

Hepatic Innervations and Nonalcoholic Fatty Liver Disease

Monika Adori, PhD¹ Sadam Bhat, MSc² Roberto Gramignoli, PhD³ Ismael Valladolid-Acebes, PhD⁴
Tore Bengtsson, PhD⁵ Mathias Uhlén, PhD^{6,7} Csaba Adori, PhD^{5,6}

¹ Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden

² Department of Molecular and Cellular Medicine, Institute of Liver and Biliary Sciences, New Delhi, India

³ Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden

⁴ Department of Molecular Medicine and Surgery, The Rolf Luft Research Center for Diabetes and Endocrinology, Karolinska Institutet, Stockholm, Sweden

⁵ Department of Molecular Biosciences, The Wenner-Gren Institute (MBW), Stockholm University, Stockholm, Sweden

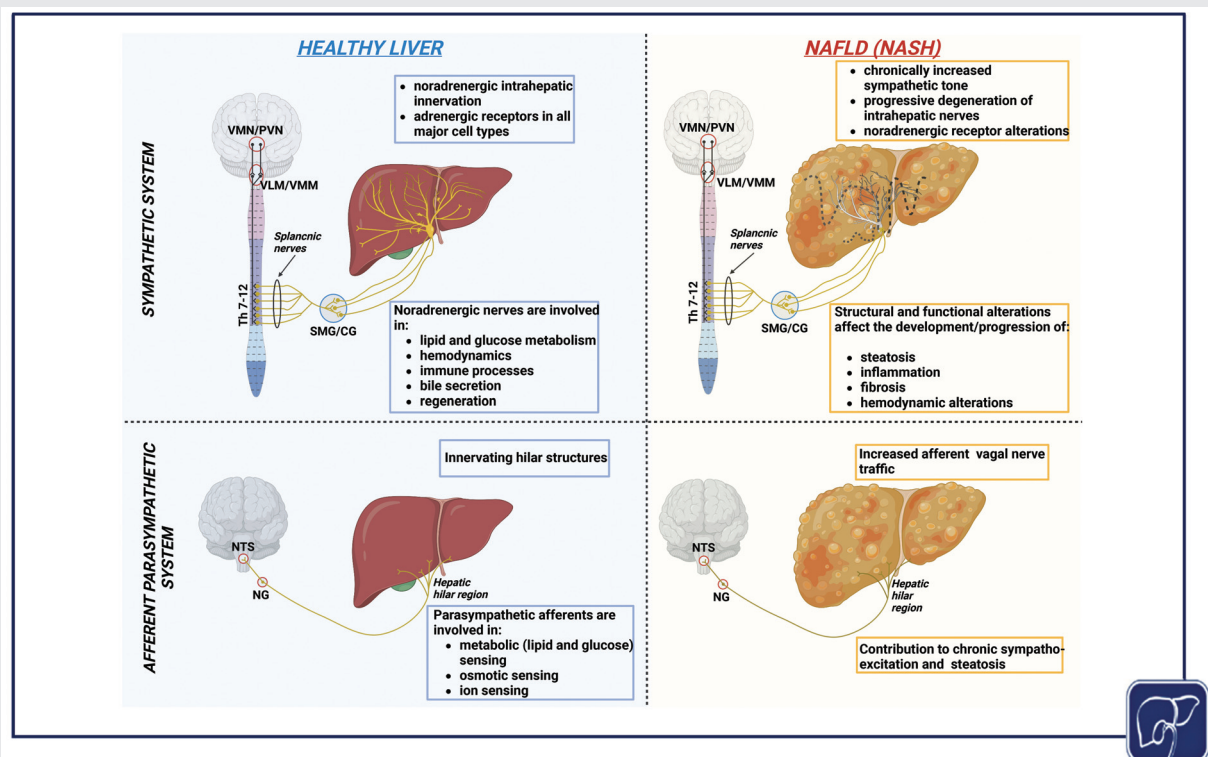
Address for correspondence Csaba Adori, PhD, Department of Molecular Biosciences - The Wenner-Gren Institute (MBW), Svante Arrhenius väg 20C, 114 18, Stockholm, Sweden; Department of Neuroscience, Karolinska Institutet, Solnavägen 9, 171 65 Solna, Sweden (e-mail: adorics@gmail.com; csaba.adori@ki.se).

⁶ Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden

⁷ Science for Life Laboratory, Royal Institute of Technology, Stockholm, Sweden

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Graphical Abstract



Abbreviations: VMN/PVN, hypothalamic ventromedial nucleus/paraventricular nucleus; VLM/VMM, ventrolateral medulla/ventromedial medulla; SMG/CG, superior mesenteric ganglion/caeliac ganglia; NTS, nucleus of the solitary tract; NG, nodose ganglion.

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Abstract**Keywords**

- ▶ nonalcoholic fatty liver disease
- ▶ sympathetic nerves
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- ▶ noradrenaline
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- ▶ inflammation
- ▶ fibrosis
- ▶ portal hypertension

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder. Increased sympathetic (noradrenergic) nerve tone has a complex role in the etiopathomechanism of NAFLD, affecting the development/progression of steatosis, inflammation, fibrosis, and liver hemodynamical alterations. Also, lipid sensing by vagal afferent fibers is an important player in the development of hepatic steatosis. Moreover, disorganization and progressive degeneration of liver sympathetic nerves were recently described in human and experimental NAFLD. These structural alterations likely come along with impaired liver sympathetic nerve functionality and lack of adequate hepatic noradrenergic signaling. Here, we first overview the anatomy and physiology of liver nerves. Then, we discuss the nerve impairments in NAFLD and their pathophysiological consequences in hepatic metabolism, inflammation, fibrosis, and hemodynamics. We conclude that further studies considering the spatial-temporal dynamics of structural and functional changes in the hepatic nervous system may lead to more targeted pharmacotherapeutic advances in NAFLD.

Lay Summary

Nonalcoholic fatty liver disease (NAFLD) is the most common hepatic disorder, with prevalence around 25% globally. Nearly 70% of patients with type-2 diabetes have fatty liver, and NAFLD is associated with heart and kidney complications. Currently, the therapeutic options are limited to lifestyle changes and no universally approved drug therapy exists for NAFLD. Nerve fibers in the liver play a complex role in liver fat and sugar metabolism, blood circulation, immune responses, and bile secretion. Previous research revealed structural and functional impairments of the liver nerves in NAFLD, which contribute to fat accumulation, inflammation, and abnormal growth of fibrous connective tissue in the diseased liver. Here, we overview the anatomy and physiology of liver nerves and discuss the nerve alterations, with their potential causes and consequences in NAFLD. We emphasize that deeper understanding of structural and functional changes in the liver nervous system in NAFLD may lead to new targeted therapeutic interventions.

Nonalcoholic Fatty Liver Disease: Symptoms, Pathogenesis, and the Role of Hepatic Innervations

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder, with a prevalence of approximately 25 to 30% in the Western countries.^{1–3} NAFLD is a systemic disease and it is considered as the hepatic manifestation of the metabolic syndrome. NAFLD is usually associated with visceral obesity, insulin resistance, increased blood pressure, fasting hyperglycemia, and dyslipidemia.³ Nearly 70% of type-2 diabetes patients have NAFLD comorbidity.⁴ Moreover, NAFLD is associated with renal and cardiovascular complications.⁵ Indeed, the most common cause of death in NAFLD is cardiovascular-related.⁶

Currently, the therapeutic options are palliative, limited to lifestyle and dietary changes, with no consensus on pharmacological therapy.

The spectrum of NAFLD may vary from early-onset steatosis (intrahepatic triglyceride accumulation), via nonalcoholic steatohepatitis (NASH; up to 30% of all NAFLD cases),^{7,8} to end-stage liver cirrhosis and hepatocellular carcinoma.⁹ Mild forms of liver steatosis can be considered as a relatively benign condition; however, NASH is a severe disease, which is characterized by large-scale steatosis, chronic lobular inflammation, hepatocellular injury, and progressive fibrosis.⁹

The pathogenesis of NAFLD implies initial metabolic disturbances, such as insulin resistance, which may lead to steatosis. The transition between steatosis and NASH could be described by a “multiple-hit” model. Namely, impaired lipid partitioning, oxidative stress caused by lipotoxicity, proinflammatory cytokine-mediated hepatocyte injury and cell death, as well as further metabolic abnormalities may contribute to the development of NASH.¹⁰ Since NAFLD is the hepatic manifestation of a multisystem metabolic disorder that is heterogeneous in its underlying causes and presentation, some authors recently suggested calling it as “metabolic dysfunction-associated fatty liver disease” (MAFLD).¹¹

The liver has a complex innervation with significant interspecies differences among mammalian species. Anatomical and physiological studies revealed that efferent hepatic innervations have a complex role in the regulation of liver lipid and glucose metabolism, hemodynamics, immune-processes, bile secretion, and tissue regeneration. Furthermore, sensory (afferent) liver nerves play important role in metabolic (glucose and lipid) sensing, osmotic sensing, and ion sensing.^{12–15}

Despite such relevance, hepatic innervations have been understudied until recently, especially concerning their potential involvement in liver pathologies. This was perhaps because of methodical limitations in manipulating and visualizing hepatic nerves, or because liver functions did not appear seriously compromised after orthotopic liver transplantation (OLT).¹⁶ However, long-term follow-up studies

described dyslipidemia, postprandial hyperglycemia, insulin resistance, and alterations in intrahepatic microcirculation after OLT.^{12,17,18} Furthermore, postprandial hyperglycemia was exaggerated and lasted longer in liver transplant patients than in kidney-transplanted individuals who also underwent immunosuppressive medications.¹⁹ This raises the possibility that the inevitable liver denervation in OLT may contribute to the development of the post-transplantation metabolic syndrome. Nonetheless, further studies are needed to examine this question in more detail.

Recent functional and structural investigations using selective stimulation of hepatic sympathetic nerves,²⁰ pharmacological methods targeting liver adrenergic receptors,²¹ as well as high-resolution and large-scale 3D visualization of hepatic nerves by state-of-the-art volume imaging^{22,23} have highlighted a significant role of liver nerves in NAFLD pathophysiology. This review first overviews the anatomy and physiology of liver nerves and the intrahepatic nerve-mediated cellular communication. Then, liver nerve structural and functional alterations in NAFLD will be discussed, and finally potential causes and consequences of all these nerve-related pathologies will be elucidated.

Hepatic Nerves and the Intrahepatic Nerve-Mediated Cellular Communication

The Roman physician and philosopher Galen already mentioned liver nerves in one of his works.²⁴ The first more detailed descriptions of mammalian hepatic innervations go back to the mid-19th century.²⁵

The liver does not contain neural crest-derived intrinsic neurons²⁶ but receives sympathetic efferent innervations from the splanchnic nerves, parasympathetic efferent and afferent innervations from the vagus nerve, and spinal afferent innervations from the dorsal root ganglia.

During fetal life, the primary role of the liver is hematopoietic, and only sparse hepatic innervations are noticed, especially in rodents.²⁶ NPY-positive (sympathetic) fibers were first found at embryonic day 19 (E19) in the mouse liver.²⁷ In another study, no intrahepatic nerves were described in the mouse at E17.5 and at postnatal day 1 (P1) by using tubulin β 3 class III (TUBB3), a pan-neuronal marker. Nerves were first noticed at P7 in the liver surrounding the intrahepatic bile ducts, and they gradually populated the liver by P21. The intrahepatic bile ducts guided the extension of nerve fibers by secreting nerve growth factor during development.²⁸ According to a recent report in mice, using another pan-neuronal marker, protein gene product 9.5 (PGP9.5), nerves first appeared at the hilum at E16.5 and gradually populated the entire lobes toward their periphery by P28. The authors concluded that the interaction between nerves, intrahepatic bile ducts, and hepatic artery plays an important role in the morphogenesis and stabilization of portal structures.²⁹ In humans, the first hepatic nerves were noticed on the 8 to 12th gestation weeks, around the portal tracts, as identified by PGP9.5, neuron-specific enolase, and neurofilament pan-neuronal markers. These nerves gradually increased in density and reached the adult level by the 32

to 33rd gestation weeks. However, intraparenchymal thin fibers appeared only at term (40th week).³⁰

Centrally, the descending liver sympathetic projections arise from pre-autonomic neurons of the ventromedial hypothalamic nucleus and paraventricular nucleus (PVN),^{31,32} projecting to the ventrolateral and ventromedial medullary reticular formation. Neurons of this medullary autonomic center project to cholinergic preganglionic neurons in the intermediolateral column of the thoracic spinal cord (Th7–Th12). Preganglionic splanchnic nerve fibers then innervate the noradrenergic superior mesenteric ganglion and celiac ganglia, which send postganglionic innervations toward the liver. The nerves enter the liver in the hilum and then follow the portal triads. The major nerve fibers localize around larger intrahepatic bile ducts and the hepatic artery. Such primary fibers give rise to a thin, spiderweb-like nerve plexus, which wraps around all the bile ducts as well as branches of the hepatic artery and portal vein (**Fig. 1A**). Spatially, this noradrenergic nerve plexus terminates approximately 100 to 200 μm ahead of the most distal parts of the portal veins, 300 to 400 μm below the organ surface.²² Fine nerve filaments follow extracellular matrix (ECM) collagen fibers within the Glisson's sheath, in close contact with vascular smooth muscle cells, endothelial cells, and biliary epithelial cells.²² No significant innervation has been detected around the central veins or the hepatic vein.³³ However, bolstered by advanced 3D imaging technology, we have been able to elucidate a limited innervation of central veins in mouse. Such nerve fibers originate from the adjacent periportal nerve plexus: bridging fibers branch again and extend for a few hundred micrometers around the central veins.²² The periportal innervation is described in all examined mammalian species; however, there are significant interspecies differences reported regarding the parenchymal innervation. Namely, while the sympathetic innervation in healthy conditions is restricted to the periportal region in mouse and rat, fine noradrenergic fibers enter the liver parenchyma in human, monkey, cat, dog, guinea pig, and rabbit.^{34–36} In our recent work, we have explored the parenchymal innervation in more detail in humans. 3D imaging showed that the periportal nerves give rise to “interlobular” nerve fibers running in the interlobular connective tissue septae (**Fig. 1B**). Such nerves give rise to 3- to 6- μm -thin fine varicose fibers that run in the perisinusoidal (Disse) space, following ECM collagen III⁺ fibers, with decreasing density toward the centrolobular zone 3.²² Previous electron microscopy (ELMI) studies revealed that these nerve filaments are nonmyelinated and frequently glial sheath-free. They form neuroeffector junctions, without synaptic specializations, with both parenchymal cells (hepatocytes) and nonparenchymal cells (endothelial-, Kupffer-, and hepatic stellate- [Ito] cells).^{27,37,38} The hepatic noradrenergic fibers co-localize with the neuropeptides NPY and galanin.^{22,39}

Centrally, the liver efferent parasympathetic projections arise from neurons in the lateral hypothalamic area, which project to the dorsal motor nucleus of the vagus and the ambiguous complex.^{31,32,40} These nuclei supply the preganglionic vagal efferents. Afferent (sensory) vagal nerves are

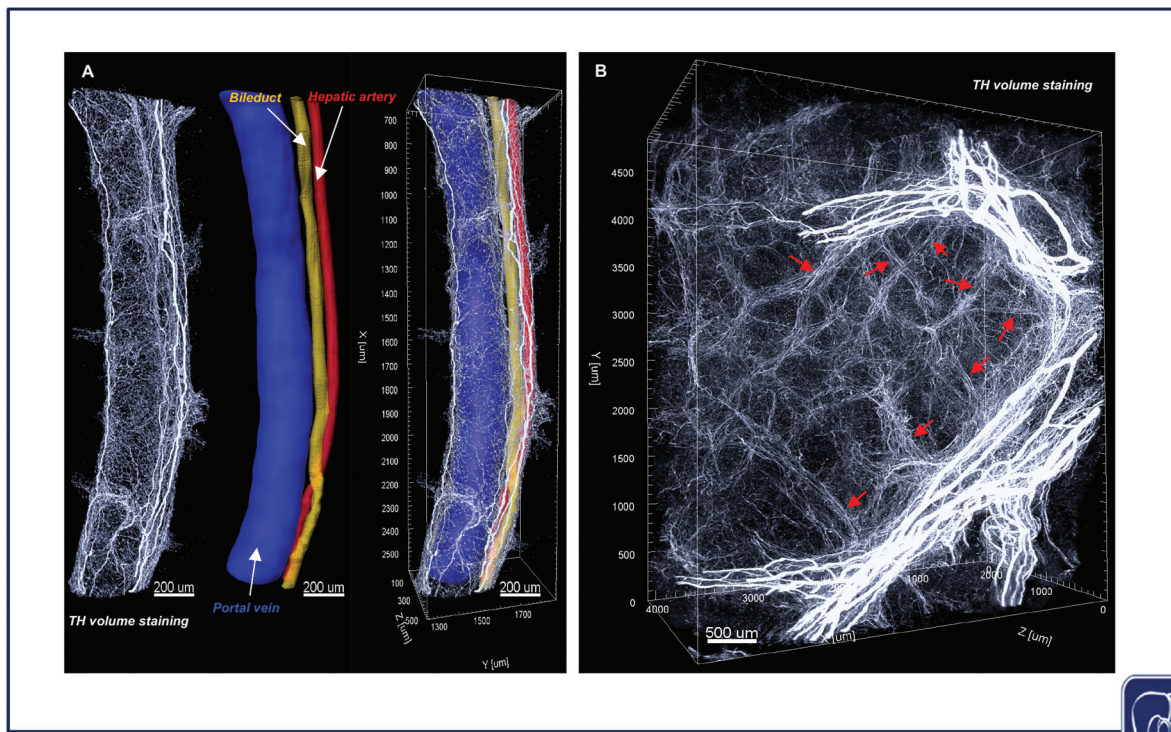


Fig. 1 Sympathetic innervation of the mouse and human liver. (A) Periportal sympathetic nerves in the mouse liver are visualized by tyrosine hydroxylase (TH) volume immunostaining. *Left panel:* TH staining; *middle panel:* elements of the portal triad are segmented and color-coded based on autofluorescence; *right panel:* sympathetic nerves around the segmented elements of the portal triad. (B) TH volume immunostaining of a $5 \times 5 \times 2$ mm piece of healthy human liver. Note the extensive thick nerve fibers around the portal triad and the interlobular branches (arrows) that arise from the periportal nerve plexus. From these interlobular nerves, fine intraparenchymal fibers target the parenchyma. (Micrographs are from Adori et al.²²)

supposed to be derived from the nodose ganglion.⁴¹ Nodose ganglion neurons then project to the medullary nucleus of the solitary tract. The common hepatic branch of the vagus bifurcates to the larger gastroduodenal branch, targeting the pancreas and gut, while the minor hepatic branch proper targets the liver hilum. So far, however, no clear documentation of intrahepatic parasympathetic ganglia has been provided. Moreover, we and others were unable to detect intrinsic parasympathetic (cholinergic) innervation, both in rodents^{22,23} and in primates or humans.²³ Earlier studies described intrahepatic cholinergic nerves based on acetylcholinesterase histochemistry.^{36,42} However, this method cannot be considered fully specific and reliable for visualizing cholinergic fibers.³⁸ More specific vesicular acetylcholine transporter immunohistochemistry or green fluorescent protein (GFP) immunostaining of choline-acetyltransferase (ChAT)-GFP mice failed to show any intrinsic hepatic cholinergic nerves.^{22,43} Based on tracing techniques, a few vagal efferent fibers were found within the fascicles of the vagal hepatic branch and in a small fraction of vagal paraganglia in the rat. Regarding vagal afferents, fibers were noticed around larger portal triads in the hilum of different liver lobes, around the extrahepatic part of the portal vein, and around almost all paraganglia adjacent to the hepatic branch of the vagus and the hilum.^{41,44} Overall, (mainly afferent) parasympathetic hepatic innervations may be restricted to hilar structures, while a direct hepatic cholinergic innervation is

doubtful. Some authors propose an indirect vagal control of the liver, by affecting the sympathetic celiac ganglion or microganglia around the celiac artery. Such hypothesis is supported by the demonstration of vagal preganglionic terminals in these structures.⁴⁵ However, further detailed and comparative studies are needed to elicit the anatomy of the liver parasympathetic innervations.

Spinal afferent innervation of the liver is provided by lower thoracic (Th7–Th12) dorsal root ganglia (DRG) neurons. The neuropeptides calcitonin gene-related peptide (CGRP) and substance P are markers to visualize these fibers.⁴⁶ CGRP volume immunostaining identified sparse innervations around portal triads, but no CGRP⁺ nerves were found in the parenchyma or around the central veins in mice.²²

Between hepatocytes, nerve signals can be propagated via gap junctions. Electric coupling of hepatocytes by gap junctions is relevant also in the human liver, but it is particularly important in rodents where the nerves are restricted to the periportal area.⁴⁷ In mice and rats, the nerves are in contact only with a few periportal hepatocytes, and then the nerve-derived signal is propagated via gap junctions. The major gap junction protein in the liver is connexin 32 (CX32).⁴⁸ Gap junction inhibitors in rats completely blocked the nerve-mediated metabolic- and hemodynamic alterations.⁴⁷ Moreover, in CX32-deficient mice, dilated bile canaliculi have been observed and a

decrease in bile flow was described after electric stimulation of sympathetic nerves.⁴⁹

G-coupled noradrenergic receptors are expressed by virtually all major cell types in the mammalian liver. Namely, α and β receptor expressions were described in hepatocytes.^{50–52} In primary cultures, hepatic stellate cells (HSCs) express α 1a, α 1b, α 1d, α 2b, β 1, β 2, β 3, and NPY receptors.^{53–55} The liver resident macrophage Kupffer cells were reported to express all the nine adrenergic receptors.⁵⁶ Liver progenitor cells express α 1 receptors.⁵⁷ Moreover, α and β receptors in the portal vein wall, as well as α receptors in all segments of the liver vasculature, were described.^{58,59} In a more detailed study, β 3 receptors were shown in the portal vein endothelium and muscle cells in mice.⁶⁰ Cholangiocytes express α 1 forms (a, b, and d)⁶¹ and β 2⁶² receptors. More detailed information concerning adrenergic receptor expressions in various hepatic cell types is reported in a single cell RNA sequencing study of the human liver⁶³ and in a recent detailed review.⁶⁴ In addition to the noradrenergic receptors, nicotinic⁶⁵ and muscarinic (M1–M5)⁶⁶ cholinergic receptors were also described in HSCs. Sensory receptive mechanisms on the molecular level are much less explored in the liver. In case of osmotic sensing, Lechner et al identified transient receptor potential channel member 1 ion channels in DRG-derived spinal sensory nerve fibers in the mouse liver, which detect physiological changes in blood osmolality.⁶⁷

Vegetative Imbalance and Its Role in NAFLD Pathogenesis

Besides sedentary lifestyle, unhealthy diet, or genetic predisposing factors, several studies highlight an autonomic imbalance, namely, an increased sympathetic nerve tone, as a key factor in the etiopathogenesis of NAFLD.^{68–70} Such increased sympathetic tone may be the consequence of enhanced excitability and overactivity of liver-related preautonomic neurons in the hypothalamic PVN.⁷¹ In guanine nucleotide exchange factor 3 (Vav3) knockout mice with chronic sympathetic hyperexcitation, the metabolic syndrome and fatty liver onset were noted already at 4 months of age, progressing to NASH without obesity after 1 year in animals kept on normal chow diet.⁷² A recent study depicted a more direct pathogenic link between liver sympathetic outflow and hepatic steatosis, the initial manifestation of NAFLD.²⁰ Namely, high fat diet-induced NAFLD in mice was associated with doubling of firing rate in efferent liver sympathetic nerves. Furthermore, the steatosis was effectively reversed with chemical sympathectomy, independently of overall weight changes, caloric intake, or adiposity.

Chronically high sympathetic tone in NAFLD was confirmed also in humans. One study on 2,000 participants concluded that increased sympathetic and decreased parasympathetic activities, rather than changes in hypothalamic–pituitary–adrenal (HPA) axis, are associated with the metabolic syndrome.⁶⁹ A recent large-scale study with 34,000 participants showed decreased parasympathetic activity and elevated sympathetic tone as an increased risk for NAFLD.⁷³ Another study in a smaller cohort of participants demon-

strated that NAFLD was associated with cardiac sympathetic/parasympathetic imbalance assessed by heart rate variability, regardless of the presence or absence of type-2 diabetes.⁷⁴

Finally, chronically elevated sympathetic tone in the liver has systemic consequences as well. It leads to increased hepatic arteriolar resistance, affecting renal sympathetic activation, with consequence of renal arteriolar vasoconstriction and renin–angiotensin–aldosterone system (RAAS) activation. This hepatorenal reflex that mediates RAAS activation further decreases the renal blood flow and glomerular filtration, and increases systemic sodium retention⁷⁵ with possible further cardiovascular consequences.

Liver Nerve Pathologies and Receptor Alterations in NAFLD

The NAFLD spectrum is associated with pathologies of the hepatic nerves. Early studies showed decreased densities of liver innervations in cirrhosis, mainly in the parenchyma.^{76–81} Moreover, liver sympathetic nerve density decreased in male macaques exposed to perinatal high fat diet (combination of high fat diet to mothers and early postnatal high fat diet to offspring).⁸²

More detailed examination of hepatic nerve pathologies was possible with the recent application of volume immunostaining and light sheet microscopy. Using such novel 3D imaging technique, we showed parallel signs of mild axonal trimming and sprouting of sympathetic nerve fibers in steatosis, which turns to a severe degeneration of nerves in steatohepatitis in mice (**–Fig. 2A–D**).²² Aberrantly sprouting fibers in steatosis showed ectopic parenchymal localization and they followed collagen fibers. In parallel, hepatic NFG expression was elevated, suggesting that hepatic sympathetic nerves exhibit an increased plasticity in the early steatosis onset. This may be the consequence of a starting reorganization of the ECM, which, in turn, destabilizes the mechanical support of collagen fibers to the nerve filaments.²² Notably, ECM reorganization is an early, critical event in fibrosis, and it starts much earlier than the cirrhotic stage in the NAFLD spectrum.⁸³ Degenerating sympathetic nerve fibers, which appeared in significant amounts typically in the more advanced steatohepatitis, showed swollen axonal pathology indicating an axo-plasmatic transport problem. Namely, fine unmyelinated varicose fibers are particularly sensitive for oxidative stress that may be a consequence of chronic nerve activity, lipotoxicity, and proinflammatory immune processes. Oxidative stress leads to the destruction of microtubules, causing axo-plasmatic transport impairments in the nerves.^{84–86} Human fatty livers showed similar nerve damages that was observed in mice, starting with the finest intraparenchymal fibers, and correlating with the severity of NAFLD pathology (**–Fig. 2E**). Chronically high sympathetic tone is a key factor in this degeneration—as we showed similar fiber degeneration in the Vav3 knockout mouse line with chronic sympathoexcitation.²² Liver sympathetic neuropathy in high fat diet-fed mice was also described by Liu et al, using volume

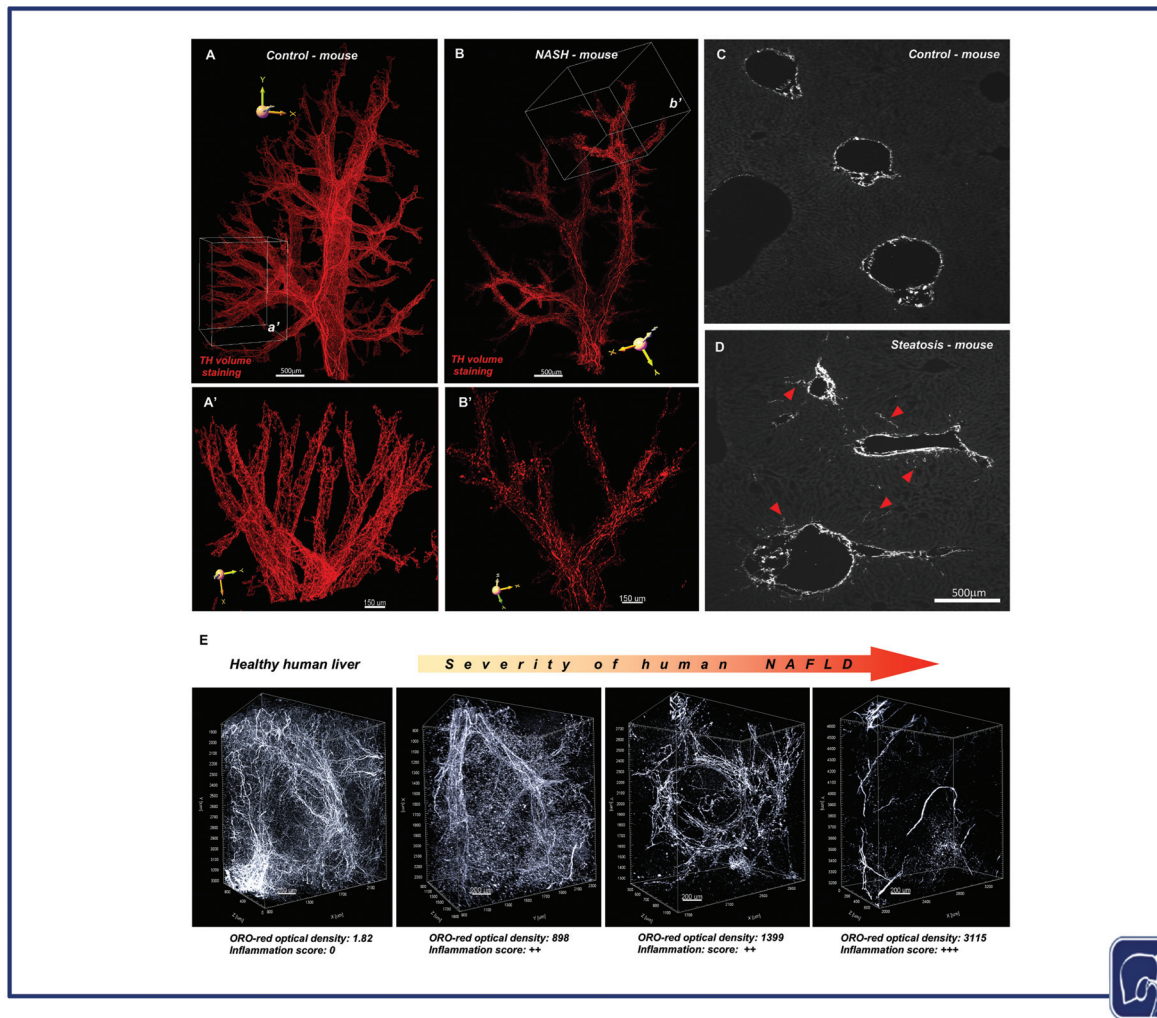


Fig. 2 Sympathetic nerve pathology in experimental and human nonalcoholic fatty liver disease (NAFLD). (A, B) tyrosine hydroxylase (TH) volume immunostaining in control mouse liver (A–A') and in experimental steatohepatitis (B–B'). Boxes in A and B indicated with a' and b' are enlarged in A' and B', respectively. Note the extensive fiber degeneration in NASH. TH immunostaining in control mouse (C) and in experimental steatosis (D). Note the ectopic, parenchymal sprouting of noradrenergic fibers in steatosis (arrowheads in D). (E) Sympathetic nerve pathology in human NAFLD. Intraparenchymal fine fibers are gradually degenerating and disappearing with the more severe NAFLD pathology. ORO-red: Oil Red O lipid staining. (Micrographs are from Adori et al.²² with slight modifications.)

imaging technique. The neuropathy was shown as a result of elevated macrophage-derived tumor necrosis factor α (TNF α) signaling through *sarm1*. The authors demonstrated that fiber dystrophy can be reversed by calorie restriction or by neutralizing TNF α antibody treatment. However, no comparison with NAFLD spectrum pathology was presented.²³

We also described decrease in several adrenergic receptor and CX32 gap junction protein transcript levels already in steatotic mice fed with Western (high fat and high carbohydrate) diet, which may be a physiological response to the chronically high sympathetic tone. These expressions were further decreased in the steatohepatitis phase.²² However, Wang et al found increased hepatic $\beta 3$ mRNA and protein expression in rats fed with a high fat diet.⁸⁷

Detailed Consequences of Hepatic Nerve Structural and Functional Impairments in NAFLD

Role in Altered Lipid Metabolism—Steatosis

A healthy liver normally stores minimal amounts of lipids. However, hepatocytes accumulate large amounts of fat with the progression of NAFLD. The fat content of hepatocytes is balanced by free fatty acid (FFA) uptake, de novo lipogenesis, hepatic β -oxidation, and triglyceride disposal (released as very low density lipoprotein [VLDL]).⁸⁸ The unbalance between the afore processes, such as increased lipid intake (FFA uptake or de novo lipogenesis) and/or decreased removal (impaired β -oxidation or VLDL secretion), leads to steatosis.

As we discussed earlier, NAFLD is characterized by a chronically high sympathetic tone. Elimination of liver

sympathetic nerves reduced FFA uptake and de novo lipogenesis, and reversed high fat diet-induced liver steatosis in mice. In contrast, no significant effect was noticed in VLDL release or β -oxidation.²⁰ Other studies in other models, however, found that sympathetic overactivity increased hepatic VLDL production in fa/fa Zucker rats, and liver sympathetic denervation decreased the VLDL secretion.^{70,89} On the molecular level, sympathetic denervation decreases the mitochondrial carnitine palmitoyltransferase (CPT I-II) activities. These transporters are responsible for transferring long-chain fatty acids into mitochondria for β -oxidation.⁹⁰

Regarding pharmacological studies targeting adrenoreceptors, more studies report increased hepatic β -receptor-mediated signaling during aging, as mediator of increasing hepatic steatosis.^{91–93} However, interestingly, chronic treatment with β_3 agonist decreased high fat diet-induced liver steatosis and inflammation in mice.⁸⁷ In another study, α -receptor agonist, but not β -receptor agonist, reduced steatosis in mice kept on a high fat diet, by stimulating fatty acid oxidation and autophagy.²¹ Using primary rat hepatocytes and human hepatoma cells, Schott et al found that treatment with the β -receptor agonist isoproterenol caused substantial lipid droplet loss via activation of cytosolic adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), indicating a β -adrenoreceptor-dependent lipolysis pathway in hepatocytes. β -Adrenergic stimulation rapidly activated the protein kinase A, which led to the phosphorylation of ATGL and HSL, and their recruitment to the lipid droplet surfaces.⁹⁴ Genetic depletion of β_2 receptor could aggravate high fat diet-induced liver lipid accumulation and liver injury in mice. Namely, β_2 -depletion significantly activated PPAR γ /CD36 signaling via inactivation of the cAMP response element-binding (CREB) protein to facilitate hepatocellular lipid deposition in mice kept on a hypercaloric diet.⁹⁵

In the case of chronic consumption of high amount of fat, vagus-derived lipid sensing in the portal vein wall is likely an important mediator in the development of steatosis. Since the absorbed substance concentration is four to five times higher in the portal vein, compared with other parts of the circulatory system, the portal vein is an ideal predictor of systemic blood substance concentration.^{14,96,97} Portal infusion of lipids significantly increases the hepatic vagal afferent nerve traffic.⁹⁸ Furthermore, the vagal afferent pathway influences neuronal activity in the rostral ventrolateral medulla, contributing to the chronic sympatho-excitation.⁹⁹ Another study showed that excessive portal venous supply of long-chain fatty acids in rat contributed to the development of insulin resistance via activation of the HPA axis and the sympathetic nervous system (SNS).¹⁰⁰

Besides, vagal efferents may influence steatosis. A recent study describes that leptin injection into the mouse brain third ventricle, or stereotaxic leptin injection into the dorsal vagus complex in the medulla, decreases hepatic triglyceride content and protect from steatosis by increasing hepatic VLDL secretion and by suppressing de novo lipogenesis, independently from calorie intake. Cutting the hepatic branch of the vagus abrogates this effect, suggesting that the efferent vagus nerve conveys the leptin signal from the brain to the liver.¹⁰¹ In a

randomized placebo-controlled crossover trial with a limited number of participants ($n = 9–13$), single metreleptin (recombinant human leptin) injection stimulated hepatic VLDL secretion and reduced hepatic lipid content. However, in contrast to the previous preclinical study, metreleptin was peripherally (subcutaneously) administered to the participants. Applying the “modified sham feeding” technique of vagus stimulus in humans, the authors observed an increase in VLDL secretion, mimicking metreleptin effects.¹⁰²

Role in Inflammation—Steatohepatitis

Approximately one-third of steatosis cases progress to steatohepatitis over the time. NASH inflammation is largely a consequence of chronically elevated FFA, free cholesterol, and oxidized cholesterol metabolites within the hepatocytes, which generates reactive oxidative species (ROS). This intracellular lipotoxic environment induces mitochondrial dysfunction and endoplasmic reticulum stress, which further escalate a ROS cascade, leading to irreversible hepatocyte damage and ultimately cell death.¹⁰³ This triggers a feed-forward loop between tissue injury and inflammation.

NASH inflammation is characterized by the activation of the innate immune system. Prominent players in this process are the liver-resident macrophage Kupffer cells, which compose 80 to 90% of all colonized macrophages in the human body.¹⁰⁴ Normally, Kupffer cells exhibit an M2-like activation phenotype and are involved in tissue repair and phagocytosis of cellular debris. However, in response to hepatocyte damage, Kupffer cells polarize toward proinflammatory M1 phenotype, secreting proinflammatory cytokines such as TNF α , IL-1b, IL-6, IL-8, and IL-12, thereby recruiting T-lymphocytes, natural killer T (NKT) cells, neutrophils, and blood-derived monocytes to the liver.^{7,104–106} NKT cells are components of the innate immune system. They accumulate in the liver and regulate local proinflammatory (T helper 1) and anti-inflammatory (T helper 2) cytokine production.¹⁰⁷ Moreover, recruited NKT cells release perforin and granules, escalating liver damage.

Within the liver sinusoid, M1 Kupffer cells interact with other immune cells such as T cells, dendritic cells, and innate lymphocytes. Activation of Kupffer cells is a central event in triggering further liver injury. Namely, activated Kupffer cells are principal sources of further ROS production.¹⁰⁸ Their TNF α secretion contributes to hepatocyte apoptosis.¹⁰⁶ Moreover, M1 Kupffer cells release transforming growth factor β (TGF- β) and other profibrogenic cytokines, which will activate HCSs.¹⁰⁹ Accordingly, experimental depletion of Kupffer cells attenuates inflammation in mice kept on methionine/choline-deficient (MCD) or choline-deficient, L-amino acid-defined (CDAA) diets.^{110–112}

Finally, it is interesting to mention that while NASH inflammation is generally considered as a sterile inflammation (i.e., inflammatory response in the absence of external antigen), accumulating data suggest that pathogen-associated molecular patterns in case of gastrointestinal dysbiosis may also exacerbate or even provoke the liver innate immune system response in NAFLD.¹¹³ Namely, in patients with fatty liver, an increased gut barrier permeability develops.¹¹⁴

Microbial molecular structures like lipopolysaccharides, lipoteichoic acid, peptidoglycan, lipoglycans, and lipopeptides are recognized by toll-like receptors that are expressed by HSCs and by a wide variety of liver innate immune cells. Toll-like receptor activation then leads to the expression and release of several proinflammatory cytokines in these cells.¹¹³

As mentioned earlier, fine intraparenchymal noradrenergic fibers in the Disse space form neuroeffector junctions with Kupffer cells in humans and in nonhuman primates, and Kupffer cells express all the nine noradrenergic receptors, which overall suggest a robust sympathetic control of these macrophages. For instance, SNS promotes hepatocarcinogenesis by activating $\alpha 1$ receptors and facilitating proinflammatory environment in Kupffer cells. Inhibition of SNS reduces IL-6 and TGF- β expression, which suppresses hepatocarcinogenesis.⁵⁶ Moreover, Kupffer cell proinflammatory cytokine secretion (TNF α , IL-1b, IL-6) is potentiated by gut-derived noradrenaline release in sepsis.¹¹⁵

Regarding the context of NAFLD spectrum, the leptin-deficient Ob/Ob mice, characterized by obesity and fatty liver, showed elevated hepatic TNF α expression, and treatment with anti-TNF antibodies improved liver histology and reduced hepatic total fatty acid content.¹¹⁶ Interestingly, Ob/Ob mice exhibit reduced noradrenaline level, due to a lower rate of noradrenaline synthesis and metabolism.¹¹⁷ This increases hepatic NKT cell apoptosis and depletes NKT cells. As a result of this, hepatic proinflammatory cytokine production is increasing, which sensitizes lipotoxicity in the fatty liver. Noradrenaline treatment reduces NKT cell apoptosis and reduces hepatic inflammation in Ob/Ob mice.¹¹⁸ The hepatic sympathetic neuropathy in mice kept in a high fat diet was mediated by TNF α derived from CD11b⁺ F4/80⁺ immune cells (Kupffer cells, macrophages).²³ In another recent study, 4-week administration of the $\beta 3$ agonist BRL37344 attenuated lobular inflammation (the number of inflammation foci) in high fat diet-fed mice.⁸⁷

The vagus nerve is also supposed to regulate hepatic inflammation in NASH, via $\alpha 7$ nicotinic cholinergic receptors on Kupffer cells. $\alpha 7$ knockout mice on MCD diet developed NASH faster than control mice, with highly activated Kupffer cells. Moreover, hepatic vagotomy aggravated the diet-induced NASH.¹¹⁹ Furthermore, stimulation of the cervical trunk of the vagus nerve in mice increases the phagocytosis activity of Kupffer cells.¹²⁰

Finally, we should also emphasize the role of HSCs in proinflammatory actions in injured liver. Namely, noradrenaline stimulates the secretion of inflammatory chemokines (RANTES and interleukin-8) in a dose-dependent manner, and triggers NF-kappaB activation in HSCs *in vitro*.⁵⁵

Role in Hepatic Regeneration and Fibrosis in NAFLD

SNS regulates liver repair by modulating HSCs (main fibrogenic cells in liver) and hepatic progenitor cells (HPCs).

The resident HPCs help regenerate the epithelial compartment after severe liver injury. The number of HPCs has been found to be increased in NAFLD.¹²¹ In general, noradrenaline and adrenoceptor agonists stimulate the proliferation of

cholangiocytes.^{61,62} Moreover, the β -receptor agonist isoproterenol promoted recovery of the HPC pool in dopamine β -hydroxylase (DBH)-deficient mice after acetaminophen-induced liver injury.¹²² Contrary, in an earlier study, the number of HPCs was increased after chemical sympathectomy by 6-hydroxydopamine (6-OHDA).⁶⁵

HSCs (or perisinusoidal cells or Ito-cells; with older terminology: lipocytes or fat-storing cells) are composed of one-third of nonparenchymal cells and 8% of all cells in the mammalian liver.¹²³ With “dendritic-like” processes, they form direct contacts with sinusoidal endothelial cells, hepatocytes, and Kupffer cells, which promote intercellular crosstalk and transport of soluble mediators among these cell types.¹²⁴ Moreover, HSCs are neuroglia-like cells,¹²⁵ expressing noradrenaline synthesizing enzymes,^{53,65} acetylcholine synthesizing enzymes, most adrenoceptors, glial fibrillary acidic protein, neural cell adhesion molecule, and synaptophysin.^{66,125–127} HSCs release noradrenaline and adrenaline in human primary cell culture⁵⁴ and murine HSC lysates contain dopamine, serotonin, and catecholamine metabolites.⁵³ A recent study, however, using single-cell RNA sequencing has reported that only a subpopulation of HSCs has the gene expression signature of noradrenaline synthesis, pointing to the heterogeneity of these cells.¹²⁸ ELMI studies confirmed that HSCs, just like Kupffer cells, are innervated by fine noradrenergic fibers.³⁷

In fibrosis, HSCs from quiescent phenotype move to an activated, myofibroblastic phenotype. They release hepatic growth factor that promotes hepatocyte proliferation and maintenance.¹²⁹ They also express alpha smooth muscle actin (α SMA) and fibrogenic matrix proteins.¹³⁰ Activation of HSCs is originally part of a regenerating process, which, in the long term, may turn to fibrosis and then ultimately to cirrhosis, due to inadequate communication between HSCs and neighboring cells.¹³¹

Exogenous noradrenaline dose-dependently increases the proliferation and collagen expression of activated HSCs. Activated HSCs in human primary culture upregulate a/b adrenoceptors as well.^{54,132} Injury-related fibrogenic response was inhibited in DBH knockout mice, evidenced by reduced expression of α SMA, decreased induction of TGF- $\beta 1$, and collagen in cultured HSCs from DBH knockout animals.⁵³ Moreover, Ob/Ob mice with low catecholamine level and lower SNS tone are resistant to liver fibrosis,¹³³ and chemical sympathectomy or $\alpha 1$ receptor antagonism inhibits carbon-tetrachloride (CCl₄)-induced liver fibrosis in rats.¹³⁴ Overall, activation of liver noradrenergic (sympathetic) nerves exacerbates liver fibrosis, while sympathetic inhibition attenuates it.

Importantly, acetylcholine also facilitates HSCs proliferation via intracellular activation of phosphoinositide 3-kinase and MEK cell survival pathway, and induces TGF- β and collagen fibrogenesis via M2 and M3 receptors.⁶⁶

Role in Hepatic Hemodynamics in NAFLD

Portal hypertension (PH; supraphysiological pressure in the portal venous system) is a severe complication in liver cirrhosis.¹³⁵ The pathophysiology of PH involves both

hepatic factors (increased intrahepatic resistance to portal venous and sinusoidal blood flow) and/or extrahepatic effects (splanchnic arterial vasodilation).^{135,136} Although PH has mostly been discussed in the context of cirrhosis, elevated portal venous pressure has also been detected in earlier phases of NAFLD, when fibrosis is less advanced, and cirrhosis is absent.^{136,137}

Numerous earlier studies reported decreased hepatic blood flow and increased hepatic arterial and portal venous pressure after electrical stimulation of the hepatic nerves in rat,¹³⁸ dog,¹³⁹ or cat.¹⁴⁰ Moreover, electrical stimulation of portal vein-associated nerves elicited α -receptor-mediated constriction of portal venules, sinusoids, and hepatic arteries, but no response could be recorded from the central vein.⁵⁸ Intraportal injection of 6-OHDA prevented the large reduction of hepatic blood volume normally seen with nerve stimulation, while the contractive response to noradrenaline infusion was not altered.¹⁴¹ In a rat study, repetitive periportal nerve stimulation was accompanied with a gradually diminished noradrenaline overflow, while the metabolic (glucose output) and hemodynamic (portal vein contraction) effects remained unchanged,¹⁴² possibly indicating an adrenergic receptor sensitization during repeated stimulation. While β 1–2 adrenoreceptors have vasoconstrictor effects, β 3 is a vasodilator receptor. β 3 agonist treatment in rats significantly and dose-dependently caused portal vein (but not central vein) relaxation and decreased portal pressure in cirrhosis (induced by CCl₄), but not in controls.⁶⁰

Early investigations showed a direct relation between the size of portal/sinusoid pressure and circulating level of noradrenaline in cirrhosis.¹⁴³ Based on advanced 3D imaging, we recently described and quantitatively characterized a portal vein stenotic alteration (abnormal narrowing) in mouse experimental steatohepatitis. Systematic 3D analysis revealed that such portal stenosis ended exactly where the already degenerating noradrenergic innervations terminated, followed by dilated distal portal branches (–Fig. 3A,B). At the same time, the central vein system that virtually lacks innervation did not show decreased volume or shrunken morphology. These findings propose that the liver periportal sympathetic nerves could contribute to a stenotic portal vein condition in advanced stages of NAFLD.²² The latter results also suggest that these morphologically impaired sympathetic fibers in NASH are still in operation, albeit presumably with impaired functionality. Taken together with the well-established chronically high sympathetic tone in NAFLD, a potential role of periportal sympathetic nerves in the development of PH is suggested.

Furthermore, HSCs are extensively involved in the regulation of hepatic sinusoidal microcirculation by contraction and relaxation.¹⁴⁴ In PH, resistance of the sinusoidal wall is increasing, leading to a decreased microcirculation. Thus, HSCs proliferation and increased contractility may also contribute to PH,¹⁴⁵ and noradrenergic signaling may serve as a significant factor in such process.¹⁴⁴

Finally, increased portal/sinusoid pressure leads to further activation of sympathetic nerves in heart and kidney,

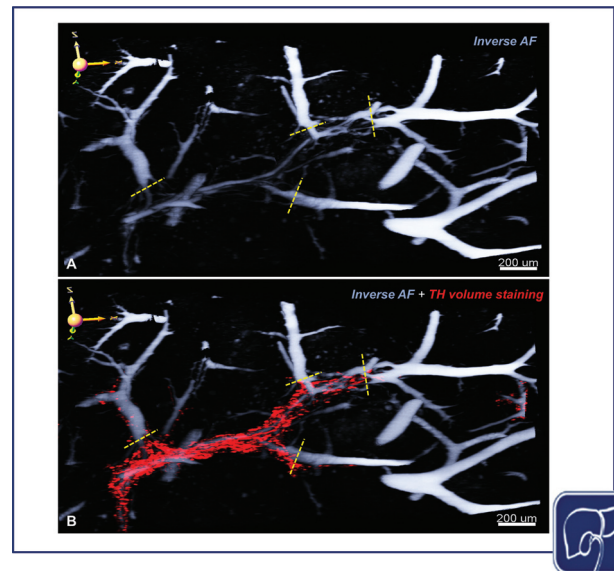


Fig. 3 Portal vein stenosis in experimental steatohepatitis and its spatial correlation with the remaining sympathetic nerves. (A, B) Liver vasculature in steatohepatitic mouse is visualized by inverted auto-fluorescence (AF) in 3D (gray channel in A and B). Portal vein stenotic alteration is correlating with the extension of the already degenerating noradrenergic nerve fibers (tyrosine hydroxylase [TH] volume immunostaining, red channel in B). Yellow dashed lines indicate the end of TH⁺ innervations. (Micrographs are from Adori et al.²²)

which is mediated by activation of non-volume-dependent hepatic baroreceptors.¹⁴⁶

Summary and Concluding Remarks

Accumulating evidence suggests that hepatic innervations have multiple roles in the pathomechanism of NAFLD. Major players are supposed to be the efferent sympathetic (noradrenergic) nerves, affecting the development/progression of steatosis, inflammation, fibrosis, and liver hemodynamical alterations. Sympathetic nerve effects on the liver are overwhelmingly complex and lots of details are still poorly understood.

Namely, while chronically high sympathetic tone seems to drive liver steatosis, more studies have shown that β -adrenoreceptor agonists actually protect against steatosis in experimental NAFLD. The extensive interaction between sympathetic innervation and Kupffer cells is doubtless, but it needs further exploration in the context of steatohepatitis. Here, the *balance* between proinflammatory M1 Kupffer cells and anti-inflammatory M2 Kupffer cells is likely crucial to regulate the occurrence and development of liver inflammation and subsequent injury.¹⁴⁷ Accordingly, limiting the amount of M1 Kupffer cells and promoting M2 Kupffer cell polarization may be a valuable therapeutic strategy in attenuating inflammation in NAFLD. Activation of various subsets of adrenergic receptors seems to produce antagonistic effects on monocytes. Namely, α -receptors are both pro- and anti-inflammatory, while β -adrenergic receptors mostly show

immunosuppressive and anti-inflammatory properties.^{148,149} Finally, based on the available data, sympathetic nerve stimuli in NAFLD are clearly profibrotic with promoting HSC activation to myofibroblastic phenotype, and mostly vasoconstrictor, albeit β_3 receptors have vasodilator effects in mice.

As discussed in this review, several noradrenergic receptors have been described in hepatocytes, cholangiocytes, and in the wall of portal vessels. Moreover, virtually all noradrenergic receptors are expressed by Kupffer cells and HSCs. In a healthy liver, orchestration of noradrenergic receptors by the sympathetic nerves maintains equilibrium in several signaling mechanisms with sometimes antagonistic effects, which overall promotes liver metabolic homeostasis. These fine-tuned balances may slowly change under metabolic stress and/or with altered nerve functionality. Specifically, in NAFLD, chronically high sympathetic tone may lead to alterations in receptor expression levels, in receptor sensitivity, and perhaps in signal transduction cascades, with a cell-autonomous spatial-temporal character. Besides, increased plasticity (disorganization, ectopic sprouting, and mild trimming) of liver sympathetic nerves in earlier phase of the disease (steatosis), and degeneration of sympathetic nerves in more advanced stages (steatohepatitis), have been described in NAFLD. These structural alterations likely come along with impaired nerve functionality and lack of adequate signaling, which further affect the receptors, as a vicious cycle of hepatic noradrenergic pathology. All these may contribute to a multiphase hepatic “catecholamine resistance” (inability of (nor)adrenaline to induce a defined response),¹⁵⁰ which should be considered when planning and evaluating pharmacological studies or pharmacotherapeutic strategies targeting the SNS in NAFLD.

Afferent vagal nerves also seem to be significant in the pathomechanism of NAFLD. Specifically, lipid sensing by vagal afferent fibers in the portal vein wall is likely an important player in the development of steatosis by influencing neuronal activity in the ventrolateral medulla and, consequently, by contributing to the chronic sympathoexcitation.

Future Perspectives

In an interesting recent study, Wang et al reported that sustained sleep deprivation promotes hepatic steatosis, which is mediated by sympathetic overactivation.¹⁵¹ Other studies also found that short sleep duration is a risk of incident NAFLD.^{152,153} Considering that sleep deprivation, poor sleep quality, and stressful lifestyle are very common in modern societies, their metabolic consequences that possibly imply hepatic steatosis due to elevated sympathetic tone may need further investigations.

Also, despite its high importance, only a few studies explored the development and integrity of hepatic nerves of offspring in case of maternal obesity or perinatal/early postnatal high fat nutrition.⁸² More information may be needed in this highly relevant field, since a potential early abnormal hepatic nerve development may have long-lasting

consequences, particularly in the case of metabolic challenges later in life.

Numerous studies examined parasympathetic nerve effects on the liver by hepatic vagotomy experiments. However, as emphasized by Berthoud, cutting the common hepatic vagal branch denervates not only the liver but also parts of the distal stomach, pylorus, duodenum, and pancreas.⁴⁰ Moreover, common hepatic branch vagotomy not only destroys vagal fibers, since approximately 30% of the nerve fibers running in this branch are of nonvagal origin.¹⁵⁴ As mentioned earlier, we and others failed to detect parasympathetic (cholinergic) nerves in rodent, nonhuman primate, and human livers by using volume imaging.^{22,23} However, cholinergic fibers were described around the liver hilum with tracing studies, and some authors speculate that parasympathetic nerves may act on the liver indirectly, by innervating hilar sympathetic nerve structures.⁴⁵ Overall, more detailed and comparative anatomical studies are needed concerning the parasympathetic liver innervations.

The pathogenesis of NAFLD is incompletely understood. Currently, the therapeutic options are limited to lifestyle changes and no approved drug therapy exists that may address both the progression of liver fibrosis and associated metabolic disturbances in NAFLD.¹⁵⁵

Hepatic nerves, primarily the efferent sympathetic (noradrenergic) nerves, have multiple subtle regulatory roles in liver glucose and lipid metabolism, bile secretion, inflammatory reaction, hemodynamics, and regeneration. However, their role may be more essential during the fight-or-flight response or when subjected to metabolic challenges, like in the case of NAFLD.¹⁵⁶ Future studies, simultaneously considering structural and functional alterations of hepatic nerves and their receptors, may reveal novel aspects in the pathophysiology of NAFLD, which could ultimately lead to pharmacotherapeutic advances.

Conflict of Interest

None declared.

Acknowledgments

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