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Reference:

van der Graaff Denise, Kw anten Wilhelmus, Francque Sven.- The potential role of vascular alterations and subsequent impaired liver blood flow and hepatic hypoxia in the pathophysiology of non-alcoholic steatohepatitis
Medical hypotheses - ISSN 0306-9877 - 122(2019), p. 188-197
Full text (Publisher's DOI): <https://doi.org/10.1016/J.MEHY.2018.11.014>
To cite this reference: <https://hdl.handle.net/10067/1556870151162165141>

Title: The potential role of vascular alterations and subsequent impaired liver blood flow and hepatic hypoxia in the pathophysiology of non-alcoholic steatohepatitis

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WK and SF received funding from the Fund for Scientific Research (FWO) Flanders (11J9513N, 1802154N). The funders had no role in preparation of the manuscript.

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) covers a spectrum of disease ranging from steatosis to steatohepatitis (NASH) and fibrosis, but the underlying pathophysiological mechanisms remain largely unknown. As there is currently no approved pharmacological therapy and the prevalence of NAFLD keeps increasing, understanding of its pathophysiology is crucial. We hypothesise that vascular alterations in early NAFLD play a role in the progression of the disease by inducing an increased intrahepatic vascular resistance and consequently relative hypoxia in the liver. Evidence of the detrimental effects of hypoxia in NAFLD has already been observed in liver surgery, where the outcomes of steatotic livers after ischaemia-reperfusion are worse than in healthy livers, and in obstructive sleep apnoea, which is an independent risk factor of NAFLD. Moreover, early histological damage in NAFLD is situated in the pericentral zone, which is also the first zone to be affected by a decreased oxygen tension because of the unique hepatic vascular anatomy that causes the pericentral oxygen tension to be the lowest. Angiogenesis is also a characteristic of NAFLD, driven by hypoxia-induced mechanisms, as demonstrated in both animal models and in humans with NAFLD. Relative hypoxia is most probably induced by impaired blood flow to the liver, caused by increased intrahepatic vascular resistance. An increased intrahepatic vascular resistance early in the development of disease has been convincingly demonstrated in several animal models of NAFLD, whereas an increased portal pressure, a consequence of increased intrahepatic vascular resistance, has been proven in patients with NAFLD. Animal studies demonstrated a decreased intrahepatic effect of vasodilators and an increased reactivity to vasoconstrictors that results in an increased intrahepatic vascular resistance, thus the presence of a functional component. Pharmacological products that target vasoregulation can hence improve the intrahepatic vascular resistance and this might prevent or reverse progression of NAFLD, representing an important therapeutic option to study. Some of the

drugs currently under evaluation in clinical trials for NASH have interesting properties related to the hepatic vasculature. Some other interesting drugs have been tested in animal models but further study in patients with NAFLD is warranted.

In summary, in this paper we summarise the evidence that leads to the hypothesis that an increased intrahepatic vascular resistance and subsequent parenchymal hypoxia in early NAFLD is an important pathophysiological driving mechanism for the progression of the disease.

ACCEPTED MANUSCRIPT

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) describes a spectrum of disease that is characterised by the intrahepatic accumulation of fat. Without signs of inflammation or fibrosis, it is referred to as isolated steatosis or non-alcoholic fatty liver (NAFL). Patients can remain in the stage of isolated steatosis, but in some cases NAFLD progresses to a state with inflammation and hepatocellular damage, known as non-alcoholic steatohepatitis (NASH). NAFLD increases the risk of fibrosis, cirrhosis and hepatocellular carcinoma that is mainly confined to NASH, whereas NAFL is believed to run a more benign course (1,2). Moreover, NAFLD patients have an important risk of development of tumours or cardiovascular morbidity and mortality (3).

So far, the exact pathophysiology remains unknown but is believed to be subject to multiple 'hits' that play either parallel or sequentially (4). However, full understanding of the pathophysiology of NAFLD is important, because of the increasing prevalence (5) and the unavailability of approved pharmacotherapy (1). A considerable amount of research has been devoted to the role of genetic factors (6), hepatic and inflammatory cells and extrahepatic tissues (4), but less attention has been paid to the potential impact of vascular alterations.

HYPOTHESIS

We hypothesise that early-onset hepatic vascular alterations play a role in the progression of NAFLD. More specifically, we hypothesise that the early vascular changes that can be observed in steatosis impair intrahepatic blood flow, which induces local tissue hypoxia, which subsequently triggers several pathways that ultimately lead to the progression of NAFL to NASH and its further hepatic and extrahepatic complications (Figure 1).

EVALUATION OF THE HYPOTHESIS

Steatosis in liver surgery

The first suggestion of a role for vascular alterations in steatosis was described in the field of liver surgery. About 26-50% of potential donor livers to date in the Western world are steatotic (7,8). Impairment of liver perfusion has been demonstrated by laser Doppler flowmetry in steatotic donor livers. The microcirculation was reduced and the perfusion rate in response to inotropic agents was decreased in steatotic compared to normal liver (9). The specific alterations in the hepatic microcirculation will be discussed further.

Steatotic donor livers appear to be more vulnerable to ischaemia-reperfusion injury compared to normal donor livers. Clinically, this is illustrated by the high risk of primary graft non-function in case of severe steatosis of the donor liver in liver transplantation (about 60% of the recipients) (7), the increased complication rate after liver transplantation of steatotic donor livers (10), more frequent elevated serum transaminases after transplantation (primary graft dysfunction) and decreased survival (10,11). Finally, the mortality and complication rate in primary liver surgery on steatotic livers is increased as well (7).

The detrimental effects of steatosis on ischaemia-reperfusion injury have been reproduced in animal models. In mice fed a high trans-fat, fructose and cholesterol diet or a Western diet, serum alanine aminotransferase (ALT) levels, pro-inflammatory tumour necrosis factor- α (TNF- α), neutrophil marker S100a8, macrophage marker CD68 and the expression of interleukin-6 (*IL-6*) gene were increased compared to low-fat diet fed mice after ischaemia-reperfusion. Histologically, the steatotic mouse livers demonstrated significantly more hepatic necrosis (12). A meta-analysis of 18 studies confirmed the poor outcome of steatotic livers after ischaemia-reperfusion injury in animal models (13).

Obstructive sleep apnoea and NAFLD

Another hint of the role of hypoxia in NAFLD is the link between obstructive sleep apnoea (OSA) and the progression of NAFLD. Several papers demonstrate that the presence of OSA, defined by nocturnal intermittent hypoxia, promotes the progression of NAFLD (14–16).

Several data point towards an independent connection between OSA and NAFLD. First, in a large retrospective study of 137 patients with OSA, in which the severity of NAFLD was assessed by the hepatorenal index on ultrasound, the apnoea/hypopnoea index (AHI) increased in relation to the severity of NAFLD (14). In another study, 101 patients who underwent bariatric surgery were included to assess the link between OSA and NAFLD. This study, in which NAFLD was classified by histology, not only showed the association between OSA and fibrosis, but also demonstrated that the AHI was increased in patients with NASH (15). Moreover, desaturation was more profound and SaO₂ decreased more in patients with NASH and fibrosis compared to healthy subjects (14,15). A more recent study compared the AHI and hypoxia in OSA patients to histological steatosis, inflammation and fibrosis and also demonstrated an association between the severity of OSA and the severity of NAFLD independent of BMI. Hypoxia was associated with both inflammation and fibrosis, although surprisingly, this association was not observed anymore in patients with the metabolic syndrome (16). A possible explanation for this is that the components of the metabolic syndrome dominate the risk of inflammation and fibrosis, thus concealing the effects of hypoxia. Altogether, these findings point towards hypoxia as a potential cause for the progression of NAFLD, mainly regarding inflammation and fibrosis.

Animal models of (intermittent) hypoxia, resembling the effects of OSA, have been shown to induce hepatocyte injury, hepatic lipid accumulation and hepatic endothelial dysfunction (17–19). In a genetic mouse model of NAFLD, animals were submitted to relative hypoxia by housing them in air mixture containing only 10% oxygen. Compared to a control group that

had a normal 21% oxygen exposure, the hypoxic mice developed more severe forms of NAFLD (17). A study in high fat and high cholesterol diet-fed mice also showed that intermittent hypoxia induced liver damage independent of obesity (18). In mice submitted to chronic intermittent hypoxia, hepatic lipid peroxidation was observed and active nuclear factor kappa B levels, which is associated with liver damage and might play a protective role against apoptosis, were increased compared to mice kept in normoxic conditions. In both groups, no histological signs of inflammation or fibrosis were detected (20).

Another observation suggesting an independent link between OSA and NASH is the decrease of serum transaminases as a result of continuous positive airway pressure (CPAP) treatment for OSA (21). This is particularly relevant, as demonstrating the causal role of a certain mechanism does not necessarily imply that a treatment that counteracts this mechanism results in relevant improvement. The evidence is limited, as NAFLD severity was only measured by transaminase levels, which is not as reliable as a liver biopsy. Moreover, in two randomized controlled trials, overall liver tests did not improve with CPAP therapy (22,23). A beneficial effect of CPAP on liver fat assessed by liver imaging was also reported, although these were found in observational studies (24) and not in randomized controlled trials (25), making these results debatable. Finally, CPAP therapy was able to ameliorate insulin resistance (IR) in a cohort of Chinese men (26).

Hypoxia is, however, not the only mechanism through which OSA might influence liver disease. IR and inflammatory effects, consequences of OSA, may also play a role in the pathophysiology of NAFLD (27). Hypoxia *per se* does not seem to induce clinically relevant steatosis, but hypoxia in steatotic livers, which appear to be more vulnerable, could stimulate the progression of NAFL to steatohepatitis and/or stimulate fibrogenesis.

Histological changes in NAFLD

Portal venules transport blood from the portal vein to the sinusoids, accounting for 70-80% of the blood supply, while hepatic arterioles supply oxygenated blood from the hepatic artery (28,29). Because the hepatic blood supply is mainly venous, the oxygen tension in the liver is already low. This makes the liver more vulnerable to small decreases in arterial oxygen tension compared to organs that have a full arterial blood supply. Blood in the liver flows from the portal vein and the hepatic artery to the central vein (28). In case of hypoxaemia, the centrilobular liver tissue will therefore be the first zone to suffer from the effects of lowered oxygen tension whereas the periportal zone will be better supplied and hence preserved (30).

In a study of 545 liver biopsies in patients with NAFLD, the most common histological pattern of steatosis was zone 3 predominant, situated around the central vein. This was especially the case in biopsies of livers that did not yet display signs of inflammation or fibrosis, indicating that these were early stages of NAFLD (31). Other histological signs of liver damage in NAFLD like Mallory-Denk bodies and fibrosis first appear in the centrilobular zone as well (32,33). The pattern of pericentral damage is comparable to what is seen when hypoxia is artificially induced in experimental models (34), underpinning the theory.

Hepatic hypoxia in NAFLD has also been observed directly by using the hypoxia marker pimonidazole in an experimental model. Mice in which NASH was induced by 16 weeks of high fat diet, exhibited more pronounced hypoxia in the pericentral region of the liver lobule, although the hypoxia was also detectable to a lesser extent around the portal vein and in the area between the central and portal vein (35).

Angiogenesis

Besides the direct observation of centrilobular injury and hypoxia in NAFLD, the observation of angiogenesis also supports the presence of hypoxia in NAFLD. Angiogenesis is a result of hypoxia, mediated by the formation of hypoxia inducible factors (HIFs) that stimulate angiogenic growth factors (36). A second pathway for the induction of angiogenesis, is the stimulation of vascular growth by reactive oxygen species (ROS) (36). ROS are produced in circumstances of oxidative stress, which might already be present in steatosis before the development of inflammation (37). Both HIFs and ROS will be discussed separately further on.

Vascular endothelial growth factor (VEGF), which plays an important role in the formation of blood vessels, and its receptor soluble VEGF receptor 1 (sVEGFR1) were shown to be increased in patients with steatosis without NASH or fibrosis compared to healthy controls. Compared to patients with NASH, serum VEGF concentration elevation and VEGF and sVEGFR1 gene expression were more pronounced in NAFL (38). Blockage of the VEGF receptor 2 before or during the induction of NASH in mice partly reversed hepatic fat accumulation (39). Angiopoietin-2, another mediator of angiogenesis, was shown to be increased in the serum of patients with NASH and in methionine choline deficient (MCD) diet fed mice (40).

Hypoxia inducible factors

Another clue to the presence of hepatic hypoxia in NAFLD is given by the detection of HIFs in animal models of steatosis. HIF proteins regulate the cellular response to hypoxia. HIF consists of a constitutively expressed HIF-1 β domain and a HIF- α domain that is degraded in normoxic conditions (41). Many studies have demonstrated a link between HIFs and the hepatic characteristics of NAFLD.

The concentration of HIF-1 α was increased in mice with hepatic steatosis after a cholesterol and cholate-rich diet. The immunohistochemical staining for HIF-1 α mainly showed overexpression in the pericentral region, in line with the observations of pericentral damage as described previously. The mRNA levels of HIF-1 α were increased as well (42). ▶

Some studies with HIF knockout mouse models or mice with activated HIF were performed to study the link between HIF and steatosis. Mice without HIF-1 α were protected from hepatic steatosis induced by alcohol, whereas alcohol administration induced the hepatic expression of HIF-1 α RNA (43). When HIF-1 α and HIF-2 α genes are simultaneously activated and their levels increased, the development of macrovesicular hepatic steatosis is seen in transgenic mice (44). The HIF-2 α subunit appears to be the main player (45). HIF-2 α was shown to decrease fatty-acid β -oxidation and lipogenesis and to increase the hepatic lipid storage. These effects led to hepatic steatosis in mice with activated HIF-2, which was suppressed by inactivation of HIF-2 α (46). Moreover, deletion of HIF-2 α in a MCD diet-fed mouse model decreased lipid accumulation, NASH and fibrosis (47).

The role of HIFs has been studied in isolated hepatocytes as well. Both the deficiency of HIF-1 α and HIF-2 α were able to increase lipid β -oxidation and diminish the increased lipid accumulation in hepatocytes that were submitted to only 1% of oxygen (48).

Even though the production HIFs are a compensation mechanism to protect the liver in the presence of hypoxia, most data seem to indicate that the absence of HIFs protects the liver from steatosis. This contradiction might be explained by the observation that steatosis itself might actually be a protective mechanism of the liver against further liver injury, caused by lipotoxicity, as suggested in a mouse model of NAFLD (49). Moreover, the data on the detrimental effects of HIF-2 are not all confirmatory. In one study, the absence of HIF-2 in a specific HIF-2 knockout mice model induced the development of steatosis instead of suppressing it (50). Furthermore, as hypoxia appears to be already present in steatosis, we

estimate that hypoxia is not significantly relevant in the development of steatosis itself but rather promotes the progression of NAFL to NASH.

Besides the effects of HIFs on steatosis, they also seem to play a role in inflammation and fibrosis. The inflammatory cytokines IL-6 and IL-1 β increased in a HIF-2 α -dependent manner in a model with conditional knockout of the Von Hippel-Lindau gene in the liver, both on the gene expression and the protein level (45). The effects of HIFs in fibrosis will be discussed separately.

Data in humans also show HIF-1 α expression in steatosis and even more pronounced expression in NASH, while immunohistochemical staining was negative in healthy livers (51). Overexpression of HIF-2 α in hepatocytes was also observed in humans with NAFLD, even before the development of significant fibrosis (47).

The evidence of HIF in NAFL supports presence of hypoxia in early NAFLD and demonstrates a potential mechanism by which hypoxia influences NAFLD pathophysiology, although it is not clear if this adds to the progression of NAFLD or protects the liver from further damage. Of course, these data are mainly based on animal models that are not completely representative for human NAFLD, but the data they provide demonstrate a link between HIFs and hepatic lipid metabolism, inflammation and fibrosis. The exact mechanisms and their direction, *i.e.* contributing or protecting to NAFLD, need to be studied in more detail.

Oxidative stress

Mitochondria are structurally altered (52) and their function is impaired in NAFLD (53). Dysfunctional mitochondria consume more oxygen, thereby further decreasing the remaining oxygen tension (35). Besides, dysfunctional mitochondria produce ROS leading to oxidative stress that has been suggested to play a critical role in the pathophysiology of NAFLD. In both

genetic and MCD-diet induced murine model of steatosis, the production of O_2^- and H_2O_2 (*i.e.* ROS) was increased compared to a control group (54). ROS can induce the activation of Kupffer cells and the production of inflammatory cytokines (55), thus stimulating the progression of NAFL to NASH.

Several studies suggest that hypoxia can induce hepatic oxidative stress and hence ROS (56). Hepatocytes in rats that were submitted to intermittent hypoxia demonstrated higher levels of ROS (57). ROS also appear to play a role in the response to hypoxia by promoting the stabilization of HIF- α (58). In children with NAFLD, a role for oxidative stress in NAFLD has been proposed as well, even though the pathophysiology in children might differ from that in adults (59).

There is also a link between ROS and hepatic vascular alterations, which will be discussed in more detail later on. Endothelial dysfunction results in an impaired nitric oxide (NO) production, which can promote the production of superoxide and hydroxyl radical intermediates (60). The other way around, ROS are able to scavenge NO, thus impairing NO even further (61).

Glucose metabolism

Hypoxia also exerts an effect on IR, leading to a diminished hepatic and extrahepatic sensitivity to insulin that is associated with NAFLD (1). Like NAFLD, diabetes is associated with OSA (62). Moreover, IR in adipose tissue is associated with the presence and severity of NAFLD (63,64). Several pathways that might explain the link between hypoxia and insulin have been studied.

Prolyl hydroxylases play a role in the stabilization of HIF in an oxygen-dependent way. PHD3 is one isoform of the prolyl hydroxylase domain-containing proteins (PHD), which degrade the HIF- α subunit in normoxic circumstances (65). Thus, HIF- α is only stable in conditions of

hypoxia. In an experiment in which PHD3 was deleted in mice, both fasting glycaemia and insulinaemia were significantly decreased. Additionally, glucose tolerance was restored in the absence of PHD3 (66). These data imply that HIF has a protective effect on glucose metabolism, as it might have for steatosis.

Besides a decrease in the insulin-dependent signalling pathway that normally activates glycogen synthesis in hepatocytes (by inhibiting serine/threonine kinase protein-kinase B (AKT) and glycogen synthase kinase-3 β), hypoxia also decreased the expression and activation of forkhead box protein O1 (FOXO1), independently of insulin, in isolated hepatocytes. FOXO1 is a regulator of the phosphoenolpyruvate carboxykinase gene (*PEPCK*), which plays a pivotal role in gluconeogenesis. However, nor the intrahepatic glycogen concentration nor the production of glucose in the hepatocytes after intermittent hypoxia were significantly impaired (67). On the other hand, other studies did show an increased glucose production in hepatocytes when the duration of intermittent hypoxia was increased (68).

VEGF might also have a detrimental effect on glucose metabolism and hence be a mediator of the effect of hypoxia on glucose metabolism (*vide supra*). The inhibition of VEGF, VEGF-A and VEGFR2 resulted in an improvement in glucose tolerance after intraperitoneal injection of glucose in mice (69).

Another mechanism explaining the link between hypoxia and insulin involves endothelin-1 (ET-1). Levels of ET-1 have been shown to be increased not only in NAFLD (70), but also in patients with OSA (71,72), in patients with diabetes (73) and in laboratory animals that were submitted to intermittent hypoxia (74). Blocking the ET_B-receptor had a beneficial effect on glucose tolerance in mice after intermittent hypoxia (75). The specific effects of ET-1 on the hepatic vasculature will be discussed later.

Fibrosis

In some patients, NAFLD can lead to significant fibrosis (mostly associated with NASH rather than with NAFL (2)), which eventually results in liver cirrhosis and its complications. The degree of fibrosis is currently also the best predictor of mortality in patients with NAFLD (76). There are data supporting that this fibrogenic evolution could be mediated by hypoxia as well. Since fibrosis is intimately linked to liver damage and inflammation, the link between hypoxia and fibrosis is at least in part to be seen in that context.

Similarly to steatosis and hepatocyte damage, the development of fibrosis in NAFLD starts in the pericentral zone, in which the oxygen tension is lowest as described above (33). In line with this, some hypothesise that release of intracellular components following hepatocellular necrosis leads to the obliteration of smaller hepatic veins that subsequently leads to fibrosis (77). Cell death is indeed more likely to occur due to hypoxia related mechanisms, whereas obliteration of the hepatic veins further impedes proper blood flow in these areas.

In high fat diet-fed mice in which OSA was mimicked, periportal collagen deposition in the livers of mice exposed to hypoxia was more pronounced after four weeks compared to controls. The same results were seen in control mice without high fat diet-induced obesity, suggesting that this effect is independent of body weight (78). As mentioned before, the link between HIFs and fibrosis has also been studied. A possible mechanism for the induction of fibrosis through hypoxia, is a HIF-1-dependent pathway. Isolated hepatocytes from HIF-1 α knockout mice after a high trans-fat diet were kept under hypoxic conditions with 1% oxygen and compared to hepatocytes at an atmospheric oxygen tension. The degree of cross-linking between collagen strands in the culture media surrounding primary hepatocytes exposed to hypoxia was lower in HIF-1 α knockout compared to wild type (79). Moreover, HIF-2 α suppressed the expression of α -smooth muscle actin (α -SMA) in murine livers, whereas the deletion of HIF-1 α increased α -SMA expression. HIF-2 α also plays a role in the expression of

several pro-fibrogenic genes (45). The effects of HIFs are rather protective in early NAFLD as described above, but these data suggest that HIFs themselves can play a pathological role when NASH progresses to the development of fibrosis.

In common bile duct-ligated (CBDL) mice, a model for hepatic fibrosis, hypoxia in the hepatic cells was observed by the hypoxia marker pimonidazole and the level of HIF-1 α was increased in hepatocytes and macrophages 3 days after the procedure. mRNA levels of fibrogenic genes, which were increased in CBDL mice, were less elevated in HIF-1 α deficient mice. In line with these findings, type I collagen and α -SMA levels were significantly lower in HIF-1 α deficient CBDL mice compared to control CBDL mice (80). In hepatic stellate cells (HSC), hypoxia did not only induce increased levels of HIF-1 α , but VEGF, placental growth factor (PGF) mRNA and prolyl-4-hydroxylase- α 2 mRNA, an enzyme involved in collagen synthesis, were all increased as well (81). These genes seem to be upregulated in a partially HIF-1 α -dependent way, as the levels were lower in hypoxia-exposed HSCs from HIF-1 α deficient mice (81,82). These data also illustrate the importance of looking at the differential effects on the different cell types present in the liver in order to understand the net resulting effect.

Vascular alterations in NAFLD

We will now discuss the alterations in hepatic blood supply that might cause hepatic hypoxia in early NAFLD and thereby promote NAFLD progression (Figure 2).

In the field of liver transplantation, changes in liver blood flow, namely sinusoidal congestion, were documented in animals after warm ischaemia (83) and impaired hepatic perfusion, as described before, was demonstrated in patients with steatosis (9). Portal hypertension has been demonstrated in patients with NAFLD (84) prior to the development of inflammation or fibrosis (85,86), and in animal models of steatosis (87–90). A combination of structural and

dynamic vascular changes in the liver can cause the intrahepatic vascular resistance (IHVR) to increase (causing hypoxia) with a subsequent impact on portal pressure, as was observed.

Data on structural vascular changes in steatosis are scarce. In liver fibrosis, a loss of fenestration (the pores in the sinusoidal wall) has been observed in models of liver fibrosis as an early morphological change, impairing molecular transport and increasing IHVR (29,91). In *in vitro* studies in a rat model of isolated steatosis, it appeared that the number of fenestrae is not diminished and endothelial cells remain morphologically unaltered while portal pressure is already increased (89), whereas in mice with non-cirrhotic NASH, the capillarisation of sinusoids (*i.e.* the formation of a naturally absent basement membrane and disposition of matrix proteins around the sinusoids, an important characteristic of the sinusoids in cirrhosis), that includes the loss of fenestrae, has been reported (92). With the process of capillarisation, an increased IHVR will develop (93).

One of the obvious structural consequences of fat accumulation in and the ballooning of the hepatocytes is swelling. Swollen hepatocytes can compress the sinusoids and hamper normal sinusoidal blood flow (94). The effects of steatosis on the hepatic blood vessels are, however, not restricted to the mechanic narrowing of sinusoids. In one study, electron microscopy of vascular casts of rat livers with histologically confirmed NAFL showed disorganisation of sinusoids. The regular pattern that was seen in normal livers was lost, and replaced by a dense network of compressed sinusoids, of which some had blunt endings (70). These findings were reproduced in NASH, where electron microscopic scanning of vascular corrosion casts demonstrated a disrupted vascular architecture with disorganized and tortuous vessels in mice fed a MCD diet (39). Further supporting structural vascular alterations in isolated steatosis, is the association between the degree of steatosis and the activation of HSCs, which have both contractile capabilities and are believed to be the drivers of perisinusoidal fibrogenesis

(39,95). These data suggest that structural vascular alterations, all impairing normal blood flow thus oxygenation, take part in early NAFLD when NAFL progresses to NASH.

Next to structural vascular alterations in the steatotic liver, dynamic vascular changes might equally impair the oxygen supply. The hepatic vascular tone is regulated by adjustment of the diameter of the sinusoidal fenestrations under the influence of ET-1 (96,97) and by contraction of the blood vessels (mainly mediated by contractile elements in the HSCs) (96). In NAFLD, an imbalance between vasoconstrictive and vasodilatory mediators and pathological responses to these mediators result in an increased intrahepatic vascular tone.

Nitric oxide (NO) is the most important vasodilator in the liver. By studying the portal pressure in an *ex vivo* liver perfusion rat model, a significantly increased IHVR was demonstrated in NAFL induced by MCD diet (70,90), high fat diet (37) and cafeteria diet (89). Steatotic livers show less reactivity to acetylcholine (70,89), an endothelium-dependent vasodilator, pointing towards endothelial dysfunction as one of the mechanisms of increased IHVR. On the other hand, administration of NO was able to correct the elevated portal pressure in steatosis (89). Decreased expression of the NO synthase gene and protein and decreased protein activity in steatosis were demonstrated as well (70,89).

Moreover, intermittent hypoxia worsened the response to acetylcholine in an animal model of cirrhosis and increased the hyperresponsiveness to vasoconstrictors (19). Furthermore, NO is known to have more effects related to the oxygen metabolism besides regulation of the vascular tone. NO is able to interact with mitochondrial respiration and may thus modulate the response to hypoxia (98). These findings point towards endothelial dysfunction not only as a cause for relative hypoxia in the liver, but also as a result, potentially causing a vicious circle.

Hydrogen sulphide (H₂S) is another vasodilator that plays a role in the hepatic vasculature. H₂S is able to decrease the IHVR after vasoconstriction by norepinephrine, both in cirrhotic

and control livers (99). In the same cirrhotic models it is shown that H₂S production is defective, while homocysteine (a precursor of H₂S) accumulates, induces HSC contraction and impairs endothelial NO-production, hence contribute to the hepatic microvascular dysfunction (100). Interestingly, increased levels of homocysteine have been demonstrated in NAFLD as well (3), so an imbalance between NO and H₂S bioavailability might also play a role in the increased IHVR in NAFLD.

Adding to decreased vasodilation in steatosis, an increased production of vasoconstrictors and hyperreactivity to vasoconstrictive agents has been demonstrated (70,90). Thromboxane synthase, an enzyme involved in the production of the vasoconstrictive thromboxane A₂, was elevated in rats with MCD diet-induced steatosis (70). These steatotic rats also had elevated serum levels and hepatic expression of ET-1 (70). Increased reactivity of the vasculature in steatotic rat liver to several vasoconstrictors like the α 1-adrenergic agonist methoxamine and ET-1 has been observed (90).

In NASH, urea cycle enzymes are impaired. The urea cycle is necessary to remove ammonia from the blood, so loss of urea cycle enzymes can lead to hyperammonaemia (101). Hyperammonaemia causes several effects in the liver itself: changes in HSC morphology, induction of HSC activation and production of ROS. In a rat model of portal hypertension, decreasing the concentration of ammonia resulted in a decreased portal pressure and decreased HSC activation (102). Thus, ammonia might play a role in the increased portal pressure in NAFLD as well.

Compensatory splanchnic vasodilation also plays a role in the regulation of portal pressure and thus can be involved in the blood supply of the liver. Arteriolar vascular dilation leads to increased perfusion of the splanchnic organs and subsequent increase in portal inflow. This is a normal postprandial physiological reaction. It is also a well-known phenomenon in cirrhosis, where it contributes to the severity of the portal hypertension, probably in an

attempt to preserve intrahepatic portal perfusion, but at the expense of the deleterious consequences of the increased portal pressure and hyperdynamic circulation that result from it. There are some clues that this phenomenon is also present in early NAFLD.

In rats with NAFL induced by MCD diet, the mean arterial blood pressure was lower compared to controls (90). In organ bath experiments, the maximal contraction force and vasoconstriction in response to phenylephrine were decreased in the abdominal aorta of rats with severe steatosis (87,90). These effects appeared to be cyclooxygenase-2-dependent (90). Arterial vasodilation, as a result of this hyporesponsiveness to vasoconstrictory molecules, leads to an increased blood flow in the splanchnic circulation, as was demonstrated by measuring flow in the mesenteric artery, with subsequently an enhanced blood supply to the liver through the portal vein. This increased blood flow contributes to the development portal hypertension (87).

The hepatic arterial buffer response (HABR) is a known compensatory mechanism where the arterial inflow increases when portal flow decreases to maintain adequate perfusion of the liver sinusoids (103). Observations in both animal models as well as in patients with NAFLD indicate that, in response to the increased IHVR, the HABR is activated in early NAFLD (104–106), which is supported by the early and prominent presence of arteries in the pericentral zone (107). Finally, it appears that increased arterial-to-portal blood flow velocities (a surrogate for HABR) in patients with NAFLD is associated with more pronounced metabolic derangements compared to those patients in whom the HABR did not yet develop (108).

CONSEQUENCES OF THE HYPOTHESIS

We conclude that the results of several studies point towards a pathophysiological role of hypoxia resulting from hepatic vascular alterations as a driving force for the evolution of

NAFLD. Consequently, since there is currently no pharmacological therapy registered for NAFLD, potential targets can be found in improving oxygen delivery to the liver.

Statins

The use of statins (5-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors), a lipid lowering therapy that is already available for the treatment of dyslipidaemia and is considered safe even in the presence of compensated cirrhosis, has been proposed as potential treatment for NAFLD. In animal models of NAFLD, several statins have improved steatosis, inflammation and fibrosis (109–111). The data on the beneficial effects of statins on NAFLD are not limited to animal studies. In humans, statin treatment in NAFLD patients was associated with significantly lower values for histological steatosis (112), steatohepatitis, presence of significant fibrosis (113) and NAFLD activity score (NAS, composite score for steatosis, lobular inflammation and ballooning) in a matched-control analysis (112). Furthermore, in a small study of 20 patients, NAFLD disappeared in 19 patients according to the NAS score when statins were combined with lifestyle modifications (114). However, these were small prospective open label (112,114) or cross-sectional retrospective (113) studies, and to our knowledge no randomized controlled trials on statins have been performed in NAFLD.

In both animal models and in humans with cirrhosis, the effects of statins on the portal pressure were studied. In a rat model of liver cirrhosis, statins significantly improved the response to vasoactive mediators. Furthermore, eNOS expression and activation were increased after treatment of cirrhotic animals with simvastatin (115). In patients with cirrhosis, IHVR was decreased after treatment with a statin, and postprandial portal pressure was less increased than without the administration of statins (116), possibly through an increase of hepatic NO release (117,118). In addition to these beneficial effects, statins also have been observed to be capable of reducing fibrogenesis by HSC inhibition (119) and

impairing angiogenesis (120). In a study of non-cirrhotic NASH, rats receiving a high fat diet for seven days were treated with a statin, their livers were submitted to cold preservation and subsequently reperfused. The portal pressure was attenuated in steatotic livers that were treated with a statin (121). These data suggest a potential beneficial effect of this drug on hepatic haemodynamics that might improve the oxygen supply to the hepatic cells and thereby counteract the progression of disease. However, most data are in cirrhotic animal models and patients and the data in early NAFLD are limited. The benefits of statins in early NAFLD, therefore, remains unclear.

PHD inhibitors

As PHDs deactivate HIFs and HIF appear to play a role in the progression of NAFLD, PHD inhibition might be a potentially interesting therapeutic target. By inhibiting PHDs, HIF will not be degraded and can pursue its response to hypoxia, stimulating angiogenesis and erythropoiesis (122), thereby increasing oxygen supply to the liver tissue. Several clinical studies in patients with chronic kidney disease have shown that PHD inhibitors are capable of increasing haemoglobin (123). Besides, a limited amount of studies has shown that PHD inhibition can lower total and low density lipoprotein (LDL) cholesterol and decrease the blood pressure, thus improving other components of the metabolic syndrome that can affect NAFLD (124). Interestingly, endothelium-specific PHD2 knockout mice on high fat diet developed more hepatic steatosis and fibrosis. However, HIF-2 α expression was unaltered, potentially because PHD produced in other tissues might have been sufficient to suppress HIF, and beneficial effects were consequently not observed (125).

Intrahepatic vascular resistance

As the increased IHVR prevents sufficient blood supply to the liver, thus inducing hypoxia, targeting the IHVR could be an interesting therapeutic option. Common therapies for clinically significant portal hypertension are non-selective beta-blockers and splanchnic

vasoconstrictors like terlipressin (93). These therapies have, however, not been considered or tested in the context of the portal hypertension early in the development of NAFLD. Furthermore, these therapies mainly focus on the extrahepatic contributors to the observed (cirrhotic) portal hypertension but target very little the dynamic components of the IHVR.

Statins appear to lower the portal pressure in NASH, as discussed above (121), but so far little research has been done to directly target the hepatic vasculature in early NAFLD. NO donors could potentially reduce the IHVR and thus improve bloodflow and decrease portal pressure/hypertension. However, NO donors lead to more pronounced systemic vasodilation and hypotension, which could, among others, result in renal failure because of renal hypoperfusion (115,126). These mechanisms result in more water and sodium retention too, adding to an overload of effective blood volume and its consequences (93). Nevertheless, tetrahydrobiopterin has been suggested as a beneficial treatment of portal hypertension. Its administration to cirrhotic rats showed a significantly decreased portal pressure following increased NOS activity (93). Moreover, liver-selective NO stimulation might be an alternative option. For example, V-PYRRO/NO is able to release NO in the liver, hence without inducing periheral vasodilation, and resulted in less steatosis in an animal model (127).

Cyclooxygenase-dependent mediators might also represent a potential target. Thromboxane synthase has been demonstrated to be increased in steatotic livers compared to control livers (70) and the cyclooxygenase inhibitor indomethacin has the ability to decrease portal pressure (37). Nitroflurbiprofen, which both releases NO and inhibits cyclooxygenase, has been demonstrated to attenuate increased portal pressure in cirrhosis (128).

In liver perfusion experiments in rats with MCD diet-induced NAFL, steatotic livers showed hyperreactivity to ET-1 (90). ET-1 has a dual working mechanism, inducing vasoconstriction

via the ET_A and ET_{B2} receptors and vasodilation via the ET_{B1} receptor (129). Preliminary data are pointing towards a potential benefit of ET_A receptor antagonists.

Furthermore, a potential role for angiotensin in increased IHVR has also been suggested (130). Circulating levels of angiotensin II, a pro-oxidant and fibrogenic vasoconstrictor, are frequently increased in chronic liver diseases (131). A significant reduction of the portal pressure directly related to a reduction of the mean arterial pressure and plasma renin activity were reported after angiotensin II blockage in patients with liver cirrhosis (132). Moreover, angiotensin receptor blockage resulted in a decrease in steatosis in high fat diet-fed mice (133). Besides its vasoconstrictive effects, blocking the angiotensin II receptor 1 attenuated the increase in nuclear factor kappa B levels, in rats fed a high fat diet (134).

Obeticholic acid, a farnesoid X nuclear receptor ligand, is currently studied as a therapeutic agent for NASH in a phase 3 trial (135). Obeticholic acid was shown to decrease portal hypertension in rats with toxic and CBDL-induced cirrhosis (136). Other promising therapies for NAFLD, such as PPAR modulation (for example elafibranor (PPAR- α/δ dual agonist) in phase 3 and lanifibranor (panPPAR agonist)), currently do not have any data on hepatic microcirculation. Experimental research demonstrated that PPAR- γ knock-out mice improve metabolically, but the vasoregulation remained impaired (137) arguing against an important role for this PPAR isotype in vasoregulation. On the other hand, PPAR- α is present in the hepatic endothelium and can inhibit ET-1 production, suggesting a role in the hepatic vasoregulation (138) that might also be of relevance in the treatment of NASH.

Effects of all these drugs on the described alterations in hepatic vasculature and reactivity in early NAFLD remain, however, to be demonstrated and the questions whether their impact improves tissue oxygenation and also subsequently impacts on NASH progression warrants further study.

Acknowledgements

WK and SF received funding from the Fund for Scientific Research (FWO) Flanders (11J9513N, 1802154N). The funders had no role in preparation of the manuscript.

Disclosures/conflicts of interest

The authors declare no conflict of interest relevant to this article

ACCEPTED MANUSCRIPT

CAPTURES TO THE FIGURES**Figure 1**

An increased intrahepatic vascular resistance (IHVR) disturbs the hepatic blood supply, leading to hypoxia in the liver, which might play a role in the progression of non-alcoholic fatty liver disease (NAFLD). The hepatic arterial buffer response acts to increase the blood supply compensatory, but its effects are insufficient for complete compensation. Increased portal pressure is first caused by an increased IHVR, a subsequent increase in splanchnic perfusion can further contribute. The latter is increased by a decreased arterial contraction force, a decreased reactivity to phenylephrine (PE) and an increased nitric oxide (NO) production. The IHVR increases because of structural alterations, like swelling of hepatocytes, disorganisation of the sinusoidal pattern and capillarisation of the sinusoids. Furthermore, functional alterations contribute to the IHVR: the reactivity to acetylcholine (ACh) and the activity of NO synthase are decreased and the reactivity to vasoconstrictors, thromboxane (TXA) synthase and concentration of endothelin-1 (ET-1) are increased, leading to relatively more vasoconstriction.

Figure 2

Regulation of the vascular tone in the hepatic sinusoids in physiological circumstances and in early NAFLD. Dynamic changes play an important role in the increased intrahepatic vascular resistance in early NAFLD, besides the presence of some early structural changes, such as the activation of hepatic stellate cells (HSC) and the deposition of fibrin. Although the exact mechanisms and the location of vasoregulation in the liver remains partly unknown, several mechanisms have been demonstrated (mainly in pre-clinical models). In physiological circumstances, (nor)epinephrine, thromboxane and endothelin-1 (ET-1) induce vasoconstriction. Acetylcholine (through the production of nitric oxide (NO)) and endothelin-

1 (via the endothelin B1 (ET_{B1}) receptor) cause vasodilation. In early NAFLD, hyperreactivity to ET-1 and α 1-adrenergic stimulation cause more vasoconstriction. Vasodilatory mechanisms are decreased because of a decreased endothelial NO synthase (eNOS) activity and a decreased ability of acetylcholine to induce vasodilation. Liver sinusoidal endothelial cell (LSEC), thromboxane receptor (TP), prostaglandin (PG), thromboxane (TX), thromboxane synthase (TXAS)

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