Review

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Role of liver sinusoidal endothelial cells in non-alcoholic fatty liver disease

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Summary

Non-alcoholic fatty liver disease (NAFLD) and its complications are an expanding health problem associated with the metabolic syndrome. Liver sinusoidal endothelial cells (LSECs) are highly specialized endothelial cells localized at the interface between the blood derived from the gut and the adipose tissue on the one side, and other liver cells on the other side. In physiological conditions, LSECs are gatekeepers of liver homeostasis. LSECs display anti-inflammatory and anti-fibrogenic properties by preventing Kupffer cell and hepatic stellate cell activation and regulating intrahepatic vascular resistance and portal pressure. This review focusses on changes occurring in LSECs in NAFLD and on their consequences on NAFLD progression and complications. Capillarization, namely the loss of LSEC *fenestrae*, and LSEC dysfunction, namely the loss of the ability of LSECs to generate vasodilator agents in response to increased shear stress both occur early in NAFLD. These LSEC changes favour steatosis development and set the stage for NAFLD progression. At the stage of non-alcoholic steatohepatitis, altered LSECs release inflammatory mediators and contribute to the recruitment of inflammatory cells, thus promoting liver injury and inflammation. Altered LSECs also fail to maintain hepatic stellate cell quiescence and release fibrogenic mediators, including Hedgehog signalling molecules, promoting liver fibrosis. Liver angiogenesis is increased in NAFLD and contributes to liver inflammation and fibrosis, but also to hepatocellular carcinoma development. Thus, improving LSEC health appears to be a promising approach to prevent NAFLD progression and complications.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of conditions including simple steatosis and non-alcoholic steatohepatitis (NASH), defined as the association of steatosis, hepatocellular damage, inflammation and varying degrees of fibrosis.[1] NAFLD is associated with obesity, insulin resistance and/or type 2 diabetes and other metabolic abnormalities, collectively termed the metabolic syndrome.[2,3] NAFLD is an expanding health problem with an estimated global prevalence of 25%.[2,4] A recent modelling approach estimated that NAFLD cases in the United States will expand from 83 million in 2015, corresponding to about 25% of the population, to 100 million in 2030, corresponding to more than 33% of the population.[4] While simple steatosis is generally benign, NASH can progress to both cirrhosis and end-stage liver disease. NASH is currently a leading cause of liver disease among adults awaiting liver transplantation in Europe and in the United States and is projected to become the most common indication for liver transplantation in the next decade.[2,4] Importantly, patients with metabolic syndrome and NASH also develop hepatocellular carcinoma (HCC) in the absence of cirrhosis.[5,6] Despite its prevalence and severity, there is no approved therapy for NASH and available treatments only aim to control associated conditions.[1] Understanding the mechanisms of NAFLD, and in particular how simple steatosis progresses to NASH and then to cirrhosis and/or liver cancer, is of the utmost importance.

The current view of the pathogenesis of NASH centres on the response of hepatocytes to insulin resistance and lipotoxicity. The immune system and hepatic stellate cell activation are regarded as secondary events.[1] The vascular endothelium, representing the interface between blood and other tissues of the body, is not only a physical barrier but is implicated in different physiological roles, such as haemostasis, metabolite transportation, inflammation, thrombosis, angiogenesis and vascular tone. The liver endothelium is mainly formed of liver sinusoidal endothelial cells (LSECs) which are highly specialized endothelial cells at the interface between blood derived from the visceral adipose tissue and the gut, on the one side, and hepatic stellate cells and hepatocytes, on the other side. LSECs have a unique phenotype in the human body as they lack a basement membrane and have a multitude of *fenestrae* organized into sieves, that regulate the transport of macromolecules, including lipids and lipoproteins, across the sinusoid.[7,8] This review will specifically focus on the role of LSECs in the pathophysiology of NAFLD and its complications.

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LSECs and simple steatosis

Role of LSECs in lipid transfer in the normal liver

Dietary lipids present in the circulation have to be transported through the vascular endothelium to be metabolized by tissues. In physiological conditions, LSECs are major regulators of the bidirectional lipid exchange between the blood and the liver parenchyma. First, LSEC *fenestrae* allow for efficient transfer of lipoproteins, chylomicron remnants (small lipoproteins derived from chylomicrons generated by enterocytes from dietary lipids), and other macromolecules, from the sinusoidal blood to the space of Disse, where they are taken up by hepatocytes.[9–11] LSEC *fenestrae* form a selective barrier for lipids. Indeed, older studies using radiolabelled lipoproteins showed that larger lipoproteins do not cross LSEC *fenestrae* and remain in the lumen of the sinusoid.[12] Moreover, when LSECs lose their *fenestrae* following vascular endothelial growth factor (VEGF) pathway disruption,[11,13,14] uptake of fluorescently labelled lipids is impaired.[11,15] Second, LSECs also regulate lipid transfer through their high capacity for endocytosis, as shown by their ability to rapidly take-up oxidized or acetylated-low density lipoproteins.[8,16,17]

LSEC capillarization occurs early in NAFLD and promotes steatosis

LSECs undergo morphological and functional changes during liver steatosis.[18–21] One of the most remarkable phenotypic changes is the loss of *fenestrae*, also called defenestration or sinusoidal capillarization, associated with the formation of a basement membrane on the abluminal surface of LSECs. Several independent groups reported that sinusoidal capillarization appears very early in NAFLD.[18–20] Miyao and colleagues observed that defenestration begins after 1 week of choline-deficient, L-amino acid-defined diet in mice.[18] Similar observations were made in rats fed a high-fat diet for 3 weeks.[20]

Triggers for sinusoidal capillarization are not fully identified, but we can speculate that excessive dietary macronutrients, including lipids, carbohydrates, and gut microbiota-derived products play a role.[19] Cogger and coworkers demonstrated in mice challenged with several diets varying in content of macronutrients and energy that LSEC porosity and *fenestrae* frequency are inversely correlated with dietary fat intake, while *fenestrae* diameter is inversely correlated with protein or carbohydrate intake.[19] In this study, authors also found a negative correlation between LSEC *fenestrae* (frequency, porosity and diameter) and circulating free fatty acid (FFA) levels.[19] *In vitro* studies suggested that defenestration occurs following excessive lipid exposure. For instance, exposure of human primary LSECs to oxidized low-density lipoprotein (ox-LDL) reduces the diameter and the porosity of the *fenestrae*.[21] The effect of FFA on *fenestrae* has also been tested in primary rat LSECs, but firm conclusions cannot be drawn as the authors did not test several concentrations of FFA but rather the presence *vs.* the absence of FFA, which does not adequately mimic *in vivo* conditions.[22] Recent evidence point to gut microbiota in the pathogenesis of NAFLD.[3,23] Cogger and colleagues showed that LSEC *fenestrae* inversely correlated with the abundance of *Bacteroidetes* in the gut and positively correlated with the abundance of *Firmicutes*.[19] Moreover, it has been shown that a single injection of endotoxin in rats induces a decrease in both diameter and number of *fenestrae* suggesting that gut microbiota-derived products may contribute to LSEC capillarization, although caution is needed since the concentration of endotoxin used in that study was high.[24]

In turn, capillarization favours liver steatosis (Fig. 1), as observed in mice deficient in plasmalemma vesicle-associated protein (PLVAP), an endothelial-specific integral membrane glycoprotein required for the formation of endothelial *fenestrae*.[25] These mice exhibit a pronounced reduction in the number of LSEC *fenestrae,* associated with a decrease in sinusoidal permeability,[25] and spontaneously develop extensive steatosis.[25] These mice also have hyperlipoproteinemia and increased triglyceridemia due to the retention of chylomicron remnants in the blood. As mentioned above, *Vegfb*$^{-/-}$ mice also exhibit a reduction in the number of LSEC *fenestrae* and less uptake of labelled oleic acid due to capillarization, but steatosis was not evaluated.[15]

A first hypothesis explaining this consequence of capillarization on steatosis could be that reduced LSEC permeability impairs the passage of hepatocyte-derived very low-density lipoprotein toward the sinusoidal lumen, thus inducing cholesterol and triglyceride retention in the liver. However, these lipoproteins may escape the liver through the lymphatic system.[26] As an alternative explanation, Herrnberger *et al.* proposed that chylomicron remnants originating from the blood, and required for synthesis of very low-density lipoprotein by hepatocytes, cannot reach hepatocytes due to LSEC capillarization; their absence in hepatocytes might then stimulate *de novo* hepatic lipid synthesis and induce steatosis as a compensatory mechanism.[25,27] However, there is no available data to ascertain this hypothesis. Similarly, Fraser and collaborators postulated that, following LSEC capillarization, chylomicron remnants and dietary cholesterol no longer cross the *fenestrae* to inhibit HMGCoA reductase, the rate limiting enzyme for hepatocyte cholesterol biosynthesis, consequently activating endogenous cholesterol synthesis in hepatocytes.[28]

Taken together, these data suggest that features of metabolic syndrome are associated with LSEC capillarization, which promotes steatosis (Fig. 1).

Key point

LSEC capillarization and dysfunction occur very early in NAFLD progression and contribute to dietary induced steatosis.

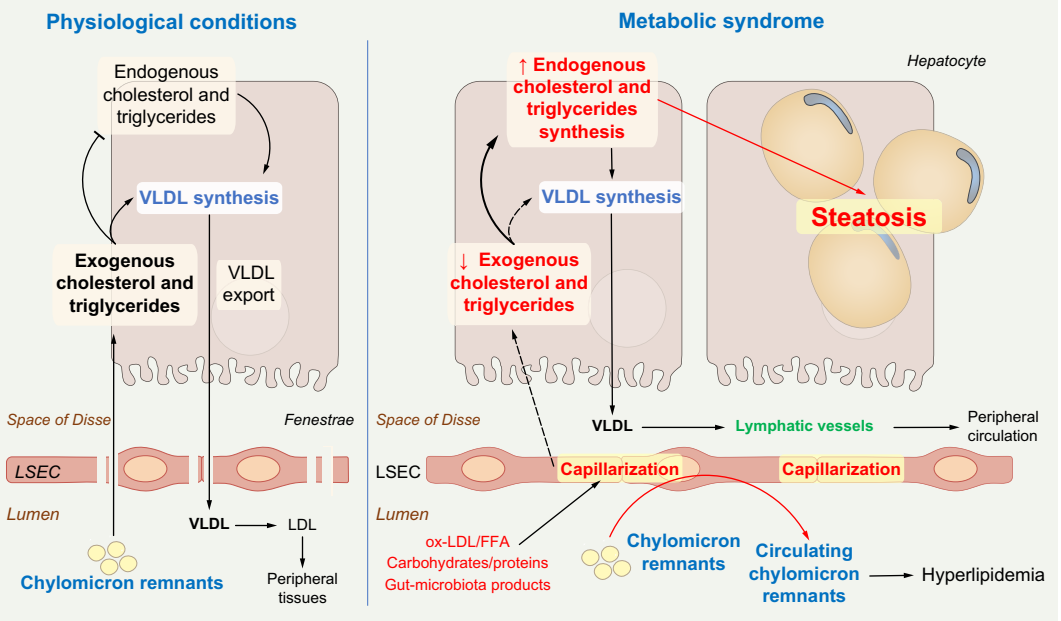

Fig. 1. LSEC capillarization promotes steatosis. *In physiological conditions,* chylomicron remnants cross LSEC *fenestrae* and provide cholesterol and triglycerides for VLDL synthesis. VLDL are then released by hepatocytes and reach blood flow through *fenestrae*. *In metabolic syndrome conditions,* LSEC capillarization arises early in the course of NAFLD, possibly because of exposure of LSECs to dietary macronutrients. In turn, LSEC capillarization promotes steatosis, possibly because capillarization blocks the transfer of chylomicron remnants to hepatocytes, thus stimulating endogenous cholesterol and triglyceride synthesis, as a compensatory mechanism for the synthesis of VLDL, which reach blood flow through the lymphatic system. FFA, free fatty acids; LDL, low-density lipoprotein; LSECs, liver sinusoidal endothelial cells; NAFLD, non-alcoholic fatty liver disease; ox-LDL, oxidized low-density lipoprotein; VLDL; very low-density lipoprotein.

Key point

Intrahepatic vascular resistance is increased even when steatosis is the only histological feature of NAFLD. This is due to the combination of a compression of sinusoids by fat-laden enlarged hepatocytes and of a dysfunction of LSECs due to reduced nitric oxide bioavailability.

LSECs dysfunction occurs early in NAFLD and promotes steatosis

Liver steatosis is associated with an increased portal pressure and increased intrahepatic vascular resistance.[29–32] In patients, hepatic venous pressure gradient correlates with the degree of steatosis.[29] Using Doppler flowmetry, Seifalian and colleagues observed impaired sinusoidal perfusion in human fatty liver grafts compared with normal liver grafts.[33] Similar results were obtained in rabbits and rats with diet-induced steatosis, with an inverse correlation between hepatic parenchymal microcirculation and the severity of steatosis.[34,35]

This increased intrahepatic vascular resistance has a mechanical and a dynamic component. The mechanical part is due to the compression of the sinusoidal lumen by enlarged fat-laden hepatocytes.[29,36–38] The dynamic part is due to liver endothelial dysfunction. Endothelial dysfunction is a pathological condition, common to all vascular beds, defined as the inability of blood vessels to dilate in response to increased blood flow. Endothelial dysfunction is generally indicated by the loss of nitric oxide bioavailability due to eNOS (also called NOS3) inhibition.[39] Several lines of evidence show that LSEC dysfunction occurs in fatty livers and is involved in the increased intrahepatic vascular resistance associated with steatosis.[40–43] eNOS activation and liver nitric oxide content are reduced in mice and rats fed a high-fat diet or a diet rich in saturated fatty acids for 4 weeks.[40,41] Isolated-perfused liver experiments performed in these animals showed augmented portal perfusion pressure and reduced vasodilatory response to acetylcholine, indicating liver endothelial dysfunction. These changes were observed in the absence of inflammation and fibrosis, suggesting that endothelial dysfunction is an early feature associated with steatosis in NAFLD.[41,42]

Several mechanisms could account for this liver endothelial dysfunction associated with steatosis (Fig. 2). First, LSECs dysfunction can be induced by overabundance of lipids during steatosis. *In vitro* experiments showed that stimulation of human primary LSECs with ox-LDL downregulates eNOS expression through the ox-LDL receptor, LOX1.[21] In addition, exposure of primary LSECs to palmitic acid also attenuates nitric oxide bioavailability through peroxynitrite production by NOX1, a nitric oxide consuming enzyme highly expressed in LSECs of mice fed a high-fat diet.[44] Second, steatosis induces insulin resistance in LSECs, leading to impairment of insulin-dependent vasodilation.[41,45,46] This effect is due, on the one hand, to the downregulation of eNOS activity,[41] and on the other hand to the upregulation of iNOS (also called NOS2), the inducible form of NOS which can cause endothelial dysfunction through increased nitro-oxidative stress.[46–48] Interestingly, V-PYRRO/NO – a diazeniumdiolate ion metabolized in the liver that spontaneously

decomposes to nitric oxide with a very short half-life at physiological pH and that triggers cyclic guanosine 3′,5′-monophosphate (cGMP) synthesis – improves hepatic microcirculation in mice with steatosis induced by a high-fat diet.[49] Third, the gut microbiota also seems to contribute to liver endothelial dysfunction. Indeed, Garcia-Lezana and colleagues demonstrated that restoration of a healthy microbiota *via* faecal transplantation normalizes portal hypertension by improving intrahepatic vascular resistance and endothelial dysfunction in rats.[43]

In turn, LSEC dysfunction favours steatosis (Fig. 2). Indeed, deficiency in nitric oxide in *eNos*$^{-/-}$ mice results in massive fat droplet deposition and increases liver triglyceride content.[40,50] Nitric oxide contributes to the regulation of hepatic lipid content by limiting citrate synthesis in mitochondria, which is involved in fatty acid production.[50] Nitric oxide also attenuates synthesis of fatty acids in isolated cultured rat hepatocytes by nitrosylating acetyl-CoA[51] and activating AMP-activated protein kinase,[52–54] an inhibitor of glycerol-3-phosphate acyltransferase and thus of triacylglycerol synthesis.[55,56] In addition, nitric oxide also allows efficient fatty acid beta-oxidation through s-nitrosylation of very long-chain acyl-CoA dehydrogenase in hepatocytes.[57] Interestingly, therapies augmenting nitric oxide availability in the liver ameliorate steatosis. The V-PYRRO/NO or the improvement of nitric oxide/cGMP signalling with the phosphodiesterase-5 inhibitor sildenafil protect against liver steatosis in mice fed a high-fat diet.[40,58,59] Moreover, treatment with simvastatin, a drug able to increase expression and activity of eNOS expression in the liver, decreases steatosis induced by a high-fat diet in rats.[60]

To summarize, steatosis is associated with LSEC dysfunction which in turn worsens steatosis (Fig. 2).

Angiogenesis and steatosis

Angiogenesis, defined as the formation of new vessels from pre-existing vessels, is a key event in the progression of NAFLD.[61–65] VEGF is the master pro-angiogenic regulator of this process supported by activation of hypoxia inducible factors (HIFs) in hypoxic areas.[64]

Serum VEGF levels are higher in patients with biopsy-proven steatosis than in healthy individuals.[62,63,65] In animal models, liver expression of VEGF and CD105, an endothelial cell marker, increase after 3 days of methionine- and choline-deficient diet in obese and diabetic *db/db* transgenic mice and after 1 week of this diet in C57BL6/J mice, before NASH appears.[63] However, 3 studies reported that new vessels develop in the livers of patients with NASH, but not in individuals with simple steatosis or normal livers.[66–68] This suggests that molecular events associated with upregulation of angiogenic factors start early in the course of NAFLD, while angiogenesis appears later, as detailed below.

LSECs in NASH

LSECs contribute to oxidative stress in NASH

In response to lipotoxicity, hepatocytes generate reactive oxygen species (ROS) and initiate a robust inflammatory response that accentuates liver injury.[1,3] In addition to hepatocytes,[1,3] the lipotoxic response also occurs in LSECs contributing to ROS generation.[44,69] Indeed, ROS have been detected not only in hepatocytes but also in sinusoidal cells in patients with NASH.[70] Circulating lipids seem to account for the oxidative stress in LSECs. Indeed, exposure of murine LSECs to palmitic acid upregulates NOX1 expression, an enzyme implicated in ROS production.[44] In addition, stimulation of human primary LSECs with ox-LDL increases ROS generation after binding to LOX1.[21] This oxidative stress in LSECs contributes to NASH. Indeed, mice with a global deficiency in NOX1, which is highly expressed in LSECs in NAFLD, had attenuated liver lesions when fed a high-fat diet, as shown by lower serum ALT level and lower hepatic cleaved caspase-3 expression.[44] Therefore, in NASH, ROS production takes place not only in hepatocytes, but also to some extent in LSECs, and seems to contribute to hepatocyte injury.

Anti-inflammatory role of LSECs at initial stages of NASH

Progression of simple steatosis to steatohepatitis is accompanied by adhesion of leukocytes to the sinusoidal endothelium followed by infiltration of leukocytes within liver parenchyma to form inflammatory foci.[71] Moderate and resolved inflammatory responses are beneficial to the liver as they promote the re-establishment of homeostasis, contribute to tissue repair and exert hepatoprotective effects.[72] However, chronic inflammation, as seen in NASH, leads to death of hepatocytes and causes damage to the liver parenchyma.[72] In physiological conditions, LSECs constitute a barrier regulating the entry of circulating leukocytes within liver parenchyma and playing an anti-inflammatory role.[73–76] At early stages of NAFLD progression, some evidence indicates that LSECs also exhibit anti-inflammatory functions.[40,77] Indeed, Tateya and colleagues elegantly demonstrated that nitric oxide derived from LSECs inhibits Kupffer cell activation in mice fed a high-fat diet for a short period of time (8 weeks).[40] *In vitro*, both human and murine LSECs exposed to FFA for a short period (16 hours) exhibit a downregulation of pro-inflammatory chemokines involved in monocyte and macrophage recruitment, through a MAPK dependent pathway.[77]

Key point

Molecular events associated with angiogenesis are initiated during simple steatosis, but angiogenesis itself is only detected in NASH.

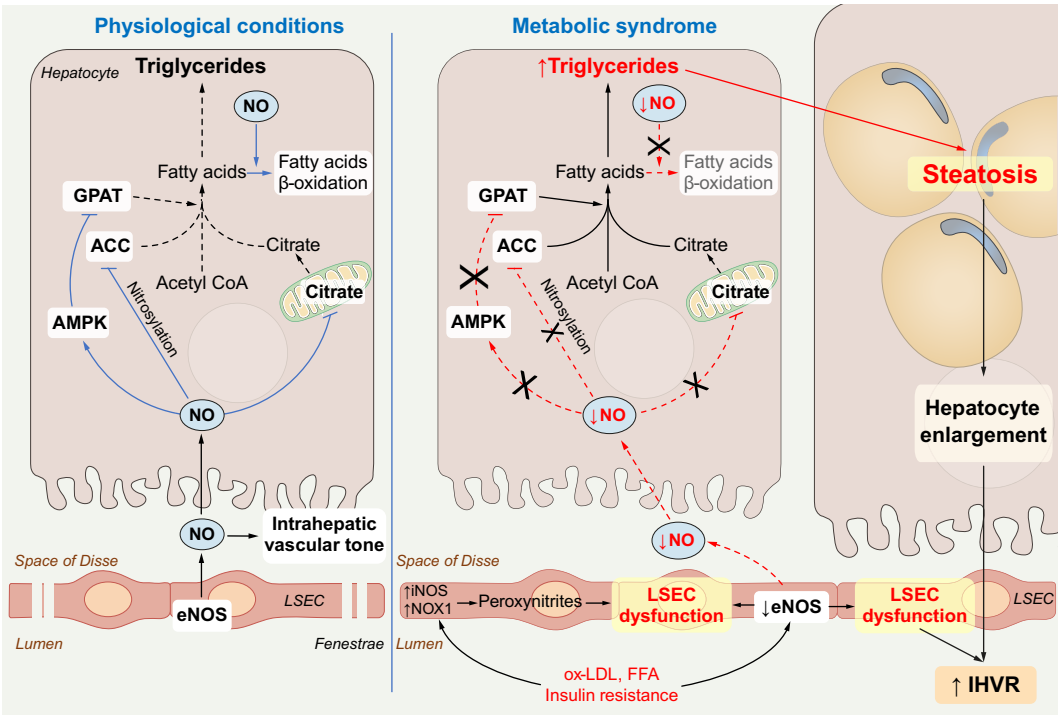

Fig. 2. LSEC dysfunction promotes steatosis. *In physiological conditions,* LSECs release NO which regulates intrahepatic vascular tone on the one hand, and hepatic lipid metabolism on the other hand. NO limits hepatic lipid content by inhibiting hepatic *de novo* lipogenesis, through a limitation of citrate synthesis in mitochondria, an inhibition of ACC and of GPAT, and by promoting fatty acids beta-oxidation. *In metabolic syndrome conditions,* overabundance of lipids and insulin resistance lead to downregulation of eNOS activity and to upregulation of iNOS and of NOX1 (a nitric oxide consuming enzyme), causing nitro-oxidative stress through peroxynitrite production and eventually endothelial dysfunction. Reduced NO availability promotes steatosis. Liver steatosis is associated with an increased intrahepatic vascular resistance which has a mechanical component, due to the compression of the sinusoidal lumen by enlarged fat-laden hepatocytes, and a dynamic component, due to a liver endothelial dysfunction. ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; eNOS, endothelial nitric oxide synthase; GPAT, glycerol-3-phosphate acyltransferase; LSECs, liver sinusoidal endothelial cells; IHRV; intrahepatic vascular resistance; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOX1, NADPH oxidase 1.

Key point

Lipotoxicity and inflammation induce endothelial inflammation. Activated LSECs release cytokines and chemokines and overexpress adhesion molecules, thus sustaining liver inflammation.

LSECs promotes liver inflammation at more advanced stages of NASH

As mentioned, LSEC alterations arise early in NAFLD progression, prior to liver inflammation.[18,41] Indeed, LSEC capillarization precedes Kupffer cell activation[18,41] and liver nitric oxide content falls before liver NF-kB activation and TNFα, IL-6 and ICAM-1 upregulation.[30,40,41] LSEC capillarization and dysfunction are permissive for establishment of liver inflammation. Indeed, mice deficient in eNOS exhibit an accelerated hepatic inflammatory response, while improving nitric oxide/cGMP signalling with the phosphodiesterase-5 inhibitor sildenafil or with simvastatin prevents liver inflammation in rodents fed a high-fat diet.[40,60]

During NALFD progression, LSECs then acquire a pro-inflammatory phenotype and functions (Fig. 3). LSECs pro-inflammatory phenotype during NASH is characterized by progressive overexpression of adhesion molecules including ICAM-1, VCAM-1 and VAP-1 (AOC3) at the surface of LSECs, as observed in mouse models of NASH.[68,78–82] LSECs also produce a number of pro-inflammatory mediators in NASH, including TNFα, IL-6, IL-1 and MCP1 (CCL2).[68,82–84]

This pro-inflammatory phenotype of LSECs in NASH is associated with pro-inflammatory functions (Fig. 3). First, dysfunctional LSECs fail to maintain Kupffer cell quiescence.[40] Second, the release of inflammatory mediators by LSECs contributes to the inflammatory response by activating neighbouring Kupffer cells, and by favouring recruitment, adhesion and transmigration of blood leukocytes.[82,85,86] The mechanisms of interaction between leukocytes and LSECs in NASH have been reviewed elsewhere in detail and are summarized in Fig. 3.[80,87–89] LSECs' expression of ICAM-1, VCAM-1 and VAP-1 is crucial for these interactions since *in vivo* and *in vitro* studies showed reduced leukocyte adhesion to hepatic sinusoids when these receptors are blocked or not functional.[80,90] Moreover, inhibition of the VCAM-1 ligand, VLA-4 (or ITGA4), on monocytes using an anti-VLA-4 antibody inhibits adhesion and transendothelial migration of monocytes across LSECs – from wild-type mice fed a high-fat diet and from *ob/ob* obese mice – and improves liver inflammation.[82]

Although the stimuli responsible for LSECs' inflammatory phenotype and functions in NASH are not firmly identified, several mediators are potential candidates. This includes products derived from the visceral adipose tissue, such as ox-LDL, FFA and adipokines. Indeed, *in vitro* studies showed that stimulation of LSECs with ox-LDL and FFA (palmitate) activate NF-kB and TLR-4, respectively.[21,44,91] Moreover, circulating concentrations of several adipokines, including TNFα and IL-6, are increased in the portal vein in the context of metabolic syndrome, and may contribute to LSECs inflammatory phenotype.[92] The gut microbiota also has an emerging role in NASH as a source of inflammatory stimuli.[1,3] Increased intestinal permeability and elevated plasma concentrations of lipopolysaccharide (LPS)[93,94] observed in NASH may also contribute to LSECs' pro-inflammatory function.[83] Besides mediators derived from the portal vein, hepatocytes and liver inflammatory cells also release inflammatory mediators in NASH that can activate LSECs.[1,95]

To summarize, while LSECs play an anti-inflammatory role in the initial stages of NAFLD, a switch towards pro-inflammatory functions occurs during the course of NAFLD development, paving the way for NASH progression.

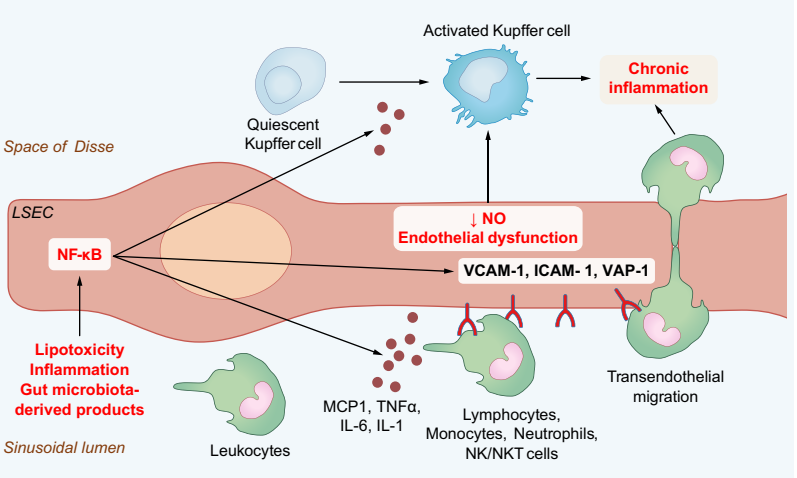

Fig. 3. LSECs acquire pro-inflammatory functions in NASH. Lipotoxicity, inflammation and gut microbiota-derived products induce LSECs' inflammatory phenotype and function mediated by NF-kB activation, which orchestrates the release of pro-inflammatory mediators and the overexpression of adhesion molecules. Inflammatory mediators and LSEC dysfunction promote Kupffer cell activation and leukocyte chemoattraction. Adhesion molecule overexpression allows adhesion and transendothelial migration of the recruited leukocytes in the hepatic parenchyma. ICAM-1, intercellular adhesion molecule-1; IL-1, interleukin 1; IL-6, interleukin 6; LSECs, liver sinusoidal endothelial cells; MCP1, monocyte chemoattractant protein-1; NASH, non-alcoholic steatohepatitis; NF-kB, nuclear factor kappa B; NK, natural killer; NO, nitric oxide; TNFα, tumor necrosis factor alpha; VAP-1, vascular adhesion protein-1; VCAM-1, vascular cell adhesion molecule-1.

Angiogenesis in liver inflammation in NASH

Pathologic angiogenesis increases with NASH[61,68,96,97] (Fig. 4). Indeed, several studies reported the formation of new vessels in the liver of patients with NASH. Moreover, serum VEGF and sVEGFR1 levels are higher in patients with steatosis and biopsy-proven NASH than in healthy individuals.[62,66–68,98] Similarly, in animal models of NASH, liver vasculature is disrupted and hepatic expression of VEGF and CD105 is increased.[63]

Several mechanisms trigger angiogenesis during NASH. First, chronic inflammation promotes angiogenesis. Indeed, chronic inflammation sustains tissue hypoxia and induces transcription of

Key point

Inflammation promotes angiogenesis that in turn worsens liver inflammation as demonstrated by the anti-inflammatory effect of anti-angiogenic therapies.

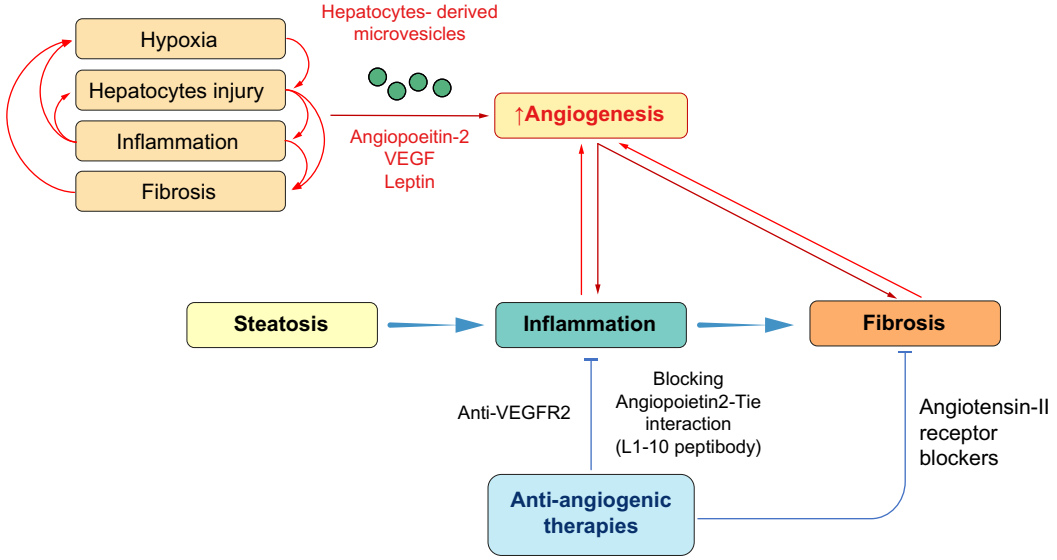

Fig. 4. Angiogenesis in NASH. Hypoxia, liver injury, lipids, oxidative stress, inflammation and fibrosis induce the release of pro-angiogenic factors, such as hepatocyte-derived microvesicles, VEGF and Angiopoietin-2, from parenchymal and non-parenchymal cells including LSECs, promoting pathologic angiogenesis. Adipokines, such as leptin, also exhibit pro-angiogenic activity contributing to pathologic angiogenesis in NASH. In turn, angiogenesis promotes liver inflammation and fibrosis as shown by anti-angiogenic therapies which prevent liver inflammation and fibrosis in experimental models of NASH. LSECs, liver sinusoidal endothelial cells; NASH, non-alcoholic steatohepatitis; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

Box 1. LSEC changes are not specific for certain animal models of NASH. LSEC, liver sinusoidal endothelial cells; NASH, non-alcoholic steatohepatitis.

LSECs alterations are observed in various dietary or genetic models of NAFLD, without apparent specificity. Indeed, LSECs capillarization is observed in animals fed a choline-deficient, L-amino acid-defined diet or a high-fat diet.[18–20] LSECs dysfunction can be induced by a high-fat diet, or a rich saturated fatty acids diet, or a high-fat high-glucose and high-fructose diet.[40,41,43] Markers of LSECs inflammatory phenotype are observed in animals fed a high-fat diet or a methionine and choline deficient diet.[68,82] Markers of angiogenesis appear after methionine and choline deficient diet in *db/db* obese mice and in C57BL/6 mice and in rats fed a choline-deficient, L-amino acid-defined diet.[63,129]

angiogenic genes modulated by HIF-1α.[61,99] Pro-inflammatory mediators also elicit a direct angiogenic response through the induction of HIF-1α transcriptional activity and VEGF production.[61] Moreover, cytokines and ROS released during NASH can activate the MAPK/ERK pathway, a signalling pathway involved in cell migration and angiogenesis.[61] Second, hepatocyte-derived microvesicles link lipotoxicity with angiogenesis. Indeed, hepatocytes exposed *in vitro* to excessive amounts of saturated FFA, that mimics steatosis, release microvesicles with a pro-angiogenic activity.[100] Likewise, mice fed a methionine- and choline-deficient diet have high circulating levels of hepatocyte-derived microvesicles able to induce angiogenesis. Third, angiopoietin-2 is another mechanism of liver angiogenesis in NASH. Angiopoietins are key regulators of angiogenesis. Although angiopoietins-1 and 2 contribute to vascular stability and quiescence in physiological conditions, angiopoietin-2 promotes pathological angiogenesis in inflammatory conditions.[101] Lefere and coworkers recently showed that serum angiopoietin-2 levels are increased in patients with NASH and correlate with liver steatosis, inflammation and hepatocyte ballooning, but not with liver fibrosis.[68] Similar findings were observed with 2 murine models of NASH, namely mice fed a methionine- and choline-deficient diet and mice with neonatal injection of streptozotocin followed by 16 weeks of western diet.[68] The main source of hepatic angiopoietin-2 was LSECs.[68] Inhibiting angiopoietin-2 levels using the angiopoietin-2/Tie2 receptor inhibiting peptibody L1-10 reduced hepatic angiogenesis and normalized vascular microarchitecture.[68]

Key point

LSEC capillarization and dysfunction precede liver fibrosis and are permissive for it, through the loss of the ability of LSECs to maintain quiescence of hepatic stellate cells.

In turn, angiogenesis promotes inflammation since various strategies of inhibition of angiogenesis all improve liver inflammation (Fig. 4). Coulon and colleagues showed in a mouse model of NASH that treatment with anti-VEGFR2 antibody improves liver vasculature and decreases liver inflammatory gene expression, both using preventive and therapeutic approaches.[63] Lefere and colleagues showed that blocking angiopoietin-2/Tie2 interaction with the L1-10 peptibody also alleviates liver injury and inflammation in mice fed a methionine- and choline-deficient diet.[68] Importantly, this effect of L1-10 therapy is at least partly mediated by an effect on LSECs since L1-10 treatment downregulates VCAM-1, ICAM-1 and MCP1 expression in liver endothelial cells isolated from mice fed a methionine- and choline-deficient diet.[68] This anti-inflammatory effect of anti-angiogenic treatment is not specific for NASH, as it is observed in most models of chronic liver disease, namely carbon tetrachloride, and bile duct ligation.[102–106]

To summarize, inflammation stimulates angiogenesis that in turn worsens inflammation, as shown by the anti-inflammatory effect of anti-angiogenic therapies (Fig. 4).

LSECs in NASH-related liver fibrosis

Liver fibrosis is defined as the excessive deposition of extracellular matrix in liver parenchyma. The main mechanism leading to liver fibrosis is a long-standing wound healing process caused by hepatocellular injury and inflammation and mediated by hepatic stellate cell activation.[1,107] Hepatic stellate cells are nonparenchymal cells close to LSECs, in the space of Disse, which store retinoids in physiological conditions and shift their phenotype to an activated myofibroblastic state during liver injury and inflammation, wherein they secrete large amounts of extracellular matrix compounds.[107] As detailed above, LSECs are major effectors of liver inflammation in NASH, and consequently also promote hepatic fibrosis. For example, LSECs overexpress VAP-1 during inflammation which, in addition to its pro-inflammatory functions in NASH, is directly involved in hepatic stellate cell activation.[80] Inhibition or deficiency in VAP-1 in mice fed a methionine- and choline-deficient diet or a high-fat diet attenuates liver fibrosis.[80] LSECs also contribute to liver fibrosis through capillarization and endothelial dysfunction, as detailed in the following sections.

LSEC capillarization promotes liver fibrosis

Capillarization is observed in patients and animal models of NASH, preceding fibrosis,[18,41,108–112] but also promoting its development (Box 1). Indeed, PLVAP deficient mice, displaying a pronounced reduction in the number of LSEC *fenestrae*, spontaneously develop perisinusoidal liver fibrosis.[25]

Experiments performed using cultured LSECs and hepatic stellate cells highlighted the importance of cross-talk between these cells types in regulating each other's phenotype. While healthy LSECs maintain hepatic stellate cell quiescence, capillarized LSECs lose this ability[112,113] (Fig. 5). A vicious cycle between LSEC capillarization and hepatic stellate cell activation then occurs during the fibrotic process.

In NASH, ballooned hepatocytes produce Hedgehog molecules.[114] The Hedgehog pathway regulates capillarization, as inhibition of Hedgehog signalling prevents capillarization and partially reverts the phenotype of LSECs from a dedifferentiated state to their differentiated

state.[115] LSECs are thus Hedgehog-sensitive cells, but they are also Hedgehog producing cells. Similarly, quiescent hepatic stellate cells are Hedgehog-sensitive cells, while activated hepatic stellate cells become Hedgehog-producing cells, which are also able to release macrovesicles loaded with Hedgehog signalling molecules that interact with LSECs.[116,117] It is thus tempting to speculate that during NASH, Hedgehog ligands are released by hepatocytes and LSECs, thus activating LSECs themselves, as well as quiescent hepatic stellate cells, by autocrine and paracrine effects. Activated hepatic stellate cells can then secrete Hedgehog molecules, promoting LSEC capillarization which in turn favours hepatic stellate cell activation, promoting the fibrogenic process.

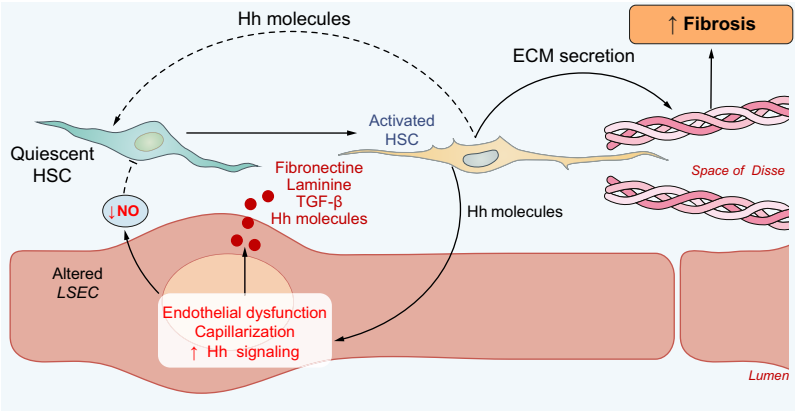

Fig. 5. LSECs in NASH-related fibrosis. Healthy LSECs are gatekeepers of liver fibrosis by maintaining HSCs quiescence through NO, while altered LSECs (following capillarization and LSEC dysfunction) lose this ability. In addition, altered LSECs release profibrogenic molecules such as TGF-β, Hedgehog molecules, laminin and fibronectin, which activate HSCs. Activated HSCs produces Hedgehog molecules reinforcing their own activation and LSEC capillarization. Activated HSCs then produce large amounts of extracellular matrix thus inducing liver fibrosis. ECM, extracellular matrix; Hh, Hedgehog; HSCs, hepatic stellate cells; LSECs, liver sinusoidal endothelial cell; NO, nitric oxide; TGF-β, transforming growth factor-β.

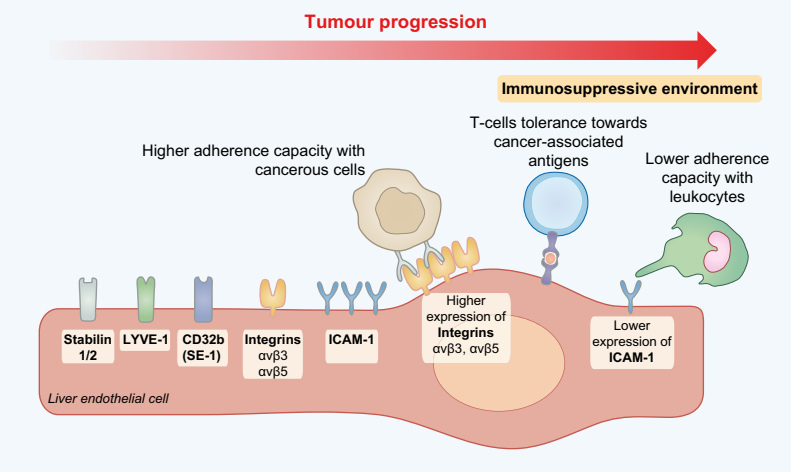

Fig. 6. Role of liver endothelial cells in HCC development in chronic liver diseases (not specific for NAFLD). During HCC progression, endothelial cells sequentially lose their specific markers including stabilin-1, stabilin-2, LYVE-1 and CD32b (SE-1). Conversely, endothelial expression of integrins increases, facilitating adhesion of liver cancer cells. In parallel, endothelial expression of ICAM-1 decreases, leading to a lower ability of leukocyte to adhere and infiltrate HCC. Endothelial cells within HCC can also alter tumour-associated immune responses *via* their ability to confer T cell tolerance towards cancer-associated antigens and to create an immunosuppressive environment. ICAM-1, intercellular adhesion molecule-1; LYVE-1, lymphatic vessel endothelial hyaluronic acid receptor 1; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease.

LSECs dysfunction promotes liver fibrosis

Endothelial dysfunction appears very early in the course of NAFLD and precedes fibrosis in animal models of NASH.[30,40,41] Several lines of evidence suggest that liver endothelial dysfunction contributes to liver fibrosis. First, in rats fed a high-fat diet, simvastatin increases liver eNOS expression and ameliorates liver fibrosis.[60] Second, eNOS inhibition using L-NAME blocks the ability of healthy LSECs to keep hepatic stellate cells quiescent.[118,119] Third, an activator of soluble guanylate cyclase, a receptor for nitric oxide, can recapitulate the effect of healthy LSECs and reverse hepatic stellate cell activation.[13] However, a limitation of these studies is that most approaches might not only act on endothelial function but also on LSEC capillarization.[112,118] Indeed, although L-NAME blocks LSEC-induced hepatic stellate cell quiescence, addition of a nitric oxide donor does not directly reverse hepatic stellate cell activation *in vitro*,[13] suggesting that additional LSEC-derived factors could be responsible for the reversion of activated hepatic stellate cells to quiescence.

To summarize, these data demonstrate that capillarization and LSEC dysfunction not only precede liver fibrosis, but also promote it (Fig. 5). In their differentiated state, LSECs are able to maintain hepatic stellate cell quiescence, making differentiated LSECs gatekeepers of fibrosis in NASH, as in other chronic liver diseases.

Liver endothelial-to-mesenchymal transition: a process promoting liver fibrosis?

Another important process that links endothelial cells to organ fibrosis is endothelial-to-mesenchymal transition, *i.e.* the mechanism by which endothelial cells convert into myofibroblasts and contribute to extracellular matrix deposition.[120,121] Endothelial-to-mesenchymal transition occurs in various fibrotic cardiovascular and pulmonary diseases.[120–122] *In vitro* studies showed that healthy LSECs produce a modest amount of collagen and fibronectin.[123] Capillarized LSECs secrete fibrogenic factors, such as TGF-β1, and extracellular matrix proteins, such as fibronectin and laminin, that may be considered as an endothelial-to-mesenchymal transition, as well as stimulating activation of neighbouring hepatic stellate cells.[8,124,125] In the liver disease field, only 1 study has described endothelial-to-mesenchymal transition *in vivo*, in patients with alcohol- or hepatitis C virus-related cirrhosis and in mice treated with carbon tetrachloride.[126] This process might also occur during liver fibrosis in NASH, but further studies are required.

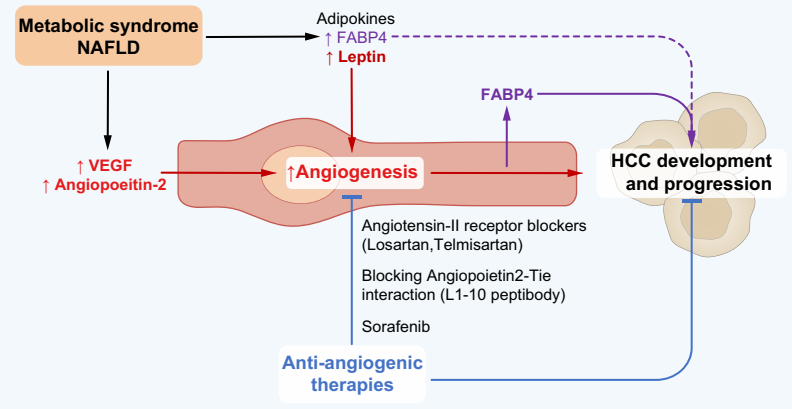

Fig. 7. Role of liver endothelial cells in hepatocellular carcinoma development in the NAFLD setting. Circulating concentrations of angiocrine factors, such as VEGF and angiopoeitin-2, and adipokines, such as leptin are increased in NAFLD. These mediators induce angiogenesis which promotes HCC growth. The adipokine FABP4 is released by adipose tissue and endothelial cells and contributes to HCC development and progression. FABP4, fatty acid binding protein 4; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease.

Key point

Liver angiogenesis correlates with the severity of liver fibrosis and promotes its development. Once established, fibrosis stimulates angiogenesis by increasing tissue hypoxia. Blocking pathologic angiogenesis prevents liver fibrosis.

Key point

Adipokines contribute to HCC development in NAFLD.

Key point

NALFD associated angiogenesis promotes HCC. Blocking pathologic angiogenesis prevents HCC development and progression.

Angiogenesis in NASH-related liver fibrosis

Liver angiogenesis correlates with liver fibrosis in patients with NASH.[66,67] The mechanisms leading to liver angiogenesis in NASH-related fibrosis include those mentioned above, namely tissue hypoxia,[61] hepatocyte-derived microvesicles[100,127] and angiopoietin-2,[68] but also leptin. Leptin concentrations are increased in the serum of patients with NAFLD.[128] This adipocytokine has both pro-angiogenic effects,[129] and direct pro-fibrogenic effects, through the upregulation of TGF-β in LSECs and Kupffer cells.[130]

In turn, angiogenesis promotes liver fibrosis since several approaches inhibiting liver angiogenesis prevent NASH-related fibrosis (Fig. 4). First, in the study by Kitade and colleagues, neither angiogenesis nor fibrosis were observed in the absence of leptin signalling in a rat model of NASH.[129] Second, blocking the release of pro-angiogenic microvesicles from fat laden-hepatocytes or inhibiting their binding to target cells protects mice from steatohepatitis-induced pathologic angiogenesis and results in a reduction in liver fibrosis.[100] Third, Zhou and coworkers recently showed that a specific deletion of the physiological inhibitor of angiogenesis, prolyl-hydroxylase-2, in endothelial cells results in an overexpression of angiopoietin-2 and TGF-β1 in the liver and promotes dietary-induced liver fibrosis in mice.[131,132] Whether this pro-fibrotic effect of endothelial prolyl-hydroxylase-2 deficiency is directly induced by promoting angiogenesis remains to be demonstrated. Fourth, 2 studies reported that inhibiting angiotensin-II receptor using telmisartan or candesartan inhibits liver angiogenesis and fibrosis in rats fed a choline-deficient, L-amino acid-defined diet.[96,97] Finally, Lefere and colleagues demonstrated that blocking angiogenesis by inhibiting the interaction between angiopoietin-2/Tie2 using the L1-10 peptibody improves liver fibrosis in preventive and therapeutic strategies in mice fed a methionine- and choline-deficient diet. Therapeutic application of L1-10 peptibody also prevents liver fibrosis in diabetic mice with NASH (streptozotocin and western diet model).[68] It should be noted that an anti-fibrotic effect of anti-angiogenic treatments has also been observed in models of chronic liver disease without NASH.[102–106,133–139]

LSECs in NASH-induced HCC

In most cases, HCC develops on a background of chronic liver disease (70–90% of all patients). The role of liver endothelial cells in HCC development, outside the NAFLD setting, has been reviewed elsewhere and is summarized in Fig. 6.[8,76,140–143]

Patients with metabolic syndrome and NAFLD also develop HCC in the absence of underlying cirrhosis, suggesting oncogenic pathways specific for NAFLD.[5,6] Adipokines and angiogenesis associated with NAFLD seem to account – at least partly – for this specific link between NAFLD and HCC.

Circulating concentrations of the adipokine FABP4 are increased in patients with NAFLD without HCC and correlate with liver inflammation and fibrosis.[144] Interestingly, Laouirem and colleagues recently demonstrated that LSECs exposed to conditions mimicking NAFLD – namely high concentrations of glucose, insulin, or VEGFA – release FABP4. They also observed that FABP4 released by LSECs exerts pro-oncogenic effects, since it induces hepatocyte proliferation. In mice fed a high-fat diet, specific inhibition of FABP4 reduces HCC growth.[145] We can speculate that FABP4 from LSECs not only contributes to HCC progression but also to HCC development in a NAFLD setting (Fig. 7).

In NAFLD, angiogenesis is highly stimulated and promotes NAFLD-associated HCC, since various inhibitors of angiogenesis all prevent HCC development. First, leptin-mediated angiogenesis has been demonstrated to be involved in HCC development as neither angiogenesis nor HCC develop in the absence of leptin signalling in Zucker rats fed a choline-deficient, L-amino acid-defined diet.[129] Second, Yoshiji and colleagues showed that a conventional anti-angiogenic treatment with sorafenib inhibits the appearance of preneoplastic lesions in rats fed a choline-deficient, L-amino acid-defined diet.[146] In this study, authors also demonstrated that a treatment combining low doses of sorafenib with the angiotensin-II receptor inhibitor losartan also successfully inhibited preneoplastic lesions.[146] Third, Tamaki and colleagues demonstrated that inhibition of angiotensin-II receptor with telmisartan inhibits HIF-1α activity and VEGF expression and prevents HCC development in the liver of rats fed a choline-deficient, L-amino acid-defined diet for 48 weeks.[96] Finally, Lefere and coworkers recently showed that therapeutic inhibition of angiopoietin-2 alleviates steatohepatitis and prevents NASH-associated HCC progression in mice.[68]

Gaps in knowledge and future directions

Even if our understanding of the role of LSECs in NAFLD has progressed over the last years, several aspects remain elusive. First, triggers responsible for LSEC alterations in NAFLD are mostly unknown. It has been suggested that mediators derived from the visceral adipose tissue and the gut are responsible, but this has not been convincingly established. Indeed, available *in vitro* studies considered each mediator individually and not in combination, as *in vivo* in the portal venous blood.[21,44,77] Second, mechanisms underlying endothelial changes in NAFLD, including capillarization, need to be defined which might provide new therapeutic targets for NAFLD. Third, the role of LSECs in NASH-related cirrhosis has not been specifically investigated. Whether LSEC function and phenotype differ in cirrhosis related to NASH from cirrhosis related to other causes remains to be assessed.[147] Fourth, although NAFLD is well recognized as favouring HCC development, we are still at a very early stage of understanding how LSEC changes might favour HCC development.

Conclusion

LSECs are gatekeepers of liver homeostasis in physiological conditions. In NAFLD, sinusoidal endothelial alterations, including capillarization and LSEC dysfunction, occur early in disease progression, at the stage of simple steatosis. These initial changes, triggered by lipotoxicity, adipokines, inflammation and gut microbiota-derived products are associated with a loss of the ability of LSECs to prevent liver inflammation and fibrosis associated with NASH. Indeed, altered LSECs fail to maintain Kupffer cells and hepatic stellate cells in a quiescent state. At the stage of NASH, altered LSECs contribute to liver angiogenesis, inflammation, fibrosis and HCC. Improving LSEC health has great therapeutic potential for NAFLD. The current challenge is the identification of strategies to specifically target LSECs in order to modulate their activity.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

A.H. and P-E.R wrote the manuscript.

Supplementary data

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Author names in bold designate shared co-first authorship

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