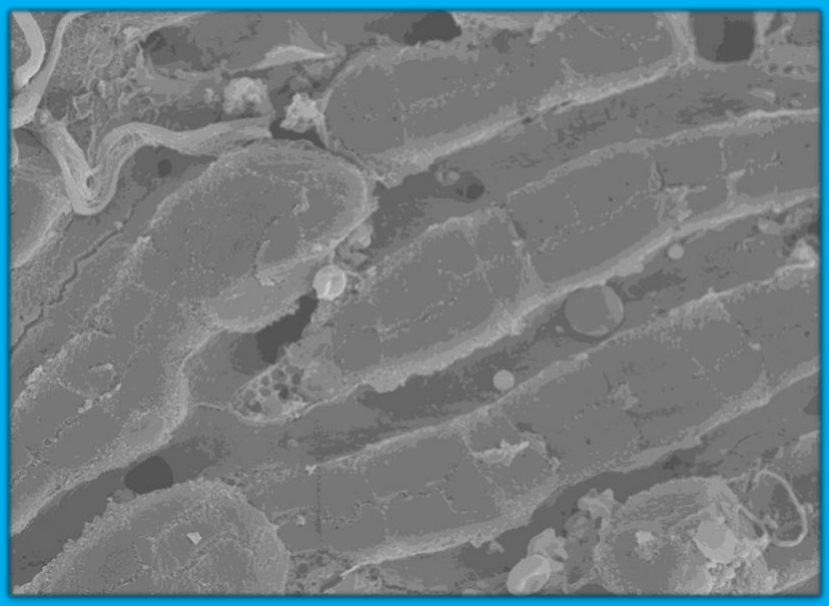
UNIVERSITAT DE BARCELONA

The hepatic sinusoid in aging: phenotypic changes and novel therapeutic approaches

Raquel Maeso-Diaz

THE HEPATIC SINUSOID IN AGING: PHENOTYPIC CHANGES AND NOVEL THERAPEUTIC APPROACHES



Doctoral Thesis presented by
Raquel Maeso Díaz

UNIVERSITAT DE BARCELONA

THE HEPATIC SINUSOID IN AGING: PHENOTYPIC CHANGES AND NOVEL THERAPEUTIC APPROACHES.

Ph.D. thesis presented by

RAQUEL MAESO-DIAZ

For the degree of Doctor in Medicine

Work performed under the supervision of **Dr. Jordi Gracia-Sancho** and **Prof. Jaime Bosch Genover**, in the laboratory of Liver Vascular Biology Research group, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Department of Medicine, Hospital Clínic of Barcelona – CEK, Barcelona.

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Ph.D. Program of Medicine and Translational Medicine
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Para mi familia:

Mis hermanos Sara y Luis

Papa y Mama

Alicia miró a su alrededor con sorpresa.

Alicia, jadeante:
"Reina Roja, ¿cómo es posible? ¡Si parece que hemos estado bajo este árbol todo el tiempo! ¡Todo está igual que antes!"

Reina Roja:
"¡Pues claro Alicia! ¿Qué esperabas?"

Alicia, jadeando aún:
"Bueno, es que en mi país... cuando se corre tan rápido como lo hemos estado haciendo y durante algún tiempo, se suele llegar a alguna otra parte..."

Reina Roja:
"¡Qué país más lento! Aquí, como ves, hace falta correr todo cuanto una pueda para permanecer en el mismo sitio. Si se quiere llegar a otra parte hay que correr por lo menos dos veces más rápido."

Lewis Carroll – Alicia a través del espejo

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Directors' report

Barcelona, 20th of November of 2019

Jordi Gracia Sancho, research group leader at a l'Institut D'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), and Jaume Bosch Genover, Professor in Medicine in the University of Barcelona and senior advisor in Hepatology at Hospital Clínic de Barcelona,

CERTIFY:

The doctoral thesis entitled "**THE HEPATIC SINUSOID IN AGING: PHENOTYPIC CHANGES AND NOVEL THERAPEUTIC APPROACHES**", presented by Raquel Maeso Díaz to obtain the title of Doctor by the University of Barcelona has been done under our supervision and accomplishes all the requisites to be defended in front of the corresponding evaluating committee.

Jordi Gracia Sancho

Jaume Bosch Genover

Acknowledgments

Acknowledgments

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Aquest llarg període de la vida ha estat molt important per mi tant a nivell formatiu com personal per tots i cada un dels seus moments. Sens dubte, una de les coses més especials d'aquest llarg viatge son les persones que he anat coneixent. Sempre he tingut la millor companyia al meu costat i per això crec es mereixen una especial menció:

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Gràcies a la PhD Community i tots els seus membres pels moments tan genials que em passat junts, fent ciència d'una manera tan diferent i alhora tan divertida.

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Abbreviations

Abbreviations

LSEC	Liver sinusoidal endothelial cells
HSC	Hepatic stellate cells
KC	Kupffer cells
PGI2	Prostaglandin I2
PGE2	Prostaglandin E2
TXA2	Thromboxane A2
ET1	Endothelin 1
NO	Nitric oxide
AA	Arachidonic acid
COX	Cyclooxygenase
LOX	Lipooxygenase
CYPP450	Cytochrome P450
eNOS	Endothelial nitric oxide synthase
BH4	Tetrahydrobiopterin
ADMA	Asymmetric dimethyl arginine
sGC	Soluble guanylate cyclase
GTP	Guanosine triphosphate
cGMP	Cyclic guanosine monophosphate
PKC	Protein kinase C
IL-6	Interleukin 6
ICAM-1	Intercellular adhesion molecule 1
VCAM-1	Vascular cell adhesion molecule 1
LPS	Lipopolysaccharides
LDL	Low density lipoprotein
Bmp-2	Bone morphogenic protein 2
HGF	Hepatocyte growth factor

ECM	Extracellular matrix
CRBP-1	Cellular retinol binding protein 1
CRBP-2	Cellular retinol binding protein 2
MMP-2	Metalloproteinase 2
MMP-9	Metalloproteinase 9
TIMP-1	Tissue inhibitors of metalloproteinase 1
TIMP-2	Tissue inhibitors of metalloproteinase 2
TGF-α	Transforming growth factor alpha
EGF	Epidermal growth factor
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
TGF- β	Transforming growth factor beta
IL-8	Interleukin 8
M-CSF	Macrophage colony stimulating factor
MCP-1	Monocyte chemotactic peptide 1
CCL21	Chemokine ligand 21
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
CCR5	Chemokine receptor 5
AT2	Angiotensin II
cAMP	Cyclic adenosine monophosphate
TNF-α	Transforming nuclear factor α
iNOS	Inducible nitric oxide synthase
IL-10	Interleukin 10
IL-1R	Interleukin 1 receptor
HVPG	hepatic venous pressure gradient
aCLD	Advanced chronic liver disease
CHC	Chronic Hepatitis C
CHB	Chronic Hepatitis B

ALD	Alcoholic liver disease
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
Q	Flow
R	Resistance
ΔP	Portal pressure gradient
n	Viscosity
L	Length of the vessel
r	Radius
VEGF	Vascular endothelial growth factor
IHVR	Intrahepatic vascular resistance
ROS	Reactive oxygen species
NFκB	Nuclear factor kappa light chain enhancer of activated B cells
Hh	Hedgehog
KLF2	Krüppel like factor 2
CXCR7- Id1	CXC chemokine receptor type 7 – DNA binding protein inhibitor
CXCR4	CXC chemokine receptor type 4
Bmp-9	Bone morphogenic protein 9
α-SMA	Alpha smooth muscle actin
5-HT	5-hidroxytryptamine
FXR	Farnesoid X receptor
PPAR	Peroxisome proliferator activated receptor
Cys-LT	Cysteinyl leukotriene
Ly-6C	Lymphocyte antigen 6 complex
SVR	Systemic vascular resistance
C/EBP	CCAAT/enhancer binding protein
GSK3β	Glycogen synthase kinase 3 beta
HDAC1	Histone deacetylase 1

SIRT1	Sirtuin1
vWF	Von Willebrand factor
TIPS	Transjugular intrahepatic portosystemic shunt
HMG-CoA	Hydroxylmethylglutaryl coenzyme A
FPP	Farnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate

Introduction

Introduction

The liver is the most resilient vital organ in adults, as demonstrated by its unique regenerative capacity. As the largest gland in the body, the liver is vital for the maintenance and protection of the organism (Arias et al. 2009). It executes unique metabolic processes, toxicants clearance, regulation of inflammation, and molecule biosynthesis. All these vital functions are accomplished by its distinctive cell types that efficiently coordinate and communicate together within the hepatic microcirculatory system.

The elementary unit of the liver is the lobule, which consists in linear cords of hepatocytes spreading out from a portal triad, composed of hepatic artery, portal vein and bile duct, to a central vein (Figure 1). The nutrients and oxygen enter the liver through a dual blood afflux, 60-70% of portal venous blood (enriched in nutrients) and 30-40% of hepatic arterial (mainly carrying the oxygen), which then mixes and flows together through a network of enlarged microvessels called liver sinusoids. The central vein is connected with the hepatic vein so that blood goes out from the liver (Marrone et al. 2016). Finally, bile flows within the canaliculus that lies between the apical surfaces of the hepatocyte towards the bile ducts and gallbladder.

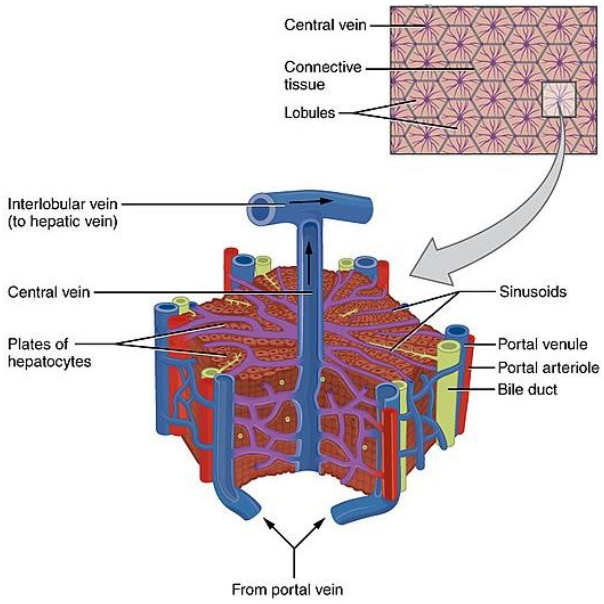


Figure 1. Microscopic anatomy of liver. Represent in this image we can observe the characteristic lobular structure of the liver. Image adapted from Anatomy & Physiology, Connexions Web site.

Blood vessels comprehend about 22% of the liver's mass/volume and the liver contains about 12% of the total blood volume under physiological conditions (RW. Brauer 1963). Therefore, the liver is considered a blood reservoir conferring its characteristic reddish color.

I. Microcirculation and the hepatic sinusoid

The hepatocytes are the main cell type of the liver contributing to its unique metabolic and synthetic capacities. Their function deeply depends on an efficient exchange of substances with the blood stream and a proper communication with other hepatic cells (Vollmar & Menger 2009). The segment of the microcirculation in which the exchange of substances occurs is the hepatic sinusoid, a special capillary bed consisting of a layer of liver sinusoidal endothelial cells (LSEC), surrounded by fat- and vitamin A-storing perisinusoidal cells, termed hepatic stellate cells (HSC), and flanked by plates of hepatocytes (parenchymal cells). Other cells lying within the sinusoids are immune cells including resident macrophages called Kupffer cells (KC), which are anchored to the luminal side of the endothelium and, thus, exposed to the bloodstream (Figure 2). Finally, the narrow space separating the sinusoidal membrane from the plasma membrane of the adjacent hepatocytes is called space of Disse (Gracia-Sancho et al. 2018). In the next pages we will focus on depicting the role of the main cells of the hepatic sinusoid and understanding why is important to protect the healthy architecture of this complex microcirculatory environment.

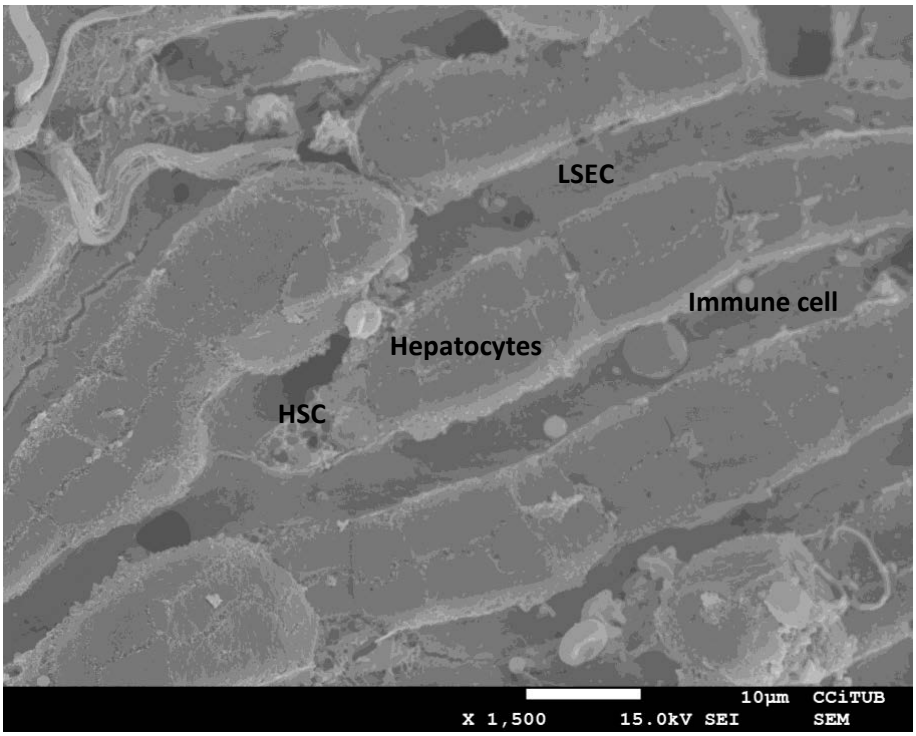


Figure 2. The hepatic sinusoids. Representative scanning electron microscopy image of the hepatic sinusoid from a healthy rat (1500X). We can observe cords of hepatocytes lining in close contact with LSEC and HSC. Immune cells are circulating or attached inside the microcirculatory vessels. This image represents the dynamic and animated environment within the sinusoids.

Note: All electron microscope images shown along this thesis have been made by Raquel Maeso Díaz and the Liver Vascular Biology Research Group.

I.1. Hepatocytes

The hepatocyte is the parenchymal and major epithelial cell of the liver (Figure 3). These cells are involved in many functions including protein synthesis (albumin, fibrinogen, clot factors and lipoproteins), carbohydrate, lipid and protein metabolisms and detoxification. Hepatocytes are polygonal cells that arranged together in unique cords. Their surface is asymmetric or polarized, which allows them to perform specialized functions in two different environments. While the basal domain is facing at least two blood sinusoids, the apical surface is exposed to the bile canaliculus (Treyer & Müsch 2013).

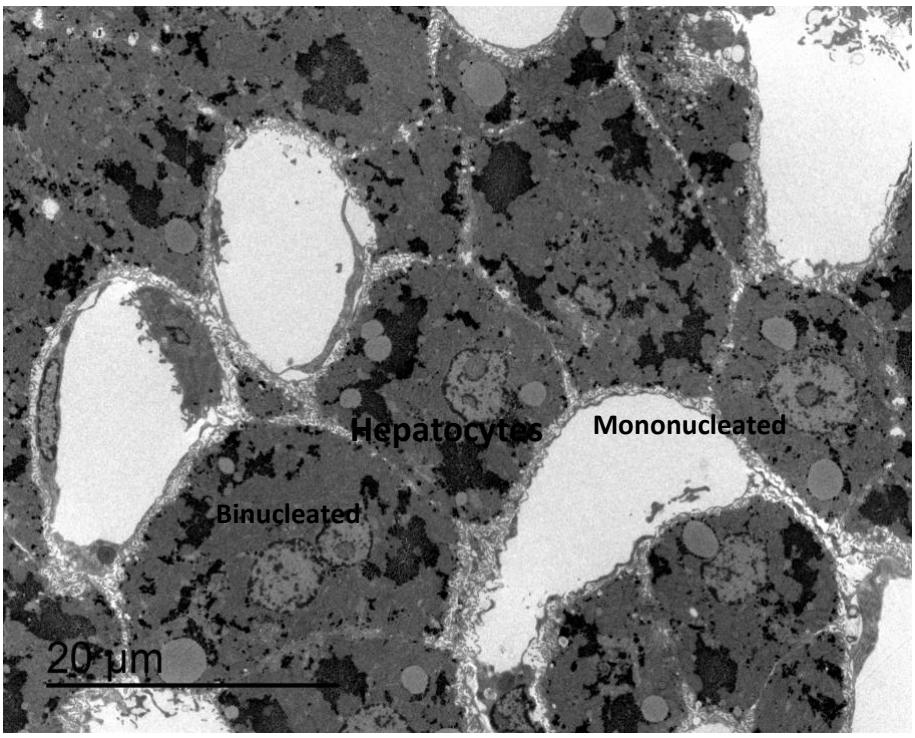


Figure 3. Hepatocytes in the liver sinusoid. Representative transmission electron microscopy image of the liver sinusoid from a healthy rat (8000X). We can observe the polygonal shape of the hepatocytes and their wide diversity in size, organelles and nucleus content. Polyploidy is very frequent in hepatocytes, which depends on the increase in the number of nuclei per cell plus the DNA content of each nucleus.

Hepatocytes not only differ in their functions within the same cell (polarity), they are a heterogeneous population along the liver with a specific metabolic patter (zonation). Hepatocytes are exposed to different gradients of oxygen, nutrients, hormones and metabolites along the liver, which causes the heterogeneity and zonation of the parenchymal cells of the liver (Kietzmann 2017). The zone 1 consists in hepatocytes located in the periportal region surrounded by the portal triad (portal vein, hepatic artery and bile duct). In zone 2 hepatocytes are positioned in the midlobular region, while in zone 3 they are situated near the efferent centrilobular vein. The consequence of this zonation is that hepatocytes present different gene expression pattern within these three zones, which determines their functionality (Birchmeier 2016). For example, hepatocytes in zone 1 are specialized in ammonia detoxification and glucose/energy metabolism (Torre et al. 2011). The Wnt/β-catenin pathway has been proposed to play a key role in the maintenance of zonation in the adult liver, which is modulated by the angiocrine signaling secreted from the liver endothelium (Benhamouche et al. 2006; Burke et al. 2009; Leibing et al. 2018).

I.2. Liver sinusoidal endothelial cells

Although someone could think that endothelial cells have only a passive role acting as a pipe where the blood can circulate in, it is quite untrue. These cells are very active and have a unique morphology and function depending on the needs of the tissue where they are located. The endothelium of capillaries may be classified as continuous or discontinuous. Continuous, the cells provide an uninterrupted lining, is the most abundant endothelium in the capillary tree. Discontinuous endothelium contains pores, fully opened or not, known as *fenestrae* and is characteristic of organs involved in filtration or secretion, such as glands, gastric and intestinal mucosa and some renal areas. The discontinuous endothelium termed as sinusoidal can only be found in certain vascular beds in the liver, bone marrow and spleen (Aird 2012). However, the morphology of these three types of sinusoidal endothelial cells is very different.

LSECs are the discontinuous endothelial cells forming the vascular bed of the liver sinusoids. LSECs are the only endothelial cells in mammals with open fenestrae and lack of organized basement membrane (Wisse 1970). Thus, the liver microcirculation has the most porous of all endothelial barriers. The cytoplasmic extensions of LSECs are very thin and perforated with circular and oval fenestrations of approximately 50–200 nm diameter covering 2-20% of the endothelial surface (Figure 4). Prof Eddie Wisse, known as the father of LSEC, cultured these cells for the first time and observed them under an electronic microscope. This experiment allowed him to discover the presence of fenestrations. In this relevant publication from 1970, he identified also

that these fenestrations are organized in clusters termed sieve plates, dynamic structures that can be altered by numerous endogenous and exogenous agents.

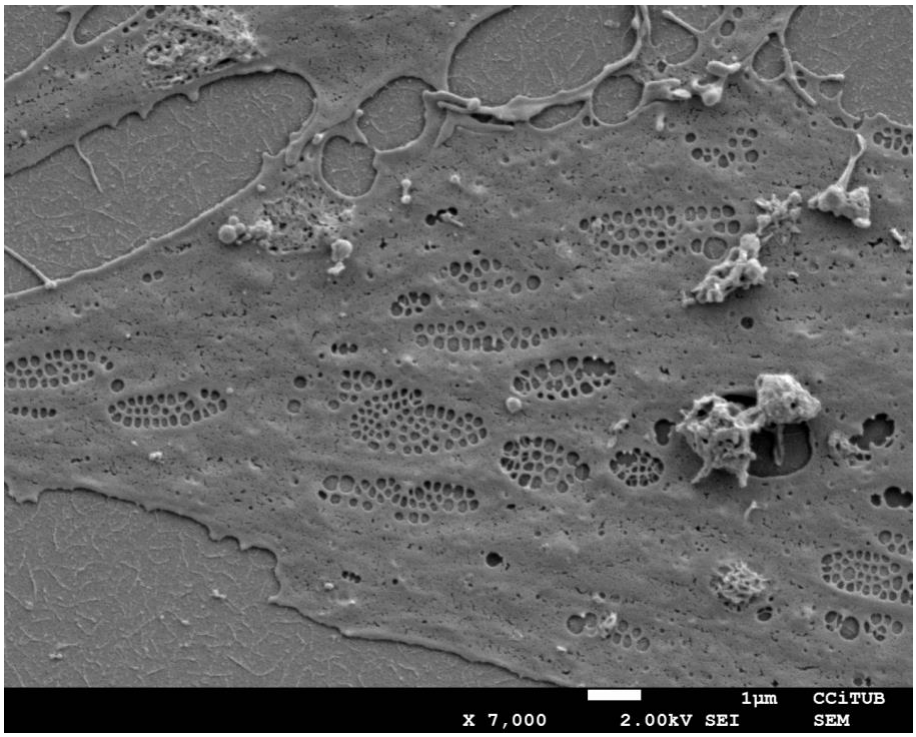


Figure 4. Liver sinusoidal endothelial cells. Representative scanning electron microscopy image of a liver sinusoidal endothelial cell isolated from a healthy rat (7000X). Fenestrations are open pores covering 2-20% of the cell area, they organized in clusters also known as sieve plates.

In addition to differences in structure, endothelial cells of the organism show remarkable heterogeneity in function. LSECs are known to contribute importantly in the regulation of the vascular tone. They also play a role in the inflammatory response and have a higher endocytic capacity compared to other endothelial cells. Let's see next very briefly the main functions of LSEC:

LSECs and vascular tone regulation: Although it has been widely demonstrated that stellate cells are the major cells regulating the hepatic blood flow because they are narrowing the sinusoidal lumen and limiting blood flow. However, *in vivo* microscopy have probed that LSECs also possessed certain contractility capacity in response to vasoactive substances (McCuskey & Reilly 1993). LSECs are more known by their indirect system of regulating the vascular tone by producing several vasoactive substances, including prostaglandin I 2 (PGI_2) and E 2 (PGE_2), thromboxane A 2 (TXA_2), as well as endothelin 1 (ET1) and nitric oxide (NO) (Wisse et al. 1996). Arachidonic acid

(AA) can be metabolized through three different enzymes: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYPP450). Depending on which pathway chooses it can generate vasodilator molecules, for example PGI_2, or vasoconstrictor like TXA_2 (Sacerdoti et al. 2015). ET1 is another important vasoconstrictor molecule that can be synthesized by LSECs (Rockey et al. 1998). Finally, NO is the main vasodilatory molecule in the liver. Endothelial nitric oxide synthase (eNOS) is the enzyme responsible for the production of NO in LSECs. The activity of eNOS is modulated by different post-translational modifications, including phosphorylation of the enzyme at its activation sites, the levels of its co-factor tetrahydrobiopterin (BH_4), and interactions with inhibitor proteins like caveolin and asymmetric-dimethyl-arginine (ADMA) (Wiest et al. 1999; Shah et al. 2001; Laleman et al. 2005; Liu et al. 2005; Matei et al. 2006). NO can also be regulated by scavenging to form peroxynitrite, mechanism mainly depending on the oxidative stress levels (Gracia-Sancho et al. 2008). Once NO is produced by LSECs, it is released into the space of Disse and incorporated by HSC, activating the soluble guanylate cyclase (sGC), enzyme that transforms guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). This second messenger triggers the C protein kinase (PKC) cascade to change the calcium levels of the cell. Ultimately the HSC relax its pressure above LSEC promoting the vasorelaxation and the blood flow increases as consequence (Iwakiri & Kim 2015). It has been well studied during the last decades that an imbalance in the synthesis of these vasodilator/vasoconstrictor molecules is associated with the dysfunction of LSECs, thus promoting the progression of chronic liver diseases (Gracia-Sancho et al. 2015). We will focus on further discuss this topic in chapter II.2.2.2 Liver sinusoidal endothelial cells dysfunction.

LSECs and immunity: LSECs, in direct contact with the portal circulation (we must remember that it is blood coming from the intestines and therefore rich in bacterial products, environmental toxins, and food antigens), serve as the first line of defense against immune and inflammatory challenges. They can produce inflammatory chemokines and cytokines to recruit inflammatory cells from the systemic circulation and lymphoid organs (Gao et al. 2008). Interleukin 6 (IL-6) is one of the most important cytokines secreted by LSEC. IL-6 is a key driver not only of infection defense but also of liver regeneration and, it is a key regulator of important metabolic functions (Wuestefeld et al. 2003). LSECs can promote the recruitment of leukocytes into the liver by up-regulating the expression of the intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in the endothelial surface after an inflammatory stimulus occurs. Both ICAM-1 and VCAM-1 are constitutively expressed in LSECs (Oteiza et al. 2011; van Oosten et al. 1995). Finally, LSECs together with KC compose the hepatic reticulo-endothelial system, which is part of

the innate immune response and the major site of the organism where removal of circulating macromolecules and microorganisms takes place (Sørensen et al. 2015).

LSECs endocytic capacity: LSECs are responsible for removal of soluble macromolecular and colloidal waste (smaller than 100 nm) from the circulation, including lipopolysaccharides (LPS), intracellular macromolecules, modified serum proteins and microbial proteins. This is possible thanks to its high endocytic capacity through the expression of several families of endocytotic receptors including: stabilin-1, stabilin-2, scavenger receptors, mannose receptor, and FcγRIIb. This endocytic capacity is relevant for regulating the lipid homeostasis in the organism. As an example, chylomicrons are passively filtered through LSEC fenestrae (Wisse 1970), and mildly oxidized low density lipoprotein (LDL) are taken up through the stabilin-1 endocytosis receptor (Li et al. 2011).

LSECs and angiocrine signaling: recently has been described LSECs as a source of angiocrine molecules including bone morphogenic protein 2 (Bmp2), angiopoietin-2, Wnt2, Wnt9b and hepatocyte growth factor (HGF) (Ding et al. 2010). LSEC-derived angiocrine molecules play a key role in liver growth, maturation and iron homeostasis in mice (Canali et al. 2017; Koch et al. 2017; Leibing et al. 2018). Also, Wnts produced by LSECs have been proposed as an important regulator of liver tissue repair following liver injury (Zhao et al. 2019).

I.3. Hepatic stellate cells

HSC are vitamin A storing cells located in the space of Disse (Figure 5). They are surrounding LSECs and represent the pericytes of the liver sinusoids (Friedman 2008). Carl von Kupffer firstly described this star-shaped cell in 1876 as liver macrophages (Geerts 2001). More than 50 years later, in 1952 Toshio Ito differentiated them from KC, after finding that these perisinusoidal cells were storing fat droplets. In honor to his discovery HSC are also named Ito cells. Lately, Kenjiro Wake noticed that these lipid droplets, characteristic of stellate cells, were composed by vitamin A (retinoid) and that in fact, over 95% body's retinoid reserves are found in these cells (Wake 1971).

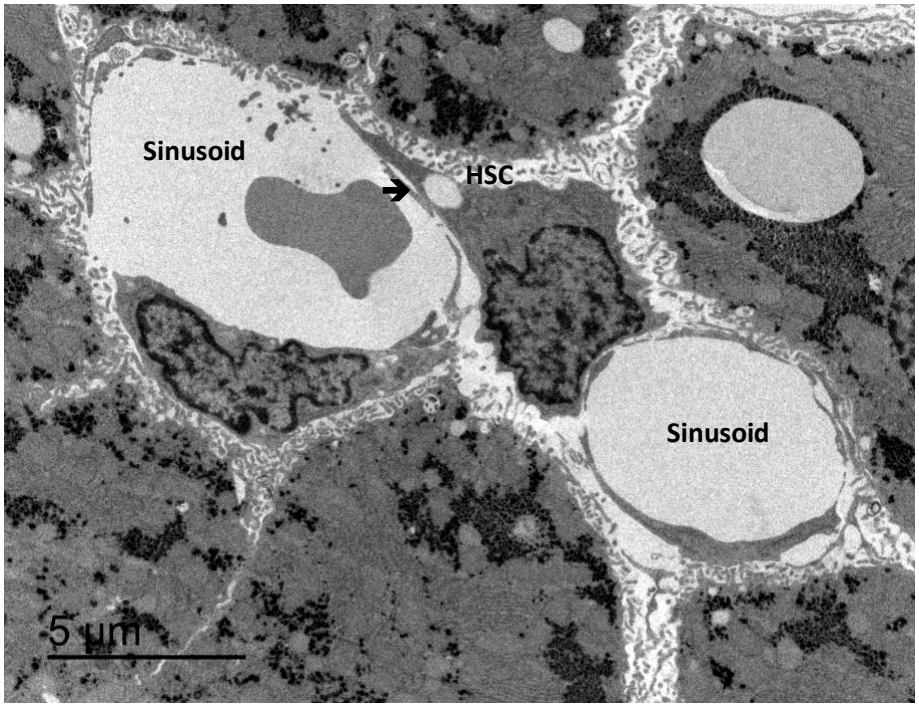


Figure 5. Hepatic stellate cell in the liver sinusoid. Representative transmission electron microscopy image of the liver sinusoid from a healthy rat (8000X). We can observe two open sinusoids and between them, inside the space of Disse, a HSC with lipid droplets in its cytoplasm. This HSC is surrounding, at least, 2 LSECs.

However, in the healthy liver, HSC are not only a storage housing for vitamin A. They play important roles in regulating the retinoid metabolism, they are the main collagen-synthetizing cells of the liver, as well as other extracellular matrix (ECM) components, and an important source of cytokines. Importantly, they are thought to be the most important cell responsible for controlling sinusoidal diameter and blood flow (Friedman et al. 1985). Here are summarized the most relevant functions of stellate cells:

HSC and retinoid metabolism. Retinols and their metabolites are essential for vision, the immune system and for the differentiation and growth of many cell types. Retinoid is absorbed and transformed to retinol in the intestines, packed inside chylomicrons and then transported to the liver (Blaner et al. 1985). These chylomicrons containing retinols are taken up by hepatocytes and then transferred to HSCs for storage. Only a small percentage remains in the hepatocytes, whereas quiescent HSCs store 80% of total liver retinols as retinyl palmitate in lipid droplets in their cytoplasm (Hendriks et al. 1985). The uptake, intracellular transport, and metabolism of retinol are regulated by cellular retinol-binding proteins (CRBP-1 and CRBP-2), which are highly expressed in HSC (Blomhoff 1990).

HSC synthetic capacity. HSC contribute to the synthesis of ECM components including fibrillar collagens, fibronectin and proteoglycans. Despite its relevance, they are not the only cells producing ECM; hepatocytes, LSECs and non-HSC myofibroblasts are also sources of ECM components (Chan & Wells 2007). The amount of ECM molecules synthesized by HSC varies depending on the hepatic microenvironment, for example when a hepatic insult occurs a fibrogenic stimulus activates and promotes a massive production of ECM components by HSC. In addition to matrix protein synthesis, HSC are also able to regulate matrix degradation by synthesizing matrix degrading metalloproteinase (MMP-2 and -9) and its inhibitors, tissue inhibitors of metalloproteinase (TIMP-1 and -2) (Iredale 1997; Takahara et al. 1995).

Stellate cells are an important source of cytokines in the liver. They are able to produce transforming growth factor α (TGF-α), epidermal growth factor (EGF) and HGF, well known hepatocyte mitogens with important roles during liver regeneration (Bachem et al. 1992; Mullhaupt et al. 1994). They also produce the most potent mitogen for themselves, the platelet-derived growth factor (PDGF), which is a dimeric subunit that activates its receptor, platelet-derived growth factor receptor (PDGFR) promoting HSC proliferation (Pinzani et al. 1995). Finally, stellate cells secrete transforming growth factor β (TGF-β) in response to liver injury, which exerts potent fibrogenic effects in both autocrine and paracrine manner (Bissell et al. 1995).

Moreover, stellate cells are mediators of the hepatic immune response. They can amplify the hepatic immune reaction by producing diverse cytokines like interleukin 8 (IL-8) and IL-6 and chemokines including macrophage colony stimulating factor (M-CSF), monocyte chemotactic peptide (MCP-1), chemokine ligand 21 (CCL21), regulated on activation normal T cell expressed and secreted (RANTES), and chemokine receptor 5 (CCR5) (Maher 2001). With all these inflammatory molecules HSC can attract and promote the infiltration of macrophages, neutrophils and other leukocytes in response to liver injury.

Vascular tone regulation. HSC together with sinusoidal endothelial cells have contractile properties and are the key regulators of the hepatic blood flow. While LSECs are well recognized by their indirect role secreting vasodilators and vasoconstrictors, HSC are the main effector cells with a contractile apparatus that respond to these stimuli. Two different types of filaments form the contractile apparatus of HSC: actin and myosin. The key for contraction is the shielding and un-shielding of the myosin-binding site on actin. Phosphorylation of the regulatory light chain subunit of myosin is sufficient for contraction. However, contraction can occur without light chain phosphorylation (Reynaert et al. 2002). There are several agents influencing HSC contractility;

ET1, thrombin and angiotensin II (AT2), which promote the contraction of HSC in a calcium-dependent manner with the implication of Rho kinase. Whereas NO and PGI_2 are promoters of the relaxation through the cGMP, cyclic adenosine monophosphate (cAMP) and Ca pathways (McCuskey 2008).

I.4. Kupffer cells

At last, KC the resident macrophages of the liver are located at the luminal side of the endothelial lining (Figure 6). In 1876, Karl Wilhem von Kupffer firstly described these cells, and thought that they were part of the endothelium. A century later, in 1989, Tadeusz Browiecz correctly identified them as macrophages (Naito et al. 1997; Ju & Tacke 2016). Nowadays, we know that KCs are the largest resident's macrophages in the organism and in fact, they are critical for the innate immune response. Together with LSECs, they constitute the reticulo-endothelial system mentioned before.

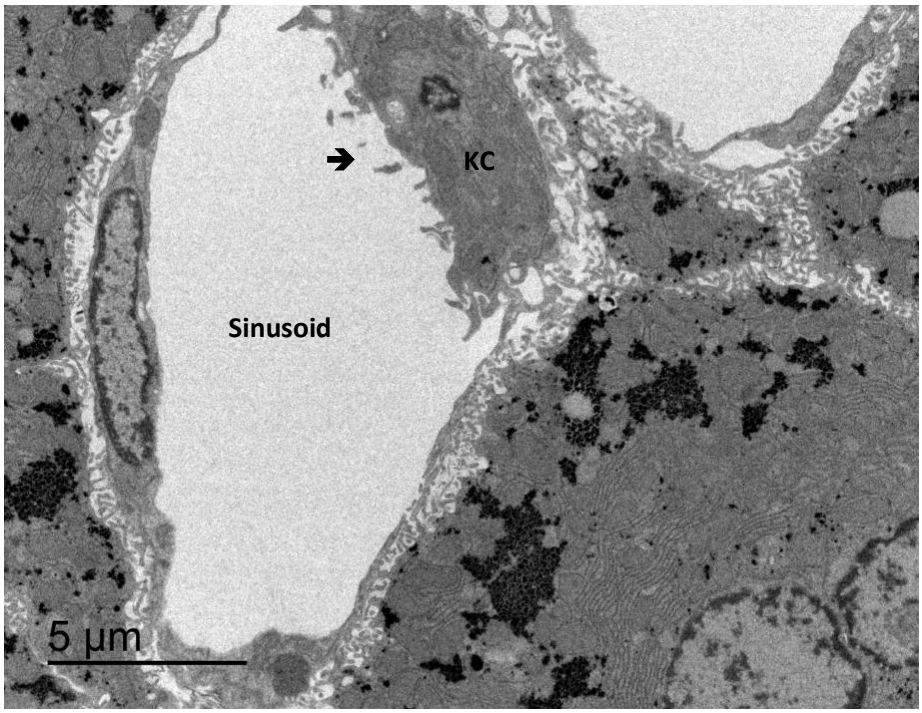


Figure 6. Kupffer cell in the liver sinusoid. Representative transmission electron microscopy image of the liver sinusoid from a healthy rat (8000X). We can observe a Kupffer cell attached to the sinusoid in the luminal side of the endothelium.

Their location, within the sinusoidal vascular space, allows them to exert efficiently their phagocytic functions of pathogens and immunoreactive material coming from the gastrointestinal tract. They prevent immunoreactive substances from portal or arterial circulation entering into the

hepatic sinusoids. Kupffer cells are also in charge of the clearance of particles, dying erythrocytes and apoptotic hepatocytes in the parenchyma. To exert this phagocytic function they need to pass through the space of Disse and establish direct contact with hepatocytes (Thomson & Knolle 2010).

KCs are very plastic cells that can change their phenotype and consequently their functional activity, depending on the metabolic and immune inputs they receive from the microenvironment. KCs are classically classified in based on their activation state or polarization (Gordon & Taylor 2005):

M1 or classically activated macrophages exhibit increased expression of proinflammatory cytokines like transforming nuclear factor α (TNF-α), IL-6 and inducible nitric oxide synthase (iNOS).

M2 or alternatively activated macrophages are characterized by predominantly expressed anti-inflammatory mediators including interleukin 10 (IL-10) and interleukin 1 receptor (IL-1R).

This classification simplifies grouping the macrophages into the most different functional categories but, exists data clearly demonstrating that macrophages are more heterogeneous, even within each of the groups (Heymann et al. 2012).

II. Cirrhosis and portal hypertension

Portal hypertension is a clinical syndrome defined by an elevation of the hepatic venous pressure gradient (HVPG) above 5 mmHg. This clinical complication results from obstruction of portal blood flow such as cirrhosis or portal vein thrombosis (Bosch 2007). In liver cirrhosis, increased intrahepatic vascular resistance to the portal blood flow is the primary factor elevating the portal pressure leading to portal hypertension (Gracia-Sancho et al. 2018). Once portal hypertension is established, it affects the extrahepatic vascular beds causing collateral vessel formation and splachnic vasodilation. This event leads to increase the portal blood inflow, which exacerbates portal hypertension and also, promotes the fully development of the hyperdynamic circulatory syndrome (Figure 7) (Iwakiri & Groszmann 2006).

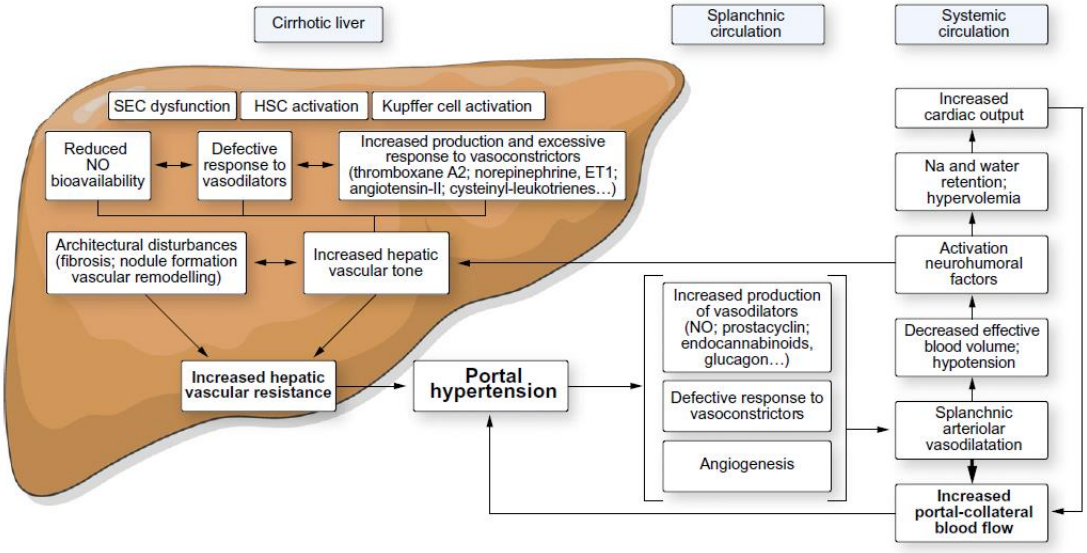


Figure 7. Pathophysiology of portal hypertension in cirrhosis. Image adapted from Journal of Hepatology 2012 vol. 57 j 458–46.

The etiology of portal hypertension is classified according to the location where the obstruction to portal blood flow occurs (Berzigotti et al. 2013):

1) Pre-hepatic. The affectation appears within the portal or splenic vein (i.e. portal vein thrombosis or congenital atresia).
2) Intra-hepatic. The problem is inner to the liver (i.e. liver cirrhosis, hepatic fibrosis, or non-cirrhotic causes such as schistosomiasis, massive fatty change and diffuse granulomatous diseases).

3) Post-hepatic. The impediment is usually in the heart, hepatic vein or inferior vena cava (i.e. hepatic vein thrombosis, inferior vena cava thrombosis, inferior vena cava congenital malformation, Budd-Chiari syndrome or constrictive pericarditis).

II.1. Epidemiology of chronic liver diseases (CLDs)

Chronic liver disease (CLD) encompasses all the chronic pathological conditions affecting the liver (inflammation, fibrosis, cirrhosis and hepatocellular carcinoma) that result from constant and long-term exposure to different hepatotoxic agents and cause clinical manifestations that last longer than 6 months. A significant percentage of patients with early CLD will develop liver cirrhosis and portal hypertension syndrome, characteristic of advanced CLD (aCLD). In fact, a report from 2015 showed that CLDs induce cirrhosis in 633.000 patients per year with a prevalence of 4.5% to 9% worldwide (Scaglione et al. 2015).

CLD represents a serious and costly problem for our society with a high prevalence and incidence. Approximately 844 million people suffer from a chronic liver condition worldwide, with a mortality rate of 2 million deaths per year, and this data may be underestimated due to the lack of information in some countries. The high impact of CLD make them comparable to other major public health problems related to chronic diseases such as cardiovascular diseases (540 million; 17,7 million deaths), diabetes (422 million; 1,6 million deaths) and pulmonary diseases (650 million; 6,17 million deaths) (Blachier et al. 2013).

The estimated worldwide prevalence of most frequent CLDs classified by its etiology is: 2,5% of chronic hepatitis C (CHC); 3,6% of chronic hepatitis B (CHB); 8,5% of alcoholic liver disease (ALD) and 25% of non alcoholic fatty liver disease (NAFLD) (Marcellin & Kutala 2018). This data suggests that nowadays, the distribution of CLD etiology is progressively changing, showing a decrease in the proportion of virus-induced CLDs with a significant increase in NAFLD and non alcoholic steatohepatitis (NASH). The recent development of the direct antivirals against hepatitis C virus explains the decrease in the prevalence of CLDs with viral etiology (Mariño et al. 2015). However, emergent metabolic CLDs, like NAFLD and NASH, are now predominant in Western and developed countries (17% to 46% for NAFLD). In fact, they are starting to be considered endemic in Europe and USA, representing an important economic burden for its countries (Younossi et al. 2018).

II.2. Pathophysiology of portal hypertension

According to the hydraulic equivalent of Ohm's Law portal pressure may be elevated by an increment in the flow (Q) or in vascular resistance (R), or a combination of both (Figure 8a). When translating this formula to the hepatic vascular system, ΔP is the portal pressure gradient, Q is the flow within the portal venous system, and R is the vascular resistance of the portal venous system, which represents the sum of the resistance of the portal vein, the hepatic vascular bed, and of the portosystemic collaterals. In liver cirrhosis, increased intrahepatic vascular resistance to the portal blood flow is the primary factor elevating the portal pressure, and is caused by both anatomical factors (fibrogenesis, angiogenesis and capillarization) and functional abnormalities (microcirculatory dysfunction, decrease in vasodilators, hyper-responsiveness to vasoconstrictors) (Gracia-Sancho et al. 2018). Once portal hypertension is developed, an increase in portal venous inflow, as a consequence of the hyperdynamic circulatory syndrome, will perpetuate and aggravate portal hypertension (García-Pagán et al. 2012).

Poiseuille's law is the formula to calculate the resistance that opposes to blood flow: $R = 8\ n\ L / r^4$. In this equation, resistance is determined by the coefficient of blood viscosity (n), the length of the vessel (L) and its radius (r). In the circulatory system, the length of the vessels and the viscosity of the blood are relatively constant factors, while the radius of the vessel is dynamic and constantly changing and therefore, it is the main influencing parameter. A decrease in the radius of the vessel causes a significant increase in the vascular resistance and consequently in the pressure gradient (figure 8b). The opposite occurs when there is an increase in the radius of the vessel (figure 8c).

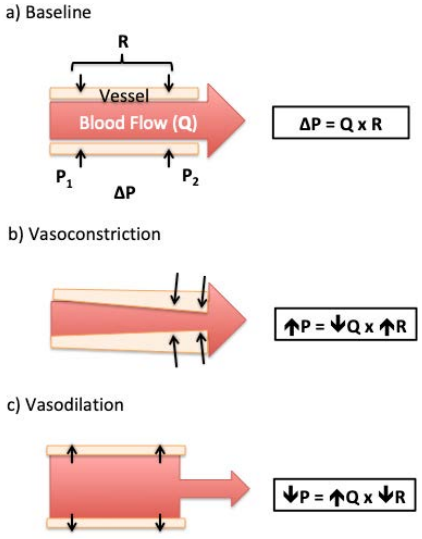


Figure 8. Ohm's law and hemodynamics. In this figure is represented Ohm's law applied to a vascular system (a) and how the parameters regulating the hemodynamics change in response to vasoconstriction (b) or vasodilation (c).

II.2.1. Intrahepatic vascular resistance and portal blood flow

As mentioned before, in cirrhosis the increased resistance to portal blood flow is the primary contributor to portal hypertension development, and this increased resistance can be located at any region of the intrahepatic circulation (Shibayama & Nakata 1985). The major contributor to increase the intrahepatic vascular resistance is the architectural abnormalities found in the cirrhotic liver (Figure 9). The structural component is a consequence of the fibrotic process, the formation of regenerative nodules, vascular occlusion and the sinusoidal capillarization (Fernández-Iglesias & Gracia-Sancho 2017).

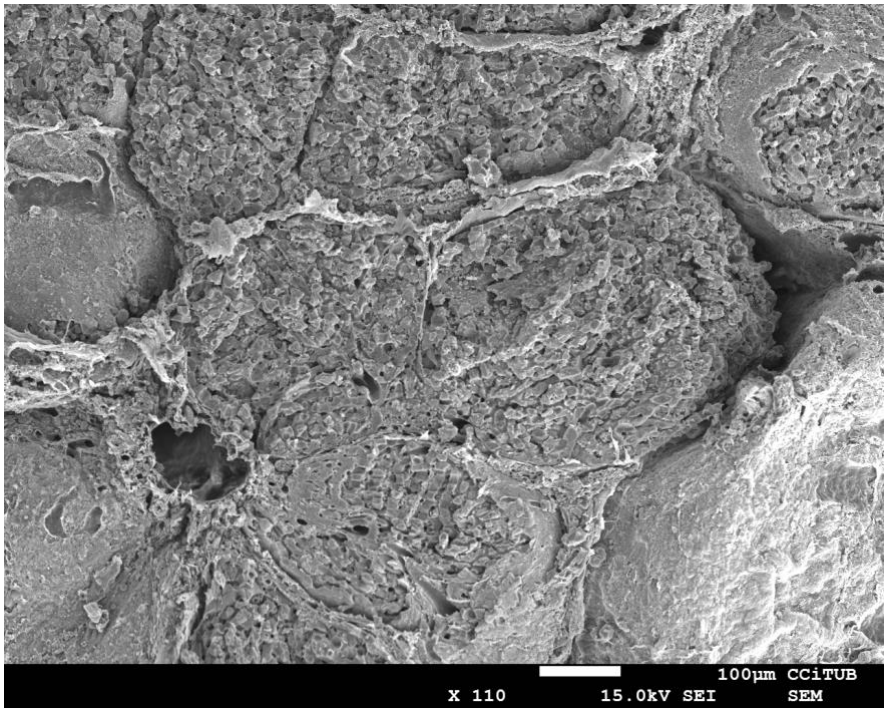


Figure 9. Cirrhotic liver. Representative transmission electron microscopy image of a rat cirrhotic liver (110X). We can observe fibrotic deposition bridging from the central vein to the portal triad and all along the hepatic sinusoids. The normal parenchymal architecture is completely lost.

Nevertheless, in 1985 a pioneer study by Bathal and Grossman revealed that also exists a non-structural, dynamic and reversible component representing up to 40% of the total increased intrahepatic vascular resistance (Bhathal & Grossman 1985). This dynamic component is consequence of the imbalance in the vasoactive factors and de-regulation of the contractile elements involved in regulating the vascular tone (Figure 10). In one hand, exists an increase in the

production of vasoconstrictors such as ET1 and TXA_2, as well as an exaggerated response to these vasoconstrictors (Gracia-Sancho et al. 2007). On the other hand, there is an insufficient availability and response to vasodilators, mainly NO and prostacyclins (Gupta et al. 1998).

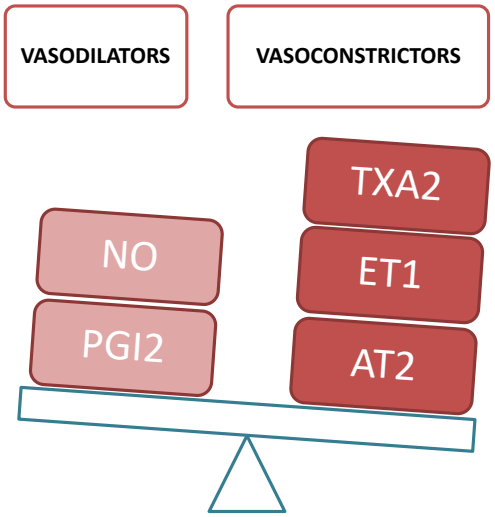


Figure 10. In cirrhosis exists an imbalance in the vasoactive factors involved in regulating the dynamic component of the intrahepatic vascular resistance. Vasoconstrictors molecules are more predominant than vasodilators and the hepatic microcirculation is sensitive to the vasoconstriction signaling.

Following the natural course of the disease, once portal hypertension is developed, an increase in portal blood flow occurs perpetuating and worsening this syndrome. The initiating mechanism of these circulatory abnormalities is an arteriolar vasodilation, which particularly occurs in the splanchnic circulation (Wiest et al. 1999). The underlying mechanisms of the splachnic hyperdynamic state are quite opposite to the intrahepatic situation. In these extra-hepatic areas there is an hyporeactivity to vasoconstrictors, increased vasodilation and formation of new vessels or angiogenesis (Fernández et al. 2009). Excessive vascular endothelial growth factor (VEGF), eNOS activity and NO availability are the main responsible for the deregulated mesenteric vasodilation (Wiest & Groszmann 2002).

II.2.2. Cellular pathophysiology of portal hypertension

In previous chapters (I.1-I.4) we have seen how important the cells of the hepatic microcirculatory system are for maintaining a healthy microenvironment and a healthy liver. Loss of function of any of these cells have dramatic consequences for the homeostasis of the organ and can lead to the development of liver diseases.

During chronic liver disease progression LSECs capillarize in other words, they become a typical endothelial cell and lose their vasodilatory synthetic capacity, hepatocytes undergo necroptosis leading to HSC activation. In this state, HSC start proliferating and synthetizing extracellular matrix and become pro-contractile. KC polarizes and acquires a pro-inflammatory phenotype. These events have a direct effect disturbing the normal liver architecture and consequently promoting a pro-inflammatory, pro-contractile and pro-thrombotic sinusoidal environment, which increases the intrahepatic vascular resistance (IHVR) and therefore portal hypertension (Gracia-Sancho et al. 2018). In the next sections we are going to overview hepatocyte dysfunction, LSEC dysfunction, HSC activation and KC polarization, and how they contribute to the pathophysiology of portal hypertension.

II.2.2.1 Hepatocyte dysfunction

The role of hepatocytes in chronic liver diseases is extremely complex. First of all, hepatocytes are the target of most hepatotoxic agents including hepatitis viruses, alcohol, metabolites and bile acids (Bataller & Brenner 2009). Accordingly, during chronic liver diseases development ,where they are exposed to a constant hepatotoxic stimulus, hepatocytes can be irreversibly damaged and consequently die (necroptosis) or on the contrary, can be stimulated to regenerate the injured hepatocytes population (regenerative nodules) (Schattenberg et al. 2012).

Both, damaged and dying hepatocytes after liver injury contribute to tissue inflammation, fibrogenesis and development of cirrhosis by releasing reactive oxygen species (ROS), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and TGF-β. These fibrogenic and inflammatory mediators induce HSC activation and trigger the inflammatory response (Seki & Schwabe 2015). In advanced stages of the disease where hepatocytes are under an hypoxic environment, TGF-β expression is extremely up-regulated perpetuating the fibrogenesis process (Jeong et al. 2004). Moreover, hepatocytes are an important source of MMPs and TIMPS, molecules clearly involved in wound-healing processes (Del Carmen García de León et al. 2006).

II.2.2.2 Liver sinusoidal endothelial cells dysfunction

In chapter I.2 we have seen the specialized morphology and function of healthy LSECs. After hepatic damage occurs LSECs become dysfunctional or dedifferentiated (Schaffner & Poper 1963). This term refers to the alterations in the phenotype of the sinusoidal endothelial cells occurring during continuous hepatic injury, in which they lose their vasoprotective phenotype. Dysfunctional LSECs suffer a characteristic change termed capillarization, which is a lack of fenestration and the appearance of organized basement membrane with consequences in their functionality and therefore, in exert the correct liver tasks (Figure 11). Moreover, dysfunctional LSEC present an impaired vasomotor control and liver regeneration properties; and they are in a pro-inflammatory, pro-thrombotic and pro-fibrogenic state (DeLeve, Wang & Guo 2008; Marrone et al. 2016). LSEC dysfunction is associated with changes in the expression of different markers, for example, increased expression of CD31, VCAM1 and ET1, and lower expression of Stabilin-1/2, CD32b and Reca1 (March et al. 2009; Graupera et al. 2005; Xie et al. 2013).

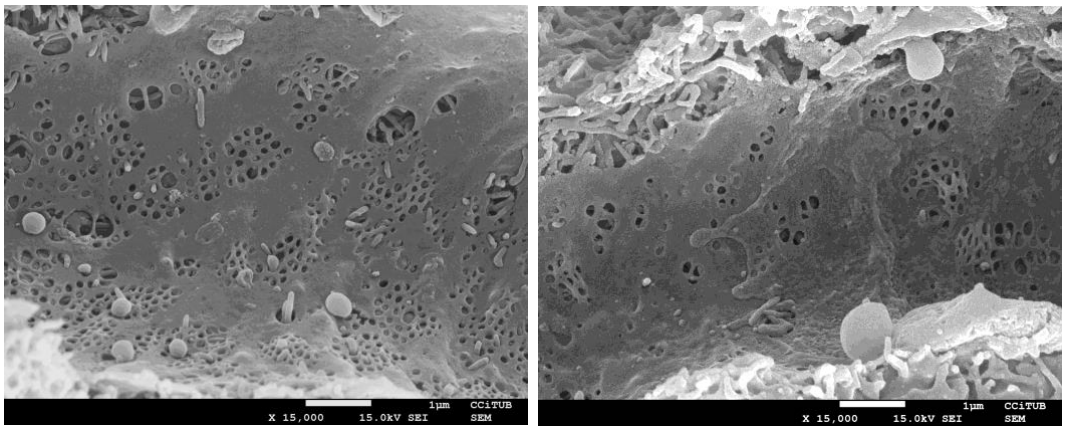


Figure 11. Liver sinusoidal endothelial cells capillarization. Representative scanning electron microscopy image of the Liver sinusoidal endothelial cell from a healthy (Left) or cirrhotic (Right) rat liver (15000X). We can observe a marked decrease in the porosity and number of fenestrations in the LSECs from cirrhotic livers compared to LSECs from healthy livers, characteristic of the capillarization process.

Since the 60s decades it is known that LSEC dysfunction is a mechanism that precedes the activation of HSC and macrophages and therefore, fibrosis development (Schaffner & Poper 1963; Mori et al. 1994; Pasarín et al. 2012). Some research studies have tried to understand the mechanisms driving LSEC dysfunction and how this is key for initiating and promoting liver injury progression (DeLeve et al. 2008; Miyao et al. 2015). So far, four main molecular pathways have been proposed: vasomotor impairment, capillarization, angiocrine signaling and angiogenesis (Poisson et al. 2017).

First of all, the *vasomotor impairment*, characteristic feature of dedifferentiated LSECs, is a consequence of decreased vasodilators and increased vasoconstrictors production. NO, the most potent vasodilator molecule is significantly diminished in cirrhotic livers (Rockey & Chung 1998). One mechanism responsible is the reduction in the production of NO by eNOS, which is inhibited by negative regulators that are up regulated during cirrhosis, as caveolin-1 (Shah et al. 1999). The second mechanisms is oxidative stress, which is increased in cirrhosis (Gracia-Sancho et al. 2008). A recent report demonstrates how Notch activation attenuates eNOS/sGC signaling and promotes LSEC dedifferentiation and accelerated fibrosis (Duan et al. 2018). Increased superoxide radicals spontaneously react with NO to form peroxynitrite (ONOO-), thereby decreasing NO bioavailability as a vasodilator. Contrarily, vasoconstrictors such as TXA2 are also increased in cirrhosis (Gracia-Sancho, Laviña, Rodríguez-Vilarrupla, Brandes, et al. 2007). The activity of COX-1 increases in LSECs from cirrhotic livers, which results in high amounts of TXA2 produced.

Regarding the *capillarization* process, Prof. Deleve et al. demonstrated how healthy LSECs phenotype is maintained through VEGF-NO-dependent or independent pathway and that restoration of LSEC healthy phenotype in vivo promoted HSC quiescence, prevention of fibrosis progression, and regression of mild fibrosis (DeLeve, Wang & Guo 2008; Xie et al. 2012). Anna Mae Diehl's lab found that Hedgehog (Hh) signaling, which is increased during liver injury, was promoting LSEC capillarization in fibrotic livers and therefore HSC activation and fibrosis progression. Inhibition of Hh both in vivo and in vitro prevented and reversed LSEC capillarization and so, they presented Hh inhibition as an attractive therapy (Choi et al. 2009; Xie et al. 2013). Studies from our group further investigated the HSC/LSEC paracrine interactions, using *in vitro* and *in vivo* experimental models of liver cirrhosis, we demonstrated that Krüppel-Like Factor 2 (KLF2)-mediated improvement in HSC phenotype paracrinally ameliorates the dysfunctional phenotype of LSEC and vice versa, leading to a significant regression of liver cirrhosis and a marked reduction in portal pressure. Hence, we proposed KLF2 as an attractive therapy for maintaining the hepatic sinusoidal milieu (Marrone et al. 2013; Marrone et al. 2015). Moreover, Dr. Guixé-Muntet showed that autophagy and the transcription factor KLF2 share a common activation pathway in the endothelium, suggesting that autophagy activation is vasoprotective for the liver endothelium (Guixé-Muntet et al. 2017). Along with this, another recent report from our colleagues proposed endothelial autophagy dysregulation as a mechanism responsible of amplifying endothelial dysfunction and HSC activation, consequently aggravating fibrosis development during mild acute liver injury (Ruart et al. 2019).

Finally, the newest mechanism involved in endothelial dysfunction is the *angiocrine signaling*.

These molecules are paracrine factors produced by LSECs, which are involved in organ homeostasis and regeneration (Ding et al. 2010). A recent study demonstrated that LSECs release divergent angiocrine signals determining and balancing liver regeneration and fibrosis. After acute liver injury, activation of C-X-C chemokine receptor type 7 - DNA-binding protein inhibitor (CXCR7-Id1) pathway in LSECs stimulates production of hepatic-active angiocrine factors leading to liver regeneration. By contrast, chronic injury causes persistent fibroblasts growth factor receptor 1 (FGFR1) activation in LSECs that perturbs CXCR7-Id1 pathway and favors a C-X-C chemokine receptor type 4 (CXCR4)-driven pro-fibrotic angiocrine response, thereby provoking liver fibrosis (Ding et al. 2014). Later, another study showed that Notch activation alters the angiocrine profile of LSECs, reduces expression of Wnt2a/9b and HGF, resulting in LSEC dedifferentiation and accelerated liver fibrogenesis (Duan et al. 2018). Finally, another report demonstrated the protective role of the bone morphogenetic protein 9 (Bmp-9) controlling LSEC fenestration. The depletion of this paracrine factor promotes LSEC capillarization and fibrosis progression (Desroches-Castan et al. 2019).

The *angiogenesis* process will be briefly discussed in section II.2.3. hyperdynamic circulation of this thesis.

After all these studies, the mechanisms underlying LSEC dysfunction and its role in initiating and driving chronic liver disease development still not well understood and therefore, more research is needed to fully comprehend this process.

II.2.2.3 Hepatic stellate cells activation

In response to acute or chronic liver injury, HSC are activated and transformed into myofibroblasts-like cells, they start to proliferate, lose their retinoid droplets and express de novo alpha-smooth muscle actin (α-SMA) (Tsuchida & Friedman 2017). TGF-β and PDGF are the most important cytokines involved in HSC activation and proliferation (Pinzani et al. 1995). Furthermore, activated HSC release proinflammatory, profibrogenic, and promitogenic cytokines, increased the production of extracellular matrix components (including collagen and proteoglicans), and present alterations in the activity of matrix proteases involved in tissue repair (Pinzani & Gentilini 1999). In acute liver damage these changes are transient, whereas after a chronic injury HSC constantly produce ECM components with a subsequent aberrant and exaggerated accumulation of these molecules, resulting in liver fibrosis and finally, cirrhosis. Importantly, HSCs become pro-contractile in an activated state (Kawada et al. 1993). They display a decreased response to vasodilators (NO), together with an increased ET1 production in cirrhosis, enhances the contraction of HSC (Perri 2006; Rockey &

Weisiger 1996). Therefore, activated HSCs are fundamental for the development and progression of portal hypertension.

The mechanisms regulating stellate cell activation have been widely study and are clearer than those promoting LSEC dysfunction. The mechanisms involved in HSC activation were organized by Prof. Friedman into core pathways, which are those that contribute to fibrosis across tissues and regulatory pathways, which are those that are tissue restricted (Mehal et al. 2011). Next are mentioned the most relevant signals driving HSC activation described so far: fibrogenic and proliferative pathways (TGF-β, PDGF, VEGF and CTGF), Hh, innate immune signals (TLRs and cytokines), adipokines, autophagy, free cholesterol, epigenetic modifications (DNA methylation and histone modifications), 5-hidroxytryptamine receptors (5-HT), AT1R, farnesoid X receptor (FXR) and peroxisome proliferator-activated receptors (PPAR).

II.2.2.4 Kupffer cells in chronic liver disease

After liver injury, Kupffer cells become activated and they start to release inflammatory chemokines and cytokines as well as to recruit large numbers of inflammatory monocytes. Activated KC contribute to the pro-contractile status by secreting vasoconstrictor substances including TXA2 and Cysteinyl leukotriene (Cys-LT) (Tacke 2017; Steib et al. 2007). Moreover, they stimulate fibrogenesis first by activating in a paracrine manner HSC, mechanism mediated by oxidative stress and IL-6 and second, because they are an important source of MMPs (Nieto 2006). Nevertheless, MMPs not only can contribute to fibrosis progression, they can also promote the removal of ECM during the resolution of fibrosis (Troeger et al. 2012). Studies have shown that macrophages can switch their fibrogenic or activated phenotype towards to a more “restorative” one that is characterized by low expression of lymphocyte antigen 6 complex (Ly-6C), high expression of anti-inflammatory mediators (HGF, IL-10) and MMP (MMP9, MMP12, MMP13) but the molecular mechanisms involved in this change of function still not well understood (Ramachandran et al. 2012).

II.2.3. The hyperdynamic circulation

As explained in chapter II.1.1 the hyperdynamic syndrome is a late consequence of portal hypertension in cirrhosis. The molecular mechanisms underlying this increased in portal blood flow

involve humoral and neural mechanisms that can determine hemodynamic changes, and lead to hyperdynamic circulation (Iwakiri & Groszmann 2006; Møller & Bendtsen 2018).

The principal hemodynamic manifestations of the hyperdynamic syndrome are high cardiac output, increased heart rate and total blood volume, accompanied by reduced total systemic vascular resistance (SVR). In patients with portal hypertension there is a marked reduction in the SVR, associated with peripheral vasodilation (Newby 2002). Arterial vasodilation in the splanchnic and systemic circulations (for example the pulmonary bed) observed in cirrhosis helps to increase the blood flow to the portal vein and is caused by an excessive release of NO in the splanchnic vascular beds. NO is the most important vasodilator molecule that contributes to excessive vasodilation observed in the arterial splanchnic and systemic circulations in portal hypertension. An increase in portal pressure increases VEGF production and triggers eNOS activation and subsequent NO overproduction (Moreau 2005; Genesca et al. 1999). In addition, mechanical stimuli (shear-stress) and pro-inflammatory cytokines (TNF-α) contribute to NO-mediated vasodilation (Lopez-Talavera et al. 1995; Muñoz et al. 1999). Hypocontractility has also been detected in the arterial splanchnic and systemic circulations in portal hypertension further aggravating the hyperdynamic syndrome. Finally, neural factors, especially through the sympathetic system, are involved in the development of hyperdynamic syndrome. Portal hypertensive rats showed sympathetic nerve atrophy/regression in the mesenteric arteries and this contributes to vasodilation and/or hypocontractility of those arteries (Coll et al. 2010).

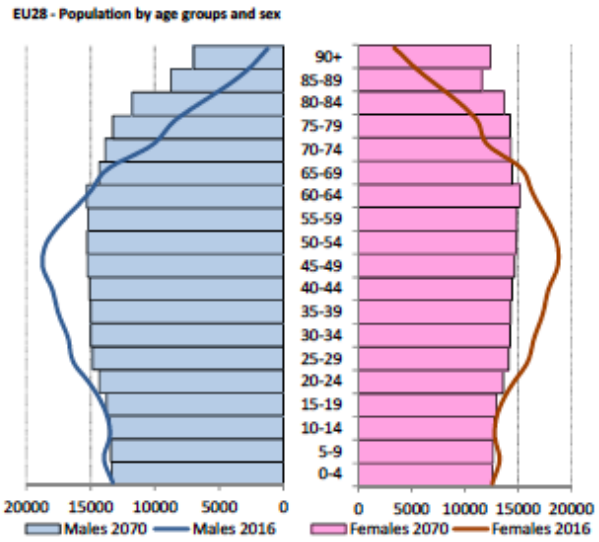
Porto-systemic collateral vessels from pre-existing or de novo (angiogenesis) develop in response to an increase in portal pressure. Portal blood flow augmentation from the splachnic circulation increases to compensate the blood escaping through these collateral vessels. Several studies have demonstrated that augmented angiogenesis in splachnic organs could be mediated through a VEGF-dependent process (Fernandez et al. 2004; Fernandez et al. 2005). Indeed, VEGF signaling blockade markedly attenuates the increase in splanchnic blood flow, as well as the increased splanchnic vascularization observed in portal hypertensive animals. Therefore, modulation of angiogenesis may represent a potential target in the treatment of portal hypertension.

The clinical consequences of the hyperdynamic syndrome which are manifest in advanced cirrhosis include risk of variceal bleeding, ascites, hepatic encephalopathy, hepatorenal syndrome, hepatopulmonary syndrome, portopulmonary hypertension and cirrhotic cadiomiopathy (Licata et al. 2014).

III. Aging at a glance

Aging is a natural and time-dependent decline in organs functionality in all-living organisms leading to death. Moreover, it is the largest risk factor for chronic diseases development including cardiovascular, neurological and liver pathologies (López-Otín et al. 2013).

During the last years, European demographic reports have warned about the increasing trend in the median age population. As we can observe in the population pyramid presented in Graph 1 according to the baseline 2015-based population projections, the structure of the EU and euro-area populations is expected to age significantly. In fact, in all age cohorts of 65 years old and above, the projected population in 2070 is higher than in 2015. Moreover, while in 2016 the largest cohort for both males and females was 45-49 years old, in 2070 the largest cohort is expected to be 70-74 years old for women and 50-54 years old for men (He et al. 2016; Uddin 2017).



Graph 1: Population by age group and gender, 2016-70 (thousands). Source: Eurostat, 2015-based population projection, 2018.

The experts in the aging field suspect that the socioeconomic and medical care improvements are the major reasons for the life expectancy increment leading to the expansion of the elderly population.

Considering these alarming reports, aging and age-associated diseases are one of the greatest socioeconomic challenges that developed and developing countries must face.

III.1. Aging and the liver

Many research studies have focused on understanding the molecular basis of aging in different organs and tried to find therapeutic strategies to prevent age-related diseases.

In 1986, Popper studied for the first time the age-related alterations in the liver. In fact, he described that the liver with age is characterized by a brown and atrophic aspect, which is attributed to the accumulation of lipofuscin pigments (Popper 1986). Since these first approaches, and along with technologies development many other studies have been able to better describe the changes occurring in the aged liver.

Aging is associated with gradual alteration of hepatic structure and function as well as various changes in liver cells (Schmucker 2005). I am going to summarize in the next lines the main alterations that have been described so far.

III.2. Aging and liver volume, blood flow, and function

Aged liver is accompanied by a reduction in hepatic liver mass and hepatic blood flow, which cause a decline in the organ functionality. Several studies have shown a gradually decrease around 20-40% in the liver volume as one gets older (Wynne et al. 1989). The alteration in this parameter is a direct consequence of the 25-35% decline in the hepatic blood flow from aged livers (Zoli et al. 1999) and, a decrease in the mass of functional liver cells (Wakabayashi et al. 2002).

More reports have analyzed and observed several alterations in single variables in the liver during aging. The serum albumin and bilirubin concentrations are slightly decrease in the aging process. The metabolism of low-density lipoprotein is decreased around 35%, while the high-density protein and cholesterol levels both in the liver and in the circulation increases over time. At last, the serum γ-glutamyltransferase and alkaline phosphatase levels are elevated with aging (Tietz et al. 1992).
Impaired drug metabolism has been associated with aging; this function is often reduced by 40-50% in old age and is specially affecting Phase I metabolism drugs (Le Couteur & McLean 1998).
The vast majority of these alterations have been confirmed in different animal models including mice and rats, non-human primates (Cogger et al. 2003) and humans (Le Couteur et al. 2001; Vollmar et al. 2002). However, still not well studied if all these changes are consequence of a decrease in the cell number, cell dysfunction or most probably, as a result of both.

III.3. Aging and the hepatic sinusoid

Most of the studies so far have focused on studying the effects of aging in liver functionality and therefore, in hepatocytes. Relatively is known about the consequences of aging in the other cells of the hepatic sinusoid. A few studies from Le Couteur's lab in Sydney suggested a morphological change in the sinusoidal vascular system (Le Couteur et al. 2008; Hunt et al. 2019). I've overviewed in the next lines the aged-related known findings in the main cells of the hepatic sinusoid.

III.3.1 Aged hepatocytes

The volume of liver cells increases with their maturity but starts to decrease gradually with aging. As mentioned before, Popper found accumulations of lipofucsin protein aggregates in aged hepatocytes. Similarly, as occurs with other aged-related diseases, like Alzheimer's, t damaged and denatured proteins by oxidative stress cannot be degraded and consequently, they accumulate inside the cell forming these highly cross-linked non-degradable protein aggregates. Lipofucsin masses cause an increased in the generation of ROS in cells reducing their survivability (Höhn & Grune 2013). A decline in the ATP levels has been observed, as a consequence of both the decreased in the number of mitochondria and a dysfunction of them (Sastre et al. 1996). Also, the area of smooth endoplasmic reticulum is markedly reduced with aging, correlating with the age-related decline in the hepatic microsomal protein synthesis and the activity of constituent enzymes from this organelle (Schmucker et al. 1990).

Polyploidy, which depends on both increases in the number of nuclei per cell plus DNA content of each nucleus, generally does not exceed 15% of the hepatocytes from adult humans. However, the fraction of polyploid hepatocytes increases to 42% in the liver from aged individuals (Kudryavtsev et al. 1993).

Finally, a major characteristic of the aging process is a reduced proliferative response, which is consequence of the significant delay in cell cycle entry and the significant reduction of the number of proliferating hepatocytes (Bucher et al. 1964; Fry et al. 1984; Timchenko et al. 1998). Some studies are trying to elucidate the molecular mechanisms for the age-dependent loss of liver regenerative capacity. Thus may involve alterations in CCAAT/enhancer-binding protein (C/EBP) family members (α, β, γ), glycogen synthase kinase 3β (GSK3β), histone deacetylase 1 (HDAC1), and sirtuin1 (SIRT1) epigenetic and signaling pathways (Gagliano et al. 2007; Jin et al. 2009; Timchenko 2009; Jiang et al. 2013).

III.3.2. Aged liver sinusoidal endothelial cells

First studies analyzing the effects of aging in LSECs found no major changes (De Leeuw et al. 1990) but, a few years later new reports described that old age is associated with changes in the structure of LSECs and the space of Disse (Jamieson et al. 2007; McLean et al. 2003; Warren et al. 2005; Ito et al. 2007). The aging sinusoidal endothelium is pseudocapillarized, meaning that aged LSECs present an increase in their thickness, a reduction in the porosity and number of fenestrations and deposition of perisinusoidal basal lamina, they become more like capillaries in other non-fenestrated vascular beds (Le Couteur et al. 2001). The term "pseudo" is added to distinguish it from the more pronounced capillarization effect observed in LSECs after chronic liver injury. The endothelial defenestration cause a negatively effect in the effective removal of substances deposited in excess in the liver (like chylomicrons) due to the reduction in the endocytosis function (Le Couteur et al. 2005). Endocytosis dysfunction in the aged LSECs causes the deposition of circulating products outside the liver increasing the risk for other aged-related diseases including diabetes, arteriosclerosis and neurodegenerative disorders (Le Couteur & McLean 1998).

Just a few studies have tried to identify alterations in the phenotype and functionality of LSECS with aging. It has been reported an increased in the expression of the glycoprotein von Willebrand factor (vWF) in old animals (Hilmer et al. 2005). The healthy endothelium does not express this protein, but after an endothelial injury occurs vWF is synthesized and secreted to mediate the attachment of platelets. Other studies showed a reduction in caveolin-1 expression (Jamieson et al. 2007) and increased in ICAM-1 expression (Ito et al. 2007), altogether suggesting alterations in LSEC biology. However, further studies are needed to understand the age-related changes in the phenotype of LSECs.

III.3.3. Aged hepatic stellate cells

Less is even known about the effect of aging in HSC. One study reported an increase in the number of desmin-positive HSC with aging, without any differences in the number of α-SMA positive HSC (Le Couteur et al. 2011). Also, has been described a variable up-regulation of collagen IV and Sirius red, markers of extracellular matrix and a marked accumulation of lipid deposition inside these cells (Figure 12).

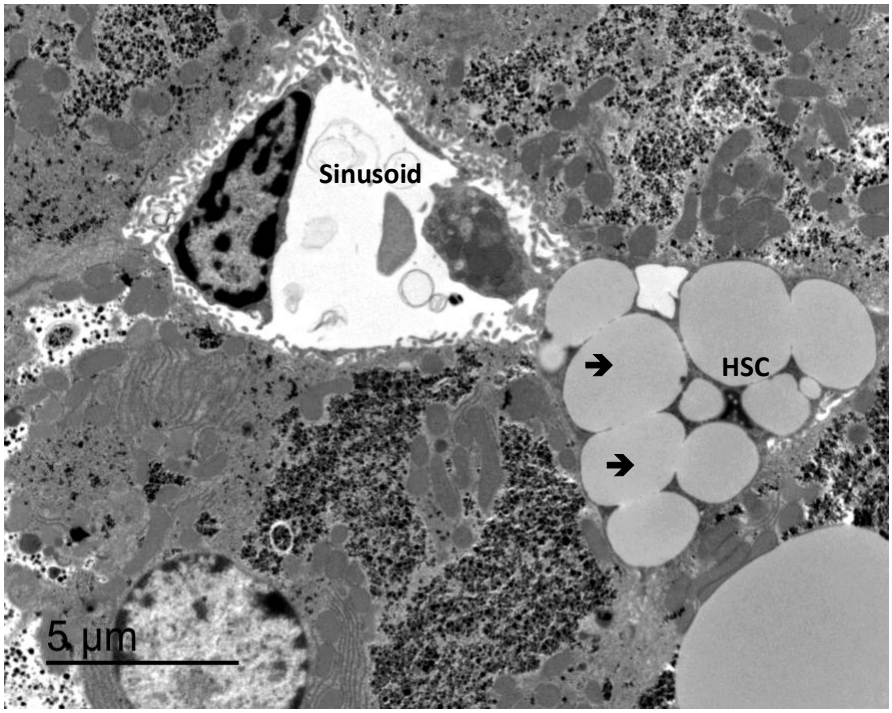


Figure 12. Hepatic stellate cells in the aged liver. Representative transmission electron microscopy image of the hepatic sinusoid from 20-months old rat liver (8000X). We can observe an open sinusoid and in the right side of it a HSC with high amounts of lipid droplets accumulated in its cytosol, typical feature of HSC from aged livers.

III.3.4. Aged Kupffer cells

The effects of aging on hepatic macrophage population dynamics, polarization, and function are not well understood. Just one report have observed that with aging the number and activation of Kupffer cells is increased (Hilmer et al. 2007). Studies performed on macrophages derived from other aged sources, such as the bone marrow, peritoneal cavity, lungs, and brain, have demonstrated other molecular alterations including reductions in autophagy and phagocytosis, dysfunction in cytokine signaling, and altered morphology and distribution, likely mediated by epigenetic changes and mitochondrial defects. Further studies are needed to confirm if these alterations are applicable to aged hepatic macrophages (Stahl et al. 2018).

III.4. Liver diseases in the elderly

Aging has been defined as a major risk factor for the development of chronic liver conditions (Tsochatzis et al. 2014; Sheedfar et al. 2013).

The incidence of aCLD increases dramatically with age, and is accompanied by worse prognosis (Angulo et al. 1999). All this epidemiological studies confirm that cirrhosis is more prevalent in the elderly (Frith et al. 2009), it progresses faster in this sub-group of patients (Poynard et al. 2001) and importantly, that decompensation is more frequent in older patients (14%) compared to younger ones (4%) (Thabut et al. 2006; Davis et al. 2010).

Moreover, aging is suspect to be a major factor in fibrosis progression (Poynard et al. 2001). In the pre-clinical scenario, little is known about the impact of aging on liver diseases. Previous work suggested higher rate of fibrosis deposition in aged rats and attributed this to alterations in the immune response (Ramirez et al. 2017; Collins et al. 2013). Importantly, and despite the impact of aging on human aCLD, most of the pre-clinical studies aimed at understanding liver disease pathophysiology have been developed in young animals, without considering age as an influencing factor for aCLD development. Therefore, it is an orphan subject that needs to be study.

IV. Current treatments and future perspectives.

The study of the molecular pathways responsible for the increment in HVR has led to the discovery of potential therapeutic strategies for the treatment of portal hypertension. Although the significant efforts on testing and validating safe and reliable strategies to reduce the HVR in cirrhotic patients still represent a pending issue (Gracia-Sancho et al. 2015). The current treatments available for portal hypertension are focused on reducing the hyperdynamic syndrome, or the prevention of portal hypertension-direct complications, mostly gastroesophageal varices and variceal hemorrhage (Vilaseca et al. 2018). Management of varices/variceal hemorrhage is based on the clinical stage of portal hypertension, which is measure using the HVPG. HVPG is a good surrogate marker of portal hypertension with a robust prognostic power (Burroughs & Thalheimer 2010). Portal hypertension is considered clinically significant when the HVPG is above 10 mmHg since patients with HVPG of less than10 mmHg had a 90% probability of not progressing to decompensation during median follow-up of 4 years (Tsochatzis et al. 2014). Nowadays, no specific treatment has shown to prevent the formation of varices. Accordingly, treatments are given to prevent the first variceal hemorrhage using β-blockers or esophageal band ligation depending on the size/characteristics of varices. While, vasoactive drugs, endoscopic band ligation, antibiotics prophylaxis and transjugular intrahepatic portosystemic shunt (TIPS) are the standard of care once an acute variceal hemorrhage has occurred (Bari & Garcia-Tsao 2012).

IV.1 Statins

Statins are a group of drugs that inhibit the action of the hydroxyl-methyl-glutaryl-coenzyme A (HMG-CoA) reductase. Statins were originally developed to lower lipid levels and treat hypercholesterolemia. However, the use of Statins to treat different conditions have shown beneficial and pleiotropic properties beside and independent of its cholesterol lowering effects (Sirtori 2014; Kopterides & Falagas 2009). The so-called pleiotropic effects of statins include improvement of endothelial dysfunction, antioxidant, anti-fibrotic, anti-inflammatory, anti-proliferative, anti-angiogenic, pro-apoptotic and immunomodulatory effects. For this reason, statins have been widely assessed for drug repurposing, including hepatology (Beckman & Creager 2006; Moctezuma-Velázquez et al. 2018).

The mevalonate pathway is an essential metabolic pathway that under physiological conditions produces the components used to make isoprenoids, a diverse class of biomolecules that are vital for

regulating cellular processes. Cholesterol, heme, vitamin K, coenzyme Q10 and steroid hormones are among the most important and known isoprenoids (Goldstein & Brown 1990). Inhibition of the mevalonate pathway prevents the formation of farnesyl-pyrophosphate (FPP), necessary for the synthesis of squalene and cholesterol, and geranylgeranyl-pyrophosphate (GGPP), which regulates the activity of the small GTPases Rho, Rac1 and Ras (Figure 13) (Casey 1995). These GTPases are essential key regulators of diverse critical cellular functions including cytoskeleton dynamics, cell cycle progression, migration, generation of reactive oxygen species and gene expression (NO synthesis, endothelin, inflammatory and thrombotic factors) (Liao 2002).

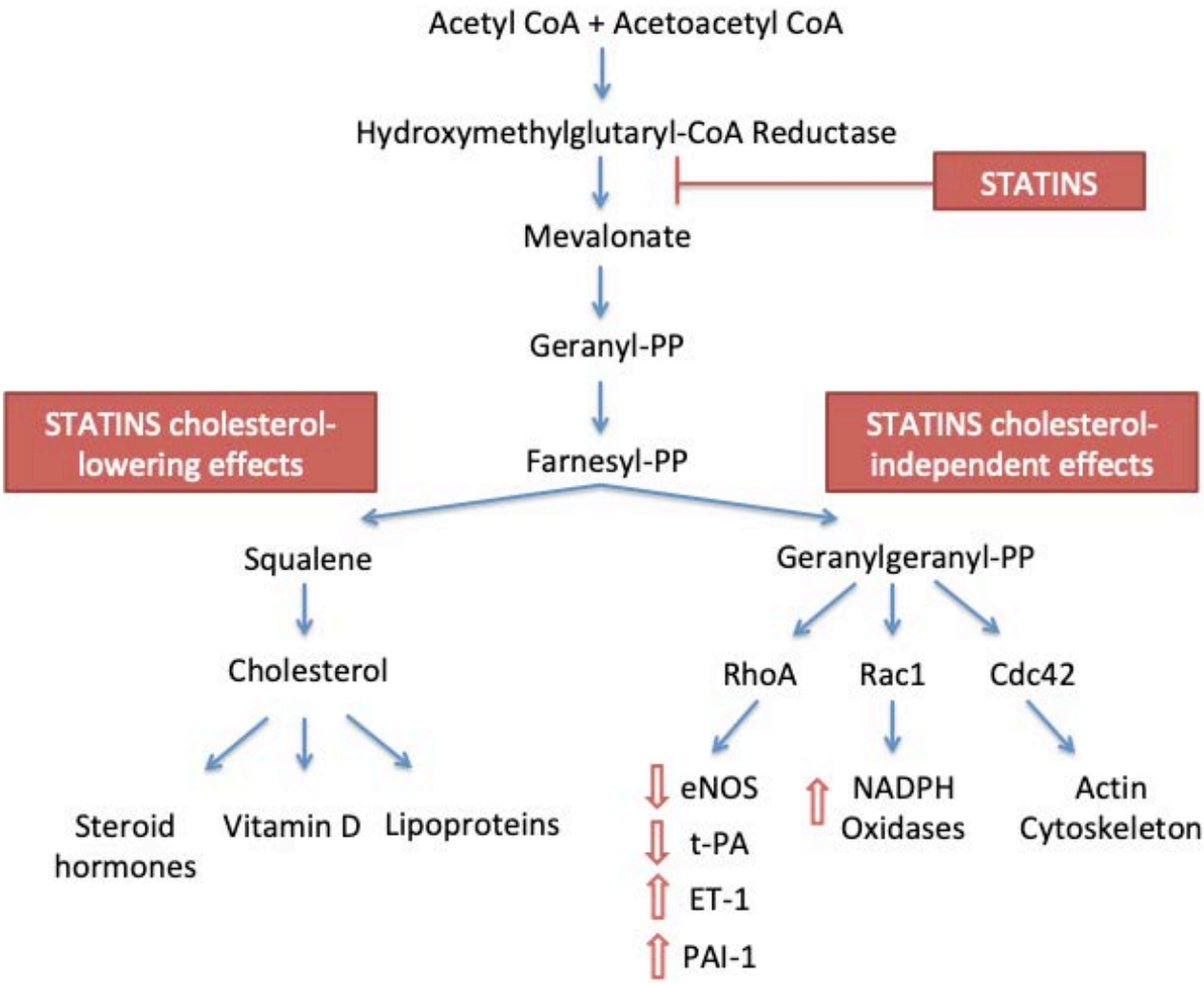


Figure 13: Effects of statins on mevalonate pathway. The beneficial effects of statins are through 1) lowering cholesterol synthesis and 2) cholesterol-independent effects related to the lack of production of the isoprenoid intermediate geranyl-geranyl-PP (GGPP).

For their pleiotropic effects Statins emerged as a good candidate for the treatment of aCLD. In fact, in 2004 our group was the pioneer administrating statins to cirrhotic patients with portal hypertension, demonstrating their beneficial effects reducing the intrahepatic vascular resistance (Zafra et al. 2004). Posterior pre-clinical and clinical studies have confirmed the benefits of statins not only on portal hypertension, but also in the development and survival of hepatocellular carcinoma (Zafra et al. 2004; Abraldes et al. 2007; Abraldes et al. 2016; Mohanty et al. 2016). Part of the underlying mechanisms of statins improving portal hypertension has been attributed to their sinusoidal-protective properties: improving the phenotype of the sinusoidal cells, activating the expression of KLF2, deactivating HSC, ameliorating the dysfunctional endothelium by promoting the increased of the NO pathway specifically in the liver and reducing oxidative stress (Trebicka et al. 2007; Marrone et al. 2015; Meireles et al. 2017; Tripathi et al. 2018).

IV.2. Treating aCLD in the elderly

We have seen along chapter III the age-related changes in the liver and in the organism and how they can affect its functionality, including a dramatic effect in drug disposition and pharmacodynamic responses. These changes have significant clinical implications for geriatrics populations, altering the risk-benefit of medical interventions in the elderly population (McLean & Le Couteur 2004). Treatment of older patients with liver disease may require different therapeutic strategies as demonstrates the few studies that have tried to address this issue. While Hepatitis C is more prevalent in the elderly population, the efficacy and safety of treating aged patients with antivirals is a source of debate, since this subgroup of patients seems to be more sensitive to develop side effects (Malnick et al. 2014). Another report showed that liver transplantation recipients aged more tan 60 years exhibited lower survival rates compared with those aged between 45 and 60 years (Randall et al. 2003).

There is accumulating evidence that statins have beneficial effects for treating chronic liver disease but, further trials and pre-clinical studies are necessary to demonstrate if statins are positive in the elderly (Schierwagen et al. 2017). In fact, the efficacy and safety of statin therapy among older people have only being addressed for treating cardiovascular diseases and even in this field, it is still on discussion due to lack of studies and information. One recent clinical trial showed that statin therapy produced significant reductions in major vascular events irrespective of age, but it was not clear the benefit of statins treatment among patients with no evidence of occlusive vascular disease (Armitage et al. 2019).

Therefore, future studies are needed to understand more about the molecular mechanism of aging and help to develop suitable treatment strategies in this subgroup of patients and block the progression of aging-induced liver diseases.

Hypothesis and aims

Hypothesis and aims

Nowadays, aging represents an important healthcare and social issue. Societies around the world are experiencing a significant increase in the elderly population every year due to extension of the lifespan as a result of socioeconomic and medical care improvements. According to the last European Aging Report, 23% of the European population will be over 65 years old in 2030 (He et al. 2016).

The liver during aging shows a physiological decline in some of their functions, as detoxification, synthetic capacities or regeneration. The molecular mechanisms explaining these alterations have not been explored in depth. Previous reports have shown single variables modified with aging including a reduction in liver mass, hepatic blood flow, or pseudocapillarization (Hunt et al. 2019). Nevertheless, studies analyzing how aging affects the hepatic sinusoidal milieu, its cellular components, and the hepatic microcirculatory function are limited.

Advanced chronic liver disease (aCLD), with 844 million people affected worldwide, is another relevant and costly problem for our society. Aging is a major risk factor for the development of chronic diseases including the ones affecting the liver. It has been shown that the incidence of aCLD increases dramatically with age, and is accompanied by worse prognosis (Carrier et al. 2019). Moreover, the risk-benefit of medical interventions in the elderly population is affected. Statins are an important therapeutic strategy proposed for the treatment of aCLD but it is unknown how the aged subgroup of patients respond to this treatment (Armitage et al. 2019). Despite the impact of aging in human aCLD, most of the pre-clinical studies designed to understand the liver disease pathophysiology and exploring the benefits of statins on aCLD have been made in young animals, ignoring this variable from the analysis (Moctezuma-Velázquez et al. 2018).

Considering all the facts mentioned before and the importance of maintaining a healthy sinusoidal phenotype for the globally protection of the liver function, the present thesis aimed to study the hepatic sinusoid in different experimental models of aging. Firstly, it was characterized the liver sinusoid during aging in healthy animals. Secondly, it was investigated the impact of cirrhosis in aged animals. Lastly, statins have been proposed as treatment for aCLD in the old population by delaying the damage on liver microcirculation and function.

- **Study 1. Effects of aging on liver microcirculatory function and sinusoidal phenotype.**

The socioeconomic and medical care improvements of the last decades have led to a relevant increase in the median age of worldwide population. Although some studies described the impact of aging in liver function, little is known about its effect in the hepatic microcirculatory status. We hypothesized that in the elderly the hepatic sinusoidal environment is mildly dysfunctional due to deregulations in the main cells of the hepatic sinusoid. That's why in this study; we aimed at characterizing the phenotype of the aged liver in a rat model of healthy aging, particularly focusing on the microcirculatory function and the molecular status of each hepatic cell type in the sinusoid.

- **Study 2. Aging influences hepatic microvascular biology and liver fibrosis in advanced chronic liver disease.**

Advanced chronic liver disease (aCLD) represents a major public health concern. As we have seen in the thesis introduction, aCLD is more prevalent and severe in the elderly, carrying a higher risk of decompensation. Considering this, we hypothesized that the cumulative mild changes we have observed in the sinusoidal milieu of healthy aged livers (Study 1) may have relevant consequences during chronic liver injury. Consequently, the present study aimed at understanding how aging may impact on the pathobiology of aCLD, specially focusing on the hepatic microcirculatory dysfunction, fibrosis and portal hypertension. Secondary aims included the evaluation of simvastatin as a therapeutic option in a pre-clinical rat model of aged cirrhosis, and to elucidate the mechanisms responsible for the possible beneficial effects of the treatment.

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Study 1:

EFFECTS OF AGING ON LIVER MICROCIRCULATORY FUNCTION AND SINUSOIDAL PHENOTYPE.

Raquel Maeso-Díaz, Martí Ortega-Ribera, Anabel Fernández-Iglesias, Diana Hide, Leticia Muñoz, Amelia J. Hessheimer, Sergi Vila, Rubén Francés, Constantino Fondevila, Agustín Albillos, Carmen Peralta, Jaime Bosch, Frank Tacke, Victoria C. Cogger and Jordi Gracia-Sancho.

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ORIGINAL ARTICLE

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Effects of aging on liver microcirculatory function and sinusoidal phenotype

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Abstract

The socioeconomic and medical improvements of the last decades have led to a relevant increase in the median age of worldwide population. Although numerous studies described the impact of aging in different organs and the systemic vasculature, relatively little is known about liver function and hepatic microcirculatory status in the elderly. In this study, we aimed at characterizing the phenotype of the aged liver in a rat model of healthy aging, particularly focusing on the microcirculatory function and the molecular status of each hepatic cell type in the sinusoid. Moreover, major findings of the study were validated in young and aged human livers. Our results demonstrate that healthy aging is associated with hepatic and sinusoidal dysfunction, with elevated hepatic vascular resistance and increased portal pressure. Underlying mechanisms of such hemodynamic disturbances included typical molecular changes in the cells of the hepatic sinusoid and deterioration in hepatocyte function. In a specific manner, liver sinusoidal endothelial cells presented a dysfunctional phenotype with diminished vasodilators synthesis, hepatic macrophages exhibited a proinflammatory state, while hepatic stellate cells spontaneously displayed an activated profile. In an important way, major changes in sinusoidal markers were confirmed in livers from aged humans. In conclusion, our study

demonstrates for the first time that aging is accompanied by significant liver sinusoidal deregulation suggesting enhanced sinusoidal vulnerability to chronic or acute injuries.

1 | INTRODUCTION

Societies in developed countries are getting older due to the increase in life expectancy. Nowadays, 14% of European citizens are aged over 65, and by 2030, they are expected to constitute 23% (He, Goodkind, & Kowal, 2016). Therefore, the pace of population aging represents an important healthcare and social issue and it is therefore essential to understand the molecular basis of aging to identify possible approaches for therapeutic intervention.

The liver plays essential roles in metabolism, toxicants clearance, regulation of inflammation, and molecule biosynthesis. To fulfill these complex tasks, it requires an adequate microcirculation and a correct coordinated function of all hepatic cell types (Arias et al., 2009). Although it is well known that hepatocytes constitute the main cell type contributing to the metabolic and synthetic capacities of the liver, their function deeply depends on an efficient exchange of substances with the blood stream and a proper communication with other hepatic cells.

Liver sinusoidal endothelial cells (LSEC), hepatic stellate cells (HSC), and Kupffer cells (KC) are the major components of the hepatic sinusoid, which collaborate to maintain the integrity and functionality of the unique liver microcirculatory system (Marrone, Shah, & Gracia-Sancho, 2016). LSEC are a very specialized fenestrated endothelial cell forming the capillary bed of the sinusoids, being separated from hepatocytes through the space of Disse. HSC are vitamin A storing cells located in the space of Disse, surrounding LSEC, and represent the liver pericytes. At last, KC, the resident macrophages of the liver, are located at the luminal side of the endothelial lining (Fernández-Iglesias & Gracia-Sancho, 2017; Friedman, 2008; Ju & Tacke, 2016).

Previous studies characterized single variables in the liver during aging and showed reduction in liver mass and hepatic blood flow, partial loss of endothelial fenestration, and possibly activation of HSC (Le Couteur et al., 2001; Vollmar, Pradarutti, Richter, & Menger, 2002). Nevertheless, studies analyzing in depth the hepatic sinusoid, its cellular components, and the hepatic microcirculatory function in aging are limited.

This study aimed at comprehensively characterizing the phenotype of the sinusoid in aged liver using a preclinical rat model of healthy aging, particularly focusing on the microcirculatory function and the molecular profile of each major liver cell type. In addition, our study further compared these findings to the key cellular modifications related to aging in livers from young and aged humans.

2 | RESULTS

2.1 | Aged rat model: Baseline characteristics and biochemical parameters

As shown in Table 1, aged rats (20 months old) presented significantly increased body and liver weight compared to 3-month young rats, however, liver-body weight ratio was moderately diminished in old rats. In addition, elderly animals exhibited certain decline in liver function as suggested by reductions in albumin levels and bile production, although no significant differences in transaminases (as a marker of liver cell injury) were observed. Evaluation of plasma lipids revealed an increase in cholesterol and LDL cholesterol, without significant changes in HDL cholesterol, triglycerides, and free fatty acids. Moreover, aged rats had higher hepatic lipid accumulation as compared to young rats by oil red O staining. Hepatic malondialdehyde (MDA) in old rats was elevated, suggesting an increase in oxidative stress secondary to lipid peroxidation process.

As expected, analysis of senescence markers exhibited differences between young and old animals (Supporting Information Figure S1). P16, a protein involved in cell cycle regulation, was up-regulated in liver tissue and in LSEC freshly isolated from aged rats. SIRT1, an enzyme related with longevity, was decreased in the aged liver. At last, we also observed hepatic telomere length attrition in old animals.

2.2 | Aged animals present mild hepatic microcirculatory dysfunction

When compared to young animals, old rats exhibited significantly higher hepatic vascular resistance (HVR) *in vivo*, associated with a reduction in liver perfusion, altogether leading to a moderately increased portal pressure (PP), without meeting criteria for portal hypertension (Figure 1). Mean arterial pressure was also significantly elevated, with no significant changes in heart rate (308 ± 9 vs. 319 ± 13 bpm).

Ex vivo perfusion experiments analyzing the liver endothelial-dependent vasodilatory capacity showed no significant differences between groups (data not shown).

Mechanisms responsible of the observed age-associated increased HVR were investigated in succeeding experiments.

2.3 | Aging is associated with mild hepatocyte injury and dysfunction

Liver architecture was overall preserved in aged animals (Supporting Information Figure S2); however, evaluation of the severity of

TABLE 1 Baseline characteristics and biochemical parameters of 3 months young and 20 months old rats

	3 months young	20 months old	% change	*p*-value
Body weight (g)	380 ± 9	678 ± 22	+78	**<0.001**
Liver (g)	9.9 ± 0.3	15.3 ± 0.4	+54	**<0.001**
Liver-body weight ratio (%)	2.6 ± 0.1	2.4 ± 0.1	−8	0.07
AST (U/L)	119.6 ± 12.2	113.2 ± 8.5	−5	>0.20
ALT (U/L)	48.9 ± 3.0	54.9 ± 6.1	+12	>0.20
Bile production (μl/min*100 g bw)	72.6 ± 6.8	31.7 ± 9.2	−56	**<0.001**
Albumin (mg/dl)	26.8 ± 0.5	24.9 ± 0.5	−7	**<0.001**
Plasma cholesterol (mg/dl)	52.1 ± 2.0	88.5 ± 8.8	+69	**<0.001**
Plasma LDL cholesterol (mg/dl)	24.3 ± 2.5	58.4 ± 5.4	+140	**<0.001**
Plasma HDL cholesterol (mg/dl)	16.3 ± 0.7	19.5 ± 2.2	+19	>0.20
Plasma triglycerides (mg/dl)	57.8 ± 9.1	53.5 ± 7.9	−7	>0.20
Plasma FFA (mg/dl)	662 ± 127	578 ± 99	−12	>0.20
Oil red O staining (%)	1.00 ± 0.18	5.31 ± 1.10	+431	**<0.001**
MDA (nmol/mg protein)	0.86 ± 0.23	2.70 ± 1.00	+213	0.09
LPS (EU/ml)	0.88 ± 0.05	1.28 ± 0.11	+45	**0.02**

Note. Data expressed as mean ± *SEM* (*n* = 12 each group) AST: aspartate transaminase; ALT: alanine transaminase; FFA: free fatty acids; MDA: malondialdehyde; LPS: lipopolysaccharide.

hepatic injury using a histological damage score revealed mild liver dysfunction in aged rats in comparison with young animals (Supporting Information Table S1). Indeed, injury analysis showed differences between groups in all of the evaluated parameters including cytoplasmic vacuolation, nuclear pyknosis, cytoplasmic hypereosinophilia, loss of intercellular borders, necrosis, neutrophil infiltration, and fat accumulation. Ultrastructural analysis of liver damage using transmission electron microscopy (TEM) evidenced a decrease in the number of sinusoids with no changes in other parameters. In addition, aged rats showed higher hepatic cell death as demonstrated by the TUNEL staining (Supporting Information Figure S3a), with no significant changes in apoptotic proteins c-caspase-3 and BAD (data not shown).

Liver tissue analyses were complemented examining the phenotype of hepatocytes freshly isolated from both groups of animals (Supporting Information Figure S3b–d). In agreement with the *in vivo* biochemical parameters, aged hepatocytes exhibited slight but not significant lower urea and albumin synthetic capacity. This was associated with deregulation in different specific markers including Oct1 and Mrp3, with no differences in Mrp2 or HNF4α. At last, hepatocyte cytochrome P4503A4 activity tended to be higher in aged animals.

2.4 | The aged hepatic endothelium is *pseudocapillarized* and procontractile

Analysis of the liver sinusoid using scanning electron microscopy confirmed pseudocapillarization of aged LSEC in this experimental model of aging. As seen in Figure 2a, fenestrae porosity was markedly diminished in the aged hepatic sinusoid. In accordance, aged livers exhibited reduced expression of VEGFR2, a protein involved in fenestrae formation and maintenance, and CD32b, a well-established marker of LSEC differentiation (Figure 2b,c).

Intrahepatic neovascularization analysis did not show differences between groups (Figure 2d). Nevertheless, the number of sinusoids seemed to be decreased in aged livers (Figure 2e), which correlates with the reduced CD32b expression described above.

Detrimental effects of aging on LSEC phenotype were not limited to markers of capillarization, but included loss of vasodilatory capacity. Indeed, aged livers presented reduced eNOS and p-eNOS protein expression (Figure 2f,g), associated with a 45% reduction in the NO-secondary messenger cGMP (Figure 2h).

Analysis of primary LSEC confirmed a remarkable dysfunction of cells isolated from aged animals (Figure 3). Although we did not observe significant changes in mRNA expression of eNOS, ED1, and CD31, aged LSEC showed alterations in angiocrine, inflammatory, scavenging, vasodilatory, and oxidative stress pathways. In fact, endothelial angiocrine molecules seemed to be decreased in aged LSEC, and the expression of different proinflammatory cytokines was up-regulated. The scavenger receptor stabilin-2 was decreased in aged LSEC. Moreover, p-eNOS expression and NO bioavailability were reduced in aged LSEC in comparison with cells isolated from young animals. In addition, aged LSEC displayed significantly more mitochondrial oxidative stress, alongside reduced expression of the anti-oxidant enzyme HO-1.

2.5 | Aged hepatic stellate cells are spontaneously activated

HSC phenotype was characterized both in liver tissue and freshly isolated cells (Figure 4). Analysis using TEM revealed that aged livers exhibit a nonsignificant increase in the number of stellate cells with higher number of intracellular lipid droplets in comparison with young. A trend for increased HSC in aged livers was supported by incremented desmin protein expression and significant increase in the proliferative HSC-related growth factor PDGFRβ. In addition, aging was associated with slight, but significant, activation of HSC as demonstrated by increments in the mRNA and protein expression of different activation markers including α-SMA, collagen1α1, collagen1α2, PDGFRβ, and p-moesin, together with changes in some matrix remodeling genes including TIMP-2 and MMP9. We observed a moderate but not significant increase in cellular and mitochondrial superoxide levels in aged HSC in comparison with young HSC. In an interesting manner, analysis of vitamin A metabolism pathways showed alterations in aged livers; indeed, PNPLA3 expression seemed to be increased while CRBP-1 protein levels were diminished.

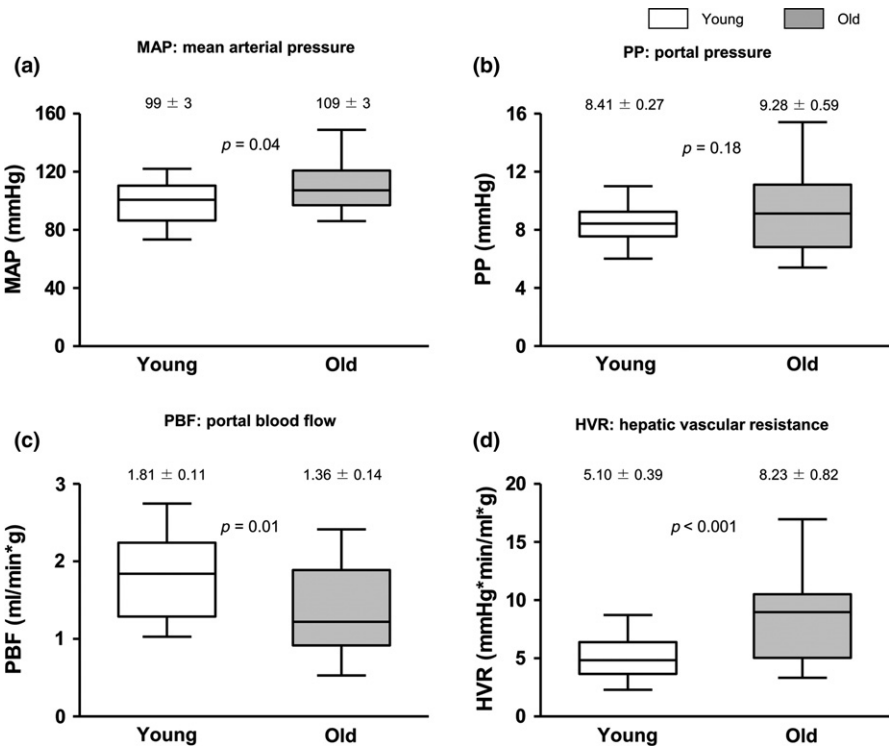


FIGURE 1 Hepatic and systemic hemodynamic of 3 months young and 20 months old rats. (a) Mean arterial pressure (MAP), (b) portal pressure (PP), (c) portal blood flow (PBF), and (d) hepatic vascular resistance (HVR) determined in 3 months young and 20 months old rats. *n* = 12 per group. Results represent mean ± *SEM*

2.6 | The aged hepatic sinusoid is in a moderate proinflammatory state

Characterization of the inflammatory status of the liver evidenced that the aged hepatic sinusoid is rather proinflammatory as suggested by elevated presence and activity of infiltrated myeloid cells including neutrophils, increased amount of CD68-positive macrophages, and diminished presence of CD163-positive cells (Figure 5a–c).

When we further analyzed the phenotype of freshly isolated aged KC, we observed an increase in the inflammatory cytokine IL-6, a key driver of liver regeneration, infection defense, and regulation of metabolic functions (Wuestefeld et al., 2013) without changes in the mRNA expression of other inflammatory molecules (Figure 5d).

2.7 | Bacterial translocation and intestinal inflammation are not affected in elderly rats

In comparison with young rats, aged animals did not present discrepancies in gut bacterial translocation to mesenteric lymph nodes, feces bacterial load, nor in the expression of the cytokines IFN-γ, TNFα, and IL17α measured in the ileum (data not shown). In contrast, a minor increase in LPS plasmatic levels was observed in aged rats (Table 1).

2.8 | The aged human liver displays similar features of a dysfunctional sinusoid

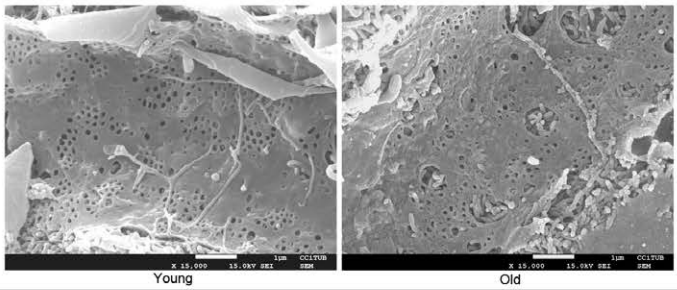
Characterization of the liver sinusoid in young and old human tissues corroborated most of the molecular changes observed in the animal model (Figure 6). Indeed, aged livers exhibited features of LSEC dedifferentiation as suggested by significant reductions in angiocrine and vasodilatory gene expression levels, together with partial activation of HSC (Supporting Information Figure S4). As expected, lipid metabolism and senescence pathways were increased in aged patients.

3 | DISCUSSION

The socioeconomic and medical care improvements occurring during the last decades have led to a relevant increase in the elderly, especially in developed countries (He et al., 2016). Therefore, it is critical to understand the molecular basis of aging and to identify possible approaches for therapeutic intervention in case major abnormalities

FIGURE 2 Endothelial dedifferentiation in the aged liver. The following markers of sinusoidal endothelial phenotype were analyzed in liver tissue from young and aged rats. (a) Representative scanning electron microscopy images and quantification of porosity, fenestration frequency, and fenestration diameter. (b) VEGFR2 protein expression normalized to GAPDH. (c) Representative images of CD32b immunohistochemistry and corresponding quantification. (d) Representative images of CD31 immunohistochemistry and corresponding quantification. (e) Representative transmission electron microscopy images and quantification of numbers of sinusoids. (f) Representative images of eNOS immunohistochemistry and corresponding quantification. (g) Representative western blots of eNOS and p-eNOS, and protein quantification normalized to GAPDH. (h) Levels of cyclic GMP. *n* = 3 (a and e) and *n* = 12 (other panels) per group. Results represent mean ± *SEM*. All images 400x, scale bar=50 μm

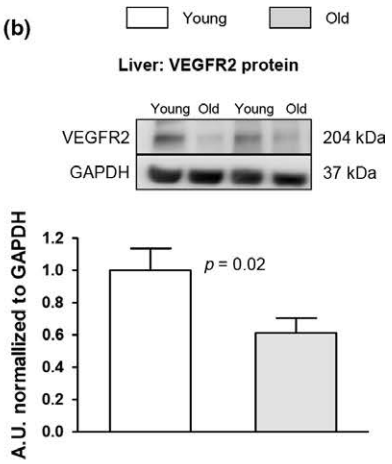
(a)



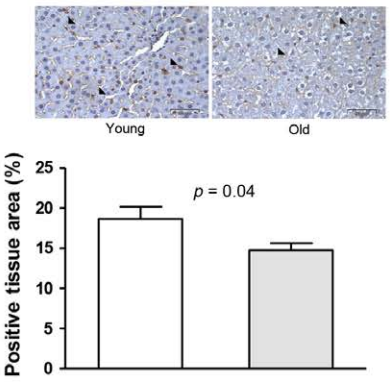
Young Old

Parameter	3 months Young	20 months Old	*p*-value
Porosity (%)	6.98 ± 0.85	4.83 ± 0.65	**0.03**
Fenestrae Frequency (number per μm²)	9.10 ± 0.89	7.91 ± 1.08	0.20
Fenestrae diameter (nm)	103.11 ± 15.77	79.46 ± 5.45	0.10

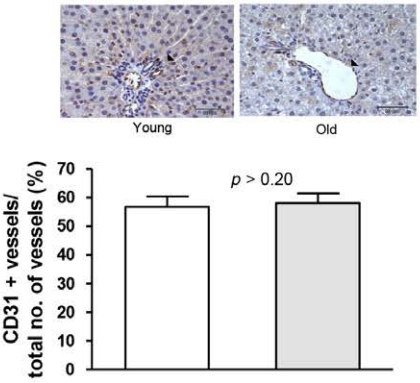
(b)



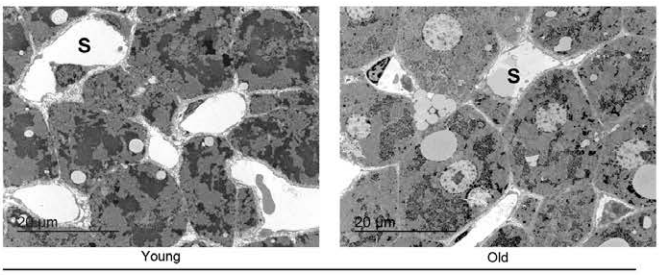
(c)



(d)

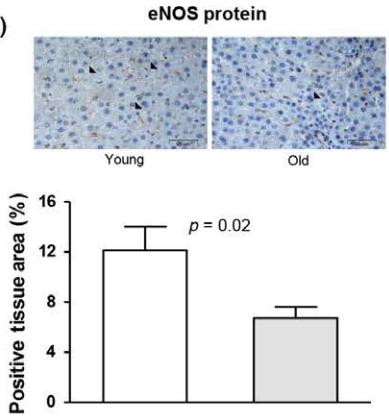


(e)

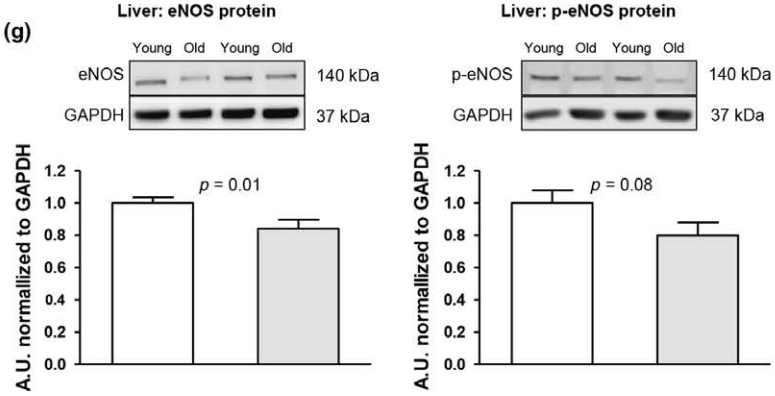


Parameter	3 months Young	20 months Old	*p*-value
No. Sinusoids	6.25 ± 0.30	5.39 ± 0.29	**0.04**

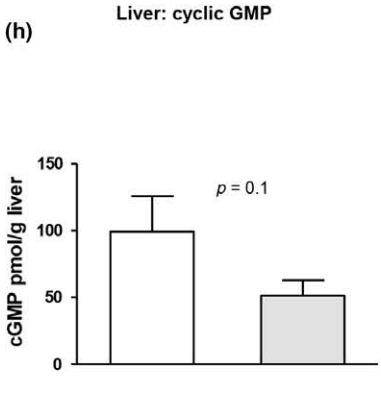
(f)



(g)



(h)



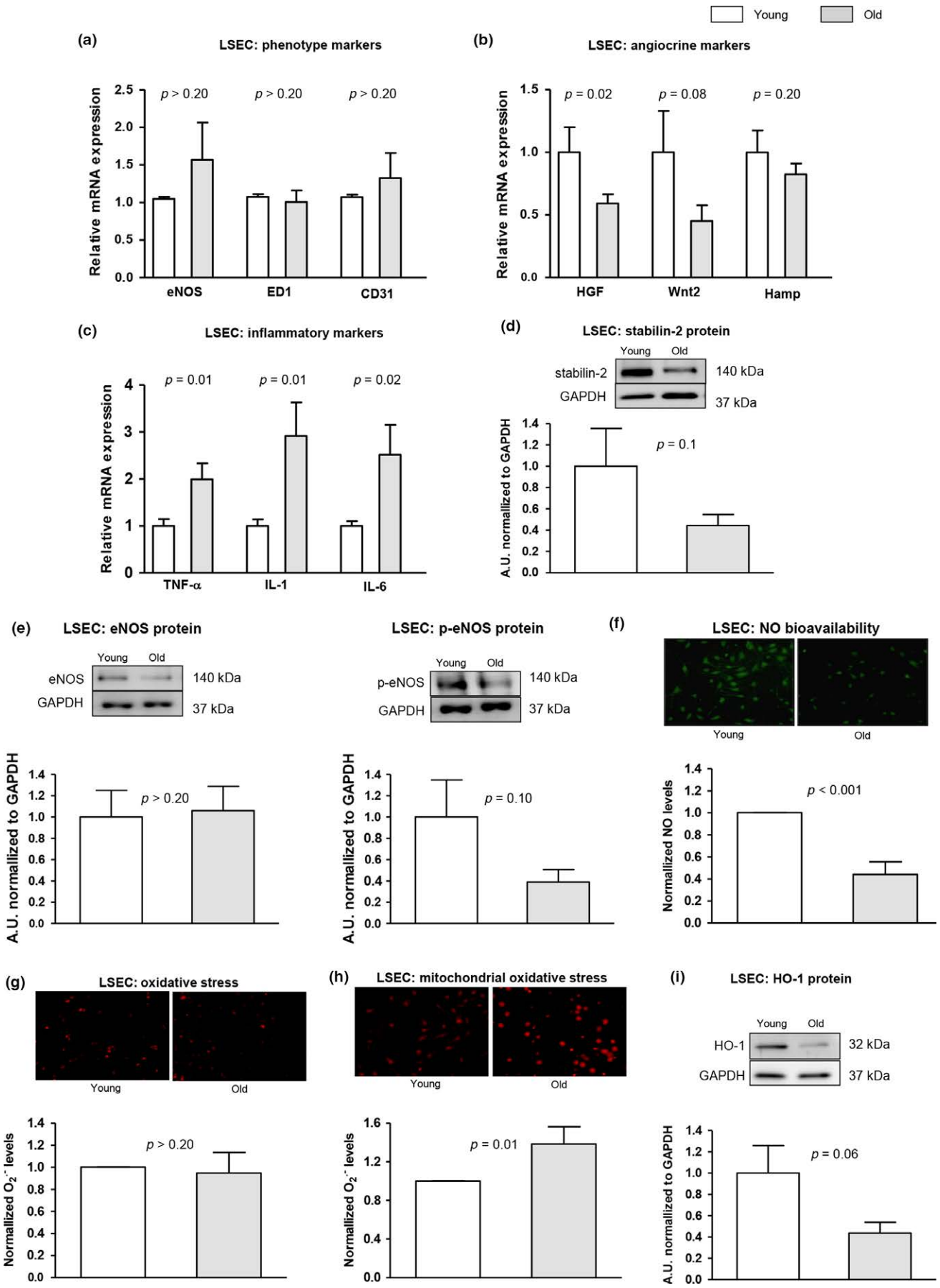

Young
Old
(a)
LSEC: phenotype markers
Relative mRNA expression
p > 0.20
p > 0.20
p > 0.20
eNOS
ED1
CD31
(b)
LSEC: angiocrine markers
Relative mRNA expression
p = 0.02
p = 0.08
p = 0.20
HGF
Wnt2
Hamp
(c)
LSEC: inflammatory markers
Relative mRNA expression
p = 0.01
p = 0.01
p = 0.02
TNF-α
IL-1
IL-6
(d)
LSEC: stabilin-2 protein
Young
Old
stabilin-2
GAPDH
140 kDa
37 kDa
A.U. normallized to GAPDH
p = 0.1
(e)
LSEC: eNOS protein
Young
Old
eNOS
GAPDH
140 kDa
37 kDa
A.U. normallized to GAPDH
p > 0.20
LSEC: p-eNOS protein
Young
Old
p-eNOS
GAPDH
140 kDa
37 kDa
A.U. normallized to GAPDH
p = 0.10
(f)
LSEC: NO bioavailability
Young
Old
Normallized NO levels
p < 0.001
(g)
LSEC: oxidative stress
Young
Old
Normallized O2- levels
p > 0.20
(h)
LSEC: mitochondrial oxidative stress
Young
Old
Normallized O2- levels
p = 0.01
(i)
LSEC: HO-1 protein
Young
Old
HO-1
GAPDH
32 kDa
37 kDa
A.U. normallized to GAPDH
p = 0.06

FIGURE 3 LSEC phenotype in aged livers. The phenotype of LSEC freshly isolated from aged and young rats was characterized as follows. (a) mRNA expression of eNOS, ED1, and CD31. (b) mRNA expression of HGF, Wnt2, and Hamp. (c) mRNA expression of TNF-α, IL-1, and IL-6. (d) Representative western blot of stabilin-2 normalized to GAPDH. (e) Representative western blots of eNOS and p-eNOS, normalized to GAPDH. (f) Representative images of nitric oxide bioavailability using DAF-FM staining (green fluorescence) and corresponding quantification. (g) Levels of $O_2^{\cdot-}$ (red fluorescence) determined using DHE. (h) Representative images of mitochondrial $O_2^{\cdot-}$ measured using mitosox (red fluorescence) and corresponding quantification. (i) HO-1 protein expression normalized to GAPDH. *n* = 6 per group. Results represent mean ± *SEM*

are detected (Longo et al., 2015; Mitchell, Morten, Longo, & Cabo, 2015). Although previous studies described the impact of aging on the vasculature of different territories, in the specific field of Hepatology, very little is known about the liver microcirculatory function and the molecular status of hepatic sinusoidal cells in aging (Le Couteur & McLean, 1998; Le Couteur et al., 2001).

In the present study, we demonstrate for the first time that healthy aged rats exhibit a significant increase in the hepatic vascular resistance, which leads to reduced liver perfusion and to a mo-derate increment in portal pressure. Although a reduction in portal blood inflow was previously reported in humans and rodents (Vollmar et al., 2002), we herein describe a deregulation in the intra-hepatic vascular system due to diverse molecular changes in the cells of the hepatic sinusoid, together with deterioration in hepatocyte function.

Analysis of hepatocytes revealed mild liver dysfunction in aged animals, which could likely be a consequence of altered oxygen and nutrient supply following the alterations in the vascular system. We observed a reduction in the liver-body weight ratio, which was accompanied by a significant decline in synthetic and detoxifying capabilities as demonstrated by reduced bile formation and decreased urea and albumin synthesis, together with dysfunctional expression of hepatocyte transporters. These findings agree with previous reports (Le Couteur & McLean, 1998; López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013; Tietz, Shuey, & Wekstein, 1992), altogether suggesting a decrease both in the amount and functional capacity of hepatocytes in aging, therefore advising a potential enhanced susceptibility to secondary insults such as ischemia/reperfusion or drug-induced liver injury. From a clinical perspective, it is important to note that these events are especially common in elderly population, which have the higher prevalence of circulatory diseases and receive multiple medications with potential hepatotoxicity.

Underlying mechanisms explaining hepatocyte dysfunction may relate to adaptations following vascular dysfunction, to other age-derived intracellular deregulation, or to cell senescence per se. Senescence, a cellular mechanism imposed by natural exhaustion of cell cycle or by stress-induced signals, was evident in aged animals as demonstrated by up-regulation in p16 along with a reduction in telomere length and SIRT1 protein. In addition, we observed an increase in hepatic oxidative stress, with multiple harmful consequences, that may derive from an imbalance between pro- and antioxidants, together with increased hepatic lipid content, a well-known source of cellular oxidative stress. In an important way, oxidative stress could also contribute to potentiate the senescence pathways, and vice versa, thus creating a deleterious vicious cycle in the aged liver (Jin, Iakova, Jiang, Medrano, & Timchenko, 2011; Sharpless & De Pinho, 2004).

Characterization of liver microcirculatory dysfunction in aged animals confirmed LSEC "pseudo-capillarization," which was defined by the decrease in the number and size of fenestrae (Le Couteur et al., 2001). Further analysis revealed a global deregulation in the hepatic endothelial phenotype in aging as demonstrated by significant decrease in key vasodilatory pathways, including nitric oxide and heme oxygenase, increment in intracellular inflammation and oxidative stress, and reduction in the expression of functional and angiocrine markers such as stabilin-2, CD32b (Xie et al., 2013), and VEGFR2 (Carpenter et al., 2005). Reduction in intrahepatic nitric oxide availability is of relevance considering its importance modulating the vascular tone (Gracia-Sancho, Maeso-Diaz, Fernandez-Iglesias, Navarro-Zornoza, & Bosch, 2015; Hori, Wiest, & Groszmann, 1998), exerting anti-inflammatory effects (Iwakiri & Kim, 2015), and maintaining neighboring cells phenotype (Marrone et al., 2016; Xie et al., 2013). Reduced nitric oxide bioavailability might, at least in part, derive from diminished eNOS activity, which may be due to reduced VEGF-p-eNOS pathway (Kroll & Waltenberger, 1998), and from increased scavenging due to elevated oxidative stress (Gracia-Sancho et al., 2008). An additional mechanism contributing to VEGF-p-eNOS pathway depletion may be the increase in the senescence marker p16, which exerts an inhibitory effect on VEGF (Kawagishi et al., 2010). Nevertheless, a previous study reported no changes in hepatic VEGF expression in aging (Le Couteur et al., 2007). Therefore, this issue would require further investigation to confirm the current findings.

Similar to nitric oxide, the observed diminution in HO-1 expression in the aged endothelium may contribute to the increased HVR (Van Landeghem et al., 2009). Altogether our results strongly suggest that vasodilatory pathways in LSEC are profoundly affected by age and this might be one of the mechanisms responsible of aged-associated increase in HVR.

A previous report described increased number of fatty HSC in the aged liver (Warren et al., 2011); however, no comprehensive analysis of stellate cells phenotype in aging has been conducted. Herein, we confirm lipid accumulation within aged HSC and additionally describe increments in other mediators of HSC activation including intracellular oxidative stress and p16 (Bataller et al., 2003). In fact, analysis of cellular phenotype revealed a slight, but significant, activation of HSC, supported by increments in different activation markers including α-SMA or collagen I. Underlying mechanisms explaining HSC activation in aging revealed no modification in different proinflammatory mediators including TLR4, NFκB, and TGFβ (data not shown), but interestingly suggested alterations in retinoid

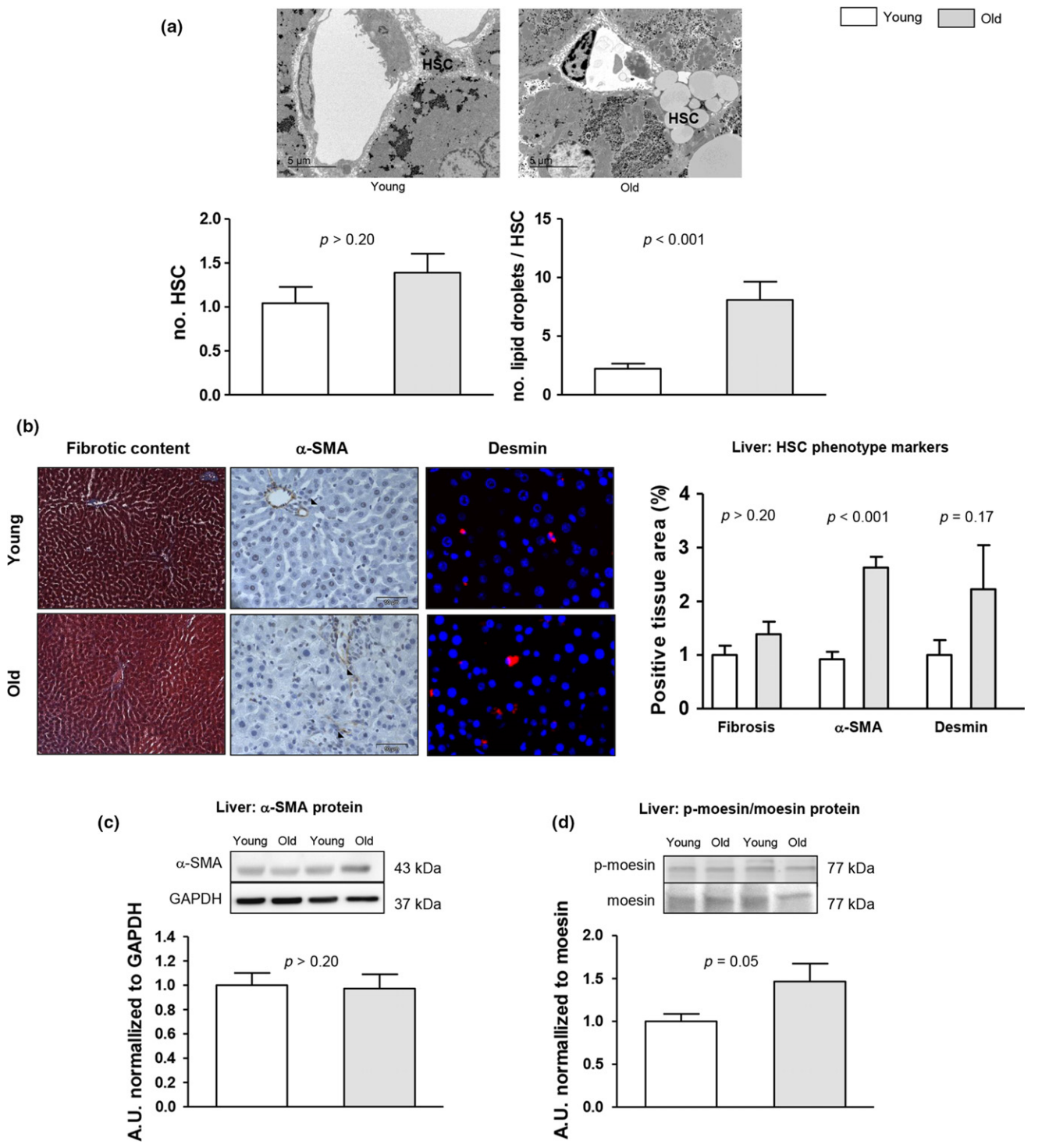


FIGURE 4 Effects of aging on HSC phenotype. The phenotype of HSC was characterized in aged and young rats as follows. (a) Quantification of HSC abundance and intracellular lipid droplets in livers using transmission electron microscopy. (b) Representative images of fibrotic content, measured as positive, are for Masson's trichromic, α-SMA, and desmin with their corresponding quantifications. (c) α-SMA protein expression in total liver tissue, normalized to GAPDH. (d) p-moesin protein expression in total liver tissue, normalized to total moesin. (e) Expression of α-SMA, collagen1α1, collagen1α2, and PDGFRβ in freshly isolated HSC. (f) Expression of TIMP1, TIMP2, MMP2, and MMP9 in freshly isolated HSC. (g) Representative images of $O_2^{\cdot-}$ levels in primary HSC (red fluorescence) and corresponding quantification. (h) Mitochondrial $O_2^{\cdot-}$ levels (red fluorescence) determined in primary HSC. (i) Expression of PNPLA3 in total liver tissue. (j) CRBP1 protein expression in total liver tissue, normalized to GAPDH. n = 3 (a), n = 12 (b–d, i–j), and n = 6 (e–h) per group. Results represent mean ± *SEM*. All images 400×, scale bar = 50 µm

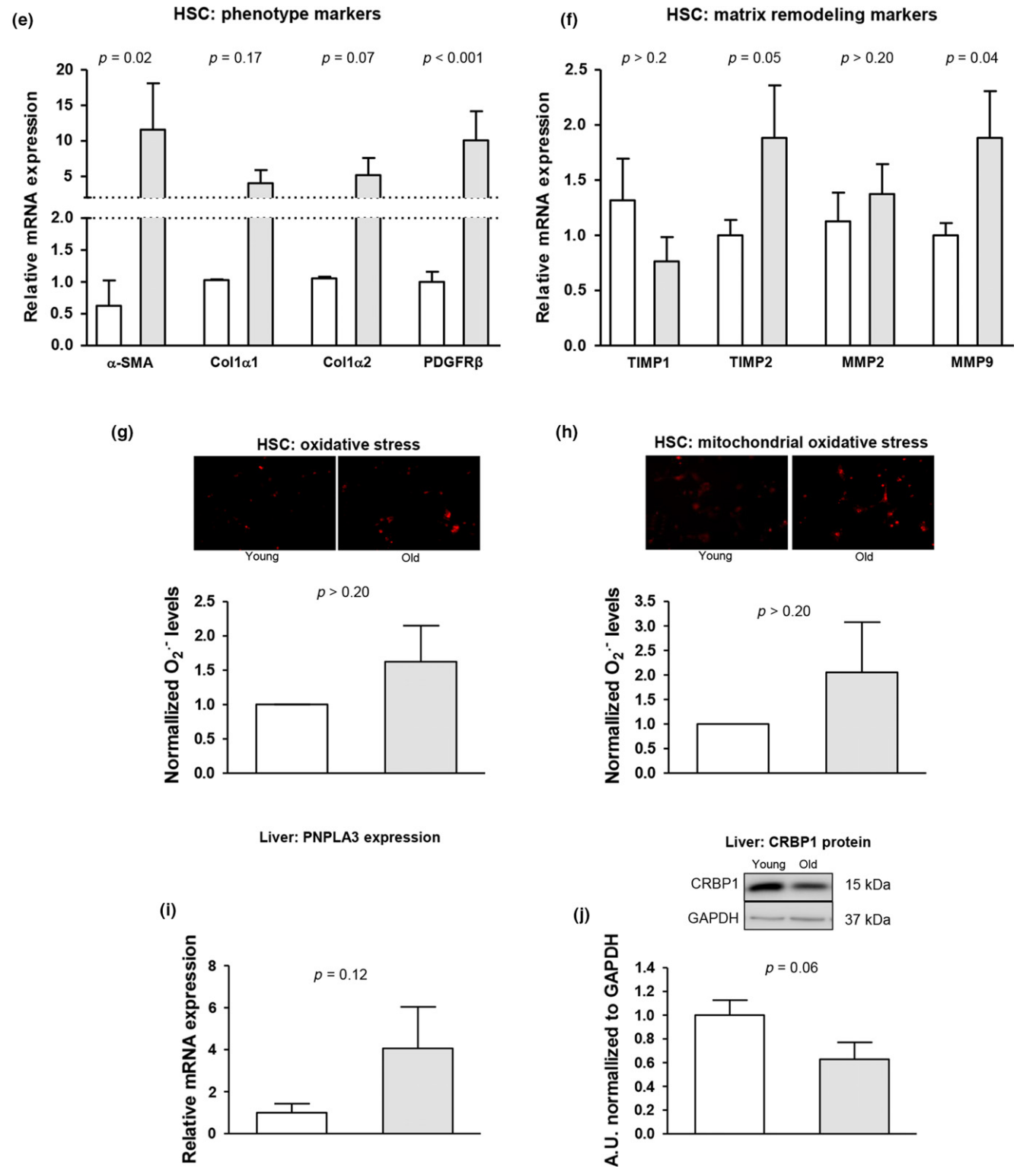


FIGURE 4 Continued

metabolism and breakdown. In fact, aged livers exhibited down-regulated CRBP-1 together with over-expressed PNPLA3, which have been associated with HSC activation and susceptibility to develop steatohepatitis (Bruschi et al., 2017; Uchio et al., 2002).

Very few data regarding inflammation and KC phenotype in aging are available, and the data are inconclusive. For instance, while one study reported that KC lose their phagocytic capacity with aging (Brouwer & Knook, 1983), a subsequent study reported increased phagocytic activity in KC from aged animals (Hilmer, Cogger, & Le Couteur, D. G., 2007). In our evaluation, we noticed an increase in the recruitment of proinflammatory cells with a concomitant increment in inflammatory markers, altogether suggesting that aging may

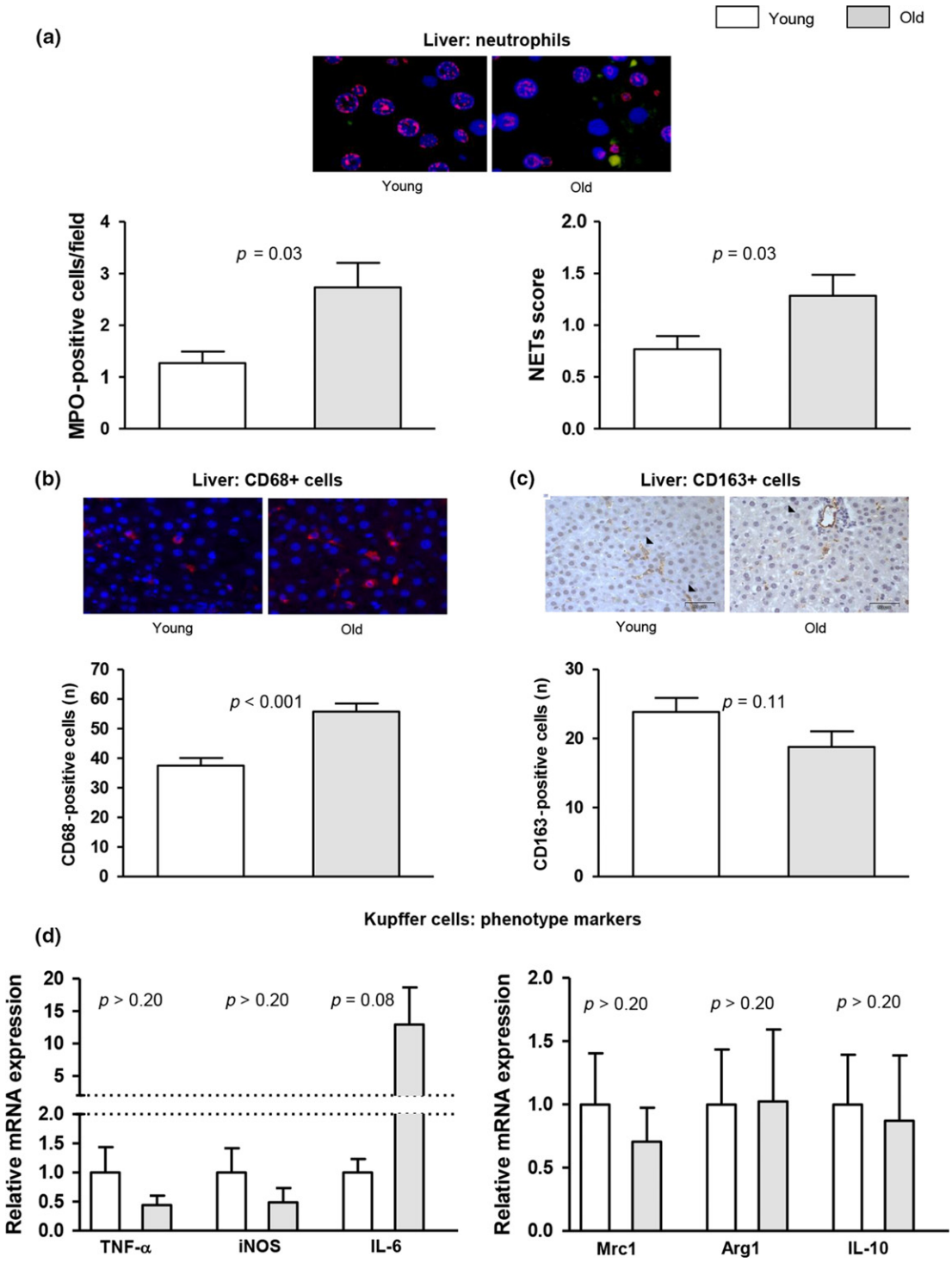


FIGURE 5 Innate immune cell activation and inflammation in aging. (a) Representative images of neutrophil immunofluorescence in liver tissue (MPO in green, histone 2B in red, nuclei in blue). *Left*, neutrophil infiltration measured as MPO-positive cells. *Right*, analysis of hepatic neutrophil extracellular traps (NETs) determined as colocalization of MPO and histone 2B. (b) Representative images of CD68 immunofluorescence in liver tissue and corresponding quantification. (c) Representative images of CD163 immunohistochemistry in liver tissue and its quantification. (d) Expression of TNF-α, iNOS, IL-6, Mrc1, Arg1, and IL-10 in Kupffer cells freshly isolated from young and old rats. n = 12 (a-c) and n = 6 (d) per group. Results represent mean ± *SEM*. All images 400×, scale bar = 50 μm

promote a moderate proinflammatory status in the liver. Kupffer cells isolated from aged rats displayed high IL-6 levels, which could possibly reflect beneficial effects of IL-6 on liver regeneration, infection defense, and regulation of metabolic functions (Schmidt-Arras & Rose-John, 2016), while traditional "polarization markers" of hepatic macrophages (Krenkel & Tacke, 2017) were not dramatically

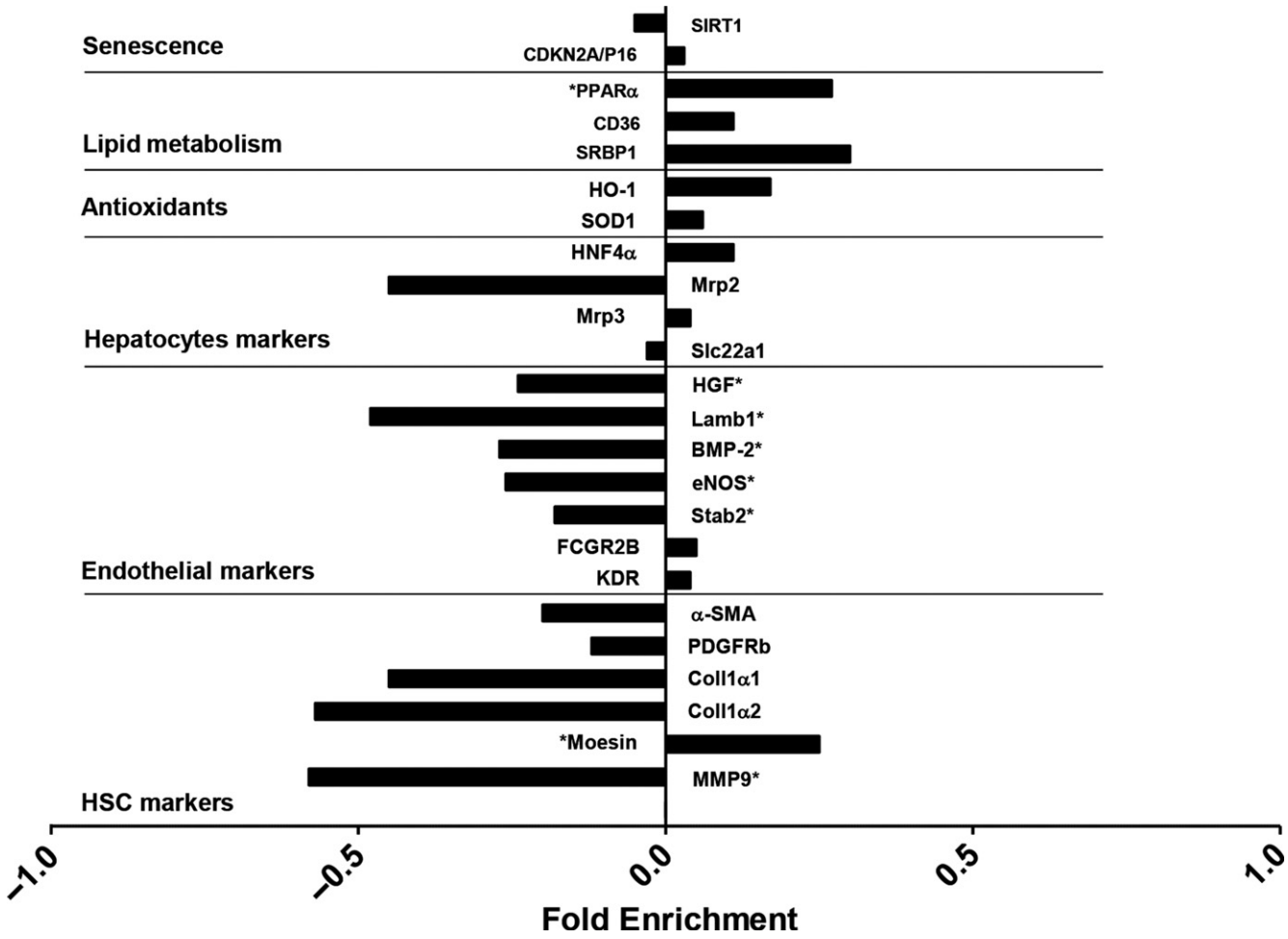


FIGURE 6 Aged-related changes in the human liver. Gene expression analysis in healthy young and old human livers. Data expressed as fold enrichment (log_2) old relative to young (0.0). The fold enrichments are plotted using positive values for transcripts that are increased or using negative values for transcripts that are decreased in old humans. *p-value <0.05, n = 14 young and 13 old livers. Clinical characteristics of donors are described in Supporting Information Table S2

altered. However, given the complexity of inflammation and immune cell subsets, further investigations are required to define, which inflammatory processes are functionally relevant during aging.

The translocation of bacterial products from the gut is the consequence of intestinal barrier disruption and may lead to bacterial infection and have consequences in terms of liver and systemic inflammation and HSC activation. Considering the hemodynamic and inflammatory changes observed in aged animals, we aimed at analyzing bacterial translocation, fecal bacterial load, intestinal inflammation, and endotoxemia in old rats. In an interesting manner, these analyses revealed no significant differences in most of these parameters when comparing both groups of rats, except for LPS plasmatic levels that were increased in aged rats. We therefore cannot discard that the observed minimal aged-related endotoxemia may contribute to the abnormalities of sinusoidal cells.

At last, and although an undeniable degree of heterogeneity was found in each group analyzed, characterization of liver biopsies from healthy young and old humans confirmed the overall trend of pathway deregulation, therefore suggesting that sinusoidal vulnerability hereby described in old rats is also of relevance in human aging.

Many efforts are currently being directed toward the development of novel therapeutic approaches for portal hypertension (Gracia-Sancho et al., 2015). Most preclinical studies on the effects of different substances and drugs on the liver circulation have been conducted in young animals. Our current work revealed striking differences between young and aged liver, including alterations in vasoactive pathways, indicating that studies performed in young animals may not entirely reflect what should be expected from the cli-nical use of most interventions, as vasoactive drugs are and will be mainly prescribed in the elderly.

We are aware that our study is descriptive in nature. However, this work represents an important cornerstone characterization of liver aging. While single variables have been examined previously, no preceding report fully described the liver microcirculatory phenotype in a preclinical model of healthy aging, additionally including validation in human samples. These data provide important evidence for the changing biology of the liver in aging and create new research avenues to comprehend the effects of acute or chronic liver injury in the elderly. It is important to acknowledge that aged animals exhibited some degree of steatosis; therefore, part of the results could be attributable to intracellular fat instead of age. Nevertheless, a

subanalysis of animals without evidence of significant steatosis confirmed the same hemodynamic and molecular results than in the group of animals exhibiting steatosis (data not shown), therefore ensuring that the results of this study describe the effects of aging per se on liver microcirculatory phenotype.

In conclusion, the present study demonstrates for the first time that aging is accompanied by significant liver sinusoidal deregulation, both in rodents and humans, suggesting sinusoidal vulnerability in front of subsequent chronic or acute injuries.

4 | EXPERIMENTAL PROCEDURES

A complete description of Materials and Methods can be found in Appendix S1.

4.1 | Animal model

Male Wistar rats at the age of 20 months old were used to evaluate aging and compared to young animals of 3 months old (n = 12 animals per group) (Steppan et al., 2014; Wang, Wehling-Henricks, Samengo, & Tidball, 2015). Animals were kept in environmentally controlled animal facilities at IDIBAPS. All procedures were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with the European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/609).

4.2 | *In vivo* hemodynamic measurements

Mean arterial pressure (MAP), portal pressure (PP), and portal blood flow (PBF) were measured in old and young rats using microcatheters and flow probes (Marrone et al., 2015). Hepatic vascular resistance (HVR) was calculated as PP/PBF.

4.3 | Hepatic cells isolation

Hepatocytes, Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC), and hepatic stellate cells (HSC) were isolated using well-established protocols (De Mesquita et al., 2017; Gracia-Sancho et al., 2007). Only highly pure and viable cells were used.

4.4 | Electron microscopy

Liver sinusoidal ultrastructure was characterized using electron microscopy as previously described (Le Couteur et al., 2001).

4.5 | Histological analysis

Liver samples were fixed in 10% formalin, embedded in paraffin, sectioned, and slides were stained with hematoxylin and eosin (H&E) to analyze the hepatic parenchyma (Hide et al. 2016), with Masson's trichrome for liver fibrosis evaluation (Gracia-Sancho et al., 2011), with oil red O for lipid quantification or with corresponding antibodies for immunohistochemistry (IHC) or immunofluorescence (IF; Marrone et al., 2015).

4.6 | Cell death

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed in deparaffinized liver sections using an In Situ Cell Death Detection Kit (Roche Diagnostics, Sant Cugat del Valles, Barcelona, Spain) according to the manufacturer's instructions (Hide et al., 2014).

4.7 | Nitric oxide and superoxide determinations

Levels of cGMP, marker of nitric oxide bioavailability, were analyzed in liver homogenates using an enzyme immunoassay following manufacturer instructions (Cayman Chemical Co., Ann Arbor, MI) (Gracia-Sancho et al., 2008). In situ superoxide and nitric oxide levels in cells were assessed with the oxidative fluorescent dye dihydroethidium (DHE 10 μM; Molecular Probes Inc., Eugene, OR) or with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA 10 μM; Molecular Probes Inc.), respectively, as described (Hide et al., 2014). Fluorescence images were obtained with a fluorescence microscope (Olympus BX51, Tokyo, Japan), and quantitative analysis of at least 20 images per condition containing equivalent number of cells was performed with Image J 1.44m software.

4.8 | Human liver samples collection for mRNA analysis

Two-by-two cm wedge sharp cuts were obtained from the liver from donors after brain death without the use of electrocautery, once the donor next of kin provided written informed consent (Document R-DON-049 02/15). Biopsies were immediately stored in diethyl pyrocarbonate solution, a nuclease inhibitor, for mRNA analysis. Ethics Committee of the Barcelona Hospital Clinic approved the experimental protocol (HCB/2011/6499). RNA extraction and gene expression analysis were performed as described in Supporting Information Appendix S1 (Experimental Procedures). Experimental groups were defined considering donors' age: young (n = 14, mean age 28 ± 2 years old, range 19–38) and old (n = 13, mean age 76 ± 0.8, range 71–85). No differences in other clinical parameters were found comparing groups (Supporting Information Table S2).

4.9 | Statistical analysis

Statistical analysis was performed with the SPSS for Windows statistical package (IBM, Armonk, New York, USA). All results are expressed as mean ± standard error of the mean (*SEM*). Comparisons between groups were performed with Student's t test. Differences were considered significant at a p value <0.05.

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CONFLICT OF INTEREST

Authors have no conflict of interest.

AUTHORS CONTRIBUTIONS

R.M.-D. designed the research, performed experiments, analyzed data, and wrote the manuscript. M.O.-R., A.F.-I., D.H., L.M., S.V., and R.F. performed experiments and analyzed data. A.H. and C.F. procured human liver tissue for the study, interpreted data and critically revised the manuscript. A.A., C.P., J.B., F.T., and V.C. interpreted data and critically revised the manuscript. J.G.-S. conceived the study, designed and directed the research, analyzed and interpreted data, wrote the manuscript, and obtained funding. All authors edited and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Effects of aging on liver microcirculatory function and sinusoidal phenotype

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Supplementary Experimental Procedures

In vivo hemodynamic

Rats were anesthetized with ketamine (100 mg/kg body weight, Imalgene 1000; Merial) plus midazolam (5 mg/kg body weight; Laboratorio Reig Jofre, S.A., Spain) intraperitoneally, fastened to a surgical board, and maintained at a constant temperature of 37 ± 0.5ºC.

A tracheotomy and cannulation with a PE-240 catheter (Portex) was performed in order to maintain adequate respiration during anesthesia. Indwelling catheters made of polyethylene tubing (PE-50; Portex, UK) were placed into the femoral artery to measure mean arterial pressure (MAP; mm Hg) and heart rate (HR; beats per minute), and to the ileocolic vein to measure PP (mmHg). PBF (mL/min) was measured with a nonconstrictive perivascular ultrasonic transit-time flow probe (2PR, 2-mm diameter; Transonic Systems Inc., USA) placed around the portal vein just before its entrance in the liver, avoiding the measurement of most portal-collateral blood flow. The flow probe and pressure transducers were connected to a Powerlab (4SP) linked to a computer using Chart v5.5.6 for Windows software (AD Instruments, Australia). Hepatic vascular resistance (HVR) was calculated as PP/PBF. Hemodynamic data were

collected after a 20-minute stabilization period (Gracia-Sancho et al. 2007). At the end of the in vivo hemodynamic study, serum samples from young and aged-rats were collected to subsequently evaluate alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, and albumin, all by standard protocols. Experiments and data collection were performed blindly.

Hepatic cells isolation

Hepatocytes, Kupffer cells (KC) and Liver Sinusoidal Endothelial Cells (LSEC) were isolated using a well established protocol (Gracia-Sancho et al. 2007). Rat livers were perfused through the portal vein with Hanks without Ca^{+2} and Mg^{+2} containing 12 mM hepes (H3375, Sigma) pH 7.4, 0.6 mM ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (E4378, Sigma) and 0.23 mM bovine serum albumin (BSA; A1391,0100, Applichem). Then, perfused for 30 min with 0.015% collagenase A (103586, Roche) Hanks containing 12 mM hepes (pH 7.4) and 4 mM $CaCl_2$. The resultant digested liver was excised and *in vitro* digestion was performed at 37°C with 0.01% collagenase A, Hank's containing 12 mM hepes (pH 7.4) and 4 mM $CaCl_2$ for 10 min. Disaggregated tissue was filtered using 100 µm nylon strainer, collected in cold Krebs' buffer and centrifuged at 50 g for 5 min. The pellet was washed three times for hepatocytes enrichment. Hepatocytes were seeded in collagen-coated wells and cultured in Dulbecco's Modified Eagle's Medium (DMEMF12; 11320074, Gibco) supplemented with 2% fetal bovine serum (04-001-1A, Reactiva), 1% L-glutamine (25030-024, Gibco), 1% penicillin plus 1% streptomycin (03-331-1C, Reactiva), 1 nM dexamethasone (D4902, Sigma), 1 µM insulin (103755, HCB) and 1% amphotericin B (03-029-1C, Reactiva). The supernatant was centrifuged at 800g for 10 min and the obtained pellet was resuspended in

Dulbecco's PBS (DPBS) and centrifuged at 800g through a two-step Percoll gradient (25-50%). The interface of the gradient was enriched in KC and LSEC. This cell fraction was diluted in DPBS and centrifuged at 800g. The cell pellet was resuspended in RPMI medium, seeded in plastic dishes and incubated for 30 min at 37ºC in humid atmosphere with 5% CO_2 in order to enhance KC purity. Non-adherent cells were seeded in collagen-coated wells and incubated for 1h (37ºC, 5% CO_2). After this time the medium was discarded and LSEC adhered cells were washed twice with DPBS and cultured in RPMI-1640 (01-100-1A, Reactiva) supplemented with 10% fetal bovine serum (04-001-1A, Reactiva), 1% L-glutamine (25030-024, Gibco), 1% penicillin plus 1% streptomycin (03-331-1C, Reactiva), 0.1 mg/ml heparin (H3393, Sigma), 0.05 mg/ml endothelial cell growth supplement (BT-203, BT) and 1% amphotericin B (03-029-1C, Reactiva). HSC were isolated through a sequential in situ perfusion of the liver with 0.195 mg/ml collagenase A (Roche), 1.5 mg/ml pronase (Roche) and 0.05 mg/ml Dnase (Roche) in Gey's Balanced Salt Solution (GBSS; Sigma), and dispersed cells were fractionated by density gradient centrifugation using 11.5% Optiprep (Sigma) (De Mesquita et al. 2017). HSC were cultured in Iscove's Modified Dulbecco's Media (IMDM, Invitrogen, Gibco) supplemented with 10% fetal bovine serum (04-001-1A, Reactiva), 1% L-glutamine (25030-024, Gibco), 1% penicillin plus 1% streptomycin (03-331-1C, Reactiva) and 1% amphotericin B (03-029-1C, Reactiva). Viability and purity were systematically over 95%.

Electron microscopy

Liver sinusoidal ultrastructure was characterized using electron microscopy as previously described (Le Couteur et al. 2001). Briefly, livers were perfused through the portal vein with a fixation solution containing 2.5% glutaraldehyde

and 2% paraformaldehyde in 0.1M cacodylate buffer 0.1% sucrose and fixed overnight at 4ºC. Samples were washed 3 times with 0.1M sodium cacodylate buffer. Liver sections were post-fixed with 1% osmium in cacodylate buffer and dehydrated in an ethanol gradient to 100%.

For scanning electron microscopy 6 to 8 liver blocks per sample were mounted on stubs, sputter coated with gold and examined using a Jeol 6380 scanning electron microscope. Measurements of fenestrae size, number and density were carried out. Fenestrations were defined as open pores with diameters <300nm. Diameter was defined as the major length of each fenestration or gap. Porosity was defined as the sum area of fenestrations/total quantified area. Frequency was defined as number of fenestrae per μm^2. At least 10 images per animal were taken.

For transmission electron microscopy, fixed liver tissue was embedded in Spurr resin, cut in 50nm ultrathin sections, counterstained with uranyl acetate and lead citrate and examined using microscope. 10 micrographs per sample were taken to estimate % of necrotic hepatocytes and % of sinusoids presenting each of the evaluated parameters.

RNA isolation and quantitative PCR

RNA from cells and tissue were extracted using RNeasy mini kit (Qiagen) and Trizol (Life Technologies), respectively. RNA quantification was performed using a NanoDrop spectrophotometer. cDNA was obtained using QuantiTect reverse transcription kit (Qiagen). Real-Time PCR was performed in an ABI PRISM 7900HT Fast Real-Time PCR System, using TaqMan predesigned probes for HNF4α (Rn04339144_m1), Slcc22a1 (Rn00562250_m1), Mrp2 (Rn00563231_m1), Mrp3 (Rn01452854_m1), eNOS (Rn02132634_s1), ED1

(Rn00561129_m1), PECAM1 (Rn01467262_m1), Col1A1 (Rn01463848_m1), α-SMA (Rn01759928_g1), PDGFRβ (Rn01491838_m1), TNF-α (Rn01525859_g1), iNOS (Rn00561646_m1), IL-1 (Rn00580432_m1), IL-6 (Rn01410330_m1), Mrc1 (Rn01487342_m1), Arg1 (Rn00691090_m1), IL-10 (Rn00563409_m1), HGF (Rn00566673_m1), Wnt2 (Rn01500736_m1), Hamp (Rn00584987_m1), TIMP1 (Rn00587558_m1), TIMP2 (Rn00573232_m1), MMP2 (Rn01538170-m1), MMP9(Rn00579162_m1), PNPLA3 (Rn01502360_m1), TGF-β (Rn_01475963_m1) and GAPDH (Rn01775763_g1) as endogenous controls. Results, expressed as $2^{-\Delta\Delta Ct}$, represent the x-fold increase of gene expression compared with the young group.

Telomere length measurement by quantitative PCR

Telomere length was measured in the genomic DNA isolated and purified from liver tissue samples using DNeasy mini kit (Qiagen). All DNA samples were tested for purity and integrity using a Nanodrop 2000 spectrophotometer. Telomere length measurement was performed using a quantitative PCR-based method with slight modifications (Cawthon 2002). Briefly, 50 ng of DNA was mixed with either the telomere or AT-1 (single-copy gene) primer and the Power SYBR Green PCR Master Mix reagent followed by qPCR Real-Time on ABI PRISM 7900HT Fast Real-Time PCR System. The primer sequences used were: (5'→3'): T1, GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT T2, TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA; AT-1 1 ACGTGTTCTCAGCATCGACCGCTACC, AT-1 2 AGAATGATAAGGAAAGGGAACAAGAAGCCC. The relative telomere length was calculated as the ratio of telomere repeats to AT1 (a single-copy gene) (T/S ratio).

Western Blotting

Liver samples were processed and western blot performed as described (Guixe-Muntet et al. 2016). Used primary antibodies: P16 (554079, BD Pharmagen), SIRT1 (ab110304, Abcam), VEGFR2 (sc-315, Santa Cruz Biotech), HO-1 (ADI-SPA-896, Enzo), phosphorylated eNOS at Ser1177 (9571, Cell Signaling), total eNOS (610297, BD Transduction Laboratories), phosphorylated Moesin at Thr558 (sc-12895, Santa Cruz), total Moesin (sc-13122, Santa Cruz), stabilin-2 (MABC76, Merck Milipore), CRBP1 (sc-271208, Santa Cruz), NFκB (6956, cell signalling) and TLR4 (sc-293072, Santa Cruz) all 1:1000. Blots were revealed by chemiluminescence and protein expression was determined by densitometric analysis using the Science Lab 2001 Image Gauge (Fuji Photo Film, Düsseldorf, Germany). Blots were also assayed for GAPDH (1:5000, Sigma-Aldrich) content as standardization of sample loading.

Histological Analysis

Liver samples were fixed in 10% formalin, embedded in paraffin, sectioned, and slides were stained with hematoxylin and eosin (H&E) to analyze the hepatic parenchyma (Hide et al. 2016), or with Masson's trichrome for liver fibrosis evaluation (Gracia-Sancho et al. 2011). Ten fields per slide were randomly taken and two independent researchers scored the hepatic histology using a semi-quantitative method (Jiménez-Castro et al. 2015), with minor modifications. Briefly, cytoplasmic vacuolation, nuclear pyknosis, cytoplasmic hypereosinophilia, loss of intercellular borders, necrosis and fat accumulation were scored as 0-abstent, 1-focal or 2-general, and neutrophil infiltration as 0-absent or 1-present, making a maximum final score of 13 points. Liver fibrosis

was quantified as the purple-stained area per total area using the Axiovision software (De Mesquita et al. 2017).

Frozen sections were cut to 10 µm and stained with Oil Red O (Sigma Aldrich) for lipid analysis. Lipid droplets were evaluated as the red-stained area per total area using ImageJ software.

Immunohistochemistry

Liver samples were fixed in 10% formalin, embedded in paraffin, sectioned and processed for immunohistochemistry (IHC) or immunofluorescence (IF) as previously described (Marrone et al. 2015).

For IHC liver sections were incubated with antibodies against CD32b (sc-13271, Santa Cruz), CD31 (sc-376764, Santa Cruz), eNOS (sc-654, Santa Cruz), α-SMA (Dako), or CD163 (MCA342R, Biorad). After incubation with corresponding secondary antibodies, color development was induced by incubation with a DAB kit (Dako) and counterstained with hematoxylin. Sections were dehydrated and mounted. The specific staining was visualized and fifteen images per liver were acquired using a microscope equipped with a digital camera and the assistance of Axiovision software. The relative volume was calculated by dividing the number of points positive in sinusoidal areas by the total number of points over liver tissue (Vilaseca et al. 2017).

For IF, liver sections were incubated with antibodies against desmin (M0760, Dako), CD68 (MCA341R, Biorad), MPO (ab9535, Abcam) and H2B (ab52484, Abcam), incubated with secondary antibodies Alexa Fluor 488 or 555 (1:400, Life technologies) and 4′,6-diamino-2-fenilindol (1;3000; DAPI, Sigma-Aldrich) and mounted in Fluoromount G medium. Ten images per sample were obtained

with a fluorescence microscope and percentage of positive area (Desmin) or positive cells per field (CD68, MPO/H2B) were quantified.

Albumin and urea production

Culture media from all experimental conditions after 24h of culture were sampled. Albumin and blood urea nitrogen (BUN) were measured using standard methods at the Hospital Clínic of Barcelona's CORE laboratory. BUN values were converted to urea as 2.1428 mg/dl BUN =1 mg/dl urea.

Cytochrome 4503A4 activity

Phase I detoxification capacity of hepatocytes was analyzed using P450-Glo™ CYP4503A4 Assay following manufacturer's instructions (V8901, Promega). Briefly, hepatocytes cultured for 24 hours were rinsed twice with DPBS and incubated with culture media containing 50 µM Luciferin-PFBE at 37°C for 4h. Then supernatant was collected and neutralized with Luciferin Detection Reagent. After incubation for 30 min at RT, plate luminescence was read in a luminometer (Orion II Microplate Luminometer, Germany). Samples luminescence was corrected subtracting background luminescence.

Conventional bacterial study of feces and mesenteric lymph nodes (MLN)

Samples of MLN and stool were plated on McConkey and blood agar (Materlab, Madrid, Spain) and inoculated in thioglycholate (Scharlab, Barcelona, Spain) for 24-48 hours at 37ºC. Specific microorganisms were identified by a manual biochemical test or automated system (Microscan, Baxter, Irvine, CA). GBT was defined by a positive bacteriological MLN culture (Úbeda et al. 2016).

Endotoxemia quantification

Endotoxemia was quantified using LAL Chromogenic Endotoxin Quantitation Kit (ThermoFisher), following manufacturer's instructions.

qPCR of cytokines in the ileum

Quantification of the expression of rat genes was performed by qPCR. A snap frozen fragment of distal ileum was lysed in 1 ml of Tri-Reagent (Ambion). Total RNA was extracted and quantified. cDNA was obtained from 2 µg of total RNA from each sample (Improm-II reverse transcriptase, Promega) and 1 µl of cDNA sample was used as template for PCR with LC Fast Start DNA Master SYBRGreen I Kit (Roche Applied Science). PCR were carried out in Lightcycler 480 equipment (Roche). For each sample and experiment, triplicates were made and normalized by 28S mRNA levels. Gene expression values were calculated based on the ΔΔCt method. The results were expressed as 2-ΔΔCt referred as fold-expression compared to young rats. Primers were rat specific and designed using sequence data and Nucleotide BLAST software from the National Center for Biotechnology Information (NCBI; Bethesda, MD) database. The primer sequences used were (5'→3') Interferon-gamma: GGATGCTATGGAAGGAAAGAG, CAAAGAGTCTGAGGTAGAAAGAG; TNF-α: CCAGGAGAAAGTCAGCCTCCT, TCATACCAGGGCTTGAGCTCA; IL-17α: CAACCTGAAAGTCCTCAACTC; CACAGAAGGATATCTATCAGGG.

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and reduces portal hypertension in cirrhotic rats. *Liver Int.,* 37, 1002–1012. https://doi: 10.1111/liv.13436.

Supplementary table 1

Severity of hepatic injury features in 3 months-young and 20 months-old rats.

Parameter	3 months-Young	20 months-Old	p-value
Cytoplasmic vacuolation	0.46 ± 0.15	1.33 ± 0.14	**< 0.001**
Nuclear pyknosis	0.31 ± 0.14	0.89 ± 0.08	**< 0.001**
Cytoplasmic hypereosinophilia	0.31 ± 0.14	0.89 ± 0.11	**< 0.001**
Loss of intercellular borders	0.08 ± 0.08	0.78 ± 0.13	0.07
Necrosis	0.15 ± 0.11	0.44 ± 0.12	**0.02**
Neutrophil infiltration	0.31 ± 0.14	0.72 ± 0.11	**< 0.001**
Fat accumulation	0.64 ± 0.22	1.33 ± 0.14	**< 0.001**
Final Score	**2.15 ± 0.64**	**6.39 ± 0.43**	**< 0.001**

Data expressed as mean ± SEM (n = 12 each group).

Supplementary table 2

Clinical characteristics of human liver donors.

Parameter	Young (n=14)	Old (n=13)	p-value
Age (years)	28.86 ± 2.00	76.14 ± 0.87	**<0.001**
Body mass index (kg/cm^2)	26.26 ± 1.48	26.19 ± 0.67	>0.2
Hepatic steatosis (%)	4.71 ± 2.33	4.24 ± 2.12	>0.2
ICU stay (h)	66.00 ± 23.07	61.60 ± 12.07	>0.2
Gender (% female)	14	30	>0.2
Cause of death (%)			
Hemorrhagic stroke	31	92	
Ischemic stroke	15	0	
Traumatic Brain Injury	23	8	
Hypoxia	23	0	
Others	8	0	

Data expressed as mean ± SEM.

Supplementary Figure Legends

Supplementary Fig.1. Senescence markers in 3 months-young and 20 months-old rats. P16 protein expression in liver tissue (A), primary LSEC (B) and primary HSC (C), normalized to corresponding GAPDH. (D) SIRT1 protein expression in liver tissue normalized to GAPDH. (E) Telomere length from young and old liver tissue measured as the ratio of telomere repeats to a single-copy gene. n=12 (A and D) and n=6 (B, C and E) per group. Results represent mean ± S.E.M.

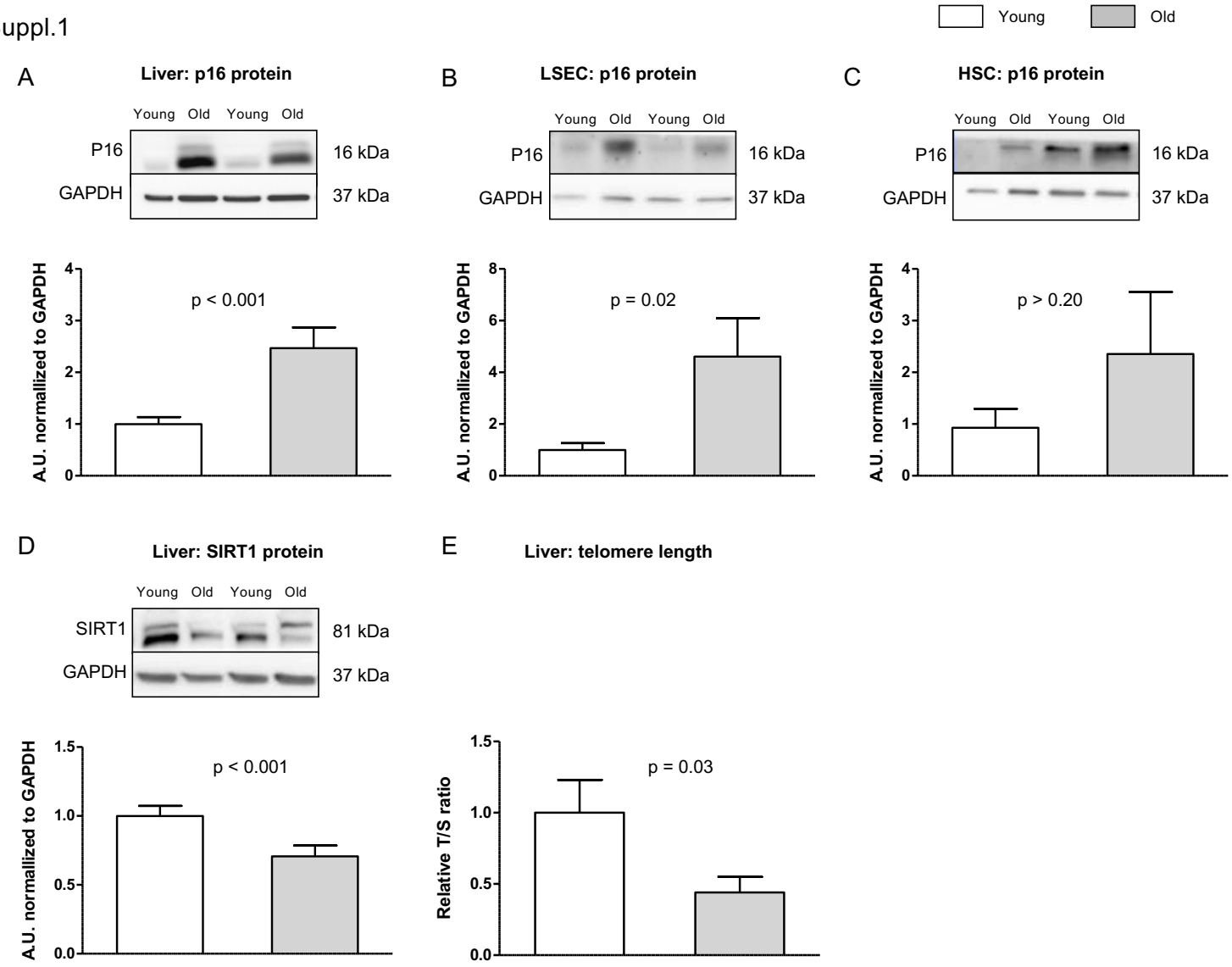
Supplementary Fig.2. Hepatic architecture in 3 months-young and 20 months-old rats. Representative images of hematoxylin & eosin staining in liver tissue (100X). n=12 per group.

Supplementary Fig.3. Liver injury and hepatocyte function in 3 months-young and 20 months-old rats. (A) TUNEL staining and quantification from young and old livers (200X). (B) Urea and albumin production from freshly isolated young and old hepatocytes. (C) HNF4α, Slc22a1, Mrp2 and Mrp3 mRNA expression in livers described in B. (D) Cytochrome P4503A4 activity from cells described in B. n=12 (A and C) and n=6 (B and D) per group. Results represent mean ± S.E.M.

Supplementary Fig.4. HSC evaluation in healthy young and old human livers. Representative images of desmin immunofluorescence (A) and α-SMA immunohistochemistry (B) with their corresponding quantification from young

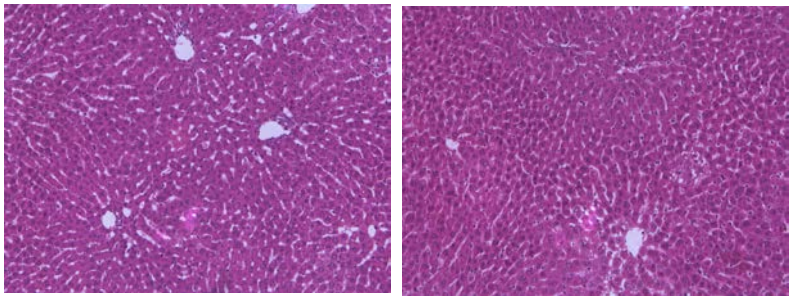
and old human livers. n=14 young & 13 old livers. Results represent mean ± S.E.M. All images 400X, scale bar=50µm.

Suppl.1


Young
Old
A
Liver: p16 protein
Young Old Young Old
P16
GAPDH
16 kDa
37 kDa
A.U. normallized to GAPDH
p < 0.001
B
LSEC: p16 protein
Young Old Young Old
P16
GAPDH
16 kDa
37 kDa
A.U. normallized to GAPDH
p = 0.02
C
HSC: p16 protein
Young Old Young Old
P16
GAPDH
16 kDa
37 kDa
A.U. normallized to GAPDH
p > 0.20
D
Liver: SIRT1 protein
Young Old Young Old
SIRT1
GAPDH
81 kDa
37 kDa
A.U. normallized to GAPDH
p < 0.001
E
Liver: telomere length
Relative T/S ratio
p = 0.03

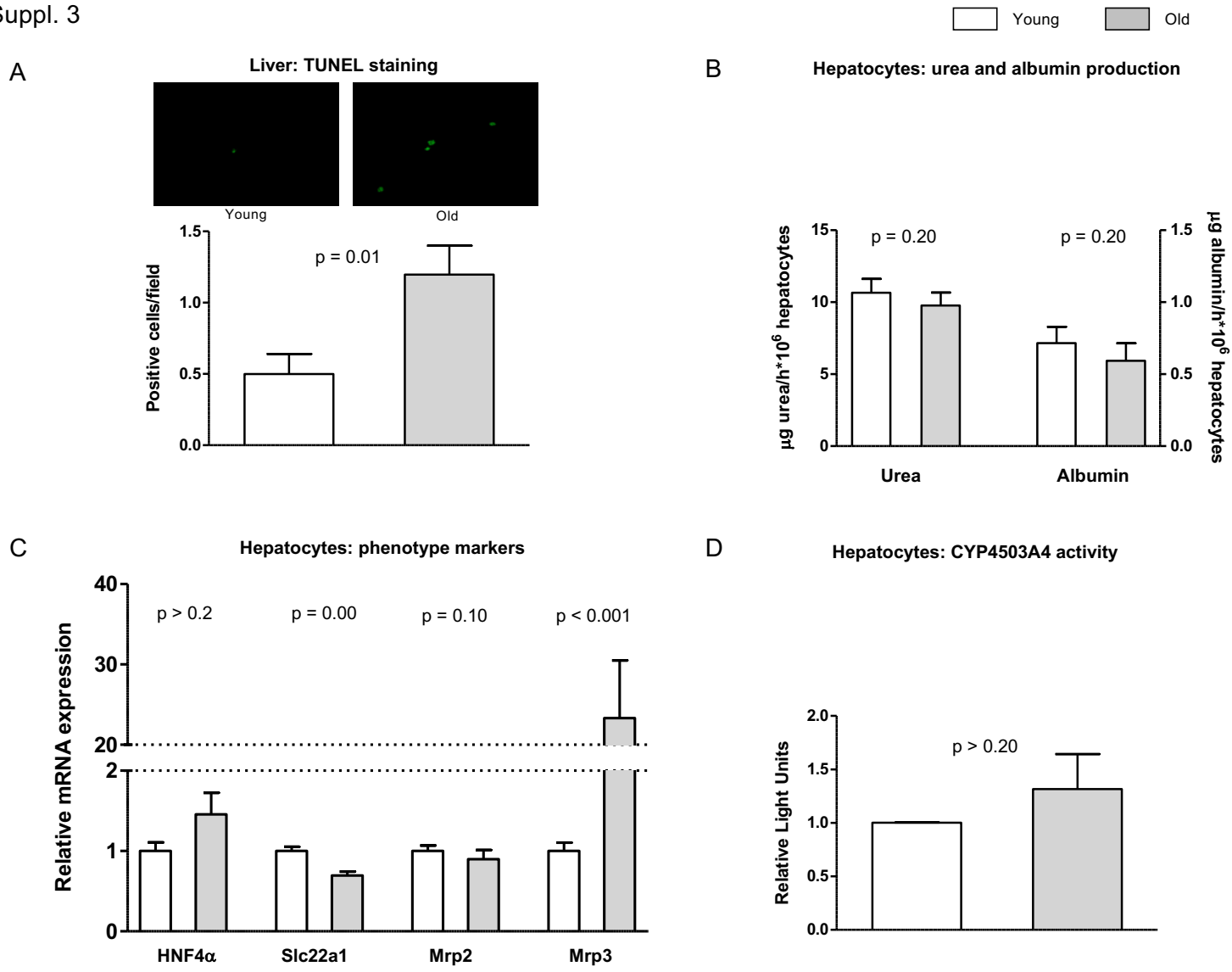
Suppl.2

Liver architecture

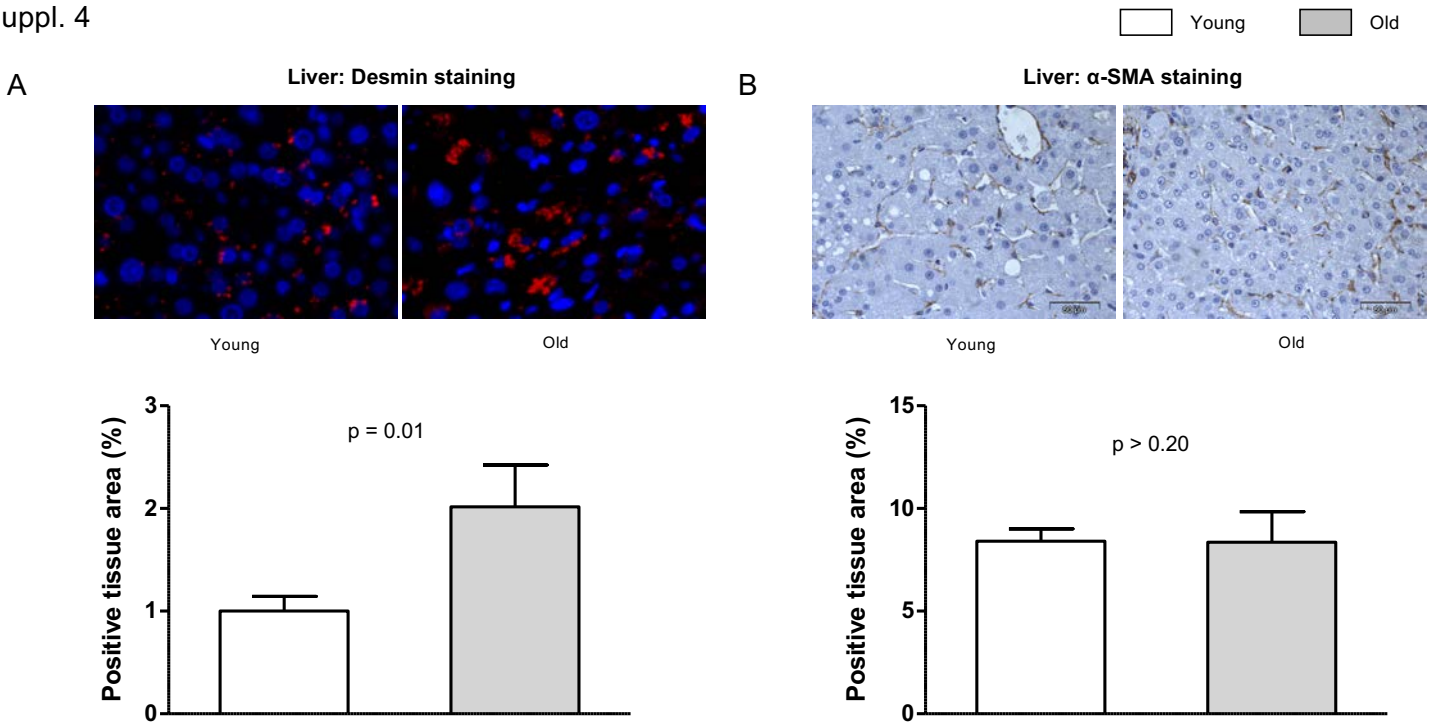


Young Old

Suppl. 3


Young
Old
A
Liver: TUNEL staining
Young
Old
Positive cells/field
1.5
1.0
0.5
0.0
p = 0.01
B
Hepatocytes: urea and albumin production
µg urea/h*10^6 hepatocytes
µg albumin/h*10^6 hepatocytes
15
10
5
0
1.5
1.0
0.5
0.0
p = 0.20
p = 0.20
Urea
Albumin
C
Hepatocytes: phenotype markers
Relative mRNA expression
40
30
20
2
1
0
p > 0.2
p = 0.00
p = 0.10
p < 0.001
HNF4α
Slc22a1
Mrp2
Mrp3
D
Hepatocytes: CYP4503A4 activity
Relative Light Units
2.0
1.5
1.0
0.5
0.0
p > 0.20

Suppl. 4


Young
Old
A
Liver: Desmin staining
Young
Old
Positive tissue area (%)
3
2
1
0
p = 0.01
B
Liver: α-SMA staining
Young
Old
Positive tissue area (%)
15
10
5
0
p > 0.20

Study 2:

AGING INFLUENCES HEPATIC MICROVASCULAR BIOLOGY AND LIVER FIBROSIS IN ADVANCED CHRONIC LIVER DISEASE.

Raquel Maeso-Díaz, Martí Ortega-Ribera, Erica Lafoz, Juan José Lozano, Anna Baiges, Rubén Francés, Agustín Albillos, Carmen Peralta, Juan Carlos García-Pagán, Jaime Bosch, Victoria C. Cogger and Jordi Gracia-Sancho.

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Original Article

Aging Influences Hepatic Microvascular Biology and Liver Fibrosis in Advanced Chronic Liver Disease

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ABSTRACT: Advanced chronic liver disease (aCLD) represents a major public health concern. aCLD is more prevalent and severe in the elderly, carrying a higher risk of decompensation. We aimed at understanding how aging may impact on the pathophysiology of aCLD in aged rats and humans and secondly, at evaluating simvastatin as a therapeutic option in aged animals. aCLD was induced in young (1 month) and old (16 months) rats. A subgroup of aCLD-old animals received simvastatin (5 mg/kg) or vehicle (PBS) for 15 days. Hepatic and systemic hemodynamic, liver cells phenotype and hepatic fibrosis were evaluated. Additionally, the gene expression signature of cirrhosis was evaluated in a cohort of young and aged cirrhotic patients. Aged animals developed a more severe form of aCLD. Portal hypertension and liver fibrosis were exacerbated as a consequence of profound deregulations in the phenotype of the main hepatic cells: hepatocytes presented more extensive cell-death and poorer function, LSEC were further capillarized, HSC over-activated and macrophage infiltration was significantly increased. The gene expression signature of cirrhosis significantly differed comparing young and aged patients, indicating alterations in sinusoidal-protective pathways and confirming the pre-clinical observations. Simvastatin administration for 15-day to aged cirrhotic rats improved the hepatic sinusoidal milieu, leading to significant amelioration in portal hypertension. This study provides evidence that aCLD pathobiology is different in aged individuals. As the median age of patients with aCLD is increasing, we propose a real-life pre-clinical model to develop more reliable therapeutic strategies. Simvastatin effects in this model further demonstrate its translational potential.

Key words: Cirrhosis, portal hypertension, hepatic sinusoid, elderly, liver microcirculation

The socioeconomic and medical care improvement during the last decades has led to a relevant increase in the elderly population around the world. This growth of older population will continue to outpace that of younger population over the next years [1]. Aging is associated with a physiological decline in most organ functions, including the liver [2]. Indeed, we have recently described the changes occurring in the liver sinusoidal cells during

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healthy aging, demonstrating that aging is accompanied by an accumulation of slight, but significant, modifications in the hepatic sinusoid that could turn into vulnerability in front of chronic or acute liver damage [3]. Advanced chronic liver disease (aCLD) represents a serious and costly problem for our society. Approximately 844 million people suffer from a chronic liver condition worldwide, making it comparable to other major public health problems related to chronic diseases such as cardiovascular diseases [4, 5]. The incidence of aCLD increases dramatically with age, and is accompanied by worse prognosis [6], consequently aging has been defined as a major risk factor for the development of chronic liver conditions [7, 8]. Importantly, and despite the impact of aging on human aCLD, most of the pre-clinical studies aimed at understanding liver disease pathophysiology have been developed in young animals, with few exceptions [9–11].

Statins have been proposed for the treatment of aCLD. Indeed, pre-clinical studies suggested sinusoidal-protective properties [12–16], which may explain the benefits of statins on portal hypertension, survival and hepatocellular carcinoma development observed in clinical and epidemiological studies [17–19]. Nevertheless, all published pre-clinical studies were made in young animals while clinical studies included patients with an advanced median age.

The present study aimed at understanding how aging may impact on the pathobiology of aCLD, specially focusing on the hepatic microcirculatory dysfunction, fibrosis and portal hypertension. We hypothesized that the cumulative mild changes observed in the sinusoidal milieu of healthy aged livers may have relevant consequences during chronic liver injury.

Secondary aims included the evaluation of simvastatin as a therapeutic option in a pre-clinical rat model of aged cirrhosis, and to elucidate the mechanisms responsible for the possible beneficial effect of the treatment.

MATERIALS AND METHODS

Additional materials and methods are included in the online supplementary information.

Induction of cirrhosis & simvastatin treatment

Advanced chronic liver disease was induced in male Wistar rats 1-month old (young group; aCLD-young) and 16-month old (aged group; aCLD-old) through chronic exposure to CCl_4 and phenobarbital [16, 20].

After approximately 14 weeks, once the animals developed ascites, CCl_4 and phenobarbital administration was discontinued. No differences in the hepatotoxicants administration period to achieve aCLD were observed comparing groups. 5 days after detection of ascites, aCLD-young and aCLD-old animals (n=7 per group) were exhaustively characterized to accomplish the first objective of the study. An additional group of aCLD-old animals (n=20) were distributed randomly to investigate the effects of simvastatin on portal hypertension, microcirculatory dysfunction and fibrosis in the aged cirrhotic model. Simvastatin (5 mg/kg) or its vehicle (PBS) was administered daily by gavage for 15 days. Hemodynamic analysis and subsequent sample processing were performed 1 hour after the last administration. Simvastatin or its vehicle was prepared by a third person, and therefore, the investigators administering the drug and performing the experiments were not aware of the treatment received by the rats. This blinding was maintained until the final analysis of results. Animals were kept in environmentally controlled animal facilities. All procedures were approved by the laboratory animal care and use committee of the University of Barcelona and were conducted in accordance with the European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/609)

In vivo haemodynamic measurements

Mean arterial pressure (MAP), portal pressure (PP) and portal blood flow (PBF) were measured in old and young rats using micro-catheters and transit-time flow probes [14]. Hepatic vascular resistance (HVR) was calculated as PP/PBF.

Liver endothelial function

After *in vivo* hemodynamic measurements, livers were quickly isolated and perfused. Liver endothelial function was determined as response to incremental doses of the endothelium-vasodilator acetylcholine [21].

Hepatic cells isolation

Hepatocytes, Kupffer cells (KC), Liver Sinusoidal Endothelial Cells (LSEC), and Hepatic Stellate Cells (HSC) were isolated using well-established protocols [22]. Only highly pure and viable cells were used.

Electron microscopy

Liver sinusoidal ultrastructure was characterized using electron microscopy as previously described [3, 23].

Histological analysis

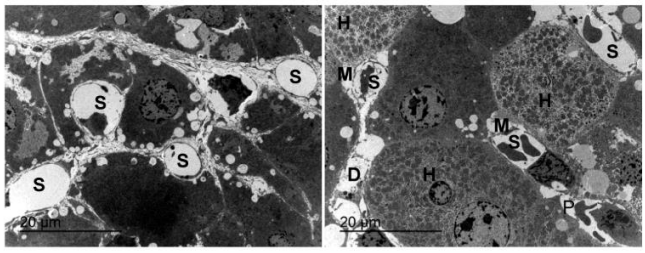
Liver samples were fixed in 10% formalin, embedded in paraffin, sectioned, and slides were stained with hematoxylin and eosin (H&E) to analyze the hepatic parenchyma, with Sirius Red for liver fibrosis evaluation, with Oil red-O for lipid quantification or with corresponding antibodies for protein immunohistochemistry (IHC) or immunofluorescence (IF).

Table 1. Biometric, biochemical and hemodynamic characteristics in aCLD-young and aCLD-old rats.

	aCLD-young 4 m.o.	aCLD-old 20 m.o.	% change	p-value
Body weight (g)	371 ± 16	701 ± 35	+88	**<0.001**
Liver (g)	10.7 ± 0.6	17.9 ± 0.9	+67	**<0.001**
Liver-body weight ratio (%)	2.88 ± 0.13	2.57 ± 0.14	-11	0.09
AST (U/L)	189 ± 11	304 ± 47	+61	**0.03**
ALT (U/L)	82 ± 8	97 ± 8	+20	0.17
Bilirubin (mg/dL)	0.51 ± 0.24	0.51 ± 0.12	0	>0.20
Bile production (µL/min*g)	30 ± 8	18 ± 3	-40	0.16
Albumin (mg/dL)	26 ± 1	20 ± 2	-24	**0.01**
Plasma cholesterol (mg/dL)	75 ± 5	106 ± 11	+41	**0.01**
Plasma LDL cholesterol (mg/dL)	45 ± 4	75 ± 8	+66	**0.007**
Plasma HDL cholesterol (mg/dL)	20 ± 2	13 ± 2	-33	**0.03**
Plasma triglycerides (mg/dL)	41 ± 8	84 ± 13	+107	**0.01**
Plasma FFA (mg/dL)	623 ± 67	504 ± 70	-19	>0.20
Oil red O-staining (%)	0.47 ± 0.14	1.83 ± 0.32	+289	**0.04**
MDA (nmol/mg protein)	2.68 ± 0.68	2.36 ± 0.28	-12	>0.20
LPS (EU/mL)	1.13 ± 0.19	2.00 ± 0.13	+77	**0.002**
PP (mmHg)	14.3 ± 0.3	16.9 ± 1.2	+18	**0.03**
PBF (mL/min*g)	1.21 ± 0.12	1.59 ± 0.23	+31	0.12
HVR (mmHg*min/mL*g)	12.7 ± 1.4	12.5 ± 2.3	-2	>0.20
***Ex vivo* HVR (mmHg*min/mL*g)**	0.23 ± 0.03	0.41 ± 0.04	+78	**0.002**
MAP (mmHg)	91 ± 6	103 ± 7	+13	>0.20
HR (bpm)	340 ± 15	338 ± 16	-0.5	>0.20

Data expressed as mean ± S.E.M. (n = 7 each group).
AST: aspartate transaminase; ALT: alanine transaminase; LDL: low density lipoprotein; HDL: high density lipoprotein; FFA: free fatty acids; MDA: malondialdehyde; LPS: lipopolysaccharide; EU: endotoxin units; PP: portal pressure; PBF: portal blood flow; HVR: hepatic vascular resistance; MAP: mean arterial pressure; HR: heart rate.

A



aCLD-Young aCLD-Old

	aCLD-Young	aCLD-Old	p-value
No Sinusoids	4.56 ± 0.41	3.43 ± 0.27	**0.007**
Lack of microvilli (%)	45 ± 6	89 ± 4	**0.03**
Big space of Disse (%)	45 ± 6	63 ± 6	>0.20
Peliosis (%)	5 ± 2	18 ± 5	**0.04**
Basal lamina (no cells)	3.40 ± 0.33	5.50 ± 0.14	0.09
Necrotic hepatocytes (%)	4 ± 2	21 ± 5	**<0.001**

B Liver: Hepatocyte phenotype markers

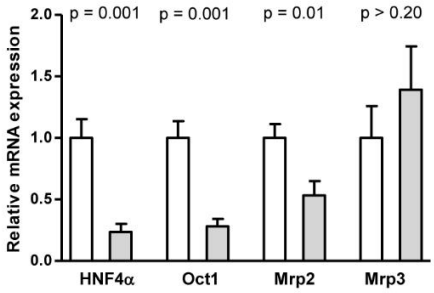


Figure 1. Hepatocyte phenotype markers in 4 months-young and 20 months-old rats with aCLD. (A) Representative transmission electron microscopy images and corresponding quantification of numbers of sinusoids *(S)*, lack of microvilli *(M)*, big space of Disse *(D)*, peliosis*(P)*, basal lamina deposition and number of necrotic hepatocytes *(H)* in liver tissue from 4 months-young and 20 months-old rats with aCLD. **(B)** HNF4α, Oct1, Mrp2 and Mrp3 mRNA expression in livers described in A. n=3 (A) and (B) n=7 per group. Results represent mean ± S.E.M. All images: 3000X, scale bar=20µm.

Nitric oxide and superoxide determinations

Levels of cGMP, marker of nitric oxide bioavailability, were analyzed in liver homogenates using an enzyme immunoassay following manufacturer instructions (Cayman Chemical Co., Ann Arbor, MI) [24]. *In situ* mitochondrial or total superoxide levels in hepatic tissue or in cells were assessed with the mitochondrial oxidative fluorescent dye MitoSOX (MitoSOX 5µM; Molecular Probes Inc., Eugene, OR) or with the total oxidative fluorescent dye dihydroethidium (DHE 10µM; Molecular Probes Inc., Eugene, OR) respectively as described [25, 26]. Fluorescence images were obtained with a fluorescence microscope (Olympus BX51, Tokyo, Japan), and quantitative analysis of at least 20 images per condition containing equivalent number of cells was performed with Image J 1.44m software.

Human liver mRNA analysis

Fractions of liver biopsy specimens obtained by transjugular route, and primarily processed for clinical pathology, were stored in diethyl pyrocarbonate (DEPC) solution for mRNA isolation using the RNeasy kit (Qiagen). 250 ng of highly pure and preserved RNA were deeply analyzed using the Illumina Whole Genome-DASL assay, which quantifies approximately 24,000 transcripts. The microarray data were deposited and stored in GEO (GSE77627).

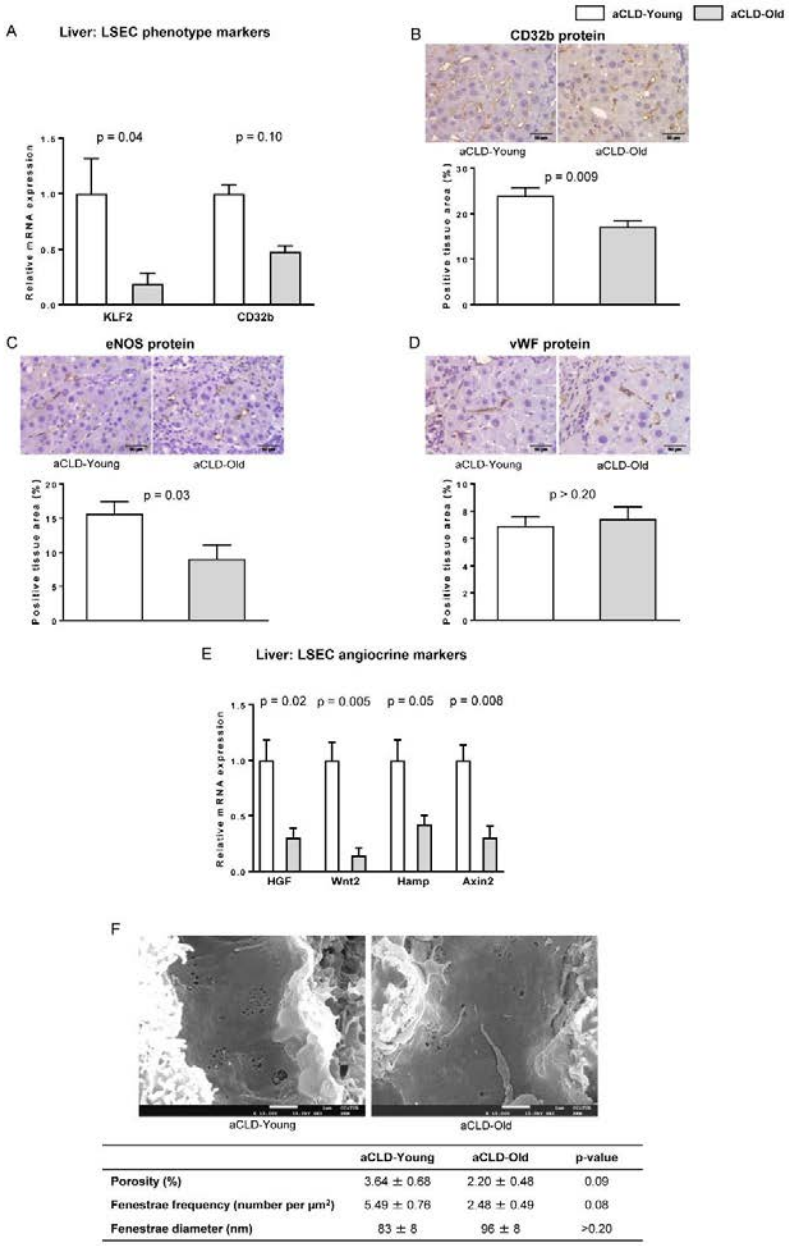
Illumina were processed using lumi package quantile normalisation [27]. Coefficient for age, derived from a linear model using probe set expression versus age and gender adjusted [28], was employed as a metric score to evaluate the influence of age in the gene expression from cirrhotic liver tissue. We performed pre-ranked gene set enrichment analysis (GSEA) using the canonical

pathways MSigDB collection signatures [29].

Ethics Committee of the Barcelona Hospital Clinic approved the experimental protocol (HCB/2011/6814). Experimental groups were defined considering patients' age: young (n=7, mean age 42 ± 5 years old, range 33-48), old (n=7, mean age 62 ± 4, range 58-70).

Statistical Analysis

Statistical analysis was performed with the SPSS for Windows statistical package (IBM, Armonk, New York, USA). All results are expressed as mean ± standard error of the mean (S.E.M.). Comparisons between groups were performed with Student's *t* test. Differences were considered significant at a p value <0.05.



	aCLD-Young	aCLD-Old	p-value
Porosity (%)	3.64 ± 0.68	2.20 ± 0.48	0.09
Fenestrae frequency (number per µm²)	5.49 ± 0.76	2.48 ± 0.49	0.08
Fenestrae diameter (nm)	83 ± 8	96 ± 8	>0.20

Figure 2. LSEC phenotype markers in aged rats with aCLD. The following markers of sinusoidal endothelial phenotype were analysed in liver tissue from 4 months-young and 20 months-aged rats with aCLD. **(A)** mRNA expression of KLF2 and CD32b. **(B)** Representative images of CD32b immunohistochemistry and corresponding quantification. **(C)** Representative images of eNOS immunohistochemistry and corresponding quantification. **(D)** Representative images of vWF immunohistochemistry and corresponding quantification. **(E)** mRNA expression of HGF, Wnt2, Hamp and Axin2. **(F)** Representative scanning electron microscopy images & quantification of porosity, fenestration frequency and fenestration diameter. n=7 (A-E) and n=3 (F) per group. Results represent mean ± S.E.M. Images from B-D: 400X, scale bar=50µm. Images from F: 15000X, scale bar=1µm.

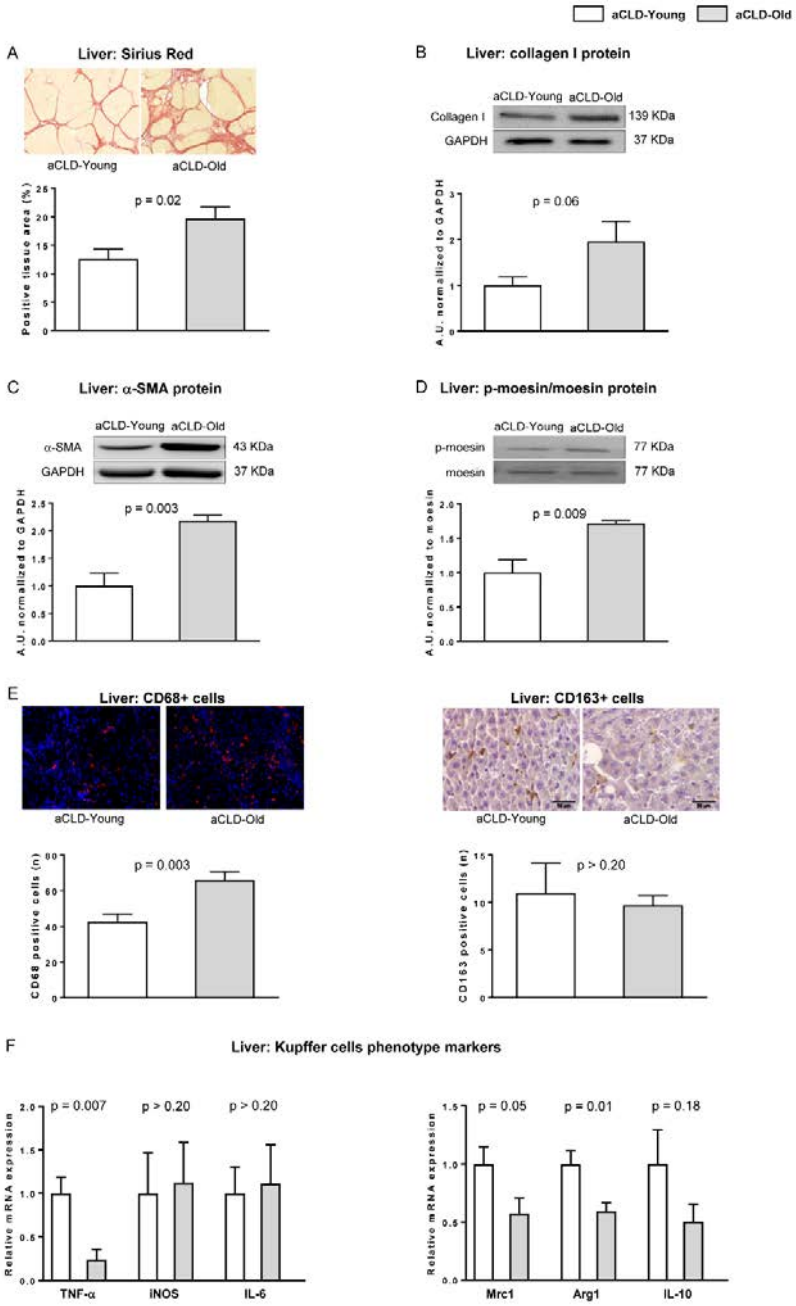


Figure 3. Aging increases fibrotic deposition, HSC activation and macrophages infiltration. Fibrotic content, HSC phenotype and macrophage infiltration and phenotype were evaluated in young and aged rats with aCLD. **(A)** Representative images of fibrotic content measured as positive area for Sirius Red with their corresponding quantifications. **(B)** Representative western blot of Collagen I normalized to GAPDH. **(C)** Representative western blot of α-SMA normalized to GAPDH. **(D)** Representative western blots of moesin and p-moesin and corresponding quantification. **(E)** *Left*, representative images of CD68 immunofluorescence in liver tissue and corresponding quantification. *Right*, representative images of CD163 immunohistochemistry in liver tissue and its quantification. **(F)** Expression of TNF-α, iNOS, and IL-6 as pro-inflammatory markers (left) and Mrc1, Arg1 and IL-10 as anti-inflammatory markers (right) in liver tissue from young and old rats with aCLD. n=7 per group. Results represent mean ± S.E.M. All images: 400X, scale bar=50μm.

RESULTS

Aged rats with aCLD: baseline biometric and biochemical characteristics.

As shown in Table 1, old rats with aCLD showed an increase in body and liver weight compared to young aCLD animals; however, liver/body-weight ratio was moderately diminished. Evaluation of hepatic function suggested a more severe deterioration in the aging group, shown by lower plasma albumin levels and bile production and higher serum transaminases.

Evaluation of plasma lipids revealed higher total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides, without significant changes in free fatty acids. Moreover, old rats with aCLD had higher hepatic lipid accumulation as compared to young rats analyzed by oil red-o-staining.

Interestingly, plasma levels of LPS were significantly higher in aged animals with aCLD, suggesting increased bacterial translocation in this group.

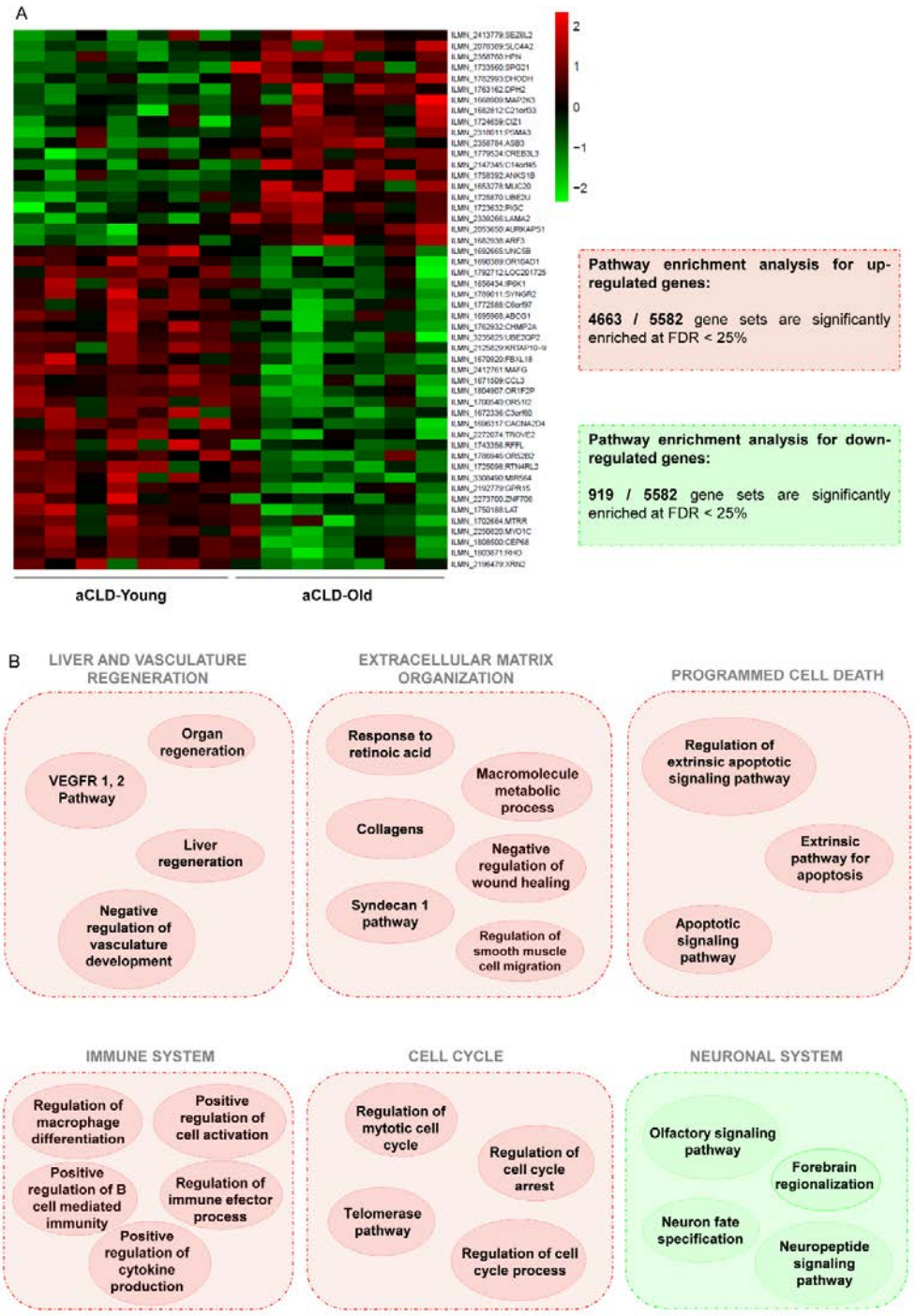
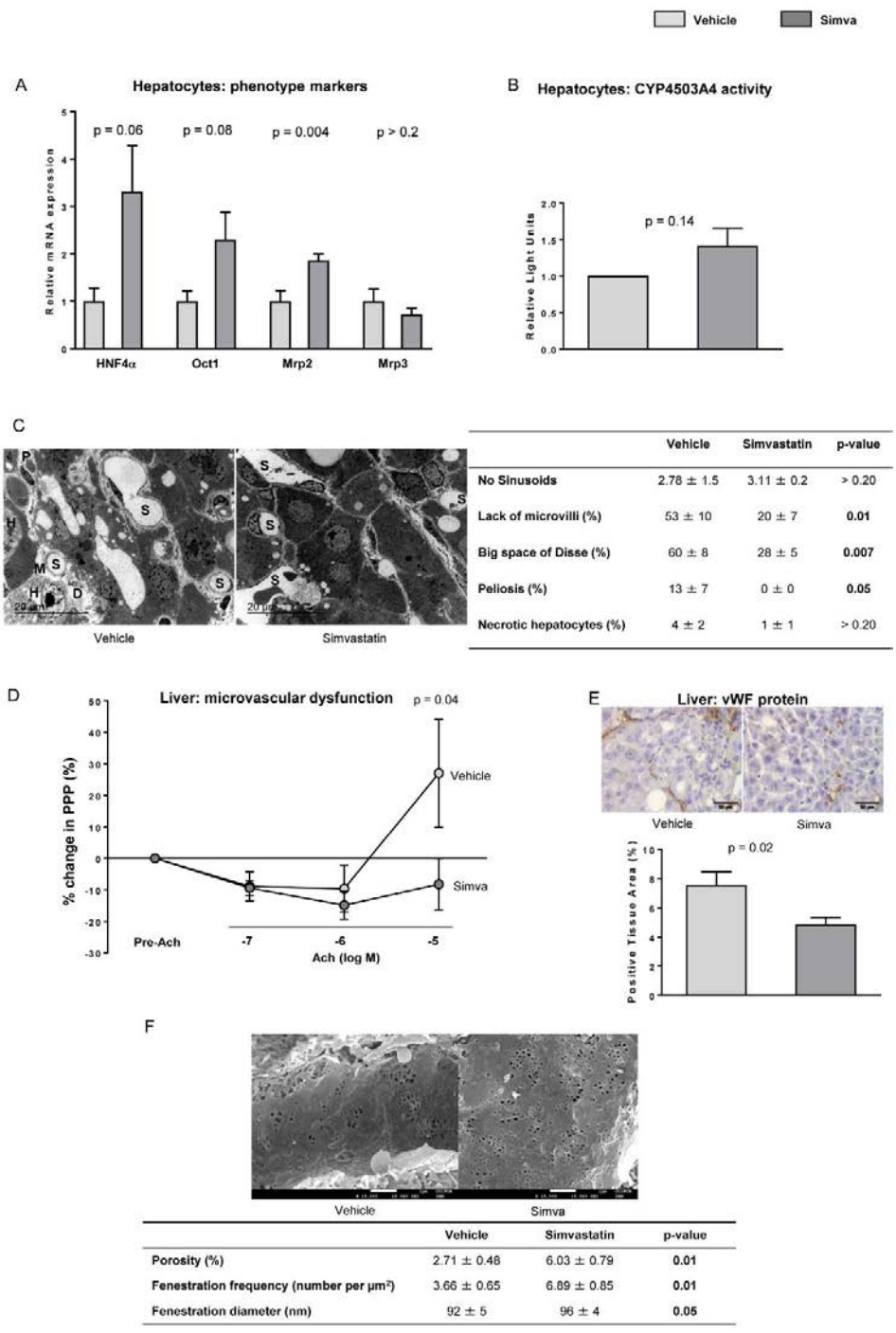


Figure 4. Aged-related changes in the gene signature of cirrhotic human liver. Gene expression analysis in cirrhotic young and old human livers. **(A)** *Left,* fold enrichments (log_2) are plotted in a heatmap using red colour for transcripts that are increased or using green colour for transcripts that are decreased in old cirrhotic humans. *Right,* pathway enrichment analysis results for genes upregulated (red) and downregulated (green) are summarized. **(B)** Representative gene sets upregulated (red) or downregulated (green) related to microcirculatory function in old cirrhotic humans, full description of top ten gene sets can be found in supplementary materials. FDR < 10%, n=7 per group. Clinical characteristics of donors are described in Supplementary table 1.



	Vehicle	Simvastatin	p-value
No Sinusoids	2.78 ± 1.5	3.11 ± 0.2	> 0.20
Lack of microvilli (%)	53 ± 10	20 ± 7	**0.01**
Big space of Disse (%)	60 ± 8	28 ± 5	**0.007**
Peliosis (%)	13 ± 7	0 ± 0	**0.05**
Necrotic hepatocytes (%)	4 ± 2	1 ± 1	> 0.20

	Vehicle	Simvastatin	p-value
Porosity (%)	2.71 ± 0.48	6.03 ± 0.79	**0.01**
Fenestration frequency (number per µm²)	3.66 ± 0.65	6.89 ± 0.85	**0.01**
Fenestration diameter (nm)	92 ± 5	96 ± 4	**0.05**

Figure 5. Effects of simvastatin on hepatocytes and microcirculatory function. (A) HNF4α, Oct1, Mrp2 and Mrp3 mRNA expression in livers from aged rats with aCLD treated with simvastatin or vehicle. **(B)** Cytochrome P4503A4 activity in hepatocytes isolated from livers described in A. **(C)** Representative transmission electron microscopy images and corresponding quantification of numbers of sinusoids *(S)*, lack of microvilli *(M)*, big space of Disse *(D)*, peliosis and number of necrotic hepatocytes *(H)*. **(D)** Microvascular function evaluation in livers from aged rats with aCLD treated with simvastatin or vehicle. **(E)** Representative images of vWF immunohistochemistry and corresponding quantification from livers described in A. **(F)** Representative scanning electron microscopy images & quantification of porosity, fenestration frequency and fenestration diameter. n=10 (A-B, D-E), n=5 (B) and n=3 (C and F) per group. Results represent mean ± S.E.M. Images from C: 3000X, scale bar=20µm. Images from E: 400X, scale bar=50µm. Images from F: 15000X, scale bar=1µm.

Portal hypertension is aggravated in aged rats with aCLD

Old animals with aCLD had significantly higher portal pressure (+18%) in comparison to young cirrhotic animals (Table 1), which was the consequence of further increases in both portal blood inflow and hepatic vascular resistance. Systemic hemodynamic parameters showed no differences between groups.

Analysis of *ex vivo* hepatic vasodilatory capacity in response to incremental concentrations of acetylcholine revealed no differences between age groups (data not shown).

Aged rats with aCLD present deterioration in hepatocyte phenotype

Ultrastructural analysis of liver architecture using transmission electron microscopy revealed that aged cirrhotic rats exhibit greater liver injury when compared to young. Indeed, and as shown in Figure 1A, old livers with aCLD presented decreased number of sinusoids, loss of hepatocyte microvilli, greater presence of erythrocytes inside the space of Disse (peliosis), further deposition of basal lamina in the sinusoid, and a significant increase in the percentage of necrotic hepatocytes. Further analysis of hepatocyte phenotype markers in liver tissue confirmed a marked deregulation in aging (Fig 1B).

The sinusoidal endothelium of aged rats with aCLD is further capillarized

As shown in Figure 2, the expression of a variety of vasoprotective, vasodilatory and angiocrine molecules, including the transcription factor KLF2, the LSEC-specific marker CD32b, and the anti-inflammatory and vasodilatory protein eNOS, were significantly deregulated in old cirrhotic livers in comparison to young. Additional LSEC analysis using scanning electron microscopy revealed a reduction in fenestrae porosity and frequency, altogether suggesting that aged cirrhotic rats undergo significantly greater sinusoidal capillarization.

Aged cirrhotic animals exhibit exacerbated fibrosis and over-activation of HSC and macrophages

Analysis of extracellular matrix deposition in aged cirrhotic livers evidenced a significant increment in hepatic fibrosis, as demonstrated by increased Sirius Red staining (Fig. 3A) and collagen I protein expression (Fig. 3B). According with this result, HSC phenotype markers α-SMA and phosphorylated moesin were overexpressed in aged cirrhotic liver tissue (Fig. 3C & D), with no differences in desmin expression (data not shown), thus suggesting over-activation of this hepatic cell type. Finally, characterization of the hepatic macrophage phenotype revealed increased infiltration of CD68+ cells (Fig. 3E) with no differences in CD163+ cells (Fig. 3E), and significant differences in the mRNA expression of different cytokines including TNFα, Mrc1 and Arg1 (Fig. 3F).

Livers from old patients display significant variations in the gene expression signature of cirrhosis

Supplementary table 1 shows the clinical and biochemical parameters of patients with aCLD included in the microarray analysis. Interestingly, older cirrhotic patients exhibited higher hepatic venous pressure gradient (HVPG) than young, with no differences in biochemical or clinical determinations except for gender. Possible gender influence in the analysis of gene expression was prevented as described in the methods section.

Analysis of gene expression in liver tissue identified 382 genes differentially expressed comparing young and aged cirrhotic patients (Fig. 4A). 204 genes were over-expressed while 178 genes were downregulated in aging.

A more comprehensive characterization of the aged cirrhotic liver gene signature was performed using pathway enrichment analysis (PEA). PEA for upregulated genes showed 4663 gene sets significantly enriched at a false discovery rate (FDR)<25%, while 52 were significantly enriched at FDR<10%. Supplementary table 2 shows the top ten significantly upregulated pathways. Focusing on the top upregulated pathways related to vascular pathobiology, these included: negative regulation of vascular development, response to retinoic acid, collagens and regulation of macrophage differentiation (Supplementary table 3 and Fig. 4B).

PEA for downregulated pathways showed 919 gene sets significantly enriched at FDR<25%, being 4 gene sets significantly enriched at FDR<10%. Top ten significantly downregulated pathways are detailed in Supplementary table 4, and included olfactory signalling pathway and neuron fate specification, among others.

Aged cirrhotic rats treated with simvastatin: biometric and biochemical characteristics

In comparison to vehicle, simvastatin-treated rats presented no differences in body weight, and a slight non-significant increase in liver mass (Table 2). Biochemical analysis showed improvements in bilirubin and albumin, which together with increased bile production and no modification in creatine kinase from muscle, suggested a global improvement in hepatic injury without toxic effects. No differences in plasma lipid spectrum were observed comparing both groups.

Simvastatin improves portal hypertension in aged rats with cirrhosis

Aged rats with aCLD treated with simvastatin exhibited a significant improvement in PP (-25%), associated with slight decreases in PBF and HVR, in comparison with vehicle (Table 2). In addition, systemic hemodynamics seemed to be ameliorated.

Simvastatin improves the hepatocyte phenotype in aged cirrhotic rats

Primary hepatocytes from old rats with aCLD treated with simvastatin showed a significant improvement in several phenotypic markers related with hepatic functionality including upregulation in HNF4α, Oct1 and Mrp2 (Fig. 5A). Additionally, the activity of cytochrome P4503A4 was slightly higher compared to hepatocytes from vehicle-treated rats (Fig. 5B). Further analysis of hepatic damage in liver tissue using transmission electron microscopy revealed a significant improvement in response to simvastatin, including maintenance of hepatocyte microvilli, a decrease in the presence of enlarged spaces of Disse, and a reduction in peliosis (Fig. 5C).

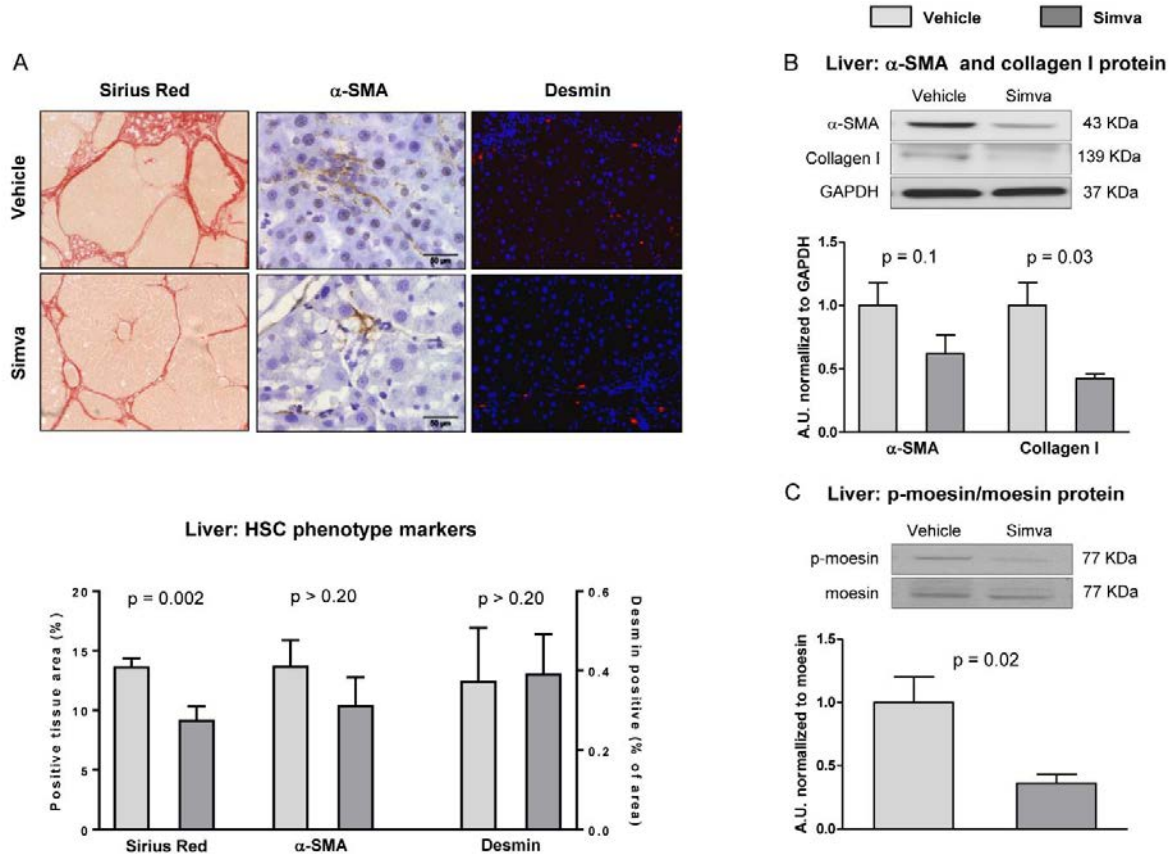


Figure 6. Simvastatin promotes decreased fibrosis deposition and HSC de-activation. (A) Representative images of fibrotic content, α-SMA and desmin with their corresponding quantifications. **(B)** α-SMA and Collagen I protein expression in total liver tissue, normalized to GAPDH. **(C)** Representative western blots of moesin and p-moesin and corresponding quantification. n=10 (A-C). Results represent mean ± S.E.M. All images 400X, scale bar=50μm.

Simvastatin improves liver endothelial dysfunction in aged rats with aCLD

Analysis of the hepatic microcirculatory function using *ex vivo* liver perfusion revealed that old cirrhotic rats treated with simvastatin exhibited a significant improvement in their endothelium-dependent vasodilatory capacity (Fig. 5D), which correlated with decreased sinusoidal expression of the capillarization marker vWF (Fig. 5E). Sinusoidal porosity as well as the number of fenestrae was significantly improved in the group of aged cirrhotic rats treated with simvastatin in comparison to those receiving vehicle (Fig. 5F). In concordance with this finding, the hepatic expression of caveolin-1, protein upregulated when fenestrae disappear [30], was significantly decreased in simvastatin-treated rats (Supplementary Fig. 1A). LSEC phenotype amelioration after simvastatin treatment was further confirmed observing up-regulation in the mRNA expression of KLF2, CD32b, Hamp and Axin2 (Supplementary Fig. 1B & C), and down-expression of CD31 (Supplementary Fig. 1D) and ICAM-1 (Supplementary Fig. 1E & F). In contrast, we did not observe differences between groups in the NO-eNOS pathway (Supplementary Fig.2).

Table 2. Biometric, biochemical and hemodynamic characteristics in aCLD-old rats treated with simvastatin or vehicle.

	Vehicle	Simvastatin	% change	p-value
Body weight (g)	655 ± 31	681 ± 18	+4	> 0.20
Liver (g)	16.6 ± 1.0	19.1 ± 1.1	+15	0.15
Liver-body weight ratio (%)	2.58 ± 0.19	2.81 ± 0.15	+9	> 0.20
AST (U/L)	188 ± 34	155 ± 28	-18	> 0.20
ALT (U/L)	61 ± 5	57 ± 6	-7	> 0.20
CKM (ng/mL)	64 ± 13	47 ± 10	-27	> 0.20
Bilirubin (mg/dL)	0.23 ± 0.06	0.10 ± 0.00	-57	**0.05**
Bile production (µL/min*100g bw)	23.5 ± 7.1	42.0 ± 16.5	+45	0.17
Albumin (mg/dL)	22.0 ± 1.1	24.2 ± 0.8	+10	0.10
Plasma cholesterol (mg/dL)	86 ± 5	79 ± 10	-8	> 0.20
Plasma LDL cholesterol (mg/dL)	64 ± 5	55 ± 7	-14	> 0.20
Plasma HDL cholesterol (mg/dL)	14.0 ± 1.5	15.6 ± 2.6	+7	> 0.20
Plasma triglycerides (mg/dL)	38.0 ± 5.1	42.1 ± 7.1	+11	> 0.20
Oil red O-staining (%)	1.33 ± 0.22	1.64 ± 0.39	+23	> 0.20
MDA (nmol/mg protein)	2.68 ± 0.74	2.35 ± 0.30	-12	0.15
LPS (EU/mL)	1.49 ± 0.18	1.62 ± 0.13	+8	> 0.20
PP (mmHg)	15.9 ± 1.4	11.9 ± 0.8	-25	**0.02**
PBF (mL/min*g)	1.28 ± 0.17	1.13 ± 0.16	-12	>0.20
HVR (mmHg*min/mL*g)	13.9 ± 2.4	12.4 ± 1.5	-11	>0.20
***Ex vivo* HVR (mmHg*min/mL*g)**	0.39 ± 0.04	0.32 ± 0.03	-18	0.20
MAP (mmHg)	97 ± 6	110 ± 5	+13	>0.20
HR (bpm)	332 ± 22	381 ± 25	+15	0.06

Data expressed as mean ± SEM (n = 10 each group).
AST: aspartate transaminase; ALT: alanine transaminase; CKM: Creatinine kinase from muscle; LDL: low density lipoprotein; HDL: high density lipoprotein; MDA: malondialdehyde; LPS: lipopolysaccharide; EU: endotoxin units; PP: portal pressure; PBF: portal blood flow; HVR: hepatic vascular resistance; MAP: mean arterial pressure; HR: heart rate.

Simvastatin decreases liver fibrosis and promotes deactivation of HSC

Aged rats with aCLD treated with simvastatin showed significantly lower hepatic fibrosis when compared to vehicle as demonstrated by significant reductions in Sirius Red staining and collagen I (Fig. 6A & B). Expression of the activation marker α-SMA decreased in response to statin (Fig. 6A & B), while we observed no differences in desmin marker indicating no changes in HSC abundance (Fig. 6A). HSC contractility was decreased by simvastatin, as suggested by reduced expression of p-moesin (Fig. 6C) and reduced *in vitro* contractility of primary human HSC (Supplementary Fig. 3A).

Oxidative stress, a well-known pro-fibrogenic stimulus, was attenuated both in liver tissue and primary HSC isolated from aged cirrhotic rats treated with simvastatin (Supplementary Fig. 3B - D).

Simvastatin ameliorates the pro-inflammatory status of aged cirrhotic rat livers

As shown in Supplementary Figure 4, CD68+ macrophage infiltration was significantly reduced in simvastatin-treated rats, with no changes in CD163+ positive cells and neutrophil infiltration (data not shown). Accordingly, the mRNA expression of several cytokines was decreased in simvastatin-treated rats.

DISCUSSION

Due to its high incidence worldwide, advanced chronic liver disease (aCLD) has become a major public health problem comparable to diabetes [4, 5]. Epidemiological data confirm that cirrhosis is more prevalent in the elderly [31], it progresses faster in this sub-group of patients [32] and importantly, that decompensation is more frequent in older patients (14%) compared to younger ones (4%) [33, 34].

In the pre-clinical scenario little is known about the impact of aging on liver diseases. Previous work suggested higher rate of fibrosis deposition in aged rats and attributed this to alterations in the immune response [9, 10]. However, this is the first study that evaluates the impact of aging on the hepatic sinusoidal milieu in a validated pre-clinical model of aCLD, focusing on the pathophysiology of the disease and its hemodynamic alterations.

The main finding of the present study is that aged animals develop a more severe form of aCLD, which can be broadly defined by poorer hepatic function and exacerbated portal hypertension, fibrosis and inflammation. Indeed, aged rats with cirrhosis exhibited serious liver damage reflected by the increase in transaminases and the reduction in serum albumin and bile production in comparison with young rats with aCLD. Interestingly, aging was also accompanied by increases in both systemic and hepatic lipid content which can be an important source of oxidative stress; a well-known detrimental factor in the pathophysiology of portal hypertension and aCLD [35]. Hepatocytes experienced profound deregulations in their phenotype and more extensive cell death, which together with increased fibrosis caused greater hepatic architecture deterioration. Indeed, increased hepatocyte necrosis could also over-stimulate HSC activation and therefore further contribute to disease exacerbation [36]. Considering the high index of cell death observed in the aged group, a caspase inhibitor may be an attractive target for the treatment of chronic liver diseases [37].

At the microcirculatory level, we describe for the first time that aged rats with aCLD show a marked aggravation in portal hypertension, which could be explained by increments in both vascular resistance and liver blood inflow. We hypothesized that the deterioration of the intrahepatic microcirculatory status is a consequence of overall deregulations in the phenotype of the main liver cells.

Although no significant differences in microvascular dysfunction were observed between young and aged cirrhotic animals, a comprehensive analysis of sinusoidal de-differentiation markers revealed alterations in LSEC phenotype including a reduction in the nitric oxide synthase pathway, depletion of angiocrine mediators and importantly, reduced porosity and frequency of fenestrae. These observations agree with previous studies reporting pseudo-capillarisation of the sinusoidal endothelium during healthy aging [3, 38], revealing that these changes are much intensified in aged livers affected by chronic injury.

In addition to LSEC, HSC play a key role modulating the hepatic vascular resistance in cirrhosis [39]. Importantly, we herein describe that old HSC undergo significant changes due to chronic liver injury, which ultimately lead to expanded deposition of extracellular matrix in comparison to young animals. Interestingly, previous pre-clinical studies suggested an increment in hepatic fibrogenesis in aging [9, 10, 40], however we describe such aggravation in a model of decompensated cirrhosis that better reflects the clinical observations [32, 41].

The role of inflammation in the progression of CLD has been widely described in the past, and recently reviewed [42], nevertheless much less is known in the context of aging [10, 40]. In our study, and in comparison, to young animals, we observe that aged cirrhotic rats exhibit further deterioration in the hepatic inflammatory phenotype as demonstrated by a significant increase in the recruitment of proinflammatory macrophages together with a decrease in the expression of pro-resolution cytokines in this cellular sub-population. The increase in the myeloid cell content in the aged cirrhotic liver may derive from the sinusoidal endothelial activation described above, and importantly, this could be another mechanism contributing to microcirculatory dysfunction and global worsening of the disease [43].

In addition, and aimed at understanding the translatability of our pre-clinical discoveries, we characterized the hepatic transcriptome in two groups of cirrhotic patients of different age. Although the cohort of patients included was small, the result of these analyses confirmed the alterations observed in the aging rat model: endothelial deregulation, HSC over-activation, enhanced fibrogenesis and immune cell activation. Altogether, the pre-clinical and clinical data obtained in this study suggest that the pathophysiology of CLD is much worse, and probably involves different molecular mechanisms, when comparing aged and young individuals. Future studies, out of the scope of the present manuscript, will be required to further comprehend the impact of the unique gene

expression signature of aged cirrhotic livers in the progression and regression of CLD.

Statins are HMGCoA reductase inhibitors originally designed as cholesterol-lowering drugs that over the years have been re-discovered for their pleiotropic effects on inflammation, fibrosis and endothelial function, among others [44]. Different studies have reported the benefits of statins in experimental models of young cirrhosis [12–14, 16] but none has evaluated the effect of this therapy in aged aCLD.

In this study we observed that 2-week administration of simvastatin to aged cirrhotic rats significantly improves liver microvascular dysfunction and portal hypertension, accompanied by slight ameliorations in the systemic hemodynamic and in hepatic function.

Underlying mechanisms explaining the global benefits of this vasoprotective compound revealed reduced disease severity in all major hepatic cell types. Hepatocytes exhibited an improved phenotype as suggested by ameliorations in their synthetic capacity, in blood tests and in the ultrastructural architecture. Importantly, we did not observe signs of toxicity due to the treatment, suggesting that previous evidence of simvastatin toxic effects [45] may derive from the experimental model used to induce CLD (common bile duct ligation) and/or be dose dependent. Indeed, in our study we selected a dose of 5 mg/day/kg in rats, which is equivalent to a dose of 80 mg/day in adult humans following Reagan-Shaw et al. dose conversion [46], and preferred to generate CLD by chronic CCl_4 with no obstruction of bile secretion, thus preventing drug accumulation.

As expected, simvastatin improved the phenotype of sinusoidal cells. The hepatic endothelium increased the expression of functional and angiocrine markers, while decreased the expression of capillarisation and inflammatory markers. Remarkably, aged cirrhotic animals treated with simvastatin for 15 days exhibited double number and frequency of fenestrae, which in fact may contribute to improve parenchymal function through a better diffusion of oxygen, nutrients, and waste products. It is important to denote that no previous study has demonstrated an improvement in sinusoidal porosity in response to statin administration *in vivo*, just one study showed amelioration in the fenestrae of the mesenteric endothelium in a rat model of arterial hypertension [47]. Although our results support that the hepatic endothelium was ameliorated due to the treatment, we could not detect differences in the nitric oxide pathway when comparing both groups of animals. Considering that we tested the effects of 15-day simvastatin, treatment period longer than in any previously published study, we cannot disregard the possibility that a partial spontaneous recovery in the vehicle-treated group may have occurred and consequently no differences in certain markers could be detected. Likewise, we cannot discard that aged rats with cirrhosis may exhibit a poor NO-signaling response to statins.

Importantly, simvastatin treatment promoted a marked regression in liver fibrosis, which may be due to deactivation of HSC, together with a reduction in oxidative stress and inflammation. Additionally, HSC phenotype improvement could also contribute to the amelioration in hepatic microvascular dysfunction. Our observations are in agreement with previous clinical observations in patients with liver disease [44].

In conclusion, this study provides evidence that aCLD has a much-aggravated pathophysiology in aged individuals, and even more importantly, that aging may activate different or additional molecular mechanisms from those observed in young. Considering the increasing age of many patients with aCLD, we propose that using closer to real-life models to investigate the pathophysiology of aCLD may allow the development of more reliable therapeutic strategies. In fact, the characterization of simvastatin in this model further recommends its applicability at the bedside.

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SUPPLEMENTARY DATA

Aging Influences Hepatic Microvascular Biology and Liver Fibrosis in Advanced Chronic Liver Disease

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Supplementary materials and methods

In vivo hemodynamic

Rats were anesthetized with ketamine (100 mg/kg body weight, Imalgene 1000; Merial) plus midazolam (5 mg/kg body weight; Laboratorio Reig Jofre, S.A., Spain) intraperitoneally, fastened to a surgical board, and maintained at a constant temperature of 37 ± 0.5ºC.

A tracheotomy and cannulation with a PE-240 catheter (Portex) was performed in order to maintain adequate respiration during anesthesia. Indwelling catheters made of polyethylene tubing (PE-50; Portex, UK) were placed into the femoral artery to measure mean arterial pressure (MAP; mm Hg) and heart rate (HR; beats per minute), and to the ileocolic vein to measure PP (mmHg). PBF (mL/min) was measured with a nonconstrictive perivascular ultrasonic transit-time flow probe (2PR, 2-mm diameter; Transonic Systems Inc., USA) placed around the portal vein just before its entrance in the liver, avoiding the measurement of most portal-collateral blood flow. The flow probe and pressure transducers were connected to a Powerlab (4SP) linked to a computer using Chart v5.5.6 for Windows software (AD Instruments, Australia). Hepatic vascular resistance (HVR) was calculated as PP/PBF. Hemodynamic data were collected after a 20-minute stabilization period [1]. At the end of the *in vivo* hemodynamic study, plasma samples from young and aged-rats were collected to subsequently evaluate alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, albumin, cholesterol, low density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL), triglycerides and free fatty acids (FFA), all by standard protocols. Experiments and data collection were performed blindly.

Liver endothelial function

Rat livers were isolated and perfused at 35 mL/min with Krebs' buffer. The perfused rat liver preparation was allowed to stabilize for 20 min before vasoactive substances were added. The intrahepatic microcirculation was pre-constricted by adding the α1-adrenergic agonist methoxamine (Mtx; 10^{-4}M; Sigma) to the reservoir. After 5 min, concentration-response curves to cumulative doses of acetylcholine (Ach; 10^{-7}-10^{-5}M; Sigma) were evaluated. The concentration of Ach was increased by 1log unit every 1.5 min interval. Responses to Ach were calculated as the percentage change in portal perfusion pressure [2]. The gross appearance of the liver, stable perfusion pressure, bile production over 0.4µl/min/g of liver and stable buffer pH (7.4 ± 0.5) were monitored during this period. If any viability or stability criteria were not satisfied, the experiment was discarded.

Hepatic cells isolation

Hepatocytes, Kupffer cells (KC) and Liver Sinusoidal Endothelial Cells (LSEC) were isolated using a well-established protocol [1]. Rat livers were perfused through the portal vein with Hanks without Ca^{+2} and Mg^{+2} containing 12 mM hepes (H3375, Sigma) pH 7.4, 0.6 mM ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetra acetic acid (E4378, Sigma) and 0.23 mM bovine serum albumin (BSA; A1391,0100, Applichem). Then, perfused for 30 min with 0.015% collagenase A (103586, Roche) Hanks containing 12 mM hepes (pH 7.4) and 4 mM $CaCl_2$. The resultant digested liver was excised, and *in vitro* digestion was performed at 37°C with 0.01% collagenase A, Hank's containing 12 mM hepes (pH 7.4) and 4 mM $CaCl_2$ for 10 min. Disaggregated tissue was filtered using 100 µm nylon strainer, collected in cold Krebs' buffer and centrifuged at 50 g for 5 min. The pellet was washed three times for hepatocytes enrichment. Hepatocytes were seeded in collagen-coated wells and cultured in Dulbecco's Modified Eagle's Medium (DMEMF12; 11320074, Gibco) supplemented with 2% fetal bovine serum (04-001-1A, Reactiva), 1% L-glutamine (25030-024, Gibco), 1% penicillin plus 1% streptomycin (03-331-1C, Reactiva), 1 nM dexamethasone (D4902, Sigma), 1 µM insulin (103755, HCB) and 1% amphotericin B (03-029-1C, Reactiva). The supernatant was centrifuged at 800g for 10 min and the obtained pellet was resuspended in Dulbecco's PBS (DPBS) and centrifuged through a two-step Percoll gradient (25-50%). The interface of the gradient was enriched in KC and LSEC. This cell fraction was resuspended in RPMI medium, seeded in plastic dishes and incubated for 30 min at 37ºC in humid atmosphere with 5% CO_2 in order to enhance KC purity. Non-adherent cells were seeded in collagen-coated wells and incubated for 1h (37ºC, 5% CO_2). After this time the medium was discarded and LSEC adhered cells were washed twice with DPBS and cultured in RPMI-1640 (01-100-1A, Reactiva) supplemented with 10% fetal bovine serum (04-001-1A, Reactiva), 1% L-glutamine (25030-024, Gibco), 1% penicillin plus 1% streptomycin (03-331-1C, Reactiva), 0.1 mg/mL heparin (H3393, Sigma),

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0.05 mg/mL endothelial cell growth supplement (BT-203, BT) and 1% amphotericin B (03-029-1C, Reactiva). HSC were isolated through a sequential in situ perfusion of the liver with 0.195 mg/mL collagenase A (Roche), 1.5 mg/mL pronase (Roche) and 0.05 mg/ml Dnase (Roche) in Gey's Balanced Salt Solution (GBSS; Sigma), and dispersed cells were fractionated by density gradient centrifugation using 11.5% Optiprep (Sigma).[2] HSC were cultured in Iscove's Modified Dulbecco's Media (IMDM, Invitrogen, Gibco) supplemented with 10% fetal bovine serum (04-001-1A, Reactiva), 1% L-glutamine (25030-024, Gibco), 1% penicillin plus 1% streptomycin (03-331-1C, Reactiva) and 1% amphotericin B (03-029-1C, Reactiva). Viability and purity were systematically over 95%.

Electron microscopy

Liver sinusoidal ultrastructure was characterized using electron microscopy as previously described [3]. Briefly, livers were perfused through the portal vein with a fixation solution containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer 0.1% sucrose and fixed overnight at 4ºC. Samples were washed 3 times with 0.1M sodium cacodylate buffer. Liver sections were post-fixed with 1% osmium in cacodylate buffer and dehydrated in an ethanol gradient to 100%.

For scanning electron microscopy 6 to 8 liver blocks per sample were mounted on stubs, sputter coated with gold and examined using a Jeol 6380 scanning electron microscope. Measurements of fenestrae size, number and density were carried out. Fenestrations were defined as open pores with diameters <300nm. Diameter was defined as the major length of each fenestration or gap. Porosity was defined as the sum area of fenestrations/total quantified area. Frequency was defined as number of fenestrae per μm^2. At least 10 images per animal were taken.

For transmission electron microscopy, fixed liver tissue was embedded in Spurr resin, cut in 50nm ultrathin sections, counterstained with uranyl acetate and lead citrate and examined using microscope. 10 micrographs per sample were taken to estimate % of necrotic hepatocytes and % of sinusoids presenting each of the following parameters: lack of microvilli, big space of Disse, peliosis and basal lamina deposition.

RNA isolation and quantitative PCR

RNA from cells and tissue were extracted using RNeasy mini kit (Qiagen) and Trizol (Life Technologies), respectively. RNA quantification was performed using a NanoDrop spectrophotometer. cDNA was obtained using QuantiTect reverse transcription kit (Qiagen). Real-Time PCR was performed in an ABI PRISM 7900HT Fast Real-Time PCR System, using TaqMan predesigned probes for HNF4α (Rn04339144_m1), Oct1 (Rn00562250_m1), Mrp2 (Rn00563231_m1), Mrp3 (Rn01452854_m1), eNOS (Rn02132634_s1), CD31 (Rn01467262_m1), KLF2 (Rn01420495_g1), CD32b (Rn00598391_m1), α-SMA (Rn01759928_g1), PDGFRβ (Rn01491838_m1), TNF-α (Rn01525859_g1), iNOS (Rn00561646_m1), IL-6 (Rn01410330_m1), Mrc1 (Rn01487342_m1), Arg1 (Rn00691090_m1), IL-10 (Rn00563409_m1), HGF (Rn00566673_m1), Wnt2 (Rn01500736_m1), Hamp (Rn00584987_m1), Axin2 (Rn00577441_m1)and GAPDH (Rn01775763_g1) as endogenous controls. Results, expressed as $2^{-\Delta\Delta Ct}$, represent the x-fold increase of gene expression compared with the young group.

Western Blotting

Liver samples were processed and western blot performed as described [4]. Used primary antibodies: phosphorylated eNOS at Ser1177 (9571, Cell Signaling), total eNOS (610297, BD Transduction Laboratories), phosphorylated moesin at Thr558 (sc-12895, Santa Cruz), total moesin (sc-13122, Santa Cruz), caveolin-1 (610059, BD Biosciences), α-SMA (A2547, Sigma Aldrich), CD31 (555027, BD Biosciences), collagen I (84336, Cell Signalling) and ICAM-1 (AF583, R&D Systems) all 1:500. Blots were revealed by chemiluminescence and protein expression was determined by densitometric analysis using the Science Lab 2001 Image Gauge (Fuji Photo Film, Düsseldorf, Germany). Blots were also assayed for GAPDH (1:1000, Sigma-Aldrich) content as standardization of sample loading.

Histological Analysis

Liver samples were fixed in 10% formalin, embedded in paraffin, sectioned, and slides were stained with Sirius Red for liver fibrosis evaluation [5].
Frozen sections were cut to 10 μm and stained with Oil Red O (Sigma Aldrich) for lipid analysis. Lipid droplets were evaluated as the red-stained area per total area using ImageJ software.

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Immunohistochemistry

Liver samples were fixed in 10% formalin, embedded in paraffin, sectioned and processed for immunohistochemistry (IHC) or immunofluorescence (IF) as previously described [6].

For IHC liver sections were incubated with antibodies against CD32b (sc-13271, Santa Cruz), eNOS (sc-654, Santa Cruz), vWF (A0082, Dako), α-SMA (M0851, Dako), or CD163 (MCA342R, Biorad). After incubation with corresponding secondary antibodies, color development was induced by incubation with a DAB kit (Dako) and counterstained with hematoxylin. Sections were dehydrated and mounted. The specific staining was visualized and fifteen images per liver were acquired using a microscope equipped with a digital camera and the assistance of Axiovision software. The relative volume was calculated by dividing the number of points positive in sinusoidal areas by the total number of points over liver tissue [7].

For IF, liver sections were incubated with antibodies against desmin (M0760, Dako) and CD68 (MCA341R, Biorad), incubated with secondary antibodies Alexa Fluor 488 or 555 (1:400, Life technologies) and 4′,6-diamino-2-fenilindol (1:3000; DAPI, Sigma-Aldrich) and mounted in Fluoromount G medium. Ten images per sample were obtained with a fluorescence microscope and percentage of positive area (desmin) or positive cells per field (CD68) were quantified.

Cytochrome 4503A4 activity

Phase I detoxification capacity of hepatocytes was analyzed using P450-Glo™ CYP4503A4 Assay following manufacturer's instructions (V8901, Promega) [8]. Briefly, hepatocytes cultured for 24 hours were rinsed twice with DPBS and incubated with culture media containing 50 μM Luciferin-PFBE at 37°C for 4h. Then supernatant was collected and neutralized with Luciferin Detection Reagent. After incubation for 30 min at RT, plate luminescence was read in a luminometer (Orion II Microplate Luminometer, Germany). Samples luminescence was corrected subtracting background luminescence.

Human HSC isolation and contraction assay

Human hepatic stellate cells were isolated from remnant liver tissue approximately weighting 20 g obtained from discarded tissue after liver transplantation (chronic ethanol etiology). Ethics Committee of the Hospital Clínic de Barcelona approved the experimental protocol (HCB/2015/0624) and in all cases patients received and agreed informed consent. Median age of liver donors for cell isolation was 70 ± 3 years.

Briefly, livers were perfused through major vessels for 10 min with Hanks without Ca^{+2} and Mg^{+2} containing 12 mM hepes (H3375, Sigma) pH 7.4, 0.6 mM ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetra acetic acid (E4378, Sigma) and 0.23 mM bovine serum albumin (BSA; A1391,0100, Applichem). Then, perfused for 30 min with 0.015% collagenase A (103586, Roche) Hanks containing 12 mM hepes (pH 7.4) and 4 mM $CaCl_2$. The resultant digested liver was excised and *in vitro* digestion was performed at 37°C with 0.01% collagenase A, Hank's containing 12 mM hepes (pH 7.4) and 4 mM $CaCl_2$ for 10 min. Disaggregated tissue was filtered using 100 μm nylon strainer, collected in cold Krebs buffer and centrifuged at 50 g for 5 min. Hepatocytes were contained in the pellet while non-parenchymal cells were found in the supernatant. HSC were fractionated by density gradient centrifugation using 11.5% Optiprep (Sigma) as previously described [8]. HSC were cultured in Iscove's Modified Dulbecco's Media (IMDM, Invitrogen, Gibco) supplemented with 10% fetal bovine serum (04-001-1A, Reactiva), 1% L-glutamine (25030-024, Gibco), 1% penicillin plus 1% streptomycin (03-331-1C, Reactiva) and 1% amphotericin B (03-029-1C, Reactiva). Viability and purity were systematically over 95%.

Contraction of aged human HSC was performed as previously described [2]. Culture plates were incubated with 1% BSA-PBS and afterwards filled with a mix of collagen (2 mg/mL) and human HSC ($1–2 \times 10^5$ cells/mL). Once the gels were solidified, serum free IMDM with 10 μM simvastatin or vehicle was added. After 24 h, contraction was induced by adding 10% FBS for 24 h. Finally, the contraction area was digitalized and measured with ImageJ software. The results are expressed as % of contraction relative to the initial area of the gel.

Endotoxemia quantification

Endotoxemia was quantified using a quantitative chromogenic limulus amoebocyte lysate (LAL) test (BioWhittaker, Nottingham, UK), following manufacturer's instructions. Due to LPS ubiquity, samples and reagents were handled in

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an airflow chamber and processed with pyrogen-free material tested by manufacturers. E. coli lyophilized endotoxin (22 UE/mL) provided by the kit was used to set standard endotoxin concentrations ranging from 5.0 UE/mL to 0.1 UE/mL. To verify the lack of product inhibition by plasma protein, a dilution/heating inactivation protocol was followed prior to endotoxin measurement. A pooled E. coli endotoxin spike solution (0.4 UE/mL) was prepared with serum samples. Dilutions ranging from 1/2 to 1/20 were performed over spiked and unspiked serum samples. All test samples were then incubated at 60°C during 30 min [9]. The LAL test was performed after this period. The non-inhibitory dilution was established when the difference between spiked and unspiked endotoxin values was equal to the known concentration of the spike ± 25%, as detailed by the manufacturer. Final sample dilutions used were 1/10 (spike recovery after correction of dilution: 0.34 UE/mL). All samples were tested in triplicate and read at 405 nm in a Tecan Sunrise microplate reader (Männedorf, Switzerland).

Supplementary references

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Supplementary table 1. Clinical characteristics of patients with liver cirrhosis included in the microarray analysis.

	Young patients (age < 50 years)	**Old patients (age > 50 years)**	**p-value**
Age (years)	42 ± 5.5	62 ± 4.1	**0.00**
Male	6 (86%)	1 (14%)	**0.01**
Cirrhosis etiology naive HCV	5 (71%)	5 (71%)	
treated HCV	2 (29%)	0	> 0.2
HCV + OH	0	1 (14%)	
cryptogenic	0	1 (14%)	
Child Pugh class A/B/C	7/0/0	4/3/0	0.19
MELD	8.5 ± 2.3	10.0 ± 3.8	> 0.2
Ascites	0	3 (42%)	0.19
Hepatic encephalopathy	0	1 (14%)	> 0.2
Gastroesophageal varices	3 (42%)	5 (71%)	> 0.2
Variceal bleeding	0	2 (29%)	> 0.2
HVPG (mmHg)	11.3 ± 4.4	16.3 ± 3.9	**0.03**
Bilirubin (mg/dL)	1.1 ± 0.7	2.1 ± 2.2	> 0.2
AST (U/L)	110.5 ± 64.0	127.8 ± 114.8	> 0.2
ALT (U/L)	116.0 ± 70.0	80.8 ± 65.3	> 0.2
Albumin (mg/dL)	42.3 ± 3.0	37.5 ± 9.1	0.13
Prothrombin time (%)	77.5 ± 6.4	81.3 ± 13.2	> 0.2

Data expressed as mean ± SD (n=7 each group).

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Supplementary table 2. Pathway enrichment analysis for genes up-regulated in aged cirrhotic patients.

Pathway	No. Genes up-regulated from total gene set	Genes	Database
Positive regulation of viral process	44/82	VPS4B, HPN, GTF2F1, LGALS1, ADARB1, SMARCA4, CDK9, CD28, TRIM11, SMARCB1, …	GO
Positive regulation of multiorganism process	68/139	VPS4B, HPN, GTF2F1, LY86, PLAU, LGALS1, ADARB1, SMARCA4, CDK9, ACVR1B, …	GO
VEGFR1,2 Pathway	36/63	MAP2K3, PTPN6, ARF1, NOS3, MAPK14, CDC42, PRKAB1, MAPK3, GRB10, AKAP1, …	PID
Endomembrane system organization	154/403	TULP1, RAB8A, ARL1, CLASP1, VPS4B, PVRL2, RPR, ZNF385A, VPS33B, RAB34, …	GO
Extrinsic apoptotic signalling pathway via death domain receptors	17/34	DAB2IP, NGF, MOAP1, SORT1, FASLG, CRADD, DDX47, TNFRSF10B, TNFRSF1A, CASP8, …	GO
Alpha beta pathway	9/25	MAP2K3, HCK, MAPK14, CDC42, PAK1, LCK, FYN, MAP2K4, RAC1, SRC	PID
Mitotic prometaphase	41/72	CLASP1, CENPH, NUP85, CCDC99, XPO1, CDC20, NUP133, NUP37, RCC2, CKAP5, …	Reactome
CXCR4 Pathway	43/93	VPS4B, HCK, PTPN6, GNAI3, INPP5D, ITGA2, CDC42, CD247, RGS1, PAK1, …	PID
Regulation of viral release from host cell	15/27	VPS4B, TRIM26, PML, TRIM11, VPS4A, CHMP4B, TRIM21, VAPA, CAV2, TSG101, …	GO
Negative regulation of extrinsic apoptotic signaling pathway via death domain RECE	16/30	NOS3, ICAM1, GPX1, SERPINE1, FASLG, RFFL, TNFRSF10B, TNFSF10, CASP8, FGG, …	GO

PID: pathway interaction database; GO: gene ontology.
Data expressed at FDR < 10% (n=7 each group).

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Supplementary table 3. Pathway enrichment analysis for genes up-regulated in aged cirrhotic patients. Top ten gene sets related to vascular biology.

Pathway	No. Genes up-regulated from total gene set	Genes	Database
VEGFR1,2 Pathway	36/63	MAP2K3, PTPN6, ARF1, NOS3, MAPK14, CDC42, PRKAB1, MAPK3, GRB10, AKAP1, ...	PID
Negative regulation of vasculature development	37/69	DAB2IP, THBS2, CXCL10, SPARC, DCN, PDE3B, PTGER4, SEMA4A, SERPINE1, XDH, ...	GO
Response to retinoic acid	37/95	TNC SP100 RET RNF2 CLK2 SOX9 CD38 FZD4 WNT2 ADNP2, …	GO
Collagens	21/37	COL7A1 COL6A2 COL16A1 COL1A2 COL5A1 COL1A1 COL8A2 COL5A2 COL11A1 COL9A2, …	Matrisome
Regulation of wound healing	59/114	CLASP1 PPAP2B VPS33B TSPAN8 ENPP4 PLAU NOS3 STX2 APCS HBEGF, …	GO
Regulation of smooth muscle cell migration	27/48	PLAU ITGA2 RPS6KB1 PTGER4 TACR1 SERPINE1 TPM1 P2RY6 F3 DOCK7, …	GO
Platelet derived growth factor receptor signaling pathway	21/31	BCR PLEKHA1 RAPGEF1 JAK2 PDGFRA GAB1 PIK3C2A IQGAP1 PTEN PDGFRB, …	GO
Positive regulation of the transforming growth factor beta production	6/14	CX3CL1 LUM LGALS9 CD46 THBS1 CD34	GO
Regulation of macrophage differentiation	12/19	CALCA C1QC PRKCA CASP8 CSF1 TRIB1 FADD RB1 ID2 INHA, …	GO
Positive regulation of cytokine production involved in immune response	11/29	FCER1G TRIM6 SEMA7A F2RL1 TNFSF4 B2M RSAD2 MALT1 CD74 CD36, …	GO

Data expressed at FDR < 10% (n=7 each group)
PID: pathway interaction database; GO: gene ontology.

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Supplementary table 4. Pathway enrichment analysis for genes down-regulated in aged cirrhotic patients.

Pathway	No. Genes down-regulated from total gene set	Genes	Database
Olfactory signaling pathway	128/282	OR9Q2 OR10AD1 OR10A3 OR51I2 OR10G9 OR4C3 OR4A5 OR5I1 OR52L1 OR5D14, …	Reactome
Olfactory transduction	158/341	PRKX OR52B2 OR9Q2 OR10AD1 OR10A3 OR51I2 OR10G9 OR4C3 OR6C74 OR4A5, …	KEGG
Sensory perception of chemical stimulus	164/405	OR52B2 OR9Q2 OR10AD1 OR10A3 OR51I2 OR5H15 SCNN1D TAS2R46 OR10G9 OR4C3, …	GO
Forebrain regionalization	15/21	SIX3 GSX2 PAX6 EOMES WNT1 EMX1 WNT7B FGF8 BMP2 BMP4, …	GO
Ventral spinal cord inter neuron differentiation	8/15	DMRT3 NKX6-1 NKX2-2 PAX6 NKX6-2 GLI2 DLL4 FOXN4	GO
Reactome FGFR1 ligand binding and activation	9/13	FGF10 FGF3 FGF9 FGF1 FGF8 FGF4 FGF17 FGF6 FGF22	Reactome
Neuron fate specification	9/24	OLIG3 DMRT3 NKX6-1 ISL1 GSX2 NKX2-2 PAX6 NKX6-2 GLI2	GO
Neuropeptide signaling pathway	35/79	SCG5 GPR139 NPW CARTPT NMUR1 NTSR2 MC2R NPVF GALR1 PPY, …	GO
Gamma Aminobutyric acid signaling pathway	10/17	PLCL1 GABRR2 GABRG3 CACNA1A GABRA5 GABRA2 GABRR1 PLCL2 ATF4 GABBR2	GO
Negative regulation of megakaryocyte differentiation	8/14	HIST2H4B HIST1H4C HIST1H4A HIST1H4B HIST1H4E HIST1H4F CIB1 HIST2H4A	GO

KEGG: Kyoto encyclopedia of genes and genomes; GO: gene ontology.
Data expressed at FDR <25% (n=7 each group).

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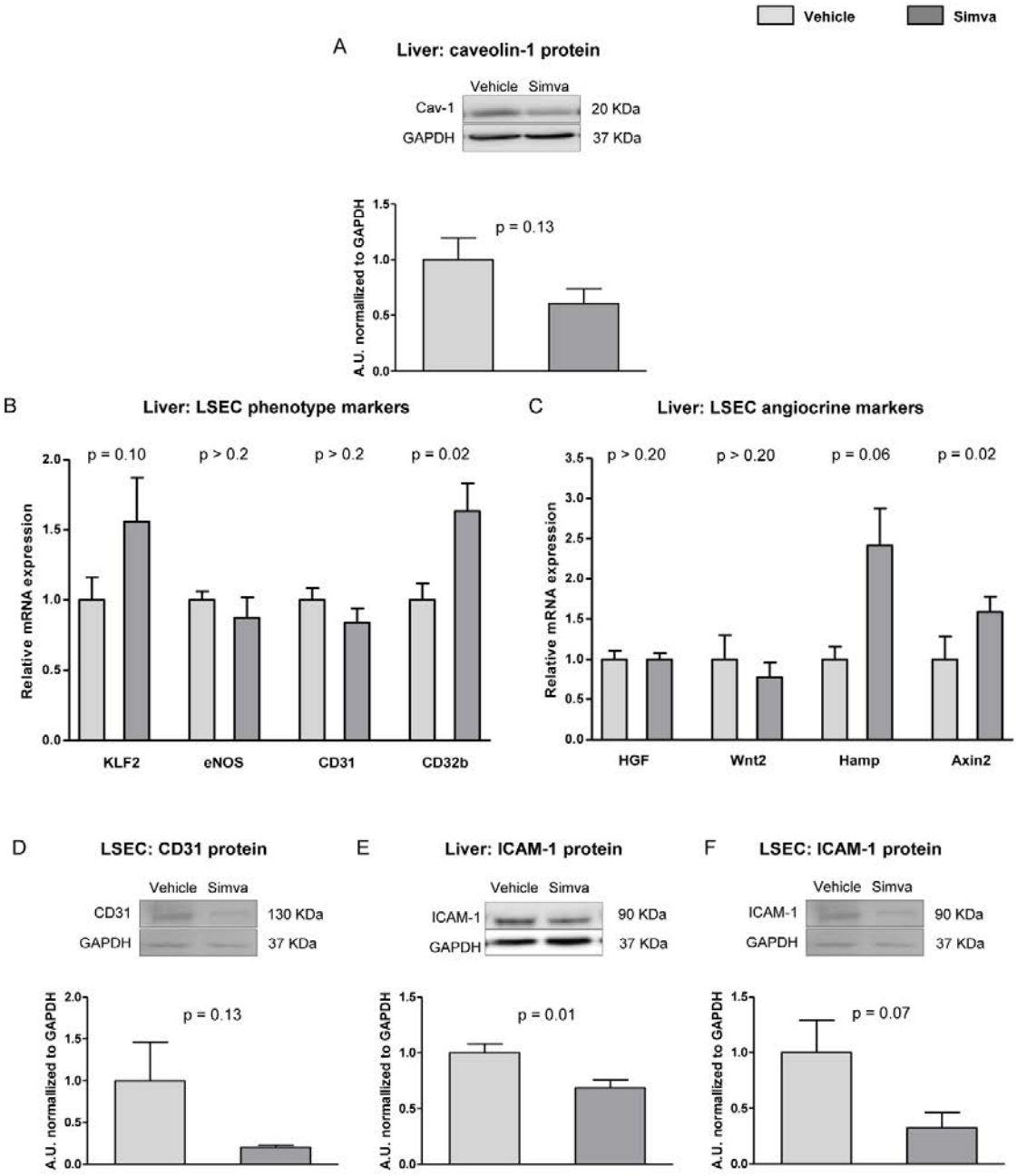

Supplementary Figure 1. Simvastatin ameliorates LSEC phenotype and microcirculatory dysfunction. (A) Caveolin-1 protein expression in total liver tissue, normalized to GAPDH. **(B)** mRNA expression of KLF2, eNOS, CD31 and CD32b. **(C)** mRNA expression of HGF, Wnt2, Hamp and Axin2. **(D)** CD31 protein expression in freshly isolated LSEC, normalized to GAPDH. **(E)** ICAM-1 protein expression in total liver tissue, normalized to GAPDH. **(F)** ICAM-1 protein expression in LSEC, normalized to GAPDH. n=10 (A-C, E) and n=5 (D, F) per group. Results represent mean ± S.E.M.

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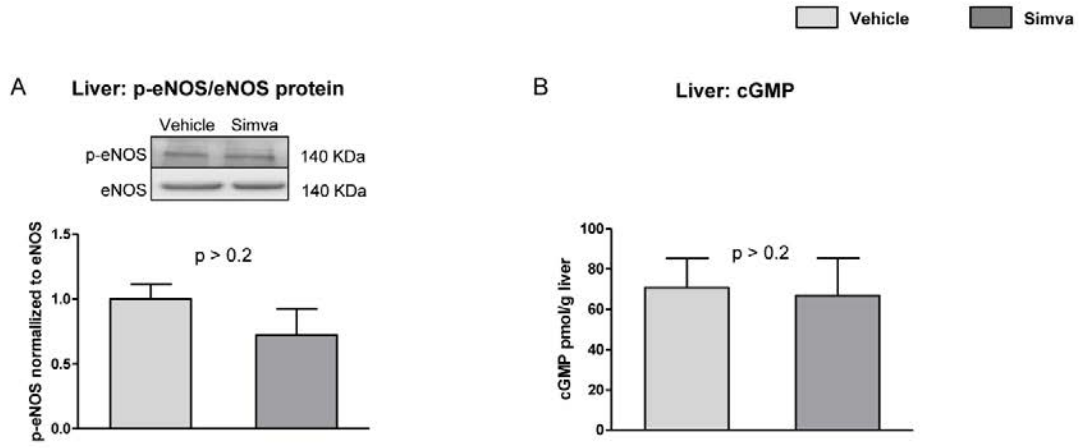
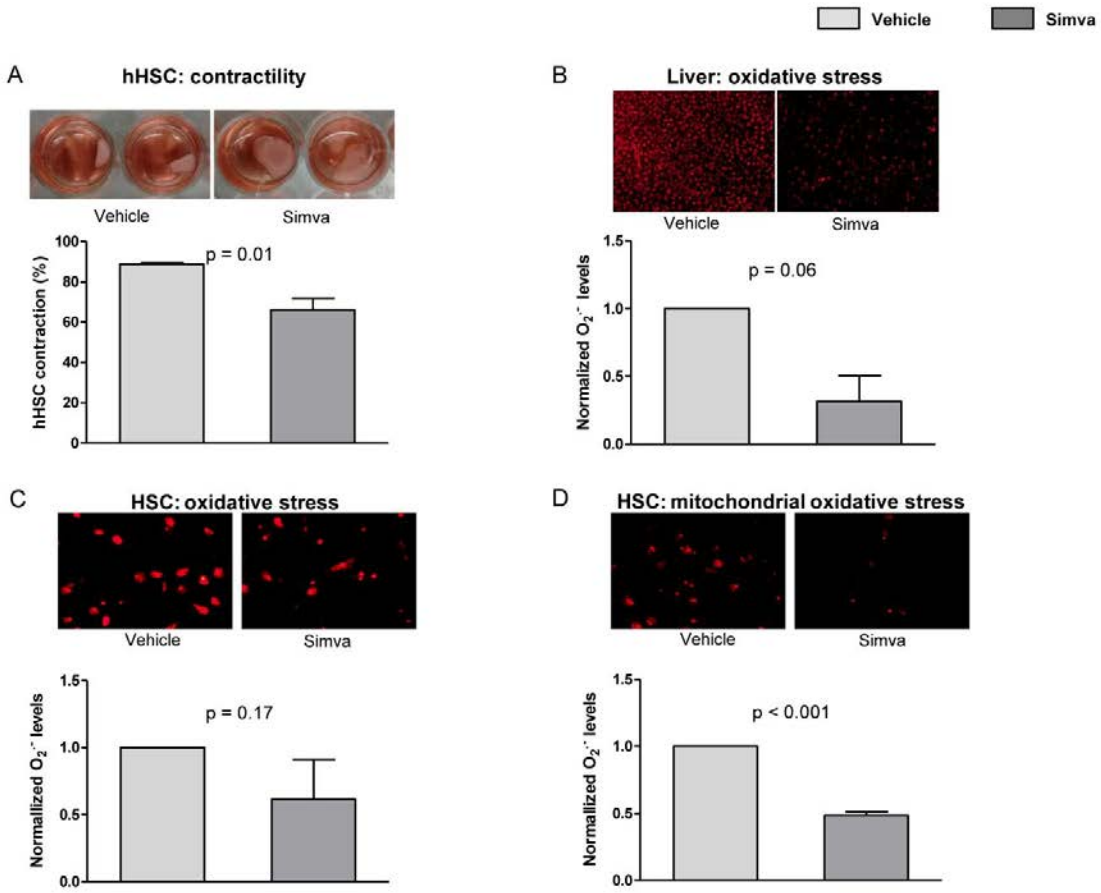


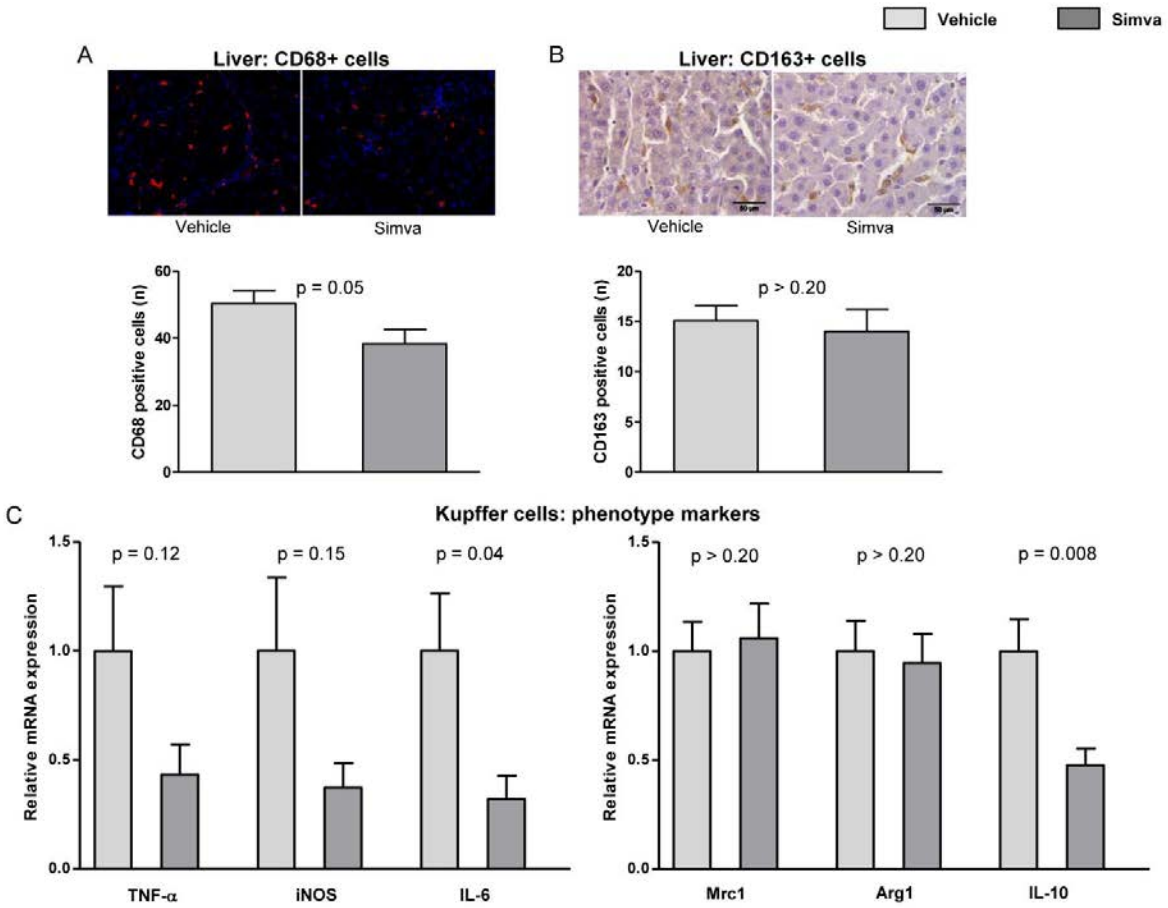
Supplementary Figure 2. NO-pathway in aged rats with aCLD treated with simvastatin or vehicle. (A) Representative blots of eNOS and p-eNOS, normalized to GAPDH in liver tissue from aged rats with aCLD treated with simvastatin or vehicle. **(B)** Hepatic levels of cyclic GMP determined in rats described in A. n=10 (A and B) per group. Results represent mean ± S.E.M.



Supplementary Figure 3. Simvastatin promotes HSC de-activation and reduction of oxidative stress. (A) Representative images of HSC contractility and their corresponding quantification in cells freshly isolated from human aged cirrhotic livers treated *in vitro* with simvastatin or vehicle. **(B)** Representative images of O_2^{-} levels in rat liver tissue (red fluorescence) & corresponding quantification. **(C)** Representative images of O_2^{-} levels in cirrhotic rat primary HSC (red fluorescence) & corresponding

SUPPLEMENTARY DATA

quantification. **(D)** Mitochondrial $O_2^{\cdot-}$ levels (red fluorescence) determined in cirrhotic rat primary HSC. n=3 (A, B) and n=5 (C, D) per group. Results represent mean ± S.E.M. All images 400X, scale bar=50μm.



Supplementary Figure 4. Simvastatin enhances macrophages deactivation. (A) Representative images of CD68 immunofluorescence in liver tissue from aged cirrhotic rats treated with simvastatin or vehicle, and corresponding quantification. **(B)** Representative images of CD163 immunohistochemistry in liver tissue from rats described in A and its quantification. **(C)** Expression of TNF-α, iNOS, IL-6, Mrc1, Arg1 and IL-10 in liver tissue from rats described in A. n=10 per group. Results represent mean ± S.E.M. All images 400X, scale bar=50μm.

Summary of results

Summary of results

Study 1. Effects of aging on liver microcirculatory function and sinusoidal phenotype.

- Aged animals present mild hepatic microcirculatory dysfunction with a moderate increase in portal pressure (PP) as a consequence of a higher hepatic vascular resistance (HVR) and an associated reduction in liver perfusion.
- Aging is associated with mild hepatocyte injury and dysfunction as shown by deficient bile flow and marked deregulation in differentiation markers (HNF4α, Slc22a1, Abcc2, and Abcc3).
- The aged hepatic endothelium is *pseudocapillarized* and procontractile. There is a clear downregulation of the NO-GMPc vasodilatory pathway, an increment in intracellular inflammatory molecules (IL-6, TNF-a, ICAM1) and oxidative stress, and a significant reduction in the expression of functional (CD32b, Stab2) and angiocrine markers (HGF, Wnt2).
- Aged hepatic stellate cells are spontaneously activated as demonstrated by increments in the expression of different activation markers including α-SMA, collagen1α1, collagen1α2, PDGFRβ, and p-moesin.
- The aged hepatic sinusoid is in a moderate proinflammatory state with a higher accumulation of neutrophils and CD68 positive macrophages, and increased expression of the pro-inflammatory cytokine IL-6.
- The aged human liver displays similar features of a dysfunctional sinusoid corroborating most of the molecular changes observed in the animal model.

Study 2. Aging influences hepatic microvascular biology and liver fibrosis in advanced chronic liver disease.

- Old animals with aCLD present higher portal pressure (+18%) in comparison to young cirrhotic animals, consequence of further increases in both portal blood inflow and hepatic vascular resistance.
- Aged hepatocytes from animals with aCLD experience profound deregulations in their phenotype and more extensive cell death, in comparison to young hepatocytes from animals with aCLD.
- The sinusoidal endothelium of aged rats with aCLD is further capillarized. Aged LSECs from rats with aCLD show reduction in the nitric oxide synthase pathway, depletion of angiocrine mediators and reduced porosity and frequency of fenestrae.
- Aged cirrhotic animals exhibit exacerbated fibrosis and over-activation of HSC and macrophages.
- The gene expression signature of cirrhotic livers from old patients is significantly divergent from the one observed in young patients.
- Simvastatin treatment in aged-rats with cirrhosis and portal hypertension decreases portal hypertension, ameliorates microcirculatory dysfunction and decreases fibrosis due to the improvement in hepatic sinusoidal cells.

Discussion

Discussion

The global population is aging, and this phenomenon has a direct social and economic impact on societies mainly through the aggravation of the burden of diseases (Kennedy et al. 2014). Aging is an influential risk factor for liver disease development: it increases the risk for drug-induced liver injury (DILI), promotes fibrosis progression, and increases the prevalence and severity of cirrhosis and liver cancer (Tsochatzis et al. 2014; Sheedfar et al. 2013; Poynard et al. 2001; Frith et al. 2009; Thabut et al. 2006; Davis et al. 2010; Stine et al. 2013; Kanwal et al. 2011).

The liver is a metabolic, synthetic, xenobiotic and endobiotic clearance center organ, which is essential for maintaining the body homeostasis (Trefts et al. 2017). In the 50s Prof. Popper started examining the effects of aging in the liver, and since then many other studies has described age-related changes in liver function and structure (Wynne et al. 1989; Sastre et al. 1996; Wakabayashi et al. 2002; Höhn & Grune 2013). Age-related changes have a direct effect in each cell type of the liver, though most research has focused on hepatocytes, ignoring about the implications for all the other hepatic sinusoidal cells. Altogether these alterations are making the aged liver vulnerable to injury, contributing to systemic susceptibility to age-related disease (Longo et al. 2015; Mitchell et al. 2015).

For this reason, studying the liver microcirculatory function and the molecular status of hepatic sinusoidal cells in uninjured and cirrhotic aged livers were the main topics of this PhD thesis. Understanding the molecular mechanisms promoting the hepatic age-related alterations, which are contributing to the susceptibility for liver disease development, are the key for identifying potential therapeutic or preventive approaches for age-associated diseases.

Previous studies have described the impact of aging on the vasculature of different territories, although no preceding report fully characterize the liver microcirculatory phenotype in a pre-clinical model of healthy aging (Hunt et al. 2019; Guzik & Touyz 2017). That's why, in the first study, we aimed at understanding the effects of aging in the specific hepatic microvasculature and the cells integrating this microenvironment.

Results from Study 1 demonstrate a de-regulation in the intrahepatic vascular system; uninjured aged rats exhibit a significant increase in the hepatic vascular resistance, which leads to reduced liver perfusion and to a moderate increment in portal pressure. Although a reduction in portal blood inflow was previously reported in humans and rodents (Vollmar et al. 2002), we herein describe age-related alterations in the hepatic microvasculature, which are consequence of molecular changes in the cells of the hepatic sinusoid.

Hepatocytes analysis revealed a significant decline in synthetic and detoxifying capabilities as demonstrated by reduced bile formation, decreased urea and albumin synthesis, and changes in the expression of hepatocyte transporters together with a decrease in the amount of this cells as suggested the decline in the relative liver mass (Le Couteur & McLean 1998; Tietz et al. 1992; López-Otín et al. 2013). The aged-related alterations in the vascular system could be affecting the oxygen and nutrient supplies entering the liver and consequently lead to a decrease in the functional capacity of hepatocytes. These finding confirmed the mild hepatic age-related dysfunction found in previous reports and, could be explaining why the aged livers are susceptible to secondary insults such as ischemia/reperfusion or drug induced liver injury, events that are especially common in elderly population. Underlying mechanisms explaining hepatocyte dysfunction may relate to senescence increased (up-regulation in p16, reduction in telomere length and SIRT1) and increase in hepatic oxidative stress. We observed an augmentation in the superoxide levels in the aged livers, which may derive from an imbalance between pro and antioxidants, together with increased hepatic lipid content, a well-known source of cellular oxidative stress. Importantly, oxidative stress could also contribute to potentiate the senescence pathways, and vice versa, thus creating a deleterious vicious cycle in the aged liver (Jin et al. 2011; Sharpless & DePinho 2004).

Our analysis of aged LSECs phenotype revealed pseudo-capillarization, which was previously defined by the decrease in the number and size of fenestrae with age (Le Couteur et al. 2001). However, this distinctiveness was not limited to morphologic changes in LSECs though was accompanied by profound de-regulations in the hepatic endothelial phenotype. We observed a significant decrease in key vasodilatory pathways, including nitric oxide and hemoxygenase in the aged endothelium, which may contribute to the increased HVR (Van Landeghem et al. 2009). The reduction in intrahepatic nitric oxide availability is of relevance (Hori et al. 1998; Gracia-Sancho, Maeso-Díaz, Fernández-Iglesias, et al. 2015) considering the roles of NO regulating the vascular tone, exerting anti-inflammatory effects (Iwakiri & Kim 2015), and maintaining neighboring cells phenotype (Marrone et al. 2016; Xie et al. 2013). Reduced nitric oxide bioavailability might, at least in part, derive from diminished eNOS activity, which may be due to reduced VEGF-p-eNOS pathway (Kroll & Waltenberger 1998), and from increased scavenging due to elevated oxidative stress (Gracia-Sancho et al. 2008). Together with the depletion in vasodilatory pathways in aged LSECs, we detected a decreased expression of several angiocrine receptors (stabilin-2, CD32b and VEGFR2) and angiocrine factors (Wnt2 and HGF) involved in the regulation of hepatocyte regeneration (Xie et al. 2013; Leibing et al. 2018; Carpenter 2005). Aging LSECs are in a moderate pro-inflammatory state as evidenced by increased CD68 positive cells, elevated expression of IL-6 and an increase intercellular adhesion molecule 1 expression, which leads to a substantial increase

in leukocyte adhesion, further contributing to reduced sinusoidal blood flow (Ito et al. 2007). Finally, there is upregulation of p16 and downregulation of SIRT1, which might influence cellular senescence. Altogether these results strongly suggest liver microcirculatory dysfunction consequence of profound deregulation of vasodilatory, inflammatory and angiocrine pathways in LSEC by age and this might be one of the mechanisms responsible of aged-associated increase in HVR.

We also analyzed the stellate cells phenotype in aging and revealed a slight, but significant, activation of HSC supported by increments in different activation markers including α-SMA or collagen I. Underlying mechanisms explaining HSC activation in aging revealed increments in intracellular oxidative stress and p16 (Uehara et al. 2013) without any changes in different pro-inflammatory mediators (TLR4, NFκB and TGFβ). Herein, we confirmed HSC lipid accumulation within aging (Le Couteur et al. 2011) and additionally describe alterations in retinoid metabolism and breakdown. In fact, aged livers exhibited down-regulated CRBP-1 protein levels together with over-expressed PNPLA3, which have been associated with HSC activation and susceptibility to develop steatohepatitis (Uchio et al. 2002; Bruschi et al. 2017). Partial activation of HSC with aging is another mechanism contributing to aged-associated increase in HVR.

Through this thesis I have explained the inflammatory roles of both LSECs and KCs. Our results revealed that LSECs are in a pro-inflammatory state with aging and along with LSECs, KC isolated from aged rats displayed high IL-6 levels, a pro-inflammatory cytokine key for liver regeneration, infection defense and regulation of metabolic functions (Schmidt-Arras & Rose-John 2016), with no alterations in other "polarization markers" of hepatic macrophages (Krenkel et al. 2018). The pro-inflammatory state of LSECs and KCs could be contributing to the recruitment of pro-inflammatory cells (neutrophils and macrophages) observed in the aged livers. LPS plasmatic levels were increased in aged rats and therefore we cannot discard that the observed systemic aged-related endotoxemia may contribute to the abnormalities observed in sinusoidal cells. The moderate pro-inflammatory status in the aged livers could be another mechanism responsible for the aged-associated increase in HVR.

Finally, we were able to confirm the overall trend of pathway de-regulation in healthy young and old humans suggesting that sinusoidal vulnerability hereby described in old rats is also of relevance in human aging.

Taken together, the data from Study 1 demonstrate for the first time that aging is accompanied by significant liver sinusoidal de-regulation affecting the phenotype of Hepatocytes, HSC, LSECs and

KCs, both in rodents and humans, suggesting sinusoidal vulnerability in front of subsequent chronic or acute injuries. Using anti-aging therapies to avoid the hepatic microcirculatory age-related dysfunction could prevent or decrease the susceptibility for the development of age-associated liver diseases.

I have cited before that aging is a major risk factor for chronic liver diseases development; in fact, cirrhosis is more prevalent, progresses faster and decompensates more frequently in the elderly (Poynard et al. 2001). In the pre-clinical scenario little is known about the impact of aging on liver diseases; two previous works suggested higher rate of fibrosis deposition in aged rats and attributed this to alterations in the immune response (Ramirez et al. 2017; Collins et al. 2013). However, in the Study 2 we evaluate for the first time the impact of aging on the hepatic sinusoidal milieu in a validated pre-clinical model of aCLD, focusing on the pathophysiology of the disease and its hemodynamic alterations.

The main finding from Study 2 is that aged animals develop a more severe form of aCLD, which is characterized by poorer hepatic function and exacerbated portal hypertension, fibrosis and inflammation. At the microcirculatory level, we describe for the first time that aged rats with aCLD show a marked aggravation in portal hypertension, which could be explained by increments in both vascular resistance and liver blood inflow. In the introduction of this thesis I have explicated in detailed that portal hypertension is a clinical syndrome consequence of an increased in HVR due to anatomical factors and functional abnormalities. We hypothesized that the deterioration of the intrahepatic microcirculatory status observed with aging is a consequence of overall deregulations in the phenotype of the main liver cells causing aggravation of both anatomical and functional components.

Indeed, aged rats with cirrhosis exhibited serious liver damage reflected by the increase in transaminases and the reduction in serum albumin and bile production in comparison with young rats with aCLD. Interestingly, aging was also accompanied by increases in both systemic and hepatic lipids content which can be an important source of oxidative stress; a well-known factor triggering HSC activation and aCLD development (Gracia-Sancho et al. 2018). Hepatocytes phenotype was significantly de-regulated and additionally, they were more affected by cell death, signaling that could be promoting HSC over-stimulation. Previous reports observed that aging is a factor promoting fibrogenesis, statement that we have confirmed in our aged rats with aCLD. In study 1 we showed that HSC are moderately activated during healthy aging and accordingly, we herein describe that old HSC undergo significant changes due to chronic liver injury in comparison to young animals. HSC are the major source of ECM and play important roles in modulating the

hepatic vascular resistance in cirrhosis (Tsuchida & Friedman 2017), which ultimately lead to expand deposition of extracellular matrix. Altogether, increased fibrotic deposition and massive cell death could be responsible for the hepatic architecture deterioration (anatomical factors) observed in the aged model of aCLD (Canbay et al. 2003).

Analysis of LSEC de-differentiation markers revealed alterations in LSEC phenotype. LSECs in aged rats with aCLD presented a major reduction in the nitric oxide synthase pathway, depletion of angiocrine mediators and importantly, reduced porosity and frequency of fenestrae. In the Study 1 we showed that LSECs during healthy aging are pseudo-capillarized and moderately dysfunctional (Maeso-Díaz et al. 2018), our findings in Study 2 suggests that these changes are much intensified in aged livers affected by chronic injury. Aggravation of LSECs capillarization could be contributing to both anatomical and functional components of portal hypertension development.

It has been widely described the role of inflammation in the progression of aCLD (Tacke 2017), but much less is known in the aging context (Mahrouf-Yorgov et al. 2011). Along with what we have observed in HSC and LSECs, during healthy aging the liver is in a moderate pro-inflammatory status in comparison to young animals, and we noticed that aged cirrhotic rats exhibit further deterioration in the hepatic inflammatory phenotype. They present a significant increase in the recruitment of pro-inflammatory macrophages together with a decrease in the expression of pro-resolution cytokines in this cellular sub-population. The increase in the myeloid cell content in the aged cirrhotic liver may derive from the sinusoidal endothelial activation described above, and importantly, this could be another mechanism contributing to microcirculatory dysfunction and global worsening of the disease (Nieto 2006).

In addition and aimed at understanding the translatability of our pre-clinical discoveries, we characterized the hepatic transcriptome in two groups of cirrhotic patients of different age. The result of these analyses confirmed the alterations observed in the aging rat model: endothelial deregulation, HSC over-activation, enhanced fibrogenesis and immune cell activation. Altogether, the pre-clinical and clinical data obtained in this study suggest that the pathophysiology of CLD is much worse, and probably involves different molecular mechanisms, when comparing aged and young individuals. This finding has implications for the discovery of new therapeutic approaches and treatment of portal hypertension and cirrhosis, both preclinical studies and clinical trials should consider age when in the experimental design.

Many efforts are currently being directed towards the development of novel therapeutic approaches for portal hypertension (Gracia-Sancho et al. 2015; Vilaseca et al. 2018). We have learnt alongside

this thesis how aging creates a distinctive hepatic milieu and because of that, drugs that show efficacy in the young may not work in the aged (McLean & Le Couteur 2004). However, most preclinical studies on the effects of different substances and drugs on the liver circulation have been conducted in young animals. Our current work revealed striking differences between young and aged liver, including alterations in vasoactive pathways, indicating that studies performed in young animals may not entirely reflect what should be expected from the clinical use of most interventions, as vasoactive drugs which are and will be mainly prescribed in the elderly.

Statins are HMGCoA reductase inhibitors, originally designed as cholesterol-lowering drugs. Over the years have been re-discovered for their pleiotropic effects on inflammation, fibrosis and endothelial function, among others (Liao 2005). Different studies have reported the benefits of statins in experimental models of young cirrhosis and in clinical trials evaluating its effects in patients with cirrhosis (Abraldes et al. 2007; Trebicka et al. 2010; Mohanty et al. 2016) but none has evaluated the effect of this therapy in aged aCLD. Actually, the efficacy and safety of statin therapy among older people have only being addressed for treating cardiovascular diseases and even in this field, it is still on discussion due to lack of studies and information regarding this topic (Armitage et al. 2019).

In this study we observed that 2-week administration of 5 mg/day/kg simvastatin to aged cirrhotic rats significantly improves liver microvascular dysfunction and portal hypertension, accompanied by slight ameliorations in the systemic hemodynamic and in hepatic function. Underlying mechanisms explaining the global benefits of this vasoprotective compound revealed reduced disease severity in all major hepatic cell types. Hepatocytes exhibited an improved phenotype as suggested by ameliorations in their synthetic capacity, in blood tests and in the ultrastructural architecture. Importantly, we did not observe signs of toxicity due to the treatment, even though has been described that old animals have a reduced detoxifying activity (Le Couteur & McLean 1998). Previous evidence of simvastatin toxic effects may derive from the experimental model used to induce CLD (common bile duct ligation) and/or be dose dependent (Rodríguez et al. 2017). Indeed, in our study we selected a dose of 5 mg/day/kg in rats, which is equivalent to a dose of 80 mg/day in adult humans following Reagan-Shaw et al. dose conversion (Reagan-Shaw et al. 2007) and preferred to generate CLD by chronic CCl_4 with no obstruction of bile secretion, thus preventing drug accumulation. In the clinical scenario, a recent study revealed that long-term statins treatment was associated with a significant increase in rhabdomyolysis toxicity events at a dose of 40 mg/kg in patients with decompensated cirrhosis but not at a dose of 20 mg/kg (Pose et al. 2019). However, in this study simvastatin is given in combination with the antibiotic rifaximin, the sample size is

limited, and most important, they do not consider age in the data analysis. Therefore, further trials are necessary to demonstrate if statins are beneficial for treating chronic liver disease specifically in the elderly subpopulation of patients.

The hepatic endothelium increased the expression of functional and angiocrine markers, while decreased the expression of capillarisation and inflammatory markers in response to simvastatin. Remarkably, aged cirrhotic animals treated with simvastatin for 15 days exhibited double number and frequency of fenestrae, which in fact may contribute to improve parenchymal function through a better diffusion of oxygen, nutrients, and waste products (Wisse et al. 1985). We could not detect differences in the nitric oxide pathway when comparing both groups of animals although our results support that the hepatic endothelium was ameliorated due to the treatment, and that previous reports have shown the effects of statins in this pathway. Further studies are required to elucidate if this is due to a partial spontaneous recovery in the vehicle-treated group after the 15 days semi-chronic treatment or that the aged rats with cirrhosis may exhibit a poor NO-signaling response to statins.

Importantly, simvastatin treatment promoted a marked regression in liver fibrosis, which may be due to deactivation of HSC, together with a reduction in oxidative stress and inflammation. Additionally, HSC phenotype improvement could also contribute to the amelioration of hepatic microvascular dysfunction (Klein et al. 2012). Our observations are in agreement with previous clinical observations in patients with liver disease.

In conclusion, this study provides evidence that aCLD has a much-aggravated pathophysiology in aged individuals, and even more importantly, that aging may activate different or additional molecular mechanisms from those observed in young. Therefore, aging should be incorporated to investigate the pathophysiology of aCLD to translate results more accurately to humans and may allow the development of more reliable therapeutic strategies. In fact, the characterization of simvastatin in this model further recommends its applicability at the bedside including the vast population affected by aCLD: the elderly.

Conclusions

Conclusions

Study 1: Effects of aging on liver microcirculatory function and sinusoidal phenotype.

- Aging is associated with increases in portal pressure and vascular resistance leading to reduce hepatic blood flow.
- The hepatic sinusoid with aging is accompanied by slight but significant alterations in all the cells of the hepatic sinusoid including hepatocytes, LSEC, HSC and KC.
- The pathway deregulations observed in aged rat livers are also present in human aging.

Aging is accompanied by significant liver sinusoidal deregulation, both in rodents and humans, suggesting sinusoidal vulnerability in front of subsequent chronic or acute injuries.

Study 2: Aging influences hepatic microvascular biology and liver fibrosis in advanced chronic liver disease.

- aCLD has a much-aggravated pathophysiology in aged rats and humans.
- The molecular profile of cirrhotic livers from old patients is different from those observed in young.
- Simvastatin treatment in aged animals with aCLD decreases portal hypertension and fibrosis, and ameliorates microcirculatory dysfunction as a result of the improvement of the cells of the hepatic sinusoid, without any toxic effect associated.

Using closer to real-life models to investigate the pathophysiology of aCLD may allow the development of more reliable therapeutic strategies. Our study further recommends the use of simvastatin for the treatment of aCLD including the subgroup of aged patients.

Future research directions

Future research directions

It is true we have provided our grain of sand to the aging field but, as it happens in science, from one contribution we have raised many other questions. In Study 1 we have seen how aging affects the phenotype of liver sinusoidal cells and how these alterations have consequences in the hepatic microcirculation and hepatic function. The next thing one would think is *when* this change occurs, during the timeline of life when there is a significant change in the organism that makes the difference and causes alterations in the phenotype of the cells and therefore in the organ functionality. Second, is *where* this happens is it a systemic alteration, as it could be the inflammaging or is it a local modification in the liver, as it could be the shortening of the telomeres in the cells of the liver. Third, is *how* and *why* it happens or in other words, which is the molecular mechanism triggering the aging switch in the liver or most probably, which ones are? These are the most general questions I would like to address in the future. However, to answer these big interrogations we must do one step at a time and focus the research in specific hypothesis. For example, is it inflammaging responsible for the age-associated microcirculatory dysfunction? Is it the microcirculatory dysfunction observed with aging equivalent to the one observed after chronic liver injury? LSEC age-related dysfunction is also preceding inflammation and HSC activation?

Hepatic age-related changes are turning cells vulnerable in front of a chronic injury as we have demonstrated in Study 2 or acute injury (Study 3 Anex). Aged livers exposed to a chronic injury exhibited an aggravated disease compared to young livers and the molecular mechanisms involved in the pathophysiology of chronic liver disease may be different because of age. In this case, more translational venues of research emerge: future preclinical and clinical studies should take age into account when searching and testing for new therapeutic approaches for treating chronic liver disease.

In conclusion, we still have a long road ahead of us in the aging liver field and we must keep pursuing the answers by doing research with the objective of finding both anti-aging therapies and treatments for chronic liver diseases.

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Other publications

Other publications

Study 3:

ISCHEMIA/REPERFUSION INJURY IN THE AGED LIVER: THE IMPORTANCE OF THE SINUSOIDAL ENDOTHELIUM IN DEVELOPING THERAPEUTIC STRATEGIES FOR THE ELDERLY.

Diana Hide, Alessandra Warren, Anabel Fernández-Iglesias, **Raquel Maeso-Díaz**, Carmen Peralta, David G. Le Couteur, Jaime Bosch, Victoria C. Cogger and Jordi Gracia-Sancho.

J Gerontol A Biol Sci Med Sci. 2019 Jan 14. doi: 10.1093/gerona/glz012.

Study 4:

NEW RAT MODEL OF ADVANCED NASH MIMICKING PATHOPHYSIOLOGICAL FEATURES AND TRANSCRIPTOMIC SIGNATURE OF THE HUMAN DISEASE.

Raquel Maeso-Díaz, Zoe Boyer-Diaz, Juan José Lozano, Martí Ortega-Ribera, Carmen Peralta, Jaime Bosch and Jordi Gracia-Sancho.

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