Liver Sinusoidal Endothelial Cells at the Crossroad of Iron Overload and Liver Fibrosis

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Liver Sinusoidal Endothelial Cells at the crossroad of Iron overload and Liver Fibrosis

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Abstract

Significance Liver fibrosis results from different etiologies and represents one of the most serious health issues worldwide. Fibrosis is the outcome of chronic insults on the liver and is associated with several factors, including abnormal iron metabolism.

Recent Advances Multiple mechanisms underlying the pro-fibrogenic role of iron have been proposed. The pivotal role of Liver Sinusoidal Endothelial Cells (LSECs) in iron level regulation, as well as their morphological and molecular de-differentiation occurring in liver fibrosis, have encouraged research on LSECs as prime regulators of very early fibrotic events. Importantly, normal differentiated LSECs may act as gatekeepers of fibrogenesis by maintaining the quiescence of Hepatic Stellate Cells (HSCs), while LSECs capillarization precedes the onset of liver fibrosis.

Critical Issues In the present review, the morphological and molecular alterations occurring in LSECs following liver injury are addressed in an attempt to highlight how vascular dysfunction promotes fibrogenesis. In particular, we discuss in depth how a vicious loop can be established in which iron-dysregulation and LSEC de-differentiation synergize to exacerbate and promote the progression of liver fibrosis.

Future Directions LSECs, due to their pivotal role in early liver fibrosis and iron homeostasis, show great promises as a therapeutic target. In particular, new strategies can be devised for restoring LSECs differentiation and thus their role as regulators of iron homeostasis, hence preventing the progression of liver fibrosis or, even better, promoting its regression.

Introduction

Liver diseases are a major health problem worldwide and it has been estimated that 2 million deaths occur per year due to cirrhosis, viral hepatitis and liver cancer (5). Liver fibrosis, characterized by the excessive deposition of extracellular matrix (ECM), is the hallmark of several liver diseases of different etiology and it is frequently associated or preceded by abnormal iron accumulation (82). Interestingly, systemic iron homeostasis is mainly orchestrated by the liver itself through the production of the peptide hormone hepcidin, which can regulate plasma iron homeostasis upon several stimuli including iron, inflammation and hypoxia (42, 89, 95, 100). The role of iron in liver injury has been recently reviewed (11, 82). Here, we focus on one of the important cellular components of the liver involved in fibrogenesis, the Liver Sinusoidal Endothelial Cells (LSECs). In particular, we review the morphological and molecular alterations occurring in LSECs at early stages of the fibrotic process and highlight the pivotal role these cells play in the regulation of both serum iron and tissue iron. Finally, we discuss the relationship between LSECs de-differentiation and liver fibrosis-related iron overload.

Liver fibrosis

The liver is the largest internal organ of the human body and performs several vital functions, such as [1] acute phase proteins synthesis, [2] detoxification, [3] carbohydrate and lipid metabolism and [4] bile production (36, 112). Liver injury triggers a wound healing response, namely fibrogenesis, involving [1] the recruitment of immune and/or inflammatory cells, [2] the secretion and reorganization of extracellular matrix (ECM) proteins and [3] hepatic regeneration (9). However, if the harmful stimulus is not promptly removed, an uncontrolled wound healing response occurs. Necrotic hepatocytes are gradually substituted by excessive ECM deposits (i.e. fibrosis) in liver parenchyma, thus disrupting its architecture and, consequently, interfering with its normal functionality. For this reason, untreated Chronic Liver

Diseases (CLDs) are characterized by fibrosis that progressively leads to the more severe clinical conditions of cirrhosis and hepatocellular carcinoma (38). Different etiologies can trigger and sustain fibrotic changes in the liver. Consistently, several diseases are associated with a high risk of fibrosis, such as Viral Hepatitis, Alcoholic Liver Disease, Non-Alcoholic Fatty Liver Disease (NAFLD), Non-Alcoholic Steatohepatitis (NASH), Diabetes and Hereditary Hemochromatosis (HH), a disorder characterized by iron overload **(Figure 1)** (82).

Fibrogenesis is a dynamic process, which involves different cell populations and a wide range of injury-dependent molecular mechanisms. At the histological level, liver fibrosis is characterized by fibrous septa, which progressively thicken and extend leading to the formation of bridges between the portal tract and the central vein (porto-central fibrosis), among portal tracts (porto-portal fibrosis) or in the perisinusoidal space (perisinusoidal fibrosis) (17). Different liver cell populations are involved in fibrogenesis. The Hepatic Stellate Cells (HSCs), located in the space of Disse around the sinusoids, are mainly responsible for perisinusoidal fibrosis following activation and myofibroblastic transformation. HSCs, as the major producers of ECM, are the most studied cell type in the fibrotic process. On the other hand, the portal myofibroblasts have been implicated the development of portal fibrosis. However, besides HSCs and portal myofibroblasts, two other non-parenchymal cellular components, situated in the sinusoidal compartment of the liver, are involved in fibrogenesis: Kupffer cells and LSECs. In particular, Kupffer cells are the resident liver macrophages with panoply of functions ranging from maintaining homeostasis to controlling inflammation and repair upon injury (12, 111). Kupffer cells participate in the early response to injury by rapidly releasing cytokines and chemokines (e.g. IL-1β and CCL2, respectively), which further induce the recruitment of other immune cells. The immune cells can then decide the fate of the liver, either by promoting liver injury, such as the Ly-6C^{hi} monocytes bearing pro-inflammatory and pro-fibrogenic activity, or by stimulating injury resolution through the Ly-6C^{low} phenotype (111). On the other hand, LSECs constitute the lining of the hepatic sinusoids (58). They perform

important filtration and endocytic functions, may act as antigen-presenting cells and secrete cytokines, eicosanoids, endothelin-1, nitric oxide and some ECM components (58). Over the years, it has been widely demonstrated that chronic liver injury leads to distinct morphological abnormalities such as loss of sinusoidal fenestration, vasoconstriction and aberrant angiogenesis, as well as molecular changes in different liver cell populations (47). Importantly, alterations in the hepatic sinusoidal microenvironment are among the first events to occur following injury, thus contributing to the onset and progression of liver fibrosis. For this reason, pharmacological inhibition of these changes, especially in LSECs, has become one of the leading strategies for anti-fibrotic drug development (68).

Iron and liver fibrosis

Iron in liver diseases: the cause or the consequence?

Hitherto, the exact cellular and molecular mechanisms underlying the onset and the progression of liver fibrosis are still not fully understood. Nevertheless, iron is an attractive candidate. In fact, it has been postulated that iron, through multiple mechanisms, exerts a pro-fibrogenic role (82). Evidence suggesting that high iron levels could be involved in the pathogenesis of liver fibrosis derives from studies on diseases characterized by iron dysregulation (40). For instance, progressive liver fibrosis is the major comorbidity and the main cause of death in patients affected by HH (40). HH is an inherited disorder of abnormal iron metabolism, due to the lack of or resistance to the iron hormone hepcidin (57). Consistently, patients affected by HH exhibit excessive amount of body iron, which, in turn, induces multi-organ injury (e.g. in the liver, pancreas and heart) (40, 57). Whilst the cellular and molecular mechanisms are still under investigation, it is well established that liver iron content correlates with hepatic fibrosis in HH patients. Over the years, multiple mechanisms

underlying the pro-fibrogenic role of iron have been proposed (24, 103) and some of these will be discussed in-depth below.

As stated above, HSCs are the most studied cell type in the fibrotic process, due to their pivotal role in the synthesis and the deposition of ECM. Interestingly, several studies showed that iron promotes the activation of HSCs (103). In particular, high iron levels within the liver may damage hepatocytes and Kupffer cells through oxidative stress-dependent mechanisms (24, 99). The damaged cells, in turn, begin to secrete pro-inflammatory and pro-fibrogenic factors (e.g. TGF-β), thus triggering liver inflammation and, most importantly, HSCs activation (24, 103). These data highlighted the importance of both cell-to-cell communication and iron–oxidative stress axis in the pathogenesis of liver fibrosis **(Figure 2)**. Consistently, defective antioxidant defenses, due to genetic disruption of Nuclear factor erythroid 2-Related Factor 2 (NRF2), promoted the transition from iron accumulation to liver fibrosis in a mouse model of HH (35). Moreover, recent studies have suggested that HSCs, once activated, become responsive to iron by starting to express Transferrin Receptor 1 (TFR1) and H-ferritin receptor on their surface (15, 81, 82, 102, 109).

However, other evidence has led researchers to question whether iron overload is the cause or can also be a consequence of CLDs and liver fibrosis. Indeed, iron metabolism dysregulation has been reported in patients with CLDs in the absence of a HH-associated genotype (11, 64). Consistently, a high number of HCV patients (20-40%) display elevated serum iron parameters, namely transferrin saturation, serum iron and ferritin levels (31). Abnormalities in serum iron parameters have also been found in subjects with NASH (44). These data, taken together, indicate that iron metabolism dysregulation is frequently observed in patients with CLD, regardless of etiology. Hence, it is reasonable that dysregulation of iron metabolism may arise consequently to a chronic liver disease. Notably, transferrin and ferritin are considered a negative and a positive acute-phase reactant, respectively (48, 113). Hence, the serum levels of these proteins are modulated not only in response to iron but also to

inflammation or liver damage. Nevertheless, despite different mechanisms can contribute to abnormal transferrin and ferritin serum levels, patients with CLDs exhibit also low hepcidin serum levels (37, 69, 114). Importantly, iron metabolism dysregulation, which arises secondary to a CLD, may accelerate the progression of liver fibrosis to cirrhosis and to hepatocellular carcinoma, due to iron pro-inflammatory and pro-fibrogenic properties. Literature-based evidence supports this speculation in both humans and rodents. For instance, Abe *et al.* recently showed that the dietary iron restriction in a rat model of NASH leads to a reduction in [1] the hepatic iron content, [2] oxidative stress, [3] inflammation and [4] hepatic fibrosis (1). Furthermore, aside from its role in accelerating the progression of early-stage CLDs, iron negatively affects the outcome of patients with an end-stage CLDs (22, 55, 63). In particular, since iron is an essential element for most microorganisms, iron overload may increase the risk of infections, and even death, following liver transplant. Consistently, HH patients with mutations in *HFE* gene, a key interactor of both TFR1 and TRF2 (21, 43), display iron overload and reduced survival after liver transplant (64). Ko *et al.* also highlighted a strong correlation between hepatic iron content and hepatocellular carcinoma, probably due to the role of iron in inducing DNA damage (60).

Overall, it is clear that iron overload, frequently observed in multiple CLDs, could have important clinical implications. However, even though CLD patients exhibit low serum hepcidin levels, the exact molecular mechanisms underlying iron overload in CLDs are still elusive. For this reason, it is of paramount importance to further address this issue in an attempt to open new windows of intervention. Along this line, several studies (discussed below) have indicated that LSECs play a key role both in the pathogenesis of liver fibrosis and in the regulation of hepcidin expression and, consequently, in the control of iron levels.

LSECs and liver fibrosis

LSECs: structure, function and molecular signature

Endothelial cells (ECs) have long been considered as passive cells delimiting blood vessels. However, in the last few years, a considerable number of studies have shown that ECs are, instead, actively involved in organ development, regeneration and homeostasis maintenance (6, 25, 65, 77). Furthermore, it is becoming increasingly clear that, at the basis of many chronic and life-threatening diseases (e.g. metabolic disorders, atherosclerosis and cancer), there is vascular dysfunction, emphasizing once again the importance of ECs in ensuring a proper organ and, consequently, body physiological function (45). In this regard, microvascular ECs undergo a process of organ-specific specialization and differentiation, i.e. vascular organotypicity (6). In our body, there are three major types of capillaries with a different grade of permeability and molecular transport (from the lowest to the highest): continuous, fenestrated and sinusoidal. Moreover, the morphological classification of capillaries is associated to a unique gene expression profile, and in particular, to a unique array of organ-specific angiocrine signals, i.e. ECs-derived paracrine factors dictating the microenvironment behavior (6, 16).

LSECs, lining the hepatic sinusoids, are liver-specific differentiated microvascular ECs, and represent about 19% of total liver cells (90). LSECs, despite falling into the category of sinusoidal capillaries with spleen and bone marrow ECs, can be regarded as unique due to the presence of open (non-diaphragmed) fenestrae and the lack of a typical basement membrane. Thus, LSECs do not represent a barrier for macromolecule transport as ECs in other organs but allow a bidirectional “open” solute exchange between blood and hepatocytes. Thanks to the pioneering work of Wisse (119) in 1970 and subsequent reports by Widmann (118) and Ogawa (93), LSECs fenestrae are considered as transcellular open pores of approximately 100-150 nm in size clustered in groups– the so-called sieve plates, thus occupying around 6–8% of the endothelial surface. Successively, it emerged that the fenestration pattern (frequency and diameter of fenestrae) is not uniform across liver lobule but changes from the periportal to the centrilobular zone: bigger but fewer fenestrae per sieve

plate to smaller but more numerous fenestrae per sieve plate, respectively (121, 123). Ultrastructural analysis of hepatic sinusoids, and specifically of LSECs, aside from fenestrae, revealed a large number of bristle-coated micropinocytotic vesicles and lysosome-like vacuoles, thus suggesting that LSECs have a high endocytic capacity (120). Overall, LSECs [1] act as a sieve, by filtering in a "passive and size-selective manner" virus, solutes and particles (e.g. chylomicron remnants) smaller than the fenestrae diameter from and to the space of Disse, and [2] have a scavenger function, by actively supporting the uptake and degradation of a diverse array of macromolecules (13, 110).

Despite these findings, several aspects of the LSEC biology remain unclear, for instance, regarding the molecular and structural mechanisms underlying the formation, maintenance and dynamic regulation of their fenestrae. This is because one of the major challenges in studying LSECs, besides the lack of specific markers for identifying differentiated LSECs or technical and methodological limitations due to the small diameter of fenestrae, is the lack of a good and reliable *in vitro* cellular model. In fact, upon isolation from the hepatic microenvironment, LSECs lose their unique morphological and functional features within very few days in culture (29). Interestingly, new cell culture systems recapitulating the complex hepatic microenvironment (e.g. co-culture and microfluidic chip) have been developed to overcome this drawback. Several groups examined the effects of extracellular matrix and neighboring cells on LSEC phenotype *in vitro* (33, 41, 54, 72, 79). They demonstrated that LSECs undergo a microenvironment-dependent differentiation program mainly orchestrated by the Vascular Endothelial Growth Factor (VEGF). In particular, DeLeve *et al.* showed that VEGF produced by HSCs regulates LSEC phenotype by stimulating Nitric Oxide (NO) production (29). Thus, the maintenance of LSECs fenestration needs both paracrine and autocrine signaling.

At the molecular level, angiodiversity remains scarcely characterized. Lately, great efforts have been made to deeply unravel the heterogeneity of organ-specific microvascular

ECs molecular signatures (50, 73, 92). To identify the LSEC-specific molecular differentiation program, Géraud *et al.* performed a cDNA microarray analysis comparing freshly isolated LSECs with both Lung Microvascular Endothelial Cells (LMECs), as common continuous ECs, and LSECs cultured for 42 hours (which undergo spontaneous de-differentiation) (50). Importantly, a microenvironment-dependent LSEC-specific repertoire of cytokines and growth factors (*Wnt2*, *Wls*, *Nrp2*), transcriptional regulators (*Gata4*, *Lmo3*, *Maf*, *Tcfec*) and scavenger- and endocytosis-related genes (*Stab1/2*, *Lyve1* and *Ehd3*) was found (50). Later on, the same authors analyzed the role of one of LSEC-associated transcription factor, GATA4, in driving liver-specific microvascular transcriptional program. By performing both *in vitro* and *in vivo* experiments, they demonstrated that GATA4 is essential for a proper LSEC phenotype and directly regulates most of the LSEC-associated genes, thus acting as the master regulator of hepatic microvascular specialization (49). Furthermore, the authors showed that LSEC-specific GATA4 deletion in mice is sufficient to induce [1] lethal embryonic anemia due to impaired liver colonization of hematopoietic progenitor cells, [2] liver hypoplasia and [3] liver fibrosis (49). Thus, this evidence further heightens the importance of LSECs fenestration in ensuring proper liver development and the pivotal role of vascular dysfunction in triggering liver fibrosis.

Among the GATA4-regulated and LSEC-specific genes, the Bone Morphogenetic Proteins (BMP) 2 gained particular attention (49, 50, 94). As further discussed below, several experimental evidence shows that LSECs, by producing BMP2 and BMP6, play an important role in the regulation of iron homeostasis (18, 19, 61, 104).

LSECs capillarization and liver fibrosis

As mentioned before, LSECs are considered unique ECs for their open fenestrae and disorganized basement membrane. Interestingly, despite their high specialization, LSECs retain considerable phenotypic and functional plasticity. In fact, LSECs fenestrae are dynamic

structures with their number and diameter capable of changing over time in the hepatic vascular beds in response to external stimuli (13).

The loss of fenestration and deposition of an organized sub-endothelial basement membrane is called “capillarization” (107). LSECs de-differentiation or sinusoidal capillarization is now considered a hallmark of liver fibrosis and cirrhosis, regardless of the cause (27, 51, 74, 101, 126). For example, Miyao *et al.* showed that Scanning Electron Microscopy (SEM) analysis of livers derived from mice fed with two different fibrogenic diets (DDC-supplemented diet and CDAA diet) presented, already at very early time-points, sinusoidal capillarization (84, 85).

Due to the crucial role of LSECs fenestration in liver physiology, its alteration could compromise parenchyma function, thus contributing to liver fibrosis onset and to hepatic failure and related complications, i.e. portal hypertension, gradually leading to cirrhosis. Consistently, several studies performed both on human samples and animal models demonstrated that normal differentiated LSECs act as gatekeepers of fibrogenesis by maintaining the quiescence of HSCs (28, 59, 124, 128) and that LSECs capillarization precedes the onset of liver fibrosis (34, 49, 124). Moreover, de-differentiated (i.e. without open fenestrae) LSECs [1] contribute to ECM deposition through the production of collagen and fibronectin (88, 125), [2] promote portal hypertension (46, 78, 105), [3] participate in the generation of the inflammatory milieu, and [4] alter intrahepatic immunity (23, 80) **(Figure 3)**.

In addition to structural alterations, during the fibrotic process, LSECs undergo a dramatic change in their expression profile. In fact, it was shown that GATA4 decreased considerably during human and rodent liver fibrogenesis (30, 115). This evidence further heightens the involvement of GATA4 in orchestrating LSEC phenotype as well as the close association between LSECs morphology and their molecular signature. In fact, de-differentiated LSECs switch on the expression of genes commonly-associated to EC, such as *CD31* and *CD34*, at the expense of LSEC-associated genes, such as *GATA4*, *STAB1/2*,

GPR182 and *LYVE1* (32, 34, 108, 115). This gene expression shift has a negative impact also on liver-specific angiocrine signals repertoire, e.g. *WNT2* and *BMP2*. All these works further highlight the importance of counteracting LSEC fenestration loss in order to prevent and/or slow-down the fibrogenic process.

LSECs and iron metabolism

LSECs regulate serum and tissue iron homeostasis

As discussed above, dysregulated iron metabolism can exacerbate the pathogenesis of fibrosis and cirrhosis in the liver due to the pro-inflammatory and pro-fibrogenic properties of iron. Multiple liver cell types contribute to determine serum iron levels by participating in iron uptake, utilization, recycling and storage. All these processes must be finely coordinated to keep iron levels within a physiological range, thus avoiding the onset of iron-related disorders(53). Importantly, recent findings suggest that LSECs, by finely communicating with hepatocytes, play a pivotal role in the coordination of these processes and in the maintenance of serum and tissue iron homeostasis. To this end, hepcidin, a peptide hormone encoded by the *HAMP* gene and secreted by hepatocytes, controls the release of iron from the cell into the circulation (91, 100). Hepcidin regulates the activity of different cell types including [1] the enterocytes, which mediate dietary iron absorption in the intestine; [2] the macrophages, responsible for the recycling of iron derived from erythrocytes' turnover; and [3] the hepatocytes, major site of iron storage (86, 89, 95, 100). At molecular level, hepcidin exerts its regulatory activity by binding the iron-exporter Ferroportin (FPN) on the surface of target cells and mediating its endocytosis and degradation, thus preventing the entry of iron into the blood **(Figure 4)**. Hepcidin expression is directly modulated by the body iron status through mechanisms that involve the BMP-SMAD signaling pathway (7, 8, 14, 20, 96, 116). BMPs belong to the Transforming Growth Factor-β (TGF-β) superfamily that includes over 30 known secreted factors (75, 117). Among them, only BMP6 and, more recently, BMP2 have been

identified as key regulators of hepcidin expression, as demonstrated by murine knockout studies and genetic analysis of patients with iron overload (4, 26, 96, 97). In particular, total BMP6 loss in mice results in a strong reduction in hepcidin expression and increased tissue iron accumulation, a phenotype resembling hereditary hemochromatosis (4). The interaction of BMPs with their type I and type II receptors on hepatocyte surface leads to the phosphorylation of SMAD1/5/8 proteins, which, after complexing with SMAD4, translocate to the nucleus, bind the hepcidin promoter and activate its expression (7, 76, 116). However, the capability of relevant BMPs to fully activate BMP-SMAD signaling pathway strictly depends on the presence of the co-receptor Hemojuvelin (HJV) on hepatocyte surface (7, 8, 62, 66). Consistently, BMP(s)-dependent hepcidin induction is inhibited by the serine protease Matriptase-2 (encoded by *TMPRSS6* gene), that is responsible for HJV cleavage (3, 39, 87). In support of the key role of Matriptase-2 in preventing inappropriate activation of BMP-SMAD signaling, it was shown that mutations in the *TMPRSS6* gene were associated to a condition of Iron-Refractory Iron-Deficiency Anemia (IRIDA) in human patients, caused by persistent hepcidin induction (87).

Importantly, while hepcidin and Matriptase-2 show a hepatocyte-restricted expression, *BMP6* mRNA levels have been detected at higher levels in liver NPCs compared to hepatocytes (104, 127). Studies utilizing conditional knockout mice led to the identification of LSECs as primary source of BMP6. In particular, Canali *et al.* showed that mice with a conditional knockout of BMP6 in LSECs exhibited significantly reduced liver *hepcidin* mRNA and, consequently, increased serum and tissues iron loading, thus resembling the phenotype of total BMP6 null mice (19). Conversely, BMP6 conditional deletion in Kupffer cells or in hepatocytes did not alter hepcidin expression or serum and tissues iron content (19). These studies pointed out the pivotal role of LSECs in the modulation of hepatic *hepcidin* mRNA expression and identified BMP6 as a key angiocrine factor involved in iron regulation. However, the specific signals that trigger BMP6 production in LSECs are still under

investigation. Recent findings showed that BMP6 expression is regulated by the transcription factor NRF2 in response to iron-induced oxidative stress (70). Interestingly, BMP6 loss in combination with HFE or TFR2 loss, both involved in HH, results in a more severe phenotype compared to single BMP6-deficient mice, with decreased SMAD5 phosphorylation and further repression of *Hamp* gene expression (67). Moreover, BMP6-null mice retain a residual ability to induce hepcidin through SMAD signaling upon iron stimulation, thus suggesting that, in the absence of BMP6, other mechanisms could occur. Some studies investigated the possible involvement of other BMPs in the regulation of *hepcidin* transcription. BMP2, a GATA4-regulated and LSEC-specific angiocrine signal, emerged as a promising candidate. Indeed, *in vitro* studies on freshly isolated murine primary hepatocytes revealed that Holo-Transferrin treatment leads to an increase in *hepcidin* mRNA levels through a HJV/BMP2/4-dependent pathway (71). Moreover, a Single Nucleotide Polymorphism (SNP) of the *BMP2* gene region, found in a cohort of human patients with hemochromatosis, has been associated to the severity of iron loading (83). A resolutive evidence of BMP2 involvement in hepcidin regulation comes from the generation of LSEC-specific conditional BMP2-null mice (18, 61). In particular, genetic inactivation of BMP2 in LSECs leads to a strong reduction in *hepcidin* expression, that in turn causes liver and tissues iron accumulation and increased serum iron levels, similar to the phenotype of LSEC-specific BMP6-null mice (61).

These data, taken together, strongly demonstrate the paramount role of endothelial BMP2 and BMP6 in the regulation of *hepcidin* transcription. However, the relative contribution of these two BMPs in activating SMAD pathway and, consequently, in stimulating hepcidin, remains uncertain. An important hint came from the study of Canali *et al.*, which essentially demonstrated that the residual ability to induce hepcidin upon iron stimulation in BMP6-null mice was lost following treatment with a neutralizing BMP2/4 antibody (18). This observation suggests that BMP2 and BMP6 work independently in activating *hepcidin* transcription. Nevertheless, the severity of the disease in both endothelial-specific BMP6- and BMP2-null

mice indicates that none of these factors can fully compensate for the absence of the other. Consistently, the up-regulation of *Bmp6* observed in mice lacking BMP2 in endothelial cells was not sufficient to mitigate their iron overload phenotype (18). Moreover, Xiao *et al.* recently showed that iron overload phenotype is not exacerbated in the double knockout of BMP2/6 compared to single BMP2 or BMP6 knockout (122). Hence, BMP2 and BMP6 may cooperate to regulate *hepcidin* expression and could be activated by different stimuli or act through different mechanisms. Notably, it has been shown that LSECs, under basal condition, mainly express *Bmp2* (that is, higher *Bmp2* mRNA copy number compared to *Bmp6*) but exhibit an iron overload-mediated positive regulation of *Bmp6* at a greater extent than *Bmp2* (18). Consistently, it may be hypothesized that BMP2 is important in conditions of basal/low iron, while BMP6 takes over when iron level increases. Furthermore, since BMPs can form both homodimers and heterodimers with a different (lower and higher, respectively) affinity for type I/type II receptors (56), it has been also proposed that BMP6 and BMP2 might work as a complex to induce *hepcidin* expression. Likewise, in the absence of BMP2, for example, homodimers of BMP6 (with a lower affinity for the receptors) are not able to sufficiently activate *hepcidin* expression and to fully compensate for the lack of BMP2. Accordingly, Canali *et al.* hypothesised that the inability of BMP6 to mitigate the iron overload phenotype due to BMP2 loss in endothelial cells, could be related to its inability to form BMP2/BMP6 heterodimers (18).

Despite the still open questions regarding hepcidin regulation, LSECs have proved to be fundamental regulators of systemic iron homeostasis, mainly through the production of the angiocrine factors BMP2 and BMP6 **(Figure 5)**.

LSECs, iron dysregulation and liver fibrosis

As previously discussed, low hepcidin serum levels, and consequently, iron overload, are common features in patients with CLDs, even in the absence of mutations in

hemochromatosis-related genes. However, the exact molecular mechanisms underlying the link between liver diseases and iron are still largely unknown.

The recent findings about the pivotal role of LSECs in iron level regulation, their morphological and, most importantly, molecular de-differentiation in liver fibrosis, prompted researchers to investigate the possible relationship between LSECs de-differentiation and liver fibrosis-related iron overload. It has been shown that cirrhotic liver-derived de-differentiated LSECs express *Bmp2* at lower levels compared to normal liver-derived fenestrated LSECs (115). Therefore, in the absence of BMP2, hepcidin synthesis and secretion may be inadequate. Moreover, Hasebe *et al.* recently described a possible mechanism underlying NAFLD-associated iron dysregulation (52). In particular, mice fed with a high-fat diet to induce NAFLD, exhibited low serum levels of hepcidin, and consequently, iron overload both in serum and in the liver. RNA sequencing analysis, performed on whole livers of these mice *versu*s controls, revealed 36 dysregulated iron metabolism-related genes, including BMP-binding Endothelial Regulator (BMPER). Consistently, mice fed a high-fat diet exhibited higher *Bmper* expression levels in the liver, as well as an increasing trend of plasma level of BMPER, which co-immunoprecipitated with BMP6. Moreover, BMPER was found strongly expressed by LSECs in the fatty/fibrotic liver (52). As previously demonstrated, a soluble form of BMPER suppresses BMP2- and BMP6-dependent hepcidin induction by binding and sequestering extracellular BMPs (98). Although there are no hints on the differentiation status of LSECs, these findings suggest that LSECs-released BMPER could impair BMPs signaling, thus reducing hepcidin expression in fatty/fibrotic livers. Hence, it can be postulated that at the basis of liver fibrosis-associated iron overload, there may be vascular dysfunction, which compromises the BMP(s)-hepcidin axis between LSECs and hepatocytes. Since altered serum iron parameters (e.g. transferrin saturation) are important drivers of liver fibrosis progression, it is of great importance to further investigate whether there is a causative association between de-differentiated LSECs and serum iron levels in liver fibrosis.

Another layer of complexity derives from the evidence that iron itself can induce the shift from sinusoidal to continuous ECs (i.e. LSECs capillarization). Addo *et al.* demonstrated that iron overload stimulates the hepatocytes to produce and secrete Nerve Growth Factor (NGF), which in turn can induce LSECs defenestration (2). Moreover, recent findings suggest that iron may also be able to directly and negatively affect LSEC phenotype. In particular, Lim *et al.* showed that iron, by promoting mitochondrial oxidative damage, activates the transcription factor NRF2, as a protective response in LSECs (70). Nevertheless, a persistent activation of NRF2 has been reported to inhibit autophagy and, since autophagy has a protective role in maintaining LSECs integrity and phenotype (i.e. fenestrae), iron-induced NRF2 activity may contribute to endothelial dysfunction (10, 106). Thus, even if further experiments are needed to validate these points, it could be hypothesized that in the liver fibrosis context, often characterized by iron accumulation, a vicious circle may exist through which de-differentiated LSECs induce iron overload, which in turn may directly and indirectly maintains and perpetuates LSECs capillarization over time, thus exacerbating liver fibrosis **(Figure 6)**.

Conclusions

Liver fibrosis represents a major world health problem due to the lack of therapeutic treatments as alternatives to liver transplantation. Due to their contribution to not only the onset and progression of liver fibrosis but also to iron homeostasis regulation, LSECs emerged as a promising and attractive therapeutic target. An early event of the fibrotic process is the loss of fenestrae in the hepatic sinusoids due to progressive de-differentiation of LSECs, a phenomenon referred to as “LSECs capillarization”. Importantly, de-differentiated LSECs undergo a dramatic change in the repertoire of secreted angiocrine factors. Some of these factors likely contribute to determine a condition of iron overload in the fibrotic liver. Nevertheless, iron overload itself has been reported to promote LSEC de-differentiation.

These findings, taken together, indicate that, in CLDs, a vicious loop can be established in which iron-dysregulation and LSEC de-differentiation synergize to exacerbate and promote the progression of liver fibrosis.

Considering the data and evidence gathered in this emerging research field, it is worth spending great efforts to develop new therapeutic strategies aimed at restoring LSECs differentiation and, consequently, at preventing the progression of liver fibrosis or, even better, at promoting its regression.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

List of Abbreviations

BMP	Bone Morphogenetic Protein
BMPER	Bone Morphogenetic Protein-binding Endothelial Regulator
BMPR	Bone Morphogenetic Protein Receptor
CLD	Chronic Liver Disease
EC	Endothelial Cell
ECM	Extracellular Matrix
FPN	Ferroportin
HAMP	Hepcidin gene
HC	Hepatocyte
HCV	Hepatitis C Virus
HGB	Hemoglobin
HH	Hereditary Hemochromatosis

HJV	Hemojuvelin
HSC	Hepatic Stellate Cell
IRIDA	Iron-Refractory Iron-Deficiency Anemia
LMEC	Lung Microvascular Endothelial Cell
LSEC	Liver Sinusoidal Endothelial Cell
MTP2	Matriptase-2
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NGF	Nerve Growth Factor
NO	Nitric Oxide
NPC	Non-Parenchymal Cell
RBC	Red Blood Cell
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
SNP	Single Nucleotide Polymorphism
TGF-β	Transforming Growth Factor β
TFR	Transferrin Receptor
TMPRSS6	Matriptase-2 gene
VEGF	Vascular Endothelial Growth Factor

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Figure legends

Figure 1. Schematic representation of the cellular events that occur when a pro-fibrotic stimulus persists over time. *LSEC, Liver Sinusoidal Endothelial Cell; HSC, Hepatic Stellate Cell; HC, Hepatocyte.*

Figure 2. Schematic representation of the harmful effects mediated by iron on liver cell populations. Iron overload induces oxidative damage in Kupffer cells and Hepatocytes, which in turn release a repertoire of pro-inflammatory and pro-fibrogenic factors. These factors promote the activation of HSCs, an early events in the fibrotic process. Iron-mediated cellular damage is then perpetuated over time and also affects HSCs that, once activated, express TFR1 on their surface. *HSC, Hepatic Stellate Cell; TFR1, Transferrin Receptor 1; TSAT, transferrin saturation.*

Figure 3. Schematic represention of structural differences between normal and cirrhotic sinusoidal milieu. In normal liver (top), the presence of open fenestrae and the lack of basement membran allow a bidirectional exchange between blood and the space of Disse, where reside quiescent HSCs. In the fibrotic liver (bottom), there is the deposition of an organized basement membrane along the sinusoids, the loss of LSECs fenestrae and the activation of HSCs, which in turn cause excessive collagen deposition whithin the space of Disse.

Figure 4. The liver-secreted peptide hormone hepcidin is a master regulator of iron homeostasis. hepcidin, by promoting the internalization of the iron exporter Ferroportin on target cells, determines the amount of iron that is released into circulation. *FPN, Ferroportin; Fe, Iron; HGB, Hemoglobin.*

Figure 5. LSECs are the major source of BMPs. Once released by LSECs, BMPs bind to their receptors expressed on the hepatocyte. The signal transduction involves SMAD proteins, which drive the transcription of *HAMP* gene. BMP-SMAD signaling pathway

requires the presence on hepatocytes surface of the co-receptor HJV. Hepcidin induction is inhibited by the serine protease MTP2, that is indeed able to cleave HJV. *BMP, Bone Morphogenetic Protein; BMPR, BMP Receptor; HJV, Hemojuvelin; MTP2, Matriptase-2; HAMP, Hepcidin gene.*

Figure 6. In the fibrotic liver, iron dysregulation and LSECs capillarization likely synergize to instaure a vicious loop that exhacerbates the progression of fibrosis. De-differentiated LSECs release high levels of BMPER and low levels of BMP2, thus inhibiting hepcidin production by the hepatocytes. Hepcidin inhibition results in iron overload. Iron overload induces the release of NGF by hepatocytes, a growth factor able to promote LSECs capillarization, thus sustaining the detrimental cycle. *LSECs, Liver Sinusoidal Endothelial Cells; NGF, Nerve Growth Factor; BMPER, BMP-binding Endothelial Regulator; BMP2, Bone Morphogenetic Protein 2; Fe, Iron.*