologies, Cat. No. 61870-044)	15 ml
	Liberase Blendzyme (Roche Diagnostics, Risch, Schweiz; Cat. No. 11814176001) 28 WU/ml	0,85 ml
	DNaseI (Roche Diagnostics, Risch, Schweiz; Cat. No. 104159, 2 mg/ml Stock solution)	0,30 ml

Table 9. ACK lysis buffer

ACK lysis buffer [1 l]	NH ₄ Cl (Carl Roth, Karlsruhe, Germany; Cat. No. P726.1) 0.15 M	1.0 g
	KHCO ₃ (Carl Roth, Karlsruhe, Germany; Cat. No. P748.1) 10mM	8.3 g
	0.5 M EDTA (Carl Roth, Karlsruhe, Germany; Cat. No. 8043.10.) 1 mM	0,2 ml
	A. d. (pH 7,2 – 7,4)	Fill up to 1000 ml

Cell staining for FACS analysis: The following antibodies (all from BioLegend, San Diego, California) and dilutions are used to characterise cell populations:

Table 10. Antibodies for FACS analysis

Channel	Blood-myelocytes	Kupfer cells	Proliferation
Pacific Blue	DAPI (1: 20000)	DAPI (1: 20000)	DAPI (1: 20000)
Alexa 488	CD45.2 FITC (1:100)	CD45.2 FITC (1:100)	BrdU FITC (1:50)
PE	CD115 PE (1:100)	CD64 PE (1:100)	CD64 PE (1:100)
PE-Cy7	Ly6C PE-Cy7 (1:200)	F4-80 PE-Cy7 (1:100)	F4-80 PE-Cy7 (1:100)
APC	CD45.1 APC (1:100)	CD45.1 APC (1:100)	CD45.1 APC (1:100)
APC-Cy7	CD11b APC-Cy7 (1:100)	CD11b APC-Cy7 (1:100)	CD11b APC-Cy7 (1:100)
Alexa 700	NA	MHCII Alexa 700 (1: 100)	MHCII Alexa 700 (1: 100)
Rat serum	2%	2%	2%

APC anti-mouse CD45.1 Antibody (Cat. No. 110713)

FITC anti-mouse CD45.2 Antibody (Cat. No. 109805)

PE/Cy7 anti-mouse F4/80 Antibody (Cat. No. 123113)

PE anti-mouse CD64 (FcγRI) Antibody (Cat. No. 139303)

Alexa Fluor® 700 anti-mouse I-A/I-E Antibody (Cat. No. 107621)

APC/Cy7 anti-mouse/human CD11b Antibody (Cat. No. 101225)

PE anti-mouse CD115 (CSF-1R) Antibody (Cat. No. 135505)

PE/Cy7 anti-mouse/human CD11b Antibody (Cat. No. 101215)

PE/Cy7 anti-mouse Ly-6C Antibody (Cat. No. 128017)

FACS analysis: The Fortessa cell analyser (Becton Dickinson, Franklin Lakes, New Jersey) is used for FACS analysis. FACS analyses are performed by Piotr Tymoszek.

Cell sorting: Same cell isolation and antibody labelling procedures are used for cell sorting of hepatic myelocytes (monocytes, neutrophils, KCs). Cell sorting is performed

by Sieghart Sopper from the Medical University of Innsbruck, Hematology and Oncology.

Isolation and labelling of neutrophils for in vivo tracking

Neutrophil isolation from bone marrow: Mice get neck dissected and the femoral and tibial bones are taken out and put onto ice (all together about 50 million bone marrow cells (with 60 % neutrophils) are isolated from one mouse). Bones get flushed with MACS buffer (PBS 0,5 % FCS, 2 μ M EDTA) for several times using a syringe and a 27 G needle and suspension is collected in a tube filled with 15 ml of MACS buffer. Afterwards, cell suspension is filtered through a 100 μ m nylon filter (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. CLS431752) into a new tube. Cell suspension is centrifuged with 300g at room temperature for 10min. During centrifugation, 3 ml of Histopaque-1119 (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. 11191) gets overloaded with 3 ml of Histopaque-1077 (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. 10771), both density gradient cell separation mediums, in a 15 ml tube. After centrifugation, supernatant is removed and cell pellet is diluted into 6 ml MACS buffer. Cell suspension is pipetted onto the Histopaque layer and centrifuged again at 700 g and room temperature for 30 min.

After centrifugation, the upper layer contains mononuclear cells which are extracted by careful pipetting. Neutrophils are contained in the well visible ring in the middle of the tube and collected by pipetting out of the tube into a new tube. Volume is filled up to 10 ml with MACS buffer and centrifuged at 300 g and room temperature for 10 min.

Supernatant is thrown away and cell pellet is dissolved in 1 ml of MACS buffer. This wash step gets repeated for a total of 3 washes.

Labelling with carboxyfluorescein succinimidyl ester (CFSE; SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. 87444): Cells are dissolved to concentration of 5 millions/ml and incubated for 10 min in a 37° C warm water bath with 1 µM CFSE concentration. After incubation, 5 parts of cold MACS buffer are added and incubated on ice for 5 min. Afterwards, cell suspension is centrifuged at 300 g for 5 min at 4° C. After centrifugation, cells get washed with 3 ml of MACS buffer and this step is repeated for a total of three washes. After fifth centrifugation, cells get dissolved in 1 ml of MACS buffer only.

Neutrophil isolation using CD11b macs beads (Miltenyi Biotec, Bergisch Gladbach, Germany; Cat. No. 130-049-601) Beads are added to cell suspension as recommended in the protocol (20 µl beads per 10 million cells) and incubated at 4° C for 15 min. After incubation, 4 ml of MACS buffer are added and centrifuged at 300 g and 10° C for 10 min. The pellet is dissolved in 500 µl of MACS buffer and cell suspension is rinsed through a LS column (Miltenyi Biotec, Bergisch Gladbach, Germany; Cat. No. 130-042-401) of a MidiMACS™ Separator ((Miltenyi Biotec, Bergisch Gladbach, Germany). Afterwards, the column is rinsed with 3 ml of MACS buffer for three times. After removing the column from the separator, magnetically labelled neutrophils (sticked within the column) are rinsed out with 6 ml of MACS buffer into a new tube. Cells suspension is centrifuged at 300 g and 10° C for 10 min and cell pellet is dissolved in 1 ml of MACS buffer.

Isolated cells are analysed by FACS and exhibit a neutrophil purity of 92 %.

For *in vivo* tracking experiments, a total of 2 million cells (50% *Lcn2*^{-/-} neutrophils and 50% WT neutrophils) in a volume of 200 µl PBS are injected intravenously in a single

injection. Livers and blood samples are analysed for labelled cells by FACS analysis 4 hours after injection.

Chemicals and Equipment

Aqua destillata (A. d.; Fresenius Kabi Austria, Graz, Austria; Cat. No. 0712221/01A)

Bovine serum albumin (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. A6003; 2,5 %)

Chloroform (or trichlormethan; Carl Roth, Karlsruhe, Germany; Cat. No. 3313.1)

EDTA ((Dinatriumsalz Dihydrat, Roth, Cat. No. 8043.1)

Ethanol (GATT-KOLLER, Absam, Austria; Cat. No. 402043342)

FCS (Biochrom, Berlin, Deutschland; Cat. No. S0115)

Formalin solution (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. HT501128-4I)

Isopropanol (Propan-2-ol; VWR, Darmstadt, Germany; Cat. No. 20842.330)

Natriumchlorid (BRAUN; Cat. No. 350 5731)

PBS (Biochrom, Berlin, Deutschland; Cat. No. L1825)

Propylene glycol (Amresco, Solon, OH; Cat. No. 0575)

Sulfuric acid (Scharlab, Sentmenat, Barcelona, Spain; Cat. No. AC 2078)

Tween20 (SIGMA-ALDRICH, St. Louis, Missouri; Cat. No. P1379)

Xylol (GATT-KOLLER, Absam, Austria; Cat. No. 402259072)

Disinfectant (Ecolab healthcare, Monheim am Rhein, Germany; Cat. No. 3004450)

Gloves (Sempercare, Singapore, Asia)

Pipettes (Gilson, Mettmenstetten, Switzerland; Pipetman)

Pipette tips:

10 µl (Starlab, Yulee, FL; Cat. No. S1120-3810)

250 µl (Mettler-Toledo, Columbus, Ohio; Cat. No. GPS-250S)

1000 µl (Mettler-Toledo, Columbus, Ohio; Cat. No. GPS-1000)

filtered 200 µl (Mettler-Toledo, Columbus, Ohio; Cat. No. 17002912)

filtered 1000 µl (Mettler-Toledo, Columbus, Ohio; Cat. No. 17002897)

Pipet aid (Drummond Scientific Company, Broomall, PA)

Pipettes:

5 ml Serological Pipet (Becton Dickinson, Franklin Lakes, New Jersey; Cat. No. 357543)

10 ml Serological Pipet (Becton Dickinson, Franklin Lakes, New Jersey; Cat. No. 357551)

25 ml SPACE SAVER Serological Pipet (Becton Dickinson, Franklin Lakes, New Jersey; Cat. No. 357525)

50 ml Serological Pipet (Becton Dickinson, Franklin Lakes, New Jersey; Cat. No. 357550)

Multipette (Eppendorf, Hamburg, Germany; Multipette® plus)

Combitips:

0,2 ml (Eppendorf, Hamburg, Germany; Cat. No. 0030 069.412)

5 ml (Eppendorf, Hamburg, Germany; Cat. No. 0030 069.250)

10 ml (Eppendorf, Hamburg, Germany; Cat. No. 0030 069.269)

Tubes:

Cryovials (SIMPORT, Beloeil, Canada; Cat. No. T311-2)

1,5 ml safe lock (Eppendorf, Hamburg, Germany; Cat. No. 0030 120.086)

2,0 ml safe lock (Eppendorf, Hamburg, Germany; Cat. No. 0030 120.094)

15 ml Polypropylene Round-Bottom Tube (Becton Dickinson, Franklin Lakes, New Jersey;
Cat. No. 352070)

50 ml Polypropylene Conical Tube (Becton Dickinson, Franklin Lakes, New Jersey; Cat.
No. 352059)

Embedding cassettes (SIMPORT, Beloeil, Canada; Cat. No. M493-6)

Tissue Embedding Molds (Polysciences, Warrington, PA; Cat. No. 18986)

Tissue disruptor T-50 (IKA® Werke, Staufen im Breisgau, Germany)

Tissue disruptor T-25 (IKA® Werke, Staufen im Breisgau, Germany)

Minishaker (IKA® Werke, Staufen im Breisgau, Germany)

Braun MultiGourmet Dampfgarer" (Braun, Neu-Isenburg, Germany)

Thermocycler „T-Gradient“ (Biometra (Göttingen, Germany)

Centrifuges (Eppendorf, Hamburg, Germany)

Photometer (Eppendorf, Hamburg, Germany) and UVette® routine pack (Eppendorf,
Hamburg, Germany; Cat. No. 6,249,345)

Biological safety cabinet (Astec Microflow, Hampshire, UK)

Incubator (Sanyo, Moriguchi, Ōsaka)

Light microscope (Olympus, Shinjuku, Tokio, Japan; Zeiss, Oberkochen, Germany)

Micro scales (Sartorius, Göttingen, Germany; BP61S)

Scale (Kern, Balingen, Germany)

Statistical analysis

Data are analysed using GraphPad PRISM (La Jolla, CA, US). An unpaired Student's *t*-test is used for analysing significance between two groups. A two-way ANOVA with Bonferroni correction is used to calculate the statistical significance between more than two groups. *P*-values less than 0.05 ($P < 0.05$) are considered as significant and results are shown as mean values with standard error of the mean (+/- SEM).

Results

LCN2 is upregulated in human ASH

To evaluate a relevant role of LCN2 in human ASH we analysed the expression of LCN2 in serum and liver biopsies of patients with this disease entity compared to patients with NAFLD (i.e. histological proven simple steatosis) taken from two cohorts published previously [8, 124]. Our analysis revealed that LCN2 levels are upregulated 2-3 fold in serum of patients suffering from ASH compared to NAFLD controls (Fig. 4A). RNA levels were induced about 6-fold in patients with ASH compared to controls (Fig. 4B).

Figure 4. LCN2 is upregulated in human ASH.

(A) Serum samples from patients suffering from ASH were analyzed for LCN2 protein using ELISA. Patients with NAFLD were used as controls. **(B)** Total mRNA was isolated from human liver tissue and reverse transcribed. LCN2 mRNA was measured by qPCR. GAPDH was used as house-keeping gene. n=8 (A, B). Data are expressed as mean $\pm$ SEM, unpaired Student's *t*-test. ***P<0.001.

LCN2 is upregulated in a murine model of ASH

Recently, it was shown that LCN2 is up-regulated in a murine model of ALD but it is still unknown whether this has an influence on disease pathogenesis [121]. To evaluate LCN2 expression in our mouse model of ALD, we fed the ethanol (EtOH-fed) or the isocaloric control (Pair-fed) Lieber De-Carli diet to C57BL/6 WT mice for 14 days. Feeding WT mice an ethanol diet induced ALT-elevation (>30 fold) indicating severe liver injury (Fig. 5A). Histopathological analysis showed that chronic alcohol feeding in WT mice induced liver inflammation (Fig. 5B, C) and steatosis (Fig. 5D, E), both characteristics of human ALD.

Figure 5. Feeding an alcohol-supplemented Lieber De-Carli diet for 2 weeks induces severe liver injury in WT mice

C57BL/6 WT mice were fed an alcohol diet or a control diet and euthanized 15 days later. (A) Serum ALT was measured to determine liver injury. (B, C) Liver inflammation was scored histologically by KC accumulation. (D, E) Steatosis was assessed by percentage of Oil-red-O positive area calculated in Photo Shop. $n = 10$ mice per group (A-E). Data are expressed as mean $\pm$ SEM, unpaired Student's t -test. *** $P < 0.001$. Histological analysis was performed by Mathias Drach.

Alcohol feeding significantly upregulated liver LCN2 protein in leukocytes as demonstrated by IHC (Fig. 6A). Further, LCN2 mRNA expression and protein measurement (Fig. 6B, C) as well as serum LCN2 levels (Fig. 6D) were induced after alcohol feeding.

Figure 6. LCN2 is upregulated in alcoholic liver disease.

C57Bl6 WT mice were fed the alcohol diet or control diet and euthanized 15 days later. (A) LCN2 was stained by immunohistochemistry of liver sections (A, black arrows, magnification 25x). (B) Total mRNA was isolated from liver tissue and reverse transcribed. *Lcn2* mRNA (D) was measured by qPCR. β -Actin was used as house-keeping gene. (C) Total liver protein was extracted from liver tissue and LCN2 protein was measured also using ELISA. (D) LCN2 protein was measured in blood using ELISA. $n = 10$ mice per group (A-D). Data are expressed as mean $\pm$ SEM, unpaired Student's *t*-test. * $P < 0.05$, ** $P < 0.01$.

To detect the main source of LCN2, hepatic myeloid cells were FACS-sorted after control and ethanol feeding. RNA was isolated and mRNA levels of *Lcn2* were compared between neutrophils, monocytes and Kupffer cells (Fig. 7). Neutrophils were found to express the highest amounts of *Lcn2* but induction of *Lcn2* by ethanol mostly occurred in KCs and monocytes.

Figure 7. Neutrophils as the main source of LCN2.

C57Bl6 WT mice were fed an alcohol diet or a control diet and euthanized 15 days later. Hepatic myeloid cells were isolated from liver tissue as described in “Material and Methods” and neutrophils (defined as CD11b⁺ Ly6C^{lo} Gr-1^{hi}), monocytes (defined as CD11b⁺ Ly6C^{hi} Gr-1^{lo}) and KCs (defined as CD11b^{lo} F4/80^{hi} CD64⁺ MHCII^{lo}) were sorted. mRNA was isolated from different cell types and reverse transcribed. *Lcn2* mRNA was measured with qPCR in neutrophils, monocytes and KCs. *β-Actin* was used as house-keeping gene. *n* = 4 mice per group. Data are expressed as mean ± SEM, unpaired Student’s *t*-test. n.s = not significant. **P* < 0.05. Sorting procedure was performed by Sieghart Sopper (Medical University of Innsbruck, Hematology and Oncology).

***Lcn2*^{-/-} mice are protected from alcohol-induced liver injury**

To test whether LCN2 contributes to inflammation and pathology of ALD, we fed C57BL/6 WT and *Lcn2*^{-/-} mice the ethanol or control diet for 14 days and sacrificed them on day 15.

First, we observed that *Lcn2*^{-/-} mice gained more weight on a control diet compared to WT mice. Contrary to our expectations, we also noted that *Lcn2*^{-/-} mice lost significantly less weight during ethanol feeding compared to WT mice suggesting a better coping with ALD (Fig. 8A). When weighing body and livers at necropsy we observed a significant higher liver-body-ratio in the alcohol-fed WT group compared to the control-fed WT group, suggesting more steatosis in alcohol-fed WT mice compared

to alcohol-fed *Lcn2*^{-/-} mice (Fig. 8B). There was no difference between pair-fed WT mice and pair-fed *Lcn2*^{-/-} mice (Fig. 8B) ruling out a difference induced by Lieber De-Carli diet itself.

Figure 8. *Lcn2*^{-/-} mice are protected from alcoholic liver disease.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and sacrificed 15 days later. **(A)** Body weight was measured every other day. **(B)** Total body weight and liver weight was measured at necropsy and liver enlargement was determined by calculating liver body ratio. *n* = 10 mice per group (A, B). Data are expressed as mean ± SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Next, we measured blood ALT levels to determine degree of liver injury. Ethanol-fed WT mice developed a severe (>30 fold) ALT-elevation compared to pair-fed WT mice which was strongly reduced in *Lcn2*^{-/-} mice (Fig. 9A). Blood ethanol concentrations were measured to index the degree of intoxication and showed that *Lcn2*^{-/-} mice exhibited lower levels of ethanol detected in the blood (Fig. 9B). Furthermore, *Lcn2*^{-/-} mice exhibited less systemic serum endotoxin, suggesting that intestinal permeability was preserved during ethanol feeding in *Lcn2*^{-/-} compared to WT mice (Fig. 9C). To rule out a bias due to different alcohol ingestion, we measured drinking amounts daily.

Drinking amounts were comparable between different genotypes in the pair-fed group and in the alcohol-fed group (Fig. 9D).

Figure 9. *Lcn2*^{-/-} mice are protected from alcoholic liver disease despite similar consumption of EtOH.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol and a control diet and sacrificed 15 days later. (A) Liver injury was evaluated by plasma ALT. (B) Plasma ethanol and (C) plasma endotoxin levels were measured at the end of the experiment to determine intoxication and intestinal permeability. (D) Drinking amount was measured every day. *n* = 10 mice per group (A-D). Data are expressed as mean ± SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

Histological analysis showed a similar normal liver histology of pair-fed WT and *Lcn2*^{-/-} mice (Fig. 10A-C). Alcohol feeding strongly induced liver steatosis and inflammatory cell infiltration in WT mice, which was significantly less pronounced in *Lcn2*^{-/-} mice (Fig. 10A-C).

Figure 10. *Lcn2*^{-/-} mice are protected from steatosis and inflammation in alcoholic liver disease.

C57Bl/6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and sacrificed 15 days later. (A) Histological steatosis and inflammation were evaluated by a pathologist (magnification 25x). (B) Liver steatosis was determined as percent area of fatty vesicles in liver tissue sections. (C) Inflammation was determined per inflammatory cells / HPF. n = 10 mice per group (A-C). Data are expressed as mean ± SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s. = not significant. *P < 0.05, **P < 0.01, ***P < 0.001. Histological analysis was performed by Mathias Drach.

To further explain the protective phenotype of *Lcn2*-deficiency in ALD, we investigated the role of LCN2 in disease-characterising biological processes such as dysfunction in gut barrier, steatosis, fibrosis, apoptosis, oxidative stress and inflammation.

Gut permeability: fewer disturbances of colonic tight junctions in *Lcn2*^{-/-} mice

Alcohol-induced dysbiosis and local ethanol and AA toxicity disrupt intestinal integrity [61-63]. To determine intestinal permeability and possibly inflammation we first analysed H&E slides of the ileum and colon. We did not detect overt inflammation in WT or *Lcn2*^{-/-} mice at baseline or after alcohol feeding in the small or large intestine (data not shown). As we previously observed endotoxemia (suggesting loss of intestinal permeability, Fig. 9C) we studied the expression of tight junctions in colonic

(Fig. 11) and ileal (Fig. 12) specimens with and without ethanol feeding, as previously reported [125]. Interestingly, mRNA expression of the tight junction proteins Claudin 1 (*Cldn1*), *Cldn3* and Occludin (*Ocln*) significantly decreased upon alcohol challenge in the colon (Fig. 11) but not in the ileum (Fig. 12). Colonic *Cldn3* (Fig. 11B), *Cldn4* (Fig. 11C), *Ocln* (Fig. 11F) expressions were higher in alcohol-fed *Lcn2*^{-/-} mice compared to alcohol-fed WT mice indicating an obtained gut barrier which goes along with diminished serum LPS levels (Fig. 9C).

Figure 11. Intestinal permeability in colon.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15 days later. (A-G) Intestinal permeability was evaluated by measuring mRNA expression of tight junction proteins. Colon RNA was extracted and levels of *Cldn1* (A), *Cldn3* (B), *Cldn4* (C), *Cldn7* (D), cytokeratin 19 (*Ck19*; E), *Ocln*; (F) and tight junction protein 1 (*Tjp*; G) were measured using qPCR. β -Actin was used as housekeeping gene. n = 10 mice per group (A-G). Data are expressed as mean $\pm$ SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 12. Intestinal permeability in ileum.

C57Bl6 WT and $Lcn2^{-/-}$ mice were fed an alcohol diet and euthanized 15 days later. (A-G) Intestinal permeability was evaluated by measuring mRNA expression of tight junction proteins. Ileum RNA was extracted and levels of *Cldn1* (A), *Cldn3* (B), *Cldn4* (C), *Cldn7* (D), *Ck19* (E), *Ocln* (F) and *Tjp-1* (G) were measured using qPCR. β -Actin was used as housekeeping gene. $n = 10$ mice per group (A-G). Data are expressed as mean $\pm$ SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. * $P < 0.05$.

Deficiency of *Lcn2* ameliorates alcoholic liver steatosis

Steatosis represents an early stage of the hepatic manifestation of ethanol exposure and is also a histopathological feature of ASH [126]. To determine the degree of steatosis we stained lipid droplets on liver sections with Oil-red-O as described in Materials and Methods. Whereas alcohol strongly induced steatosis in WT mice, $Lcn2^{-/-}$ mice were protected from these processes (Fig. 13A, B), as already observed

on H&E sections (Fig. 10). To quantify steatosis, lipids were extracted from liver tissue and liver triglycerides were measured. Alcohol feeding elevated TG levels in WT and *Lcn2*^{-/-} mice but there were no differences between both phenotypes in the alcohol- and in the pair-feeding groups detectable (Fig. 13C).

Figure 13. *Lcn2*^{-/-} mice are protected from steatosis in alcoholic liver disease.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15days later. (A) Liver slides were stained with Oil-Red-O and (B) steatosis was assessed by percentage of Oil-red-O positive area calculated in Photo Shop. (C) To quantify liver steatosis lipids were extracted from liver tissue and triglycerides were measured. *n* = 10 mice per group (A-C). Data are expressed as mean ± SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

To evaluate lipogenesis and β -oxidation also on molecular levels we measured mRNA levels of master regulator proteins of lipid metabolism. We detected a strong increase of spliced *Xbp-1*, a lipogenic transcription factor, which is activated in a variety of liver diseases secondary to stress in the endoplasmic reticulum [30], after ethanol feeding in WT mice, which was completely abrogated in *Lcn2*^{-/-} mice (Fig. 14A, B). Anti-steatogenic enzymes such as *Sirt-1* [42], *Ppara* [36] and *Pgc1 α* [42] were expressed at lower level after ethanol feeding compared to pair-feeding in both genotypes (Fig. 14C-E). More importantly, we detected a significant lower reduction of *Sirt-1* and *Ppara* mRNA in *Lcn2*^{-/-} mice compared to WT mice (Fig. 14C, D). We did not observe

differential expression of *Adipoq*, *Srebp1c*, *Fas* and *Lcad* between WT and *Lcn2*^{-/-} mice after alcohol feeding (Fig. 14F-I).

Figure 14. *Lcn2*^{-/-} mice are protected from steatosis in alcoholic liver disease.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15 days later. (A-I) To assess lipogenesis and β -oxidation mRNA was isolated and reverse transcribed. cDNA was used for *Xbp1* splicing PCR and PCR products were separated by gel electrophoresis (A, lower band= 171bp, spliced *Xbp*; upper band= unspliced/total *Xbp*). The ratio spliced to unspliced *Xbp* was calculated by Image J (B). mRNA levels of *Sirt-1* (C), *Ppara* (D), *Pgc1 α* (E), *Adipoq* (F), *Srebp1c* (G), *Fas* (H), and *Lcad* (I) were measured using qPCR. β -Actin was used as housekeeping gene. $n=10$ mice per group (A-I). Data are expressed as mean $\pm$ SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

***Lcn2*^{-/-} mice are protected from alcohol-induced fibrosis**

Fibrosis is a histological change that occurs at progressed stages of liver disease. To determine alcohol-induced fibrotic changes, we stained hepatic sections for chromotrop-anilinblue (CAB). CAB trichrome staining was determined blinded to sample identity using a fibrosis severity score ranging from 0-5 (Fig. 13B, 0=absent, 1=marginally, 2=mild, 3=moderate, 4=severe, 5=very severe). CAB trichrome scoring nicely demonstrated that alcohol feeding induced moderate fibrosis in WT mice, but only mild fibrosis in *Lcn2*^{-/-} mice (Fig. 15A, B). Additionally, mRNA levels of the pro-fibrotic cytokine *Tgf-β* were measured in hepatic tissue from both genotypes with and without alcohol exposure. *Tgf-β* expression was induced by ethanol feeding in both genotypes, without differences between WT and *Lcn2*^{-/-} mice (Fig.15C).

Figure 15. Fibrosis is diminished in *Lcn2*^{-/-} mice after alcohol feeding.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15 days later. (A) Liver slides were stained with Trichrome to detect fibrotic areas and (B) fibrosis was assessed by histological scoring. (C) Additionally liver RNA was extracted and mRNA levels of *Tgf-β* were measured using qPCR. *β-Actin* was used as housekeeping gene. *n* = 10 mice per group (A-C). Data are expressed as mean ± SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s. = not significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Alcohol-induced apoptosis of hepatocytes is unaffected by *Lcn2*-deficiency

As described previously, apoptosis, as a consequence of ROS and TNF α induction, is another feature of alcoholic liver disease [53]. LCN2 was shown to be involved in apoptosis previously [115]. To test for programmed cell death and the influence of LCN2, we labelled nicked DNA of liver sections by TUNEL (TdT-mediated dUTP-biotin nick end labelling). Ethanol feeding induced hepatocyte cell death (as indicated by increased TUNEL positive hepatocytes) in both, WT and *Lcn2*-deficient mice to a comparable extent (Fig. 16A, B). As such, despite protection from ASH *Lcn2*-deficiency (Fig. 8, 9, 10, 11, 15) does not protect from hepatocyte death (Fig. 16). These data suggested that lack of *Lcn2* in hepatocytes might not be responsible for the protection from alcohol-induced disease, as hepatocytes were equally affected in both genotypes. Rather, we hypothesized that LCN2 function in immune cells might be regulating susceptibility to disease.

Figure 16. Apoptosis is comparable in WT and *Lcn2*^{-/-} in ALD.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15days later. **(A)** Liver slides were stained with TUNEL to detect apoptotic cells. **(B)** Apoptosis was assessed by percentage of positive area calculated in Photo Shop. *n*= 10 mice per group (A, B). Data are expressed as mean $\pm$ SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. *P < 0.05, **P<0.01, ***P<0.001.

Oxidative stress is comparable in WT and $Lcn2^{-/-}$ mice

Because LCN2 was shown to be involved in oxidative stress we next investigate its role in alcohol-induced oxidative stress [112, 127, 128]. Therefore, we measured various cytokines known involved in ROS generation (Cyp2E1, Cat, iNOS) and degradation (HO-1, SOD-1, GPX) (Fig. 17 A-F). We observed that ethanol feeding downregulated *Cyp2e1* as well as *Cat* and the anti-oxidative enzymes *Sod-1* and *Gpx-1*. KC-specific *iNos* and hepatic *Ho-1* were upregulated by alcohol feeding. Notably, alcohol-induced *Cyp2e1* downregulation was restored by *Lcn2*-deficiency probably explaining diminished Ethanol serum levels in *Lcn2^{-/-}* mice (Fig. 9B) because of a higher ethanol degradation rate through CYP2E1. Expression levels of other oxidant or anti-oxidant enzymes were comparable between WT and *Lcn2^{-/-}* mice after alcohol feeding (Fig. 17B-F). Therefore, against previous data, in our model alcohol-induced anti-oxidative mechanisms to cope with oxidative stress are unaffected by LCN2. Accordingly to previous data, we only observed less *Ho-1* expression in *Lcn2^{-/-}* compared to WT mice in the pair-fed group (Fig. 17D).

Figure 17. Enzymes involved in oxidative stress are comparable in WT and $Lcn2^{-/-}$ in ALD.

C57Bl6 WT and $Lcn2^{-/-}$ mice were fed an alcohol diet and euthanized 15 days later. (A-F) Oxidative stress was evaluated by measuring mRNA expression of oxidative and anti-oxidative enzymes. Liver RNA was extracted and mRNA levels of *Cyp2e1* (A), *Cat* (B), *iNos* (C), *Ho-1* (D), *Sod1* (E), *Gpx* (F) were measured using qPCR. β -Actin was used as housekeeping gene. $n=10$ mice per group (A-F). Data are expressed as mean $\pm$ SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Hepatic inflammatory cytokine patterns are comparable in WT and $Lcn2^{-/-}$ mice

As described previously, liver inflammation is a hallmark of human alcoholic steatohepatitis. To further test whether $Lcn2$ -deficient hepatocytes were unresponsive and impaired in their ability to secrete pro-inflammatory cytokines upon alcohol exposure, we measured hepatic cytokines which are known to be regulated in ALD. Alcohol feeding increased protein levels of pro-inflammatory cytokines such as TNF-

α , IL-1 β , IL-6, CXCL-1 and IL-10 (Fig. 18A-F) which was transcriptionally regulated (Fig. 19A-F). Importantly, we did not observe differences between WT and *Lcn2*-deficient mice, suggesting that *Lcn2* in extra-hepatic cells (e.g. of immune cells) drives ASH in WT mice in our model.

Figure 18. Hepatic cytokines in *Lcn2*^{-/-} mice are comparable to WT mice in ALD.

C57Bl/6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15days later. (A-F) For hepatic protein analysis, total protein was extracted from liver tissue and of IL-1 β (A), TNF- α (B), IL-6 (C), KC (D), MPO (E) and IL-10 (F) were measured by ELISA. $n = 10$ mice per group (A-F). Data are expressed as mean $\pm$ SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 19. Hepatic transcriptional regulation of cytokines in *Lcn2*^{-/-} mice is comparable to WT mice in ALD.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15days later. (A-F) For RNA expression analysis, mRNA was extracted from liver tissue and mRNA levels of *Il-1 β* (A), *Tnf- α* (B), *Il-6* (C), *Kc* (D), *Mcp-1* (E) and *Il-10* (F) were measured using qPCR. *β -Actin* was used as housekeeping gene. $n=10$ mice per group (A-F). Data are expressed as mean $\pm$ SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s. = not significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Systemic cytokine levels are comparable in WT and *Lcn2*^{-/-} mice

Alcoholic liver disease is further characterised by circulating cytokines such as TNF- α and IL-1 β demonstrating the systemic nature of this disease [86, 87]. Surprisingly, detection of circulating cytokines in serum samples of alcohol fed WT and *Lcn2*-deficient mice by Multiplex FACS analysis demonstrated similar levels of IL-1 β , TNF α , IL-6, KC, MCP-1 and IL-10, all implicated in a systemic response in human (Fig. 20A-

F). These data suggested that inflammation is still restricted to the liver after 2 weeks of alcohol exposure.

Figure 20. Inflammation in *Lcn2*^{-/-} mice is comparable to WT mice in alcoholic liver disease.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15days later. (A-F) Systemic inflammation was assessed by multiplex analysis of IL-1 β (A), TNF- α (B), IL-6 (C), KC (D), MCP-1 (E) and IL-10 (F). n.s = not significant. $n = 10$ mice per group (A-F). Data are expressed as mean $\pm$ SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. Bio-Plex analysis procedure was performed by Viktoria Pedit.

Reduced hepatic infiltration of inflammatory cells after alcohol feeding in

***Lcn2*^{-/-} mice**

Human ASH is histologically characterised by massive leukocyte infiltration [126]. Neutrophils and cells of monocytic origin (KCs, monocytes) have been implicated in the pathogenesis of ALD and are hall marks of human alcoholic steatohepatitis as previously described [75]. To understand why *Lcn2*-deficient mice were protected from disease, we characterised hepatic immune cell populations. We analysed numbers of MAC-2⁺ (myeloid cell marker) [129] and LY-6B⁺ (neutrophil marker) [130] by immunolabelling on paraffin sections [77]. Chronic alcohol feeding increased monocytes/macrophages (Fig. 21A, B) and neutrophils (Fig. 22A, B) in WT mice. In contrast, the robust infiltration of inflammatory cells of the innate immunity was strongly reduced in alcohol-fed *Lcn2*^{-/-} mice (Fig. 21A, B; Fig. 22A, B).

Figure 21. Diminished myeloid cells in livers of *Lcn2*^{-/-} mice compared to WT mice in alcoholic liver disease.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15 days later. Liver monocytes and macrophages were detected by (A) MAC2 immunohistochemistry and (B) MAC2 positive area was calculated with ImageJ. Images (25x magnification) are representative for $n=10$ (A, B). Data are expressed as mean $\pm$ SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant, ** $P<0.01$, *** $P<0.001$.

Figure 22. Diminished neutrophils in livers of *Lcn2*^{-/-} mice compared to WT mice in alcoholic liver disease.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15 days later. (A) Liver neutrophils were stained using a Ly-6B antibody and (B) counted per HPF. Images (25x magnification) are representative for $n=10$ (A, B). Data are expressed as mean $\pm$ SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant, **P < 0.01.

To better understand the distribution and potential impact of inflammatory cells in our model that goes beyond histological analysis, we performed FACS analysis of homogenised liver to characterise monocyte, neutrophil and KC populations.

First, we observed that the majority of hepatic immune cells that we assessed in WT mice were KC cells. Importantly, the number of KCs was reduced in *Lcn2*-deficient mice on a pair-fed diet which we also observed after alcohol exposure (Fig. 23A). Resident monocytes were diminished after alcohol feeding (Fig. 23B) whereas inflammatory monocytes were found at higher levels (Fig. 23C). Numbers of both monocyte subtypes were comparable in WT and *Lcn2*-deficient mice. Strikingly, alcohol induced the numbers of neutrophils in the liver of WT mice, which was completely abrogated in *Lcn2*-deficient mice (Fig. 23D).

To rule out a general KC maturation defect in *Lcn2*^{-/-} mice we analysed KC fractions in WT and *Lcn2*-deficient mice fed a chow diet. Here, we did not observe this effect (Fig. 23E) suggesting that the Lieber De-Carli diet *per se* modulates KC numbers.

Figure 23. Diminished KC and neutrophil populations in livers of *Lcn2*^{-/-} mice compared to WT mice in alcoholic liver disease.

(A-C) C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15days later. Hepatic myelocytes were isolated from liver tissue and numbers of (A) Kupffer cells (CD45⁺, CD11b^{lo} F4/80^{hi}), (B) resident (CD45⁺ CD11b⁺ Ly6C^{hi} Gr-1^{lo}) and (C) inflammatory (CD45⁺ CD11b⁺ Ly6C^{hi} Gr-1^{hi}) monocytes, (D) neutrophils (CD45⁺, CD11b⁺ Ly6C^{lo} Gr-1^{hi}) were analysed using FACS. *n*= 8 per group. (E) C57Bl6 WT and *Lcn2*^{-/-} mice were fed a chow diet and KC fraction (CD45⁺, CD11b^{lo} F4/80^{hi}) was analyzed using FACS. *n*= 4 per group. Data are expressed as mean ± SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. **P* < 0.05, ***P* < 0.01. FACS analysis was performed by Piotr Tymoszek.

Lcn2-deficient mice exhibit reduced hepatic macrophages with a potential shift towards M1 polarisation.

Because previous studies have shown that LCN2 promotes macrophage inactivation, and we observed decreased numbers of MAC2⁺ macrophages in alcohol-fed *Lcn2*-deficient mice, we analysed the macrophage phenotype in liver tissue by measurement of differentiation markers. Similar to a reduction of MAC2⁺ cells in *Lcn2*-deficient mice compared to WT mice after alcohol exposure, we observed reduced expression of the macrophage marker *Cd68* in liver tissue (Fig. 24A). We then isolated Kupffer cells from the liver of alcohol- or pair-fed WT or *Lcn2*-deficient mice and FACS-sorted Kupffer-cells (defined as CD45⁺, CD11b^{lo} F4/80^{hi}) which are thought to be differentiated hepatic macrophages [131]. We observed increased expression of *Socs3* (but not *iNos*) in *Lcn2*-deficient KCs after alcohol exposure, suggesting M1 polarization of macrophages (Fig. 24B, C) [77]. Conversely, the M2 macrophage polarisation marker *Cd206* (*Mrc1*) [77] was expressed at lower levels in *Lcn2*-deficient KCs compared to KCs isolated from WT mice after exposure to ethanol-diet (Fig. 24D). We did not observe any difference in *Il-10*, a marker and released cytokine of deactivated macrophages, between WT and *Lcn2*^{-/-} KCs after alcohol feeding (Fig. 24E) [108]. Despite previous data showing that LCN2 promotes macrophage deactivation, our data suggests that less KCs are existent in *Lcn2*^{-/-} mice but existing *Lcn2*-deficient KCs are shifted towards an M1 differentiation phenotype.

Figure 24. Macrophage phenotype markers are comparable in *Lcn2*^{-/-} and WT mice in alcoholic liver disease.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15days later. (A) Total liver RNA was extracted and mRNA levels of *Cd68* were evaluated for comparing macrophage infiltration in total liver. (B-E) To compare macrophage phenotype hepatic myeloid cells were isolated from liver tissue and KCs were sorted. For RNA expression measurements, mRNA was isolated and (B) *Socs3*, (C) *iNos*, (D) *Cd206* and (E) *Il-10* were measured using qPCR. *β-Actin* was used as housekeeping gene. *n*= 10 mice per group (A-E). Data are expressed as mean ± SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. **P* < 0.05, ***P*<0.005, ****P*<0.001.

***Lcn2*-deficient mice exhibit reduced systemic (circulating) neutrophils after alcohol exposure**

Next, we focused on neutrophils which were shown to be highly upregulated after alcohol exposure in WT but not in *Lcn2*^{-/-} mice. These reduced numbers of neutrophils in the liver of *Lcn2*-deficient mice could be due to a defect in the generation (or half-

life) of *Lcn2*-deficient neutrophils or due to a defect in the recruitment of these cells. To better understand these processes, we analysed the number of circulating neutrophils in the blood using the same FACS analysis as for the hepatic characterisation (Fig. 23). As we demonstrated for liver populations, we did not detect any significant changes in blood monocytes before and after alcohol exposure (Fig. 25A). Strikingly, alcohol feeding strongly induced the circulation of peripheral neutrophils in WT mice which we did not observe in *Lcn2*^{-/-} mice (Fig. 25B). Importantly, WT and *Lcn2*-deficient mice demonstrated similar levels of circulating neutrophils when fed the control diet.

Figure 25. Diminished systemic neutrophils in alcohol-fed *Lcn2*^{-/-} compared to WT mice.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15 days later. To characterize peripheral blood leukocytes, blood specimens were analyzed for (A) monocytes (CD11b⁺ Ly6C^{hi} Gr-1^{lo}) and (B) neutrophils (CD11b⁺ Ly6C^{lo} Gr-1^{hi}) using FACS. *n* = 8 mice per group (A, B). Data are expressed as mean ± SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. ***P < 0.001. FACS analysis was performed by Piotr Tymoszek.

Intrinsic Lcn2 is necessary for hepatic tissue infiltration of neutrophils

Neutrophil recruitment and infiltration depends on (A) the release from the bone marrow, (B) cell viability in the circulation and (C) the recruitment to the site of inflammation (i.e. “chemotaxis”) and tissue infiltration where they are thought to exert their detrimental actions in the chronic setting of alcohol exposure [81]. Our findings in the FACS analysis suggested three possibilities for reduced neutrophil numbers in alcohol-fed *Lcn2*^{-/-} mice: (A) alcohol-induced granulopoiesis was impaired in *Lcn2*-deficient mice, (B) the half-life of *Lcn2*-deficient neutrophils was reduced in the setting of alcohol exposure or (C) *Lcn2*-deficient neutrophils for some reason get caught in tissue vessels not able infiltrating tissue.

To answer this question, we established an *in vivo* neutrophil tracking experiment: As demonstrated in Fig. 26A we isolated CD45.1 neutrophils from the bone marrow of C57Bl/6 WT mice (white coloured) and CD45.2 neutrophils from the bone marrow of *Lcn2*^{-/-} mice (blue coloured) with gradient centrifugation and Macs bead separation in a purity 92% (Fig. 26B) [132]. We labelled these neutrophils with PKH-26 *ex vivo* and transferred them in a 50/50 ratio into WT and *Lcn2*^{-/-} mice (both on a CD45.2 background) on day 10 after starting the control or alcohol feeding.

Figure 26. Adoptive transfer of WT and *Lcn2*^{-/-} neutrophils in alcohol-fed *Lcn2*^{-/-} and WT mice.

(A) Neutrophils were isolated from WT (CD45.1 background; white coloured) and *Lcn2*^{-/-} mice (CD45.2 background, blue coloured) and injected intravenously into C57Bl6 WT and *Lcn2*^{-/-} mice which were fed an alcohol diet for 10 days. (B) Isolated neutrophils exhibited a purity of 92%. FACS analysis was performed by Piotr Tymoszek.

Four hours post-injection, we analysed blood counts of PKH-26-labelled neutrophils that were either *Lcn2*-sufficient (CD45.1) or *Lcn2*-deficient (CD45.2) with FACS. We found that up to 0,5% of the analysed blood were PKH-26⁺ (i.e. transferred) in both control- and alcohol-fed mice, the rest was host-derived (i.e. not labelled for PKH-26) (Fig. 27A). When analysing CD45 background, we found about 50% WT and 50% *Lcn2*-deficient neutrophils in both pair-fed genotypes. Strikingly, when we analysed the alcohol-fed mice we found about 30 to 50% decrease in *Lcn2*-deficient neutrophils in both, WT and *Lcn2*-deficient mice (Fig. 27B).

Figure 27. Analysis of blood samples after adoptive transfer.

Neutrophils were isolated from WT (CD45.1 background) and *Lcn2*^{-/-} mice (CD45.2 background) and injected intravenously into C57Bl6 WT and *Lcn2*^{-/-} mice which were fed either a control or an alcohol diet for 10 days. (A) Blood was analyzed 4 hours after transfer for PKH positive cells. (B) Among PKH positive cells, cells were further analyzed for CD45.1 (that means WT) or CD45.2 (that means *Lcn2*-deficient) background (B). *n* = 3 mice per pair-fed group, *n* = 6 per EtOH-fed group (A, B). Data are expressed as mean ± SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. ***P* < 0.01, ****P* < 0.001. FACS analysis was performed by Piotr Tymoszek.

In a next step, we analysed liver tissue for PKH-26⁺ (i.e. transferred and therefore tissue infiltrating), which represented ~3 to 6% of CD45⁺ leukocytes (Fig. 28A). We found that WT (CD45.1) neutrophils similarly infiltrated livers of WT and *Lcn2*^{-/-} mice (Fig. 28B), suggesting that the hepatic inflammatory milieu in WT and *Lcn2*-deficient mice is potentially attracting WT neutrophils in a similar fashion after alcohol exposure (compare Fig. 18, 19). This is supported by the similar expression of chemokines that recruit neutrophils (*Cxcl-1*, *Mip-1*) in the liver of WT and *Lcn2*-deficient mice after alcohol exposure (Fig. 29A, B). When we analysed *Lcn2*-deficient (CD45.2) neutrophils labelled by PKH-26, we found ~35-50% less *Lcn2*^{-/-} compared to WT neutrophils in the livers of pair- and alcohol-fed mice (Fig. 28B). These data further support the assumption that neutrophil specific (i.e. intrinsic) LCN2 is necessary for cell viability and/or tissue infiltration.

Figure 28. Analysis of liver samples after adoptive transfer.

Neutrophils were isolated from WT (CD45.1 background) and *Lcn2*^{-/-} mice (CD45.2 background) and injected intravenously into C57Bl6 WT and *Lcn2*^{-/-} mice which were fed either a control or an alcohol diet for 10 days. **(A)** Liver was analyzed 4 hours after transfer for PKH positive cells. **(B)** Among PKH positive cells, cells were further analyzed for CD45.1 (that means WT) or CD45.2 (that means *Lcn2*-deficient) background (B). *n* = 3 mice per pair-fed group, *n* = 6 per EtOH-fed group (A, B). Data are expressed as mean ± SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. ***P* < 0.01, ****P* < 0.001. FACS analysis was performed by Piotr Tymoszek.

Figure 29. Chemokine patterns are comparable in *Lcn2*^{-/-} and WT mice in alcoholic liver disease.

C57Bl6 WT and *Lcn2*^{-/-} mice were fed an alcohol diet and euthanized 15 days later. Liver chemokine patterns were assessed by mRNA measurements. For RNA expression, mRNA was extracted from liver tissue and mRNA levels of (A) *Cxcl-1* and (B) *Mip-1* were measured using qPCR. *β-Actin* was used as housekeeping gene. *n* = 10 mice per group (A, B). Data are expressed as mean ± SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

The results approaching from the adoptive transfer experiments, especially in the control-fed animals (less *Lcn2*-deficient neutrophils in the liver although same amount in the blood) highlighted that *Lcn2*-deficient neutrophils have less potential to infiltrate into hepatic tissue. When analysing the alcohol-fed mice (less *Lcn2*-deficient neutrophils in the blood and in the liver), we could also assume that *Lcn2*-deficient neutrophils are prone to cell death in an alcohol-induced toxic environment.

To test whether *Lcn2*-deficient neutrophils exert a reduced half-life, we isolated neutrophils from WT and *Lcn2*-deficient mice and stimulated them with alcohol, pro-inflammatory cytokines or left them untreated. We then measured cell death and apoptosis by detection of AnnexinV and propidium iodide. Unstimulated *Lcn2*-deficient neutrophils did not exhibit higher levels of cell death compared to WT neutrophils and ethanol, TNFα, IL1β or LPS did not induce apoptotic processes after exposure for 1 or

4 hours (Fig. 30). As such, we suggest that *Lcn2*-deficiency in neutrophils is not associated with less viability to various inflammatory stimuli (i.e. alcohol and alcohol-induced cytokines), but is indispensable for neutrophil migration.

Figure 30. Apoptosis upon exogenous stimulation in *Lcn2*^{-/-} compared to WT neutrophils.

Neutrophils were isolated from bone marrow of WT and *Lcn2*^{-/-} mice and stimulated with LPS (100ng/ml), TNF α (50ng/ml), IL-1 β (20ng/ml) and Ethanol (0,5%). Apoptosis was analysed using Annexin positivity after 1 and 4 hours stimulation period.

Neutrophils are initial mediators of hepatic inflammation

As previously shown LCN2 critically regulates neutrophil life-span in the blood of alcohol-fed mice and also neutrophil migration to hepatic tissue. To investigate the relevance of neutrophils as initial mediators of hepatic inflammation, we administered a single dose of ethanol to WT and *Lcn2*^{-/-} mice and analysed hepatic leukocytes with FACS four hours post oral gavage. Here, we observed a 5-fold increase of blood neutrophils (Fig. 31A) and a 7-fold increase of hepatic neutrophils (Fig. 31B) after acute

alcohol gavage indicating that neutrophils are part of the initial hepatic injury in ALD. Notably, in *Lcn2*-deficient mice we found less systemic and hepatic neutrophils after the alcohol challenge compared to WT mice. While ALT was increased in WT mice after acute alcohol administration, ALT-elevation in *Lcn2*^{-/-} mice failed (Fig. 31C) further demonstrating the pro-inflammatory nature of LCN2 in ALD and also early alcohol-induced liver injury.

Figure 31. *Lcn2*^{-/-} are protected from early neutrophilia, neutrophilic liver infiltration and hepatic injury.

C57Bl6 WT and *Lcn2*^{-/-} mice underwent a control or alcohol gavage and euthanized 4 hours later. (A) Blood neutrophils were analyzed by FACS. (B) Hepatic neutrophils were analyzed by FACS. (C) Serum ALT was measured. *n* = 3-6 mice per group (A-C). Data are expressed as mean ± SEM, two-way ANOVA with post-hoc Bonferroni correction. n.s = not significant. **P* < 0.05, ***P* < 0.01.

Discussion

Alcoholic liver disease harbours a spectrum of hepatic disorders that are characterised by chronic inflammation emanating from chronic alcohol abuse [5]. Host genetic factors, the environment and microbiota appear to impact on the susceptibility to disease and its progression [15]. Here, we show that LCN2, a protein predominantly produced in neutrophils [100], is elevated in human AH.

Next, we analysed WT mice in a model of ALD, where we also show that LCN2 is upregulated in the liver after chronic alcohol feeding (Fig. 6). More specifically, LCN2 was increased in hepatic monocytes, KCs and neutrophils with the predominant expression in the latter (Fig. 7). To dissect the pathogenic role of LCN2 in ALD, we subjected *Lcn2*^{-/-} mice to the alcohol model. Here, we found that LCN2 is required for the alcohol-induced phenotype of our mouse model. More specifically, alcohol-induced disturbances in this model include loss of intestinal permeability, systemic inflammation (i.e. neutrophilia) and liver injury characterised by steatosis, fibrosis, apoptosis and leukocyte infiltration. *Lcn2*-deficiency protects against these deleterious consequences of chronic alcohol consumption: *Lcn2*^{-/-} mice lose significantly less weight (Fig. 8), develop less ALT elevation (Fig. 9A) and are histologically protected from steatosis (Fig. 13) and liver inflammation as demonstrated by reduced numbers of hepatic leukocytes (Fig. 21-23).

Considering the known pathogenic pathways in ALD and the multiple functions of LCN2, I addressed these issues (starting from anti-infective functions to its role in oxidative stress, steatosis, apoptosis, fibrosis and in the innate immune system especially macrophage polarization and neutrophil function) to find a correlation of the protective function of LCN2 and the pathogenesis of ALD.

First, changes in the gut microbiota and intestinal permeability have been linked to the development of ASH as this facilitates the entry of bacteria or bacterial products such as LPS which might perpetuate systemic inflammation [61-63, 69]. Considering the impact on LCN2 on the intestinal microbiota (Moschen et al. unpublished data) and its role in infections and sepsis [105, 133], we first investigated the role of LCN2 in alcohol-induced intestinal barrier function and endotoxinaemia. Here, we found restored expression of the gut-barrier defining proteins *Cldn3*, *Cldn4* and *Ocln* in alcohol-fed *Lcn2*-deficient (Fig. 11). Further, serum endotoxin levels were increased in WT mice after alcohol feeding, but not in *Lcn2*^{-/-} mice (Fig. 9C) assuming an extraintestinal protection by *Lcn2*-deficiency in ALD.

Previous studies demonstrated that in a model of non-alcoholic steatohepatitis *Lcn2*-deficient mice are prone to lipid accumulation and therefore steatosis (induced by an MCD diet, which represents a model for non-alcoholic steatohepatitis) [118]. When we examined liver steatosis, we found that *Lcn2*-deficiency protects from steatosis as demonstrated by less histological micro- and macrovesicular lipid accumulation (H&E and Oil-Red-O staining, Fig. 10, 13). In line with this, we observed increased splicing of *Xbp1* and induction of *Sirt-1* and *PPARα* expression (Fig.14A-D). The conflicting observation that *Lcn2*-deficient mice appear protected from alcohol-induced but not MCD-induced hepatitis may be explained by the different nature of the dietary modulation. More specifically, MCD and alcohol diets may have divergent effects on cellular inflammatory and metabolic pathways involved in liver damage (e.g. ROS generation during detoxification of alcohol). Thus, our observations suggest that LCN2 plays different roles in metabolic and alcoholic liver diseases.

During ethanol degradation ROS are produced as toxic by-products. It is known that excessive alcohol consumption increases CYP2E1 activity and decreases anti-oxidant

enzymes such as HO-1 and SOD1 [112]. Previous studies have shown that LCN2 exerts anti-oxidative functions by inducing HO-1 and SOD-1 [111, 112]. To examine the role of LCN2 in oxidative stress and ALD, we analysed expression of oxidative and anti-oxidative enzymes. Contrary to our expectations, *Ho-1* and *Sod1* expression was unaffected by *Lcn2*-deficiency (Fig. 17D, E). Surprisingly, we measured a significant reduction of *Cyp2e1*, the main ethanol metabolizing enzyme after chronic alcohol feeding in WT mice (Fig. 17A). In *Lcn2*^{-/-} mice *Cyp2e1* expression was restored in association with significant lower serum ethanol levels compared to WT mice (Fig. 9B) despite a similar food intake. We suggest that *Lcn2*-deficiency in the liver (directly or indirectly) restores *Cyp2e1* expression and therefore ethanol is potentially metabolized and detoxified further protect from ALD.

Apoptosis and fibrosis (as a consequence of ROS accumulation and lipid peroxidation) are regarded as pathophysiological mechanisms in the progression of ALD [115]. It has already been shown that LCN2 induces apoptosis in a human hepatoma cell line and since alcoholic steatohepatitis is a disease in which hepatocyte death appears to be a driving factor of disease progression, we investigated whether *Lcn2*-deficient hepatocytes would be protected from apoptosis and thus disease progression such as fibrosis [115]. Neither TUNEL labelling of hepatocytes (Fig. 16) nor FACS analysis of hepatic myeloid cells revealed a difference in protection from cell death after alcohol challenge. Despite hardly any difference in hepatic cell death of alcohol-fed WT and *Lcn2*-deficient mice, we found that *Lcn2*^{-/-} mice were protected from fibrosis (Fig. 15) maybe deriving from restored *Cyp2e1* expression (Fig. 17A) and assumed improved alcohol detoxification (Fig. 9B).

As discussed previously KC activation (presumably through LPS and the TLR-pathway) plays a key role in the pathogenesis of ASH as depletion of KCs protected

mice from disease [75]. Here, we immunohistologically and also quantitatively (by FACS analysis) demonstrate that the KC fraction is diminished in liver tissue of *Lcn2*^{-/-} mice after alcohol diet and interestingly, also after control diet (Fig. 23A). To rule out a general defect in KC development due to *Lcn2*-deficiency, we also analysed WT and *Lcn2*^{-/-} mice on a chow diet (Fig. 23E). There was no difference in the KC fraction suggesting that dietary factors contained in the Lieber De-Carli diet (independent of ethanol supplementation) diminished KC fraction in *Lcn2*^{-/-} mice. Although the origin of Kupffer cells is still incompletely understood, they are considered to be a macrophage lineage with similar polarization patterns [131]. Wan and colleagues [79] showed that M2 polarisation of KCs in liver injury promotes apoptosis of M1 polarized KCs thereby protecting from tissue damage [79, 108]. As Warszawska *et al.* [99] demonstrated that LCN2 can influence the macrophage phenotype into a deactivated state, we investigated this as a possible mechanism by which *Lcn2*-deficiency could be further protective in our model. We observed that *Lcn2*^{-/-} mice may exert a shift to M1 polarisation as suggested by increased expression of *Socs3* and decreased expression of *Cd206* in isolated KCs (Fig. 24). Whereas recent studies by others suggested a detrimental effect of M1 polarized macrophages, we speculate that loss of KC numbers in liver tissue of *Lcn2*^{-/-} mice, rather than polarization, impacts on disease severity.

Hepatic neutrophil infiltration directly correlates with mortality in human disease [80, 81]. A recent study by Bertola *et al.* [82] demonstrated that depletion of neutrophils by a specific antibody protected mice from ASH. Schroll *et al.* [110] suggested that LCN2 exerts chemotactic functions comparable to CXCL-1 which is one of the most important chemoattractants for neutrophils. A further study demonstrated that LCN2 is indispensable for neutrophil function, as *Lcn2*-deficient neutrophils show structural

abnormalities through cytoskeletal dysfunctions and defective chemotaxis activities [134]. In our study, we observed reduced numbers of neutrophils upon alcohol-challenge in livers of *Lcn2*-deficient mice (Fig. 22, Fig. 23D). Because this effect to us seemed with the highest impact in the pathogenesis of ALD, we established an *in vivo* model to track neutrophils to dissect the role of LCN2 and neutrophils.

Here, we demonstrate a strongly diminished migration of *Lcn2*-deficient neutrophils in WT and *Lcn2*-deficient recipients fed the alcohol diet and interestingly also in recipients fed the control diet (Fig. 28B). This effect was independent on existing LCN2 as chemoattractant (as we also observed reduced *Lcn2*-deficient neutrophil in WT recipients with functional LCN2) and other chemokines such as CXCL-1 and MIP-2 (Fig. 29A, B). These data suggest that neutrophil-intrinsic LCN2 is indispensable for neutrophil migration into the liver. We further demonstrate that *Lcn2*-deficient neutrophils disappear in the circulation of alcohol-fed mice (Fig. 27B). To investigate whether *Lcn2*-deficient neutrophils are prone to cell death, we stimulated WT and *Lcn2*-deficient neutrophils with cytokines known to exist systemically in high amounts of alcohol-fed mice (Fig. 20, 30). Here, we did not observe any differences in neutrophil apoptosis regarding to *Lcn2*-deficiency (Fig. 30) suggesting that other factors in the alcohol-toxic milieu limit viability of *Lcn2*-deficient neutrophils. To sum up these transfer data, we suggest that the inability to launch an appropriate systemic neutrophilic response (as reduced *Lcn2*-deficient neutrophils in the blood of alcohol-fed mice (Fig. 27B)) and the reduced capacity of *Lcn2*-deficient neutrophils to infiltrate an inflamed liver (Fig. 28B) protects *Lcn2*^{-/-} mice from ALD. As we also observed reduced neutrophils in a model of a single alcohol gavage (Fig. 31), we suggest that this is the main effect of the observed protection of *Lcn2*^{-/-} mice and that further hepatic effects such as diminished steatosis and fibrosis are consequences of reduced hepatic

neutrophils. Therefore, these data further substantiate neutrophils as drivers for ALD and the absolute necessity of LCN2 in neutrophil function.

In conclusion, LCN2 has previously been described to act protective in inflammation of various disease models. [116, 118, 133] Here, we provide evidence that LCN2 promotes disease severity as *Lcn2*-deficiency protects from neutrophil recruitment and Kupffer cell accumulation, both hallmarks of human disease. Thus, we demonstrate that LCN2 is not only an alcohol-induced acute phase protein but substantially contributes to the pathogenesis of this disease. We would suggest that LCN2 is a critical regulator of disease progression in human alcoholic steatohepatitis which might pave novel avenues for therapeutic approaches in the future.

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List of abbreviations

AA	Acetaldehyde
A.d.	Aqua destillata
ACC	Acetyl-CoA carboxylase
ADH	Alcohol dehydrogenase
ADIPOQ	Adiponectin
ADIPOR	Adiponectin receptor
AH	Alcoholic hepatitis
ALD	Alcoholic liver disease
ALDH	Acetaldehyde dehydrogenase
AMPK	Adenosine monophosphate-activated protein kinase
AOX	Acyl-CoA oxidase
AP	Activator protein
ASH	Alcoholic liver disease
BSA	bovine serum albumin
Cat	Catalase
CB	Cannabinoid CB2 receptor
CCL	Chemokine (C-C motif) ligand
CCl4	Tetrachloromethane
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CK	Cytokeratin
CLDN	Claudin
CoPP	Cobalt protoporphyrin
CPT1	Carnitine palmitoyltransferase I
Cxcl-1	Chemokine (C-X-C motif) ligand 1
CXCR	Chemokine (C-X-C motif) receptor
CYP2E1	Cytochrome P450 2E1
DF	Discriminant Function
EMT	Epithelial to mesenchymal transition
EtOH	Ethanol

FACS	Fluorescence-activated cell sorting
FAEE	Fatty acid ethyl esters
FAS	Fatty acid synthase
FCS	Fetal calf serum
GABA	Gamma-aminobutyric acid
GdCl ₃	Gadolinium chloride
HCC	Hepatocellular carcinoma
Hh	Hedgehog
HIF	Hypoxia inducible factor
HO	Heme oxygenase
HSC	Hepatic stellate cells
IL	Interleukin
KC	Kupffer cell
LAL	Limulus Amebocyte Lysate
LBP	Lipopolysaccharide binding protein
LCN	Lipocalin
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
MAC	Macrophage-1 antigen
MCD	Malonyl Co-A decarboxylase
MCD	Methionine choline-deficient
MCP-1	Monocyte chemoattractant protein-1 (also Ccl2)
MD	Myeloid differentiation factor
MEOS	Microsomal ethanol-oxidizing system
MPO	Myeloperoxidase
mtDNA	Mitochondrial DNA
NAD ⁺	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate
NF- κ B	Nuclear factor κ B
OCLN	Occludin
PBMC	Peripheral blood mononuclear cells
PGC	PPAR gamma coactivator

PLIN	Perilipin
PNPLA	Patatin-like-Phospholipase
PPAR	Peroxisome proliferator-activated receptor
PYCARD	PYD and CARD domain containing
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SCD	Stearoyl-coenzyme A desaturase
SOD	Cu/Zn-superoxide dismutase
SIRT	Sirtuin
SREBP	Sterol regulatory element-binding protein
TLR	Toll like receptor
TG	Triglycerides
TGF- β	Transforming growth factor beta
TJP	Tight junction protein
TNF- α	Tumour necrosis factor alpha
VCAM	Vascular cell adhesion protein
VEGF	Vascular endothelial growth factor
VLA	Very Late Antigen
WT	Wild-type

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■ List of publications

Original articles:

Wieser V, Tymoszyk P, Adolph TE, Grander C, Grabherr F, Enrich B, et al. Lipocalin 2 drives neutrophilic inflammation in alcoholic liver disease. J Hepatol. 2015. pii: S0168-8278(15)00797-7. doi: 10.1016/j.jhep.2015.11.037. [Epub ahead of print]

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