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1 **Role of liver sinusoidal endothelial cells in nonalcoholic fatty liver disease**

2

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20 **Key words:** endothelium; steatosis; capillarization; endothelial dysfunction;

21 angiogenesis

22

23

1 **Abbreviations:** cGMP: cyclic guanosine monophosphate; eNOS: endothelial nitric oxide
2 synthase; FABP4: fatty acid binding protein 4; FFA: free fatty acids; HCC: hepatocellular
3 carcinoma; HIF: hypoxia inducible factors; ICAM-1: intercellular adhesion molecule 1; IL:
4 interleukin; iNOS: inducible nitric oxide synthase; KLF: krüppel-like Factor; LSECs: liver
5 sinusoidal endothelial cells; MAPK: mitogen activated protein kinase; NAFLD:
6 nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; NK: natural killer;
7 ox-LDL: oxidized low density lipoprotein; PLVAP: plasmalemma vesicle-associated
8 protein; ROS: reactive oxygen species; TGF: transforming growth factor; TLR: toll like
9 receptor; TNF: tumor necrosis factor; VAP-1: vascular adhesion protein 1; VCAM-1:
10 vascular cell adhesion molecule 1; VEGF: vascular endothelial growth factor; VEGFR:
11 vascular endothelial growth factor receptor.

12

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22

23

1 **Summary:** (233 words)

2 Nonalcoholic fatty liver disease (NAFLD) and its complication is an expanding health
3 problem associated with the metabolic syndrome. Liver sinusoidal endothelial cells
4 (LSECs) are highly specialized endothelial cells localized at the interface between the
5 blood derived from the gut and the adipose tissue on the one side, and other liver cells
6 on the other side. In physiological conditions, LSECs are gatekeepers of liver
7 homeostasis. LSECs display anti-inflammatory and anti-fibrogenic properties by
8 preventing Kupffer cells and hepatic stellate cells activation and regulate intrahepatic
9 vascular resistance and portal pressure. This review focuses on changes occurring in
10 LSECs in NAFLD and on their consequences on NAFLD progression and complications.
11 Capillarization, *i.e.* loss of LSECs *fenestrae*, and LSECs dysfunction, *i.e.* the loss of the
12 ability of LSECs to generate vasodilator agents in response to increased shear stress, are
13 two events occurring early in NAFLD. These LSECs changes favor steatosis development
14 and set the stage for NAFLD progression. At the stage of non-alcoholic steatohepatitis,
15 altered LSECs release inflammatory mediators and contribute to the recruitment of
16 inflammatory cells, thus promoting liver injury and inflammation. Altered LSECs also fail
17 to maintain hepatic stellate cells quiescence and release fibrogenic mediators, including
18 Hedgehog signaling molecules, promoting liver fibrosis. Liver angiogenesis is increased
19 in NAFLD and contributes to liver inflammation and fibrosis, but also to hepatocellular
20 carcinoma development. Improving LSECs health thus appears as a promising approach
21 to prevent NAFLD progression and complications.

22

1 **Lay summary**

2 Liver sinusoidal endothelial cells are highly specialized endothelial cells integrating
3 signals derived from the gut and the adipose tissue, and interacting with other liver
4 cells.

5 In nonalcoholic liver disease, liver sinusoidal endothelial cells alterations occur early in
6 disease progression, at the stage of simple steatosis.

7 In nonalcoholic liver disease, changes in liver sinusoidal endothelial cells contribute to
8 liver angiogenesis, inflammation, fibrosis and to hepatocellular carcinoma.

9

1 Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of conditions
2 including simple steatosis and nonalcoholic steatohepatitis (NASH), defined as the
3 association of steatosis, hepatocellular damage, inflammation and varying degree of
4 fibrosis [1]. NAFLD is associated with obesity, insulin resistance and/or type 2 diabetes
5 and other metabolic abnormalities, collectively termed the metabolic syndrome [2,3].
6 NAFLD is an expanding health problem with an estimated global prevalence of 25%
7 [2,4]. A recent modeling approach estimated that NAFLD cases in the United States will
8 expand from 83 million in 2015, corresponding to about 25% of the population, to 100
9 million in 2030, corresponding to more than 33% of the population [4]. While simple
10 steatosis is generally benign, NASH can progress to both cirrhosis and end-stage liver
11 disease. NASH is currently a leading cause of liver disease among adults awaiting liver
12 transplantation in Europe and in the United States and is projected to become the most
13 common indication for liver transplantation in the next decade [2,4]. Importantly,
14 patients with metabolic syndrome and NASH also develop hepatocellular carcinoma
15 (HCC) in the absence of cirrhosis [5,6]. Despite its prevalence and severity, there is no
16 approved therapy for NASH and available treatments only aim to control associated
17 conditions [1]. Understanding the mechanisms of NAFLD, and in particular how simple
18 steatosis progresses to NASH and then to cirrhosis and/or liver cancer, is of utmost
19 importance.

20 Current view of the pathogenesis of NASH centers on the response of hepatocytes to
21 insulin resistance and lipotoxicity. Immune system and hepatic stellate cell activation
22 are regarded as secondary events [1]. Vascular endothelium, representing the interface
23 between blood and other tissues of the body, is not only a physical barrier but is
24 implicated in different physiological roles, such as hemostasis, metabolites

1 transportation, inflammation, thrombosis, angiogenesis and vascular tone. Liver
2 endothelium is mainly formed of liver sinusoidal endothelial cells (LSECs) which are
3 highly specialized endothelial cells at the interface between blood derived from the
4 visceral adipose tissue and the gut, on the one side, and hepatic stellate cells and
5 hepatocytes, on the other side. LSECs have a unique phenotype in the human body as
6 they lack a basement membrane and have a multitude of *fenestrae* organized in sieves,
7 that regulate the transport of macromolecules, including lipids and lipoproteins, across
8 the sinusoid [7,8]. This review will specifically focus on the role of LSECs in the
9 pathophysiology of NAFLD and its complications.

10

11 **LSECs and simple steatosis**

12 *Role of LSECs in lipid transfer in the normal liver*

13 Dietary lipids present in the circulation have to be transported through the vascular
14 endothelium to be metabolized by tissues. In physiological conditions, LSECs are major
15 regulators of the bidirectional lipid exchange between the blood and the liver
16 parenchyma in two manners. First, LSECs *fenestrae* allow for efficient transfer of
17 lipoproteins, chylomicron remnants (small lipoproteins derived from chylomicrons
18 generated by enterocytes from dietary lipids), and other macromolecules, from the
19 sinusoidal blood to the space of Disse, where they are taken up by hepatocytes [9–11].
20 LSECs *fenestrae* form a selective barrier for lipids. Indeed, older studies using
21 radiolabeled lipoproteins showed that larger lipoproteins do not cross LSECs *fenestrae*
22 and remain in the lumen of the sinusoid [12]. Moreover, when LSECs lose their
23 *fenestrae* following VEGF pathway disruption, [11,13,14], uptake of fluorescently

1 labelled lipids is impaired [11,15]. Second, LSECs also regulate lipid transfer through
2 their high endocytosis capacity, as attested by their high ability to rapidly take-up
3 oxidized or acetylated-low density lipoproteins [8,16,17].

4 *LSECs capillarization occurs early in NAFLD and promotes steatosis*

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

13 Triggers for sinusoidal capillarization are not fully identified, but we can speculate that
14 excessive dietary macronutrients, including lipids, carbohydrates, and gut microbiota-
15 derived products play a role [19]. Cogger and coworkers demonstrated in mice
16 challenged with several diets varying in content of macronutrients and energy that
17 LSECs porosity and *fenestrae* frequency are inversely correlated with dietary fat intake,
18 while *fenestrae* diameter is inversely correlated with protein or carbohydrate intake
19 [19]. In this study, authors also found a negative correlation between LSECs *fenestrae*
20 (frequency, porosity and diameter) and circulating free fatty acids (FFA) levels [19]. *In*
21 *vitro* studies suggested that defenestration occurs following excessive lipids exposition.
22 For instance, exposure of human primary LSECs to oxidized-LDL (ox-LDL) reduces the
23 diameter and the porosity of the *fenestrae* [21]. The effect of FFA on *fenestrae* has also

1 been tested in primary rat LSECs, but firm conclusions cannot be drawn since authors
2 did not test several concentrations of FFA but rather presence vs. absence of FFA, which
3 does not adequately mimic *in vivo* conditions [22]. Recent evidence point to gut
4 microbiota in the pathogenesis of NAFLD [3,23]. Cogger and colleagues showed that
5 LSECs *fenestrae* inversely correlated with the abundance of *Bacteroidetes* in the gut and
6 positively correlated with the abundance of *Firmicutes* [19]. Moreover, it has been
7 shown that a single injection of endotoxin in rats induces a decrease in both diameter
8 and number of *fenestrae* suggesting that gut microbiota-derived products may
9 contribute to LSECs capillarization, although caution is needed since concentration of
10 endotoxin used in that study was high [24].

11 In turn, capillarization favors liver steatosis (Figure 1), as observed in mice deficient in
12 plasmalemma vesicle-associated protein (PLVAP), an endothelial-specific integral
13 membrane glycoprotein required for the formation of endothelial *fenestrae* [25]. These
14 mice exhibit a pronounced reduction in the number of LSECs *fenestrae*, associated with a
15 decrease in sinusoidal permeability [25] and spontaneously develop extensive steatosis
16 [25]. These mice also have hyperlipoproteinemia and increased triglyceridemia due to
17 chylomicron remnants retention in the blood. As mentioned above, *vegfb*^{-/-} mice also
18 exhibit reduction in the number of LSECs *fenestrae* and less uptake of labeled oleic acid
19 due to capillarization, but steatosis was not evaluated [15].

20 A first hypothesis explaining this consequence of capillarization on steatosis could be
21 that reduced LSECs permeability impairs the passage of hepatocytes-derived very low-
22 density lipoprotein toward the sinusoidal lumen, thus inducing cholesterol and
23 triglycerides retention in the liver. However, these lipoproteins may escape the liver
24 through the lymphatic system [26]. As an alternative explanation, Herrnberger and

1 collaborators proposed that chylomicron remnants originating from the blood and
2 required for synthesis of very low-density lipoprotein by hepatocytes can not reach
3 hepatocytes due to LSECs capillarization; their absence in hepatocytes might then
4 stimulate hepatic *de novo* lipid synthesis and induce steatosis, as a compensatory
5 mechanism [25,27]. However, there is no available data to ascertain this hypothesis.
6 Similarly, Fraser and collaborators postulated that, following LSECs capillarization,
7 chylomicron remnants and dietary cholesterol do no longer pass *fenestrae* and inhibit
8 HMGCoA reductase, the rate limiting enzyme for hepatocyte cholesterol biosynthesis,
9 consequently activating endogenous cholesterol synthesis in hepatocytes [28].
10 Taken together, these data suggest that features of metabolic syndrome are associated
11 with LSECs capillarization, which promotes steatosis (Figure 1).

12 *LSECs dysfunction occurs early in NAFLD and promotes steatosis*

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

20 This increased intrahepatic vascular resistance has a mechanical and a dynamic
21 component. The mechanical part is due to the compression of the sinusoidal lumen by
22 enlarged fat-laden hepatocytes [29,36–38]. The dynamic part is due to a liver
23 endothelial dysfunction. Endothelial dysfunction is a pathological condition, common to

1 all vascular beds, defined as the inability of blood vessels to dilate in response to
2 increased blood flow. Endothelial dysfunction is generally indicated by the loss of nitric
3 oxide bioavailability due to eNOS inhibition [39]. Several lines of evidence show that
4 LSECs dysfunction occurs in fatty livers and is involved in the increased intrahepatic
5 vascular resistance associated with steatosis [40–43]. eNOS activation and liver nitric
6 oxide content are reduced in mice and rats fed a high-fat diet or a rich saturated fatty
7 acids diet for 4 weeks [40,41]. Isolated-perfused liver experiments performed in these
8 animals showed augmented portal perfusion pressure and reduced vasodilatory
9 response to acetylcholine, attesting liver endothelial dysfunction. These changes were
10 observed in the absence of inflammation and fibrosis, suggesting that endothelial
11 dysfunction is an early feature associated with steatosis in NAFLD [41,42].

12 Several mechanisms could account for this liver endothelial dysfunction associated with
13 steatosis (Figure 2). First, LSECs dysfunction can be induced by overabundance of lipids
14 during steatosis. *In vitro* experiments showed that stimulation of human primary LSECs
15 with ox-LDL downregulates eNOS expression through the ox-LDL receptor, LOX1 [21].
16 In addition, exposure of primary LSECs to palmitic acid also attenuates nitric oxide
17 bioavailability through peroxynitrite production by NOX1, a nitric oxide consuming
18 enzyme highly expressed in LSECs of mice fed a high-fat diet [44]. Second, steatosis
19 induces insulin resistance in LSECs, leading to impairment of insulin-dependent
20 vasodilation [41,45,46]. This effect is due, on the one hand, to the downregulation of
21 eNOS activity [41], and on the other hand to the upregulation of iNOS, the inducible form
22 of NOS which can cause endothelial dysfunction through increased nitrooxidative stress
23 [46–48]. Interestingly, V-PYRRO/NO, a diazeniumdiolate ion metabolized in the liver
24 that spontaneously decomposes to nitric oxide with a very short half-life at physiological

1 pH and triggers cyclic guanosine 3',5'-monophosphate (cGMP) synthesis, improves
2 hepatic microcirculation in mice with high-fat diet-induced steatosis [49]. Third, gut
3 microbiota also seems to contribute to liver endothelial dysfunction. Indeed, Garcia-
4 Lezana and colleagues demonstrated that restoration of a healthy microbiota via fecal
5 transplantation normalizes portal hypertension by improving intrahepatic vascular
6 resistance and endothelial dysfunction in rats [43].

7 In turn, LSECs dysfunction favors steatosis (Figure 2). Indeed, deficiency in nitric oxide
8 in *eNos^{-/-}* mice results in massive fat droplets deposition and increases liver triglyceride
9 content [40,50]. Nitric oxide contributes to the regulation of hepatic lipid content by
10 limiting citrate synthesis in mitochondria, which is involved in fatty acids production
11 [50]. Nitric oxide also attenuates synthesis of fatty acids in isolated cultured rat
12 hepatocytes by nitrosylating acetyl-CoA [51] and activating AMP-activated protein
13 kinase [52–54], an inhibitor of glycerol-3-phosphate acyltransferase and thus of
14 triacylglycerol synthesis [55,56]. In addition, nitric oxide also allows efficient fatty acids
15 beta-oxidation through s-nitrosylation of very long-chain acyl-CoA dehydrogenase in
16 hepatocytes [57]. Interestingly, therapies augmenting nitric oxide availability in the liver
17 improve steatosis. The V-PYRRO/NO or the improvement of nitric oxide/cGMP signaling
18 with the phosphodiesterase-5 inhibitor sildenafil protect against liver steatosis in high-
19 fat diet-fed mice [40,58,59]. Moreover, treatment with simvastatin, a drug able to
20 increase expression and activity of eNOS expression in the liver, decreases high-fat diet-
21 induced steatosis in rats [60].

22 To summarize, steatosis is associated with LSECs dysfunction which in turn worsens
23 steatosis (Figure 2).

1 Angiogenesis and steatosis

2 Angiogenesis, defined as the formation of new vessels from preexistent vessels, is a key
3 event in the progression of NAFLD [61–65]. Vascular endothelial growth factor (VEGF) is
4 the master pro-angiogenic regulator of this process supported by activation of hypoxia
5 inducible factors (HIF) in hypoxic areas [64].

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

15 **Key points:**

- 16 • *LSECs capillarization and dysfunction occur very early in NAFLD progression and*
17 *contribute to dietary induced steatosis.*
- 18 • *Intrahepatic vascular resistance is increased even when steatosis is the only*
19 *histological feature of NAFLD. This is due to the combination of a compression of*
20 *sinusoids by fat-laden enlarged hepatocytes and of a dysfunction of LSECs due to*
21 *reduced nitric oxide bioavailability.*
- 22 • *Molecular events associated with angiogenesis are initiated during simple steatosis,*
23 *but angiogenesis itself is only detected in NASH.*

1

2 **LSECs in NASH**

3 *LSECs contribute to oxidative stress in NASH*

4 In response to lipotoxicity, hepatocytes generate reactive oxygen species (ROS) and
5 initiate a robust inflammatory response that accentuates liver injury [1,3]. In addition to
6 hepatocytes [1,3], lipotoxic response also occurs in LSECs contributing to ROS
7 generation [44,69]. Indeed, ROS have been detected not only in hepatocytes but also in
8 sinusoidal cells in patients with NASH [70]. Circulating lipids seem to account for this
9 LSECs oxidative stress. Indeed, exposure of murine LSECs to palmitic acid upregulates
10 NOX1 expression, an enzyme implicated in ROS production [44]. In addition, stimulation
11 of human primary LSECs with ox-LDL increases ROS generation after binding to LOX1
12 [21]. This LSECs oxidative stress contributes to NASH. Indeed, mice with a global
13 deficiency in NOX1, which is highly expressed in LSECs in NAFLD, and fed a high-fat diet
14 display attenuated liver lesions attested by lower serum ALT level and lower hepatic
15 cleaved caspase-3 expression [44]. Therefore, in NASH, ROS production takes place not
16 only in hepatocytes but also to some extent in LSECs and seems to contribute to
17 hepatocyte injury.

18 *Anti-inflammatory role of LSECs at initial stages of NASH*

19 Progression of simple steatosis to steatohepatitis is accompanied with adhesion of
20 leukocytes to the sinusoidal endothelium followed by infiltration of leukocytes within
21 liver parenchyma to form inflammatory foci [71]. Moderate and resolved inflammatory
22 responses are beneficial to the liver as they promote the re-establishment of
23 homeostasis, contribute to tissue repair and exert hepato-protective effects [72].

1 However, chronic inflammation, as seen in NASH, is accompanied with death of
2 hepatocytes and causes damage to liver parenchyma [72]. In physiological conditions,
3 LSECs constitute a barrier regulating the entry of circulating leukocytes within liver
4 parenchyma and play an anti-inflammatory role [73–76]. At early stages of NAFLD
5 progression, some evidence indicates that LSECs also exhibit anti-inflammatory function
6 [40,77]. Indeed, Tateya and colleagues elegantly demonstrated that nitric oxide derived
7 from LSECs inhibits Kupffer cells activation in mice fed a high-fat diet for a short period
8 of time (8 weeks) [40]. *In vitro*, both human and murine LSECs exposed to FFA for a
9 short period (16 hours) exhibit a downregulation of pro-inflammatory chemokines
10 involved in monocyte and macrophage recruitment, through a MAPK dependent
11 pathway [77].

12 *LSECs promotes liver inflammation at more advanced stages of NASH*

13 As mentioned above, LSECs alterations arise early in NAFLD progression, prior to liver
14 inflammation [18,41]. Indeed, LSECs capillarization precedes Kupffer cells activation
15 [18,41] and liver nitric oxide content falls before liver NF-kB activation and TNF α , IL6
16 and ICAM-1 up-regulation [30,40,41]. LSECs capillarization and dysfunction are
17 permissive for establishment of liver inflammation. Indeed, mice deficient in eNOS
18 exhibit an accelerated hepatic inflammatory response, while improving nitric
19 oxide/cGMP signaling with the phosphodiesterase-5 inhibitor sildenafil or with
20 simvastatin prevents liver inflammation in rodents fed a high-fat diet [40,60].

21 During NAFLD progression, LSECs then acquire pro-inflammatory phenotype and
22 functions (Figure 3).

1 LSECs pro-inflammatory phenotype during NASH is characterized by progressive
2 overexpression of adhesion molecules at LSECs surface including ICAM-1, VCAM-1 and
3 VAP-1, as observed in mouse models of NASH [68,78–82]. LSECs also produce a number
4 of pro-inflammatory mediators in NASH, including TNF α , IL-6, IL-1 and MCP1 [68,82–
5 84].

6 This pro-inflammatory phenotype of LSECs in NASH is associated with pro-
7 inflammatory functions (Figure 3). First, dysfunctional LSECs fail to maintain Kupffer
8 cells quiescence [40]. Second, the release of inflammatory mediators by LSECs
9 contributes to the inflammatory response by activating neighboring Kupffer cells, and by
10 favoring blood leukocytes recruitment, adhesion and transmigration [82,85,86].
11 Mechanisms of the interactions between leucocytes and LSECs in NASH have been
12 reviewed elsewhere in detail and are summarized in Figure 3 [80,87–89]. LSECs
13 expression of ICAM-1, VCAM-1 and VAP-1 is crucial for these interactions since *in vivo*
14 and *in vitro* studies showed reduced leukocytes adhesion to hepatic sinusoids when
15 these receptors are blocked or not functional [80,90]. Moreover, inhibition of the VCAM-
16 1 ligand, VLA-4, on monocytes using an anti-VLA-4 antibody inhibits adhesion and
17 transendothelial migration of monocytes across LSECs from wild-type mice fed a high-
18 fat diet fed mice and fom *ob/ob* obese mice, and improves liver inflammation [82].

19 Although stimuli responsible for LSECs inflammatory phenotype and functions in NASH
20 are not firmly identified, several mediators are potential candidates. This includes
21 products derived from the visceral adipose tissue, such as ox-LDL, FFA and adipokines.
22 Indeed, *in vitro* studies showed that stimulation of LSECs with ox-LDL and FFA
23 (palmitate) activates NF-kB and toll-like receptor-4 (TLR-4), respectively [21,44,91].
24 Moreover, circulating concentrations of several adipokines, including TNF α and IL6, are

1 increased in the portal vein in the context of metabolic syndrome, and may contribute to
2 LSECs inflammatory phenotype [92]. Gut microbiota also has an emerging role in NASH
3 as a source for liver inflammation [1,3]. Increased intestinal permeability and elevated
4 plasma concentrations of lipopolysaccharide (LPS) [93,94] observed in NASH may also
5 contribute to LSECs inflammation [83]. Besides mediators derived from the portal vein,
6 hepatocytes and liver inflammatory cells also release inflammatory mediators in NASH
7 that can activate LSECs [1,95].

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

11 Angiogenesis in liver inflammation in NASH

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

18 Several mechanisms trigger angiogenesis during NASH. First, chronic inflammation
19 promotes angiogenesis. Indeed, chronic inflammation sustains tissue hypoxia and
20 induces transcription of angiogenic genes modulated by HIF-1 α [61,99]. Pro-
21 inflammatory mediators also elicit a direct angiogenic response through the induction of
22 HIF-1 α transcriptional activity and VEGF production [61]. Moreover, cytokines and ROS
23 released during NASH can activate the MAPK/ERK pathway, a signaling pathway

1 involved in cell migration and angiogenesis [61]. Second, hepatocyte-derived
2 microvesicles link lipotoxicity with angiogenesis. Indeed, hepatocytes exposed *in vitro* to
3 excessive amounts of saturated FFA, that mimics steatosis, release microvesicles with a
4 pro-angiogenic activity [100]. Likewise, mice fed a methionine and choline deficient diet
5 have high circulating levels of hepatocyte-derived microvesicles able to induce
6 angiogenesis. Third, angiopoietin-2 is another mechanism of liver angiogenesis in NASH.
7 Angiopoietins are key regulators of angiogenesis. Although angiopoietins-1 and 2
8 contribute to vascular stability and quiescence in physiological conditions, angiopoietin-
9 2 promotes pathological angiogenesis in inflammatory conditions [101]. Lefere and
10 coworkers recently showed that serum angiopoietin-2 levels are increased in patients
11 with NASH and correlate with liver steatosis, inflammation and hepatocyte ballooning,
12 but not with liver fibrosis [68]. Similar findings were observed with two murine models
13 of NASH, namely mice fed a methionine and choline deficient diet and mice with
14 neonatal injection of streptozotocin followed by 16 weeks of western diet [68]. The main
15 source of hepatic angiopoietin-2 was LSECs [68]. Inhibiting angiopoietin-2 levels using
16 the angiopoietin-2/Tie2 receptor inhibiting peptibody L1-10 reduced hepatic
17 angiogenesis and normalized vascular microarchitecture [68].

18 In turn, angiogenesis promotes inflammation since various strategies of inhibition of
19 angiogenesis all improve liver inflammation (Figure 4). Coulon and colleagues showed
20 in a mouse model of NASH that treatment with anti-VEGFR2 antibody improves liver
21 vasculature and decreases liver inflammatory gene expression, both using preventive
22 and therapeutic approaches [63]. Lefere and colleagues showed that blocking
23 angiopoietin-2/Tie2 interaction with the L1-10 peptibody also alleviates liver injury and
24 inflammation in mice fed a methionine and choline deficient diet [68]. Importantly, this

1 effect of L1-10 therapy is at least partly mediated by an effect on LSECs since L1-10
2 treatment downregulates VCAM-1, ICAM-1 and MCP1 expression in liver endothelial
3 cells isolated from mice fed a methionine and choline deficient diet [68]. This anti-
4 inflammatory effect of anti-angiogenic treatment is not specific for NASH, as it is
5 observed in most models of chronic liver disease, namely carbon tetrachloride, and bile
6 duct ligation [102–106].

7 To summarize, inflammation stimulates angiogenesis that in turn worsens
8 inflammation, as attested by the anti-inflammatory effect of anti-angiogenic therapies
9 (Figure 4).

10 ***Key points:***

- 11 • *Lipotoxicity and inflammation induce endothelial inflammation. Activated LSECs*
12 *release cytokines and chemokines and overexpress adhesion molecules, thus*
13 *sustaining liver inflammation.*
- 14 • *Inflammation promotes angiogenesis that in turn worsens liver inflammation as*
15 *attested by the anti-inflammatory effect of anti-angiogenic therapies.*

16

17 **LSECs in NASH-related liver fibrosis**

18 Liver fibrosis is defined as the excessive deposition of extracellular matrix in liver
19 parenchyma. The main mechanism leading to liver fibrosis is a long-standing wound
20 healing process caused by hepatocellular injury and inflammation and mediated by
21 hepatic stellate cells activation [1,107]. Hepatic stellate cells are nonparenchymal cells
22 close to LSECs, in the space of Disse, which store retinoids in physiological conditions

1 and shift their phenotype to an activated myfibroblastic state during liver injury and
2 inflammation to secrete large amounts of extracellular matrix compounds [107]. As
3 detailed above, LSECs are major effectors of liver inflammation in NASH, and
4 consequently also promote hepatic fibrosis. For example, LSECs overexpress VAP-1
5 during inflammation which, in addition to its pro-inflammatory functions in NASH, is
6 directly involved in hepatic stellate cells activation [80]. Inhibition or deficiency in VAP-
7 1 in mice fed a methionine and choline deficient diet or a high-fat diet attenuates liver
8 fibrosis [80]. LSECs also contribute to liver fibrosis through capillarization and
9 endothelial dysfunction, as detailed below.

10 *LSECs capillarization promotes liver fibrosis*

11 Capillarization is observed in patients and animal models of NASH and precedes fibrosis
12 [18,41,108–112], but also promotes its development. Indeed, PLVAP deficient mice,
13 displaying a pronounced reduction in the number of LSECs *fenestrae*, spontaneously
14 develop perisinusoidal liver fibrosis [25].

15 Experiments performed using cultured LSECs and hepatic stellate cells highlighted an
16 important cross-talk between LSECs and hepatic stellate cells to regulate each other
17 phenotype. While healthy LSECs maintain hepatic stellate cells quiescent, capillarized
18 LSECs lose this ability [112,113] (Figure 5). A vicious cycle between LSECs
19 capillarization and hepatic stellate cells activation then occurs during the fibrotic
20 process.

21 In NASH, ballooned hepatocytes produce Hedgehog molecules [114]. Hedgehog pathway
22 regulates capillarization, as inhibition of Hedgehog signaling prevents capillarization
23 and partially reverts LSECs phenotype from dedifferentiated to their differentiated state
24 [115]. LSECs are thus Hedgehog-sensitive cells, but they are also Hedgehog producing

1 cells. Similarly, quiescent hepatic stellate cells are Hedgehog-sensitive cells, while
2 activated hepatic stellate cells become Hedgehog-producing cells able also to release
3 microvesicles loaded with Hedgehog signaling molecules that interact with LSECs
4 [116,117]. It is thus tempting to speculate that during NASH, Hedgehog ligands are
5 released by hepatocytes and LSECs, thus activating LSECs themselves, as well as
6 quiescent hepatic stellate cells, by autocrine and paracrine effects. Activated hepatic
7 stellate cells can then secrete Hedgehog molecules, promoting LSECs capillarization
8 which in turn favors hepatic stellate cells activation, promoting the fibrogenic process.

9 *LSECs dysfunction promotes liver fibrosis*

10 Endothelial dysfunction appears very early in the course of NAFLD and precedes fibrosis
11 in animal models of NASH [30,40,41]. Several lines of evidence suggest that liver
12 endothelial dysfunction contributes to liver fibrosis. First, in rats fed a high-fat diet,
13 simvastatin increases liver eNOS expression and ameliorates liver fibrosis [60]. Second,
14 eNOS inhibition using L-NAME blocks the ability of healthy LSECs to keep hepatic
15 stellate cells quiescent [118,119]. Third, an activator of soluble guanylate cyclase, a
16 receptor for nitric oxide, can recapitulate the effect of healthy LSECs and reverse hepatic
17 stellate cells activation [13]. A limitation of these studies is however that most
18 approaches might not only act on endothelial function but also on LSECs capillarization
19 [112,118]. Indeed, although L-NAME blocks LSECs-induced hepatic stellate cells
20 quiescence, addition of a nitric oxide donor does not directly reverse hepatic stellate
21 cells activation *in vitro* [13], suggesting that additional LSECs-derived factors could be
22 responsible for the reversion of activated hepatic stellate cells to quiescence.

1 To summarize, these data demonstrate that capillarization and LSECs dysfunction not
2 only precedes liver fibrosis, but are also permissive for it (Figure 5). LSECs are able to
3 maintain hepatic stellate cells quiescent as long LSECs are differentiated, so that
4 differentiated LSECs are gatekeepers for fibrosis in NASH, like in other chronic liver
5 diseases.

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

7 Another important process that links endothelial cells to organ fibrosis is endothelial-to-
8 mesenchymal transition, *i.e.* a mechanism by which endothelial cells convert into
9 myofibroblasts and contribute to extracellular matrix deposition [120,121]. Endothelial-
10 to-mesenchymal transition occurs in various fibrotic cardiovascular and pulmonary
11 diseases [120–122]. *In vitro* studies showed that healthy LSECs produce a modest
12 amount of collagen and fibronectin [123]. Capillarized LSECs secrete fibrogenic factors,
13 such as TGF- β 1, and extracellular matrix proteins, such as fibronectin and laminin, that
14 may be considered as an endothelial-to-mesenchymal transition, and also stimulates
15 activation of neighboring hepatic stellate cells [8,124,125]. *In vivo*, in the liver disease
16 field, only one study described endothelial-to-mesenchymal transition in patients with
17 alcohol or hepatitis C virus-related cirrhosis and in mice treated with carbon
18 tetrachloride [126]. This process might also happen during liver fibrosis in NASH, but
19 will deserve further studies.

20 *Angiogenesis in NASH-related liver fibrosis*

21 Liver angiogenesis correlates with liver fibrosis in patients with NASH [66,67]. The
22 mechanisms leading to liver angiogenesis in NASH-related fibrosis include those
23 mentioned above, namely tissue hypoxia [61], hepatocytes-derived microvesicles

1 [100,127] and angiopoietin-2 [68], but also leptin. Leptin concentrations are increased
2 in the serum of patients with NAFLD [128]. This adipocytokine has both pro-angiogenic
3 effects [129], and direct pro-fibrogenic effects, through the upregulation of TGF- β in
4 LSECs and Kupffer cells [130].

5 In turn, angiogenesis promotes liver fibrosis since several approaches inhibiting liver
6 angiogenesis prevents NASH-related fibrosis (Figure 4). First, in the study by Kitade and
7 colleagues, neither angiogenesis nor fibrosis was observed in the absence of leptin
8 signaling in a rat model of NASH [129]. Second, blocking the release of pro-angiogenic
9 microvesicles from fat laden-hepatocytes or inhibiting their binding to their target cells
10 protects mice from steatohepatitis-induced pathologic angiogenesis and results in a
11 reduction in liver fibrosis [100]. Third, Zhou and coworkers recently showed that a
12 specific deletion of the physiological inhibitor of angiogenesis, prolyl-hydroxylase-2, in
13 endothelial cells results in an overexpression of angiopoietin-2 and TGF- β 1 in the liver
14 and promotes dietary-induced liver fibrosis in mice [131,132]. Whether this pro-fibrotic
15 effect of endothelial prolyl-hydroxylase-2 deficiency is directly induced by promoting
16 angiogenesis remains to be demonstrated. Fourth, two studies reported that inhibiting
17 angiotensin-II receptor using telmisartan or candesartan inhibits liver angiogenesis and
18 fibrosis in rats fed a choline-deficient, L-amino acid-defined diet [96,97]. Finally, Lefere
19 and colleague demonstrated that blocking angiogenesis by inhibiting angiopoietin-
20 2/Tie2 interaction using the L1-10 peptibody improves liver fibrosis in preventive and
21 therapeutic strategies in mice fed a methionine and choline deficient diet. Therapeutic
22 application of L1-10 peptibody also prevents liver fibrosis in diabetic mice with NASH
23 (streptozotocin and western diet model) [68]. It should be noted that an anti-fibrotic

1 effect of anti-angiogenic treatments has also been observed in models of chronic liver
2 disease without NASH [102–106,133–139].

3 ***Key points:***

- 4 • *LSECs capillarization and dysfunction precede liver fibrosis and are permissive for*
5 *it, through the loss of the ability of LSECs to maintain quiescence of hepatic stellate*
6 *cells.*
- 7 • *Liver angiogenesis correlates with the severity of liver fibrosis and promotes its*
8 *development. Once established, fibrosis stimulates angiogenesis by increasing tissue*
9 *hypoxia. Blocking pathologic angiogenesis prevents liver fibrosis.*

10

11 **LSECs in NASH-induced hepatocellular carcinoma**

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

16 Patients with metabolic syndrome and NAFLD also develop HCC in the absence of
17 underlying cirrhosis, suggesting oncogenic pathways specific for NAFLD [5,6].

18 Adipokines and angiogenesis associated with NAFLD seem to account –at least partly–
19 for this specific link between NAFLD and HCC.

20 Circulating concentrations of the adipokine FABP4 are increased in patients with NAFLD
21 without HCC and correlate with liver inflammation and fibrosis [144]. Interestingly,

1 Laouirem and colleagues recently demonstrated that LSECs exposed to conditions
2 mimicking NAFLD -namely high concentrations of glucose, insulin, or VEGFA- release
3 FABP4. They also observed that FABP4 released by LSECs exerts pro-oncogenic effects,
4 since it induces hepatocyte proliferation. In mice fed a high-fat diet, specific inhibition of
5 FABP4 reduces HCC growth [145]. We can speculate that FABP4 from LSECs not only
6 contributes to HCC progression but also to HCC development in a NAFLD setting.

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

22 ***Key point:***

- 23 • *Adipokines contribute to HCC development in NAFLD.*

- 1 • *NAFLD associated angiogenesis promotes HCC. Blocking pathologic angiogenesis*
2 *prevents HCC development and progression.*

3

4 **Gaps in knowledge and future directions**

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

17

18 **Conclusion**

19 LSECs are gatekeepers of liver homeostasis in physiological conditions. In NAFLD,
20 sinusoidal endothelial alterations, including capillarization and LSECs dysfunction, occur
21 early in disease progression, at the stage of simple steatosis. These initial changes,
22 triggered by lipotoxicity, adipokines, and inflammation and gut microbiota-derived

1 products are associated with a loss of the ability of LSECs to prevent liver inflammation
2 and fibrosis associated with NASH. Indeed, altered LSECs fail to maintain Kupffer cells
3 and hepatic stellate cells quiescence. At the stage of NASH, altered LSECs contribute to
4 liver angiogenesis, inflammation, fibrosis and HCC. Improving LSECs health has high
5 therapeutic potential for NAFLD. Current challenge is the identification of strategies to
6 target specifically LSECs to modulate their activity.

7

8

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1 **Figure legend**

2 **Figure 1: LSECs capillarization promotes steatosis. In physiological conditions,**
3 chylomicron remnants cross LSECs *fenestrae* and provide cholesterol and triglycerides
4 for VLDL synthesis. VLDL are then released by hepatocytes and reach blood flow
5 through *fenestrae*. **In metabolic syndrome conditions,** LSECs capillarization arises
6 early in the course of NAFLD, possibly because of exposure of LSECs to dietary
7 macronutrients. In turn, LSECs capillarization promotes steatosis, possibly because
8 capillarization blocks the transfer of chylomicron remnants to hepatocytes, thus
9 stimulating endogenous cholesterol and triglycerides synthesis, as a compensatory
10 mechanism for the synthesis of VLDL, wich reach blood flow through the lymphatic
11 system. FFA, free fatty acids; LDL, low-density lipoprotein; LSECs, liver sinusoidal
12 endothelial cells; ox-LDL, oxidized low-density lipoprotein; VLDL; very low-density
13 lipoprotein.

14 **Figure 2: LSECs dysfunction promotes steatosis. In physiological conditions,** LSECs
15 release NO which regulates intrahepatic vascular tone on the one hand, and hepatic lipid
16 metabolism on the other hand. NO limits hepatic lipid content by inhibiting hepatic *de*
17 *novo* lipogenesis, through a limitation of citrate synthesis in mitochondria, an inhibition
18 of ACC and of GPAT, and by promoting fatty acids beta-oxidation. **In metabolic**
19 **syndrome conditions,** overabundance of lipids and insulin resistance lead to
20 downregulation of eNOS activity and to upregulation of iNOS and of NOX1 (a nitric oxide
21 consuming enzyme), causing nitrooxidative stress through peroxynitrites production
22 and eventually endothelial dysfunction. Reduced NO availability promotes steatosis.
23 Liver steatosis is associated with an increased intrahepatic vascular resistance which
24 has a mechanical component, due to the compression of the sinusoidal lumen by

1 enlarged fat-laden hepatocytes, and a dynamic component, due to a liver endothelial
2 dysfunction. ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; eNOS;
3 endothelial nitric oxide synthase; GPAT, glycerol-3-phosphate acyltransferase; LSECs,
4 liver sinusoidal endothelial cells; IHRV; intrahepatic vascular resistance; iNOS, inducible
5 nitric oxide synthase; NO, nitric oxide; NOX1, NADPH oxidase 1.

6 **Figure 3: LSECs acquire pro-inflammatory functions in NASH.** Lipotoxicity,
7 inflammation and gut microbiota-derived products induce LSECs inflammatory
8 phenotype and function mediated by NFkB activation, which orchestrate the release of
9 pro-inflammatory mediators and the overexpression of adhesion molecules.
10 Inflammatory mediators and LSECs dysfunction promote Kupffer cells activation and
11 leukocytes chemoattraction. Adhesion molecules overexpression allows adhesion and
12 transendothelial migration of the recruited leukocytes in the hepatic parenchyma. ICAM-
13 1, intercellular adhesion molecule-1; LSECs, liver sinusoidal endothelial cells; NF-kB,
14 nuclear factor kappa B; NO, nitric oxide; VAP-1, vascular adhesion protein-1; VCAM-1,
15 vascular cell adhesion molecule-1.

16 **Figure 4: Angiogenesis in NASH.** Hypoxia, liver injury, lipids, oxidative stress,
17 inflammation and fibrosis induce the release of pro-angiogenic factors, such as
18 hepatocyte-derived microvesicles, VEGF and Angiopoietin-2, from parenchymal and
19 non-parenchymal cells including LSECs, therefore promoting pathologic angiogenesis.
20 Adipokines, such as leptin, also exhibits pro-angiogenic activity contributing to
21 pathologic angiogenesis in NASH. In turn, angiogenesis promotes liver inflammation and
22 fibrosis as attested by anti-angiogenic therapies which prevent liver inflammation and
23 fibrosis in experimental models of NASH. VEGF, vascular endothelial growth factor;
24 VEGFR2, vascular endothelial growth factor receptor2.

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

10 **Figure 6: Role of liver endothelial cells in hepatocellular carcinoma (HCC)**
11 **development in chronic liver diseases (not specific for NAFLD).** During HCC
12 progression, endothelial cells sequentially lose their specific markers including stabilin-
13 1, stabilin-2, LYVE-1 and CD32b (SE-1). Conversely, endothelial expression of integrins
14 increases, facilitating adhesion of liver cancer cells. In parallel, endothelial expression of
15 ICAM-1 decreases, leading to a lower ability of leukocyte to adhere and infiltrate
16 HCC. Endothelial cells within HCC can also alter tumor-associated immune responses *via*
17 their ability to confer T cell tolerance towards cancer-associated antigens and to create
18 an immunosuppressive environment.

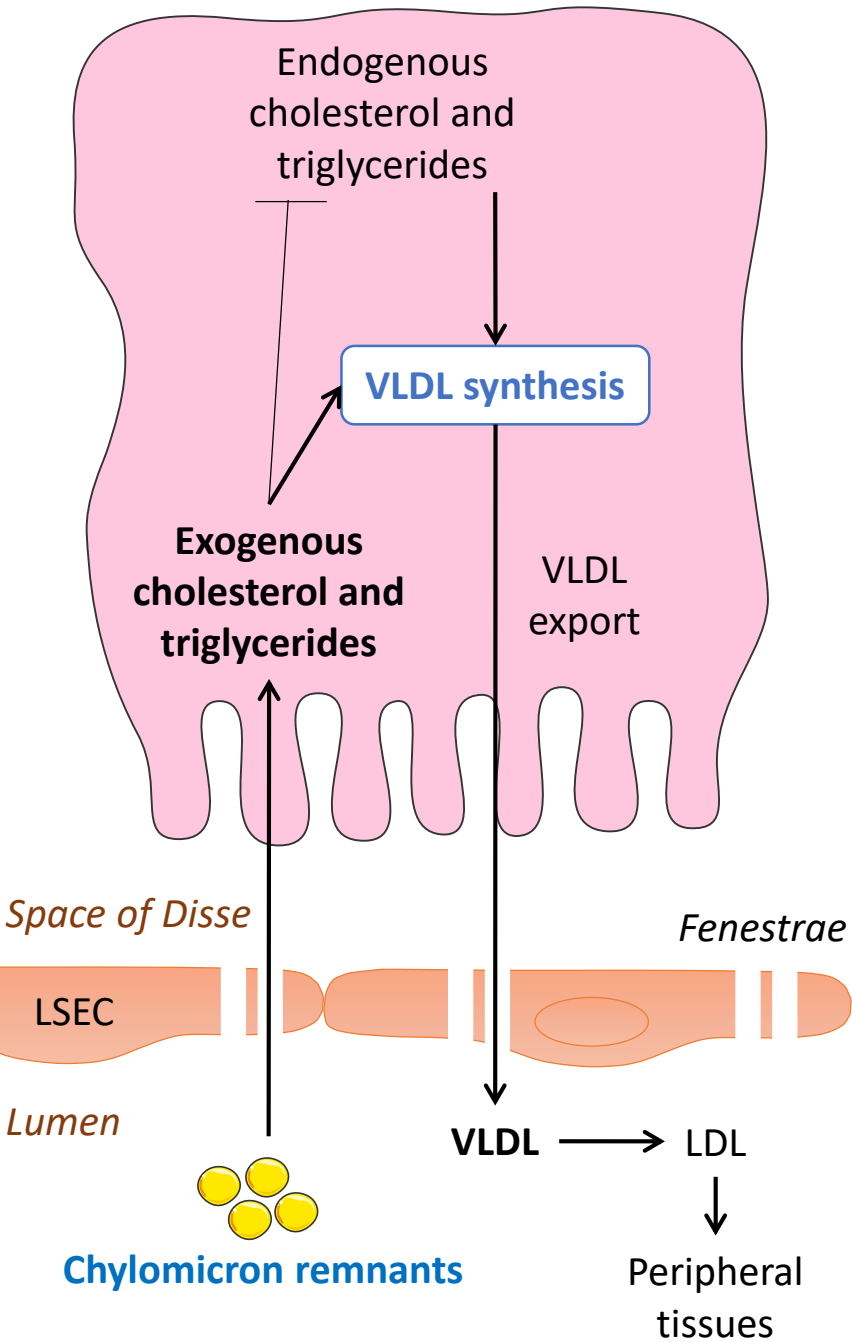
19 **Figure 7: Role of liver endothelial cells in hepatocellular carcinoma development**
20 **in the NAFLD setting.** Circulating concentrations of angiocrine factors, such as VEGF
21 and angiopoietin-2, and adipokines, such as leptin are increased in NAFLD. These
22 mediators induce angiogenesis which promotes HCC growth. The adipokine FABP4 is
23 released by adipose tissue and endothelial cells and contributes to HCC development
24 and progression.

1 **BOX: Liver sinusoidal endothelial cell (LSEC) changes are not specific for certain**
2 **animal models of NASH**

3 LSECs alterations are observed in various dietary or genetic models of NAFLD, without
4 apparent specificity. Indeed, LSECs capillarization is observed in animals fed a choline-
5 deficient, l-amino acid-defined diet or a high-fat diet [18-20]. LSECs dysfunction can be
6 induced by a high-fat diet, or a rich saturated fatty acids diet, or a high-fat high-glucose
7 and high-fructose diet [40,41,43]. Markers of LSECs inflammatory phenotype are
8 observed in animals fed a high-fat diet or a methionine and choline deficient diet
9 [68,82]. Markers of angiogenesis appear after methionine and choline deficient diet in
10 db/db obese mice and in C57BL/6 mice and in rats fed a choline-deficient, l-amino acid-
11 defined diet [63,129].

Figure 1

Physiological conditions



Metabolic syndrome

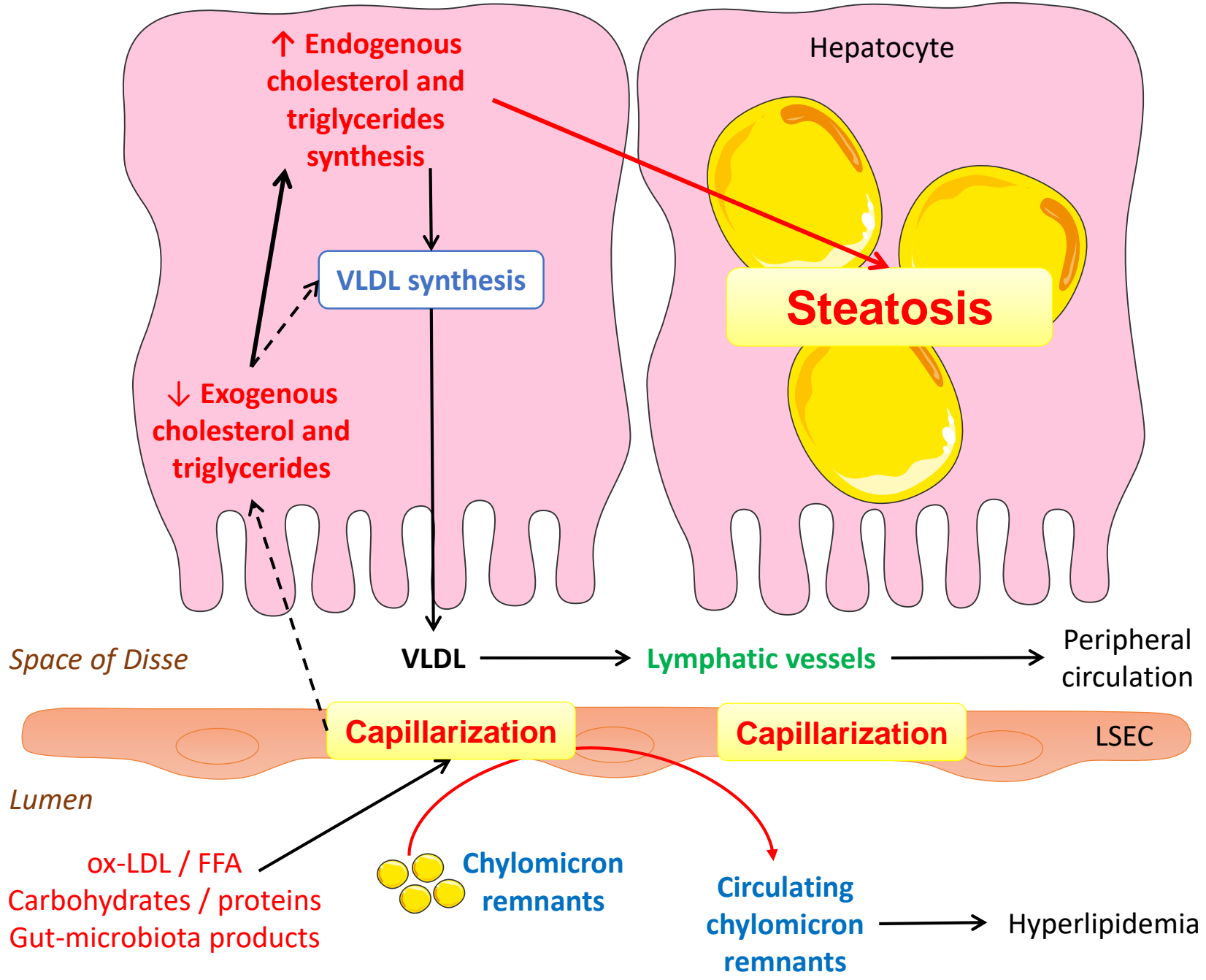


Figure 3

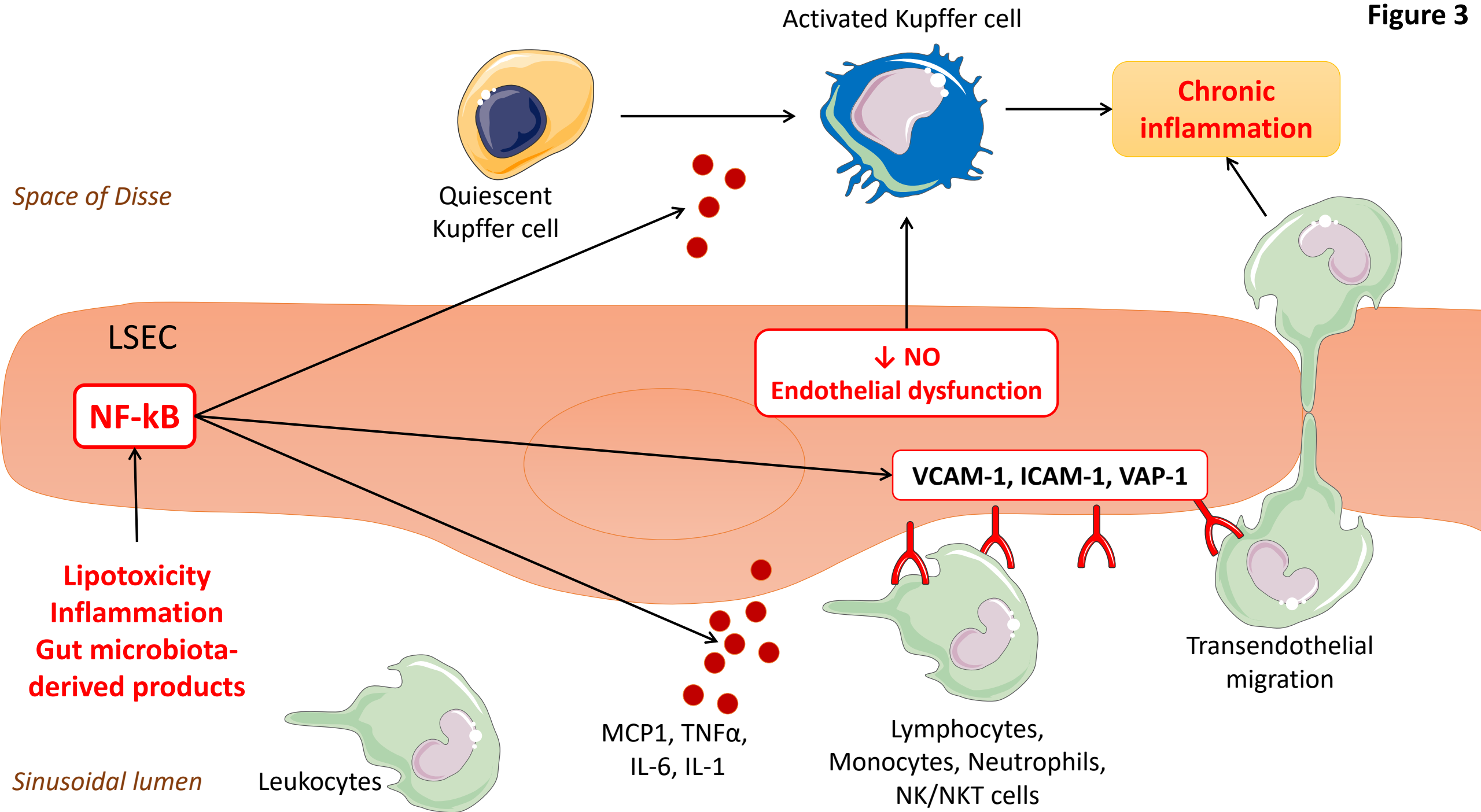


Figure 4

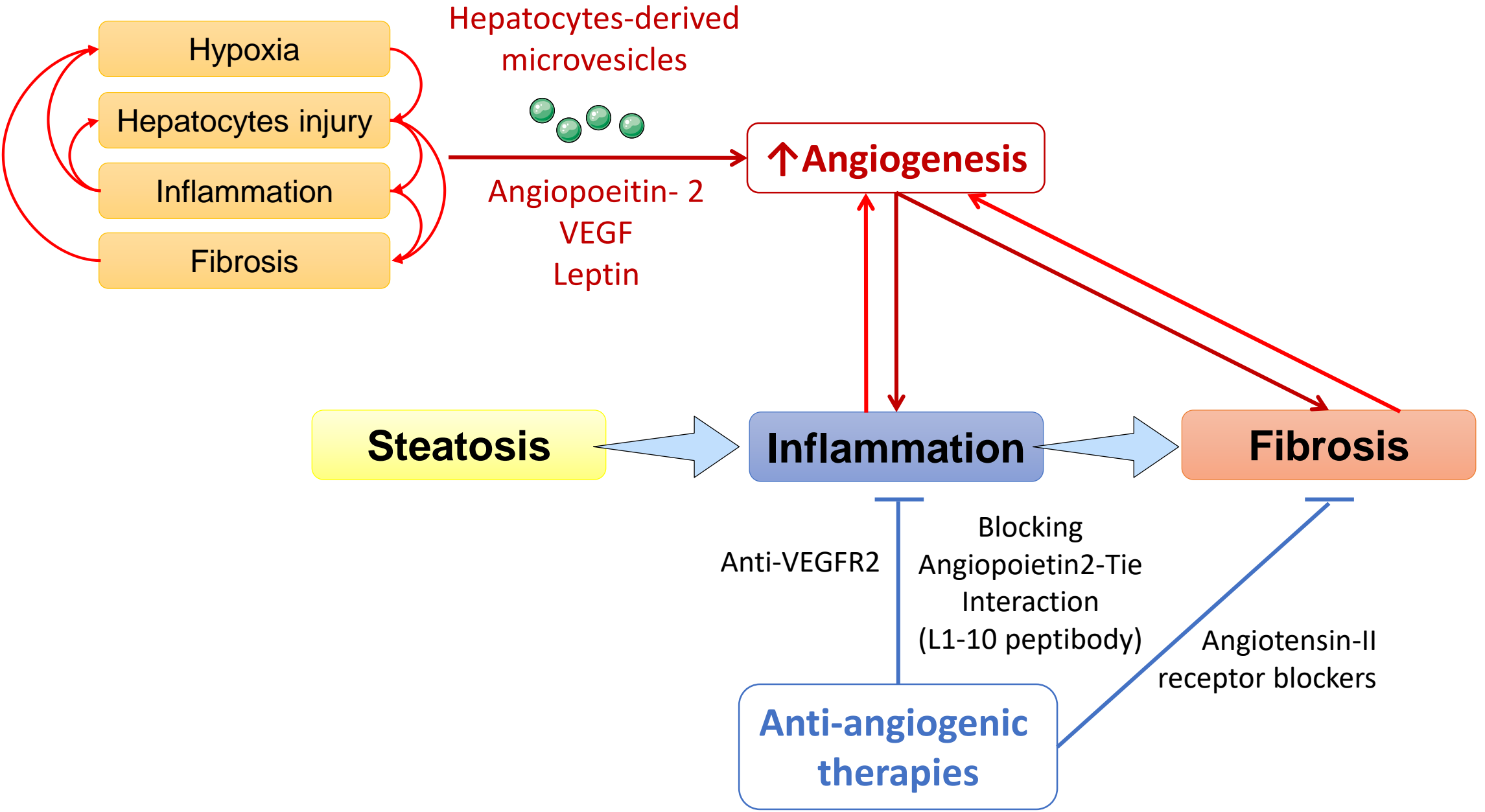
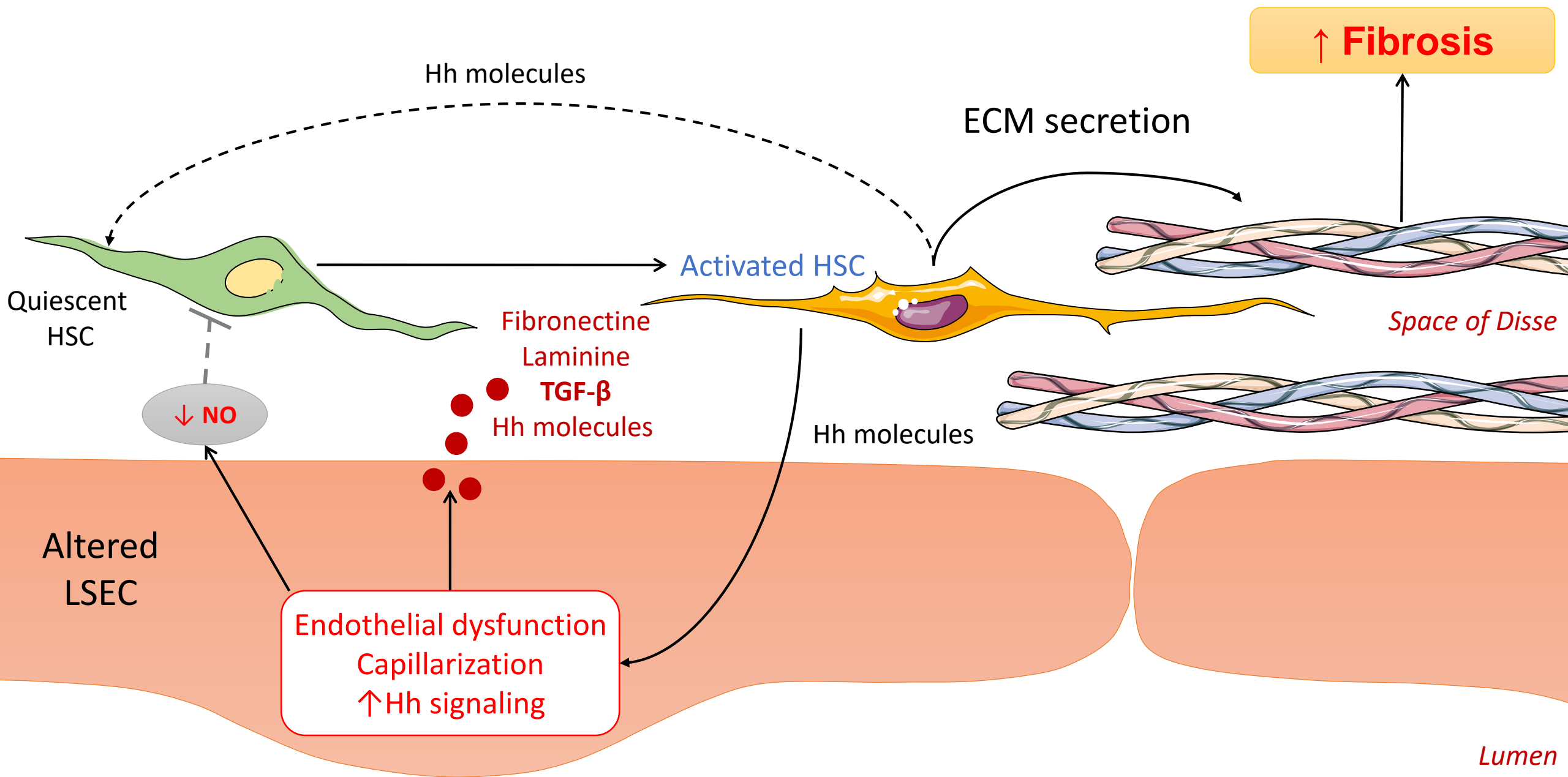


Figure 5



Tumor progression

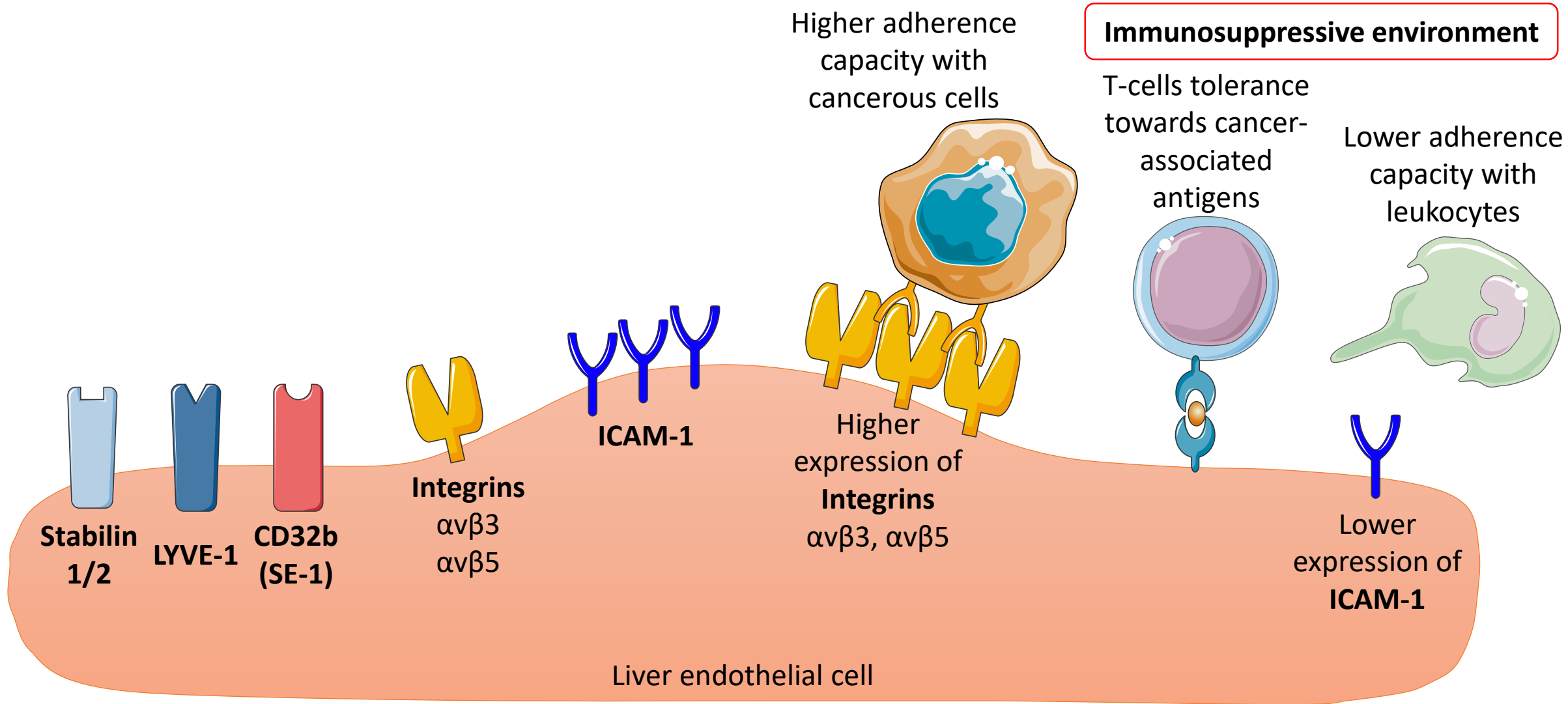
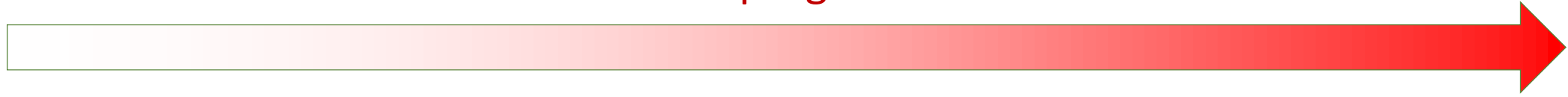


Figure 7

